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Endogenous Release of Tissue Factor Pathway Inhibitor by Topical Application of an Ointment Containing Mucopolysaccharide Polysulfate to Nonhuman Primates

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Abstract

Several studies have shown that tissue factor pathway inhibitor (TFPI) is released after the intravenous and subcutaneous administration of heparin and heparin-related drugs. Mucopolysaccharide polysulfate (MPS) is a preparation of glycosaminoglycans (GAGS) derived from mammalian cartilage, which has several structural and functional properties similar to heparin. Previous reports have shown that MPS is capable of releasing TFPI after intravenous administration. Therefore, this investigation was performed to determine the ability of topically administered MPS to release TFPI in a nonhuman primate model. A group of four monkeys were administered 3% MPS ointment in a dosage of 0.5 g/kg corresponding to 15 mg MPS/kg; another four monkeys were administered placebo ointment at a dosage of 0.5 g/kg once a day for 5 days in a period of 10 days. No effect of MPS was observed on the coagulation assays activated partial thromboplastin time (APTT), thrombin time (TT) and Heptest or on the platelet count. However, both the total and free TFPI levels were significantly and progressively elevated over the 10-day period in comparison to the placebo control group ($P < .05$). It is proposed that the ability of the topically administered MPS to increase the free and total TFPI levels may be one of the modes of action that contributes to the anticoagulant and anti-inflammatory actions of this agent.

Key Words: Mucopolysaccharide polysulfate; Ointment; Tissue factor pathway inhibitor; Anti-inflammatory; Anticoagulant

Trauma and physical exercise often result in soft tissue and joint injury leading to localized inflammation, pain and edema. Several studies have been conducted over the past few years on the efficacy of intramuscular (im) or intra-articular administration of the polysulfated glycosaminoglycan (GAGPS) (synonym: mucopolysaccharide polysulfate, MPS) in the treatment of joint disorders [1–3]. In addition, several ointments of varying composition are available for the treatment of these conditions [4–6]. In particular, these ointments have been

Abbreviations: APTT, activated partial thromboplastin time; MPS, mucopolysaccharide polysulfate; TFPI, tissue factor pathway inhibitor; TF, tissue factor; TT, thrombin time; GAGPS, glycosaminoglycan polysulfate.

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useful in the management of sports-related injuries and painful rheumatic conditions [7]. One particular component known as MPS is often used as ointment or gel in these conditions [4–7]. However, the mechanisms of its therapeutic effect are not fully understood.

MPS represents a preparation of GAGS derived from mammalian cartilage, which is highly sulfated and mimics many of the properties of heparin [8]. However, its anticoagulant actions are weaker than heparin, whereas other properties such as the anti-inflammatory actions are equivalent or better [8,9]. In addition to the anti-inflammatory actions, heparins are capable of releasing tissue factor pathway inhibitor (TFPI) from the vascular endothelium [10]. TFPI inhibits the factor VIIa–tissue factor (TF) complex and factor Xa directly, thereby causing an additional anticoagulant/antithrombotic effect [11]. In addition, TFPI is capable of binding TF released from the activated cells and inhibiting the inflammatory process.

This study was designed to test the hypothesis that topical administration of an ointment containing MPS is capable of mobilizing endogenous TFPI. Previous studies have demonstrated that this preparation is able to produce anti-inflammatory and antithrombotic actions after topical administration [12,13]. Since primates have a similar coagulation system to humans and many of the proteins involved in the hemostatic process cross-react with antihuman antibodies, the primate model was used to quantitate the level of TFPI after repeated topical administration of MPS for 10 days [14].

1. Materials and Methods

MPS ointment 3% and ointment base (placebo) were obtained from Sankyo Pharma, Munich, Germany. Male and female nonhuman primates (*Macaca mulatta*) included in this study are from a colony housed at the AAALAC approved Animal Research Facility at Loyola University Chicago, Maywood, IL.

The ointments were administered to nonhuman primates (*Macaca mulatta*) using the following protocol. A group of four monkeys (two male and two female) were assigned to the active group and another group of four to the placebo group. Each monkey was weighed prior to the experiment. The abdominal area (10 × 12 cm) and lower legs of the primates were shaved and cleaned. The animals were anesthetized with 10 mg/kg ketamine im. Plastic syringes with 21-gauge butterfly needles were used to draw baseline blood samples. The blood samples were

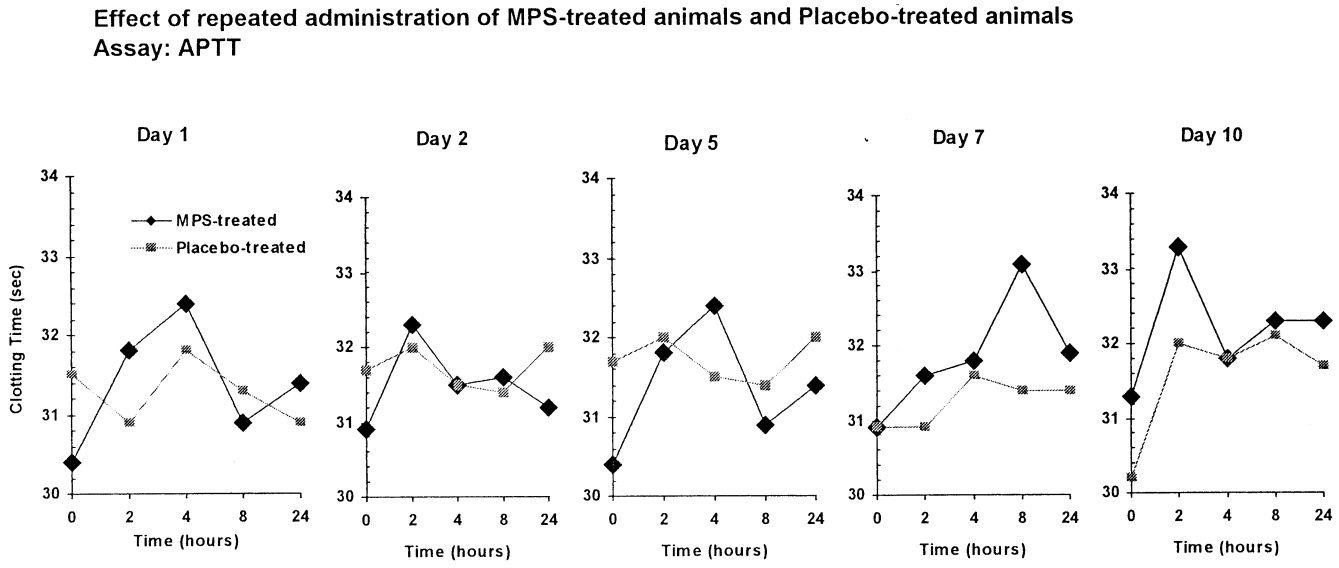

Fig. 1. MPS (3%) or placebo ointment was administered to primates ($n = 4$) on Days 1, 2, 5, 7 and 10. Blood samples ($n = 4$) were collected in 3.2% sodium citrate. Blood samples were drawn at 2, 4, 8 and 24 h after the application of 3% MPS or placebo and the APTT assay was performed. All results represent the mean ± 1 S.D.

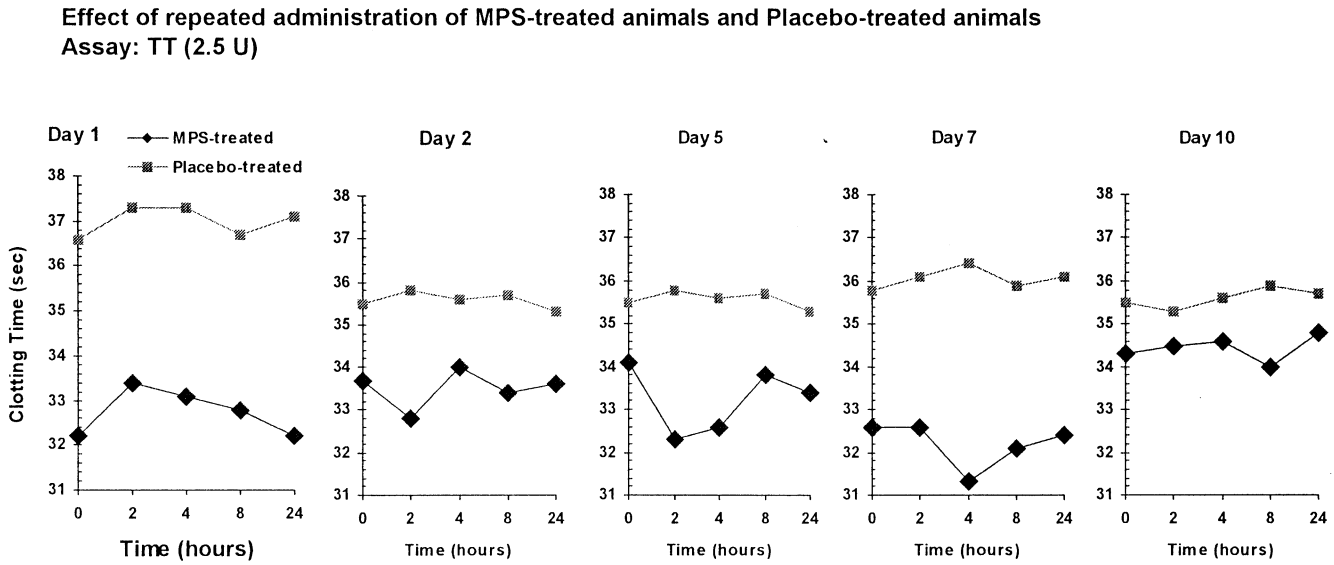

Fig. 2. MPS (3%) or placebo ointment was administered to primates ($n = 4$) on Days 1, 2, 5, 7 and 10. Blood samples ($n = 4$) were collected in 3.2% sodium citrate. Blood samples were drawn at 2, 4, 8, 24 h after the application of 3% MPS or placebo and the TT (2.5 U) assay was performed. All results represent the mean ± 1 S.D.

placed in 3.2% sodium citrate (9:1 ratio). Blood was drawn via lower leg saphenous vein puncture. The MPS ointment was applied to the skin of the abdomen at a dosage of 0.5 g ointment/kg corresponding to 15 mg MPS/kg. It was massaged into the skin with rubber gloves for 3–5 min, until all of the ointment was absorbed by the skin. The ointment application was repeated at Days 2, 5, 7 and 10. Blood draws were performed at 2, 4, 8 and 24 h after the application of MPS on Days 1, 2, 5, 7 and 10.

A platelet count was performed and the coagulation assays activated partial thromboplastin time (APTT), thrombin time (TT), Heptest and free and total TFPI were performed on the plasma samples. Blood samples were centrifuged immediately at 3000 rpm at 4°C for 15 min. The plasma was aliquoted and frozen at −70°C until

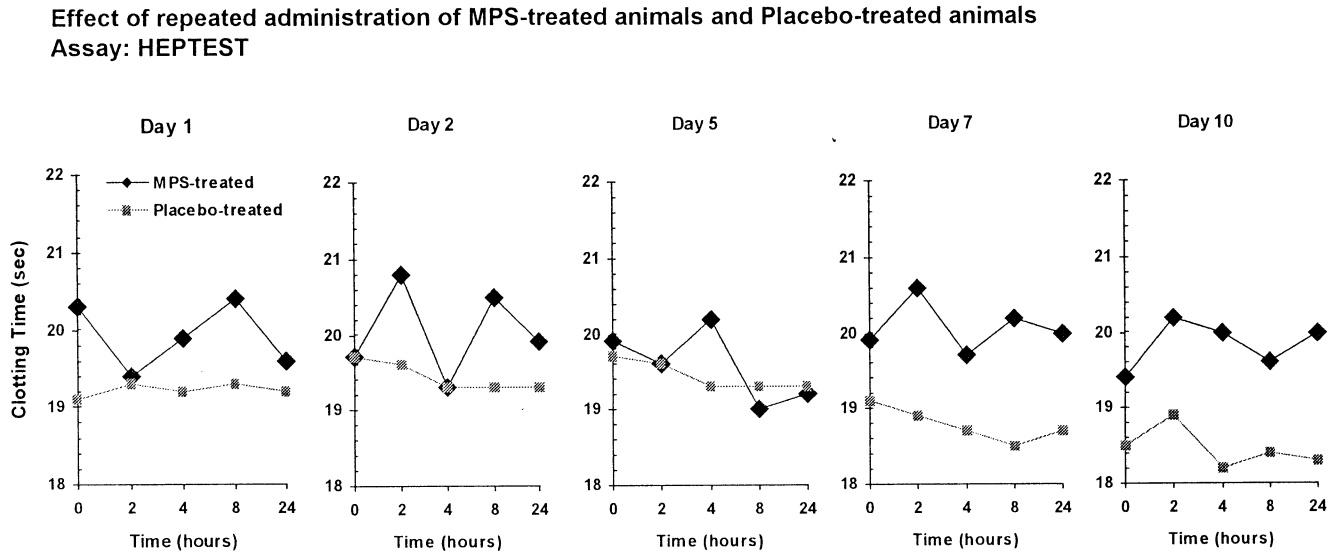

Fig. 3. MPS (3%) or placebo ointment was administered to primates ($n = 4$) on Days 1, 2, 5, 7 and 10. Blood samples ($n = 4$) were collected in 3.2% sodium citrate. Blood samples were drawn at 2, 4, 8, 24 h after the application of 3% MPS or placebo and the Heptest assay was performed. All results represent the mean ± 1 S.D.

Table 1. Effect of MPS and placebo ointment on the platelet counts

	Platelet count ($\times 10^3/\mu l$)
Placebo, Day 1, baseline	325 ± 21
Placebo, Day 1, 4 h	311 ± 17
3% MPS treatment, Day 1, baseline	333 ± 21
3% MPS treatment, Day 1, 4 h	329 ± 19
Placebo, Day 10, baseline	345 ± 25
Placebo, Day 10, 4 h	354 ± 24
3% MPS treatment, Day 10, baseline	328 ± 19
3% MPS treatment, Day 10, 4 h	349 ± 29

analyzed. The reagents used for the clotting assays were obtained from Organon Teknika, Durham, NC, (APTT), Ortho Diagnostics, Raritan, NJ (TT) and Hemachem, St. Louis, MO (Heptest). Free and total TFPI ELISA kits were obtained from Serbio (Gennevillers, France).

2. Results

Figs. 1–3 show the results of the coagulation assays APTT, TT and Heptest over the 10-day period in monkeys treated with 3% MPS or placebo. In the MPS group there was no statistically significant increase or difference to the placebo group in the APTT, TT or Heptest over the 24-h period on Days 1 through 10. Thus, neither a prolongation of coagulation nor an accumulative effect was detectable in either group. After 10 days there was also no difference in the platelet count compared to baseline or between the groups ($P > .05$) as shown in Table 1.

The results of the TFPI assays are shown in Figs. 4 and 5. There was a progressive increase in the free TFPI levels (Fig. 4) noted in the 3% MPS-treated group. The baseline levels of 6.8 ± 2.1 ng/ml on Day 1 increased to 12.0 ± 4.8 ng/ml on Day 10 ($P < .05$). Beginning at 4 h at Day 5 until Day 10 the free TFPI levels were significantly increased compared to baseline ($P < .05$). There was no elevation in the free TFPI levels in the placebo-treated animals (baseline: 8.3 ± 2.2 ng/ml; Day 10: 7.3 ± 1.7 ng/ml). Comparisons between groups demonstrated a statistically significant increase in free TFPI after the topical administration of 3% MPS at Day 7 (2 h after application) and Day 10 (2 to 24 h after application) ($P < .05$).

Similar results were obtained with the total TFPI assay (Fig. 5). A progressive increase in the

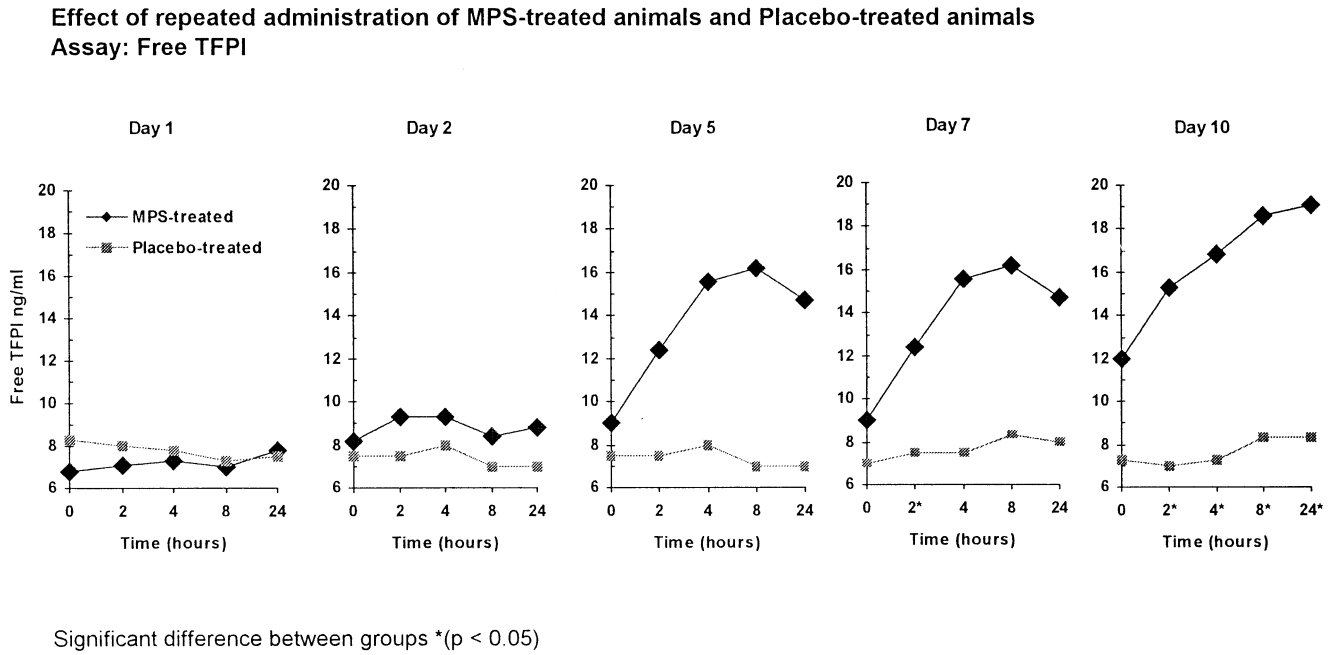

Fig. 4. MPS (3%) or placebo ointment was administered to primates ($n = 4$) on Days 1, 2, 5, 7 and 10. Blood samples ($n = 4$) were collected in 3.2% sodium citrate. Blood samples were drawn at 2, 4, 8, 24 h after the application of 3% MPS or placebo and the free TFPI ELISA assay was performed. All results represent the mean ± 1 S.D.

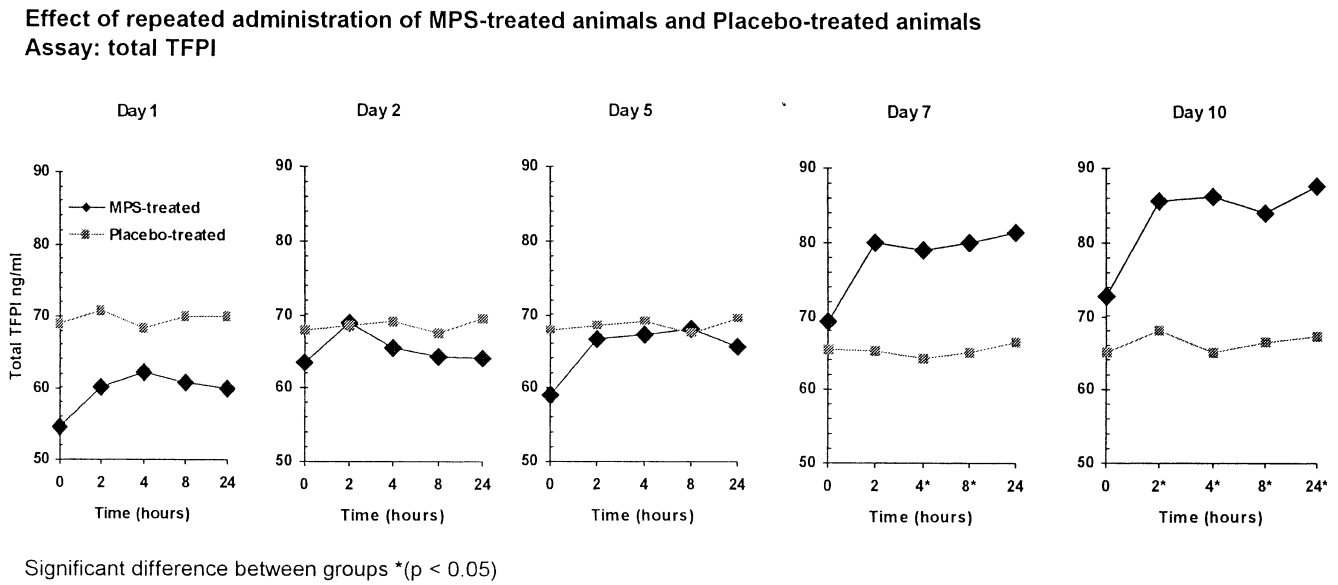

Fig. 5. MPS (3%) or placebo ointment was administered to primates ($n = 4$) on Days 1, 2, 5, 7 and 10. Blood samples ($n = 4$) were collected in 3.2% sodium citrate. Blood samples were drawn at 2, 4, 8, 24 h after the application of 3% MPS or placebo and the total TFPI assay was performed. All results represent the mean ± 1 S.D.

total TFPI levels was observed in the animals treated with 3% MPS. On Day 1 the baseline levels were 54.6 ± 4.7 ng/ml and on Day 10 baseline levels were increased to 72.8 ± 9.8 ng/ml ($P < .05$). On Days 7 and 10 there was a significant increase in total TFPI compared to baseline after the topical administration of 3% MPS ($P < .05$). The placebo control group showed no increase in total TFPI over the 10-day period (baseline: 69.0 ± 6.8 ng/ml; Day 10: 65.0 ± 5.0 ng/ml).

Comparisons between groups demonstrated a statistically significant increase in total TFPI after the topical administration of 3% MPS at Day 7 (4 and 8 h after application) and Day 10 (2 to 24 h after application) ($P < .05$).

3. Discussion

Heparin and low molecular weight heparins (LMWHs) are known to release TFPI after intravenous and subcutaneous administration in a dose-dependent manner [15]. Several other heparin derivatives have also shown to release TFPI to varying degrees [16]. MPS is a mucopolysaccharide polysulfate with many properties similar to heparin. Therefore, it has been suggested that this agent may be capable of releasing TFPI. In a previous study on the pharmacodynamics of MPS from our group, we have shown that MPS given intravenously is capable of releasing both free and total TFPI (personnel communication). In addition, intravenous administration of MPS has a systemic effect that can be measured by the clot-based APTT, Heptest, thrombin time and amidolytic anti-IIa assays.

Ointments with active ingredients such as MPS are often used to treat traumatic and rheumatic disorders that may lead to inflammation. The exact mechanism of this therapeutic action has not yet been fully elucidated. One hypothesis is that these topically administered agents are capable of releasing TFPI from the vascular sites, which produces a local anti-inflammatory and antithrombotic effect. During an inflammatory process cells become activated and several inflammatory cytokines and mediators including tissue factor are released. TFPI is capable of binding to the tissue factor, thereby inhibiting its effects. Furthermore, TFPI also exhibits anti-Xa and antiprotease effects, which are similar to other kunitz inhibitors. This study was designed to investigate the ability of topically applied MPS to release TFPI from endogenous sites.

Topically administered 3% MPS or placebo ointments showed no effect on the platelet counts and the coagulation assays APTT, TT or Heptest

at the individual time points over the 10-day period. In addition, there was no bleeding or oozing observed in the primates treated with MPS. These results suggest that MPS at this high dosage does not cause any bleeding complications. Neither was there any measurable systemic anticoagulant at these dosages. However, in the TFPI assays, in contrast to the placebo group, there was a statistically significant and progressive increase in both the free and total TFPI values from Days 5 to 10, which peaked on Day 10 in the MPS group. One explanation for the delay in the increase of the TFPI levels is that it takes approximately 3–5 days to saturate the sites with MPS at this dosage due to the absorption characteristics of MPS. After 3 days, enough MPS is present to release TFPI from the vasculature. However, the amount of TFPI released by this compound is modest and is too low to have any effect on the clotting test. The amount of TFPI needed to increase the clotting test is relatively high (>500 ng/ml).

It is concluded that one of the mechanisms by which MPS mediates its therapeutic effect is by releasing TFPI, which causes inhibition of the extrinsic pathway via inhibiting factor Xa directly and the TF–FVIIa complex. This effect of MPS may also contribute to the local antithrombotic and anti-inflammatory actions of several ointments and creams that are in current usage for various clinical indications.

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