行政院國家科學委員會專題研究計劃成果報告

敗血症引起血小板減少之機轉探討及治療方法之研究(3/3)
Investigation of pathogenic mechanisms and treatments of thrombocytopenia (3/3)

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一、中文摘要

血小板減少症常發生在格蘭氏陰性菌感染的初期。Triflavin是一種含Arg-Gly-Asp的disinteqrin，它可競爭性的干擾血液中的纖維蛋白原(fibrinogen)結合到血小板細胞膜上的纖維蛋白原受體(醣蛋白IIb/IIIa, glycoprotein IIb/IIIa complex)，而抑制血小板凝集反應。本研究計畫主要探討當敗血症發生初期引起血小板減少的作用機轉；同時並評估這類含Arg-Gly-Asp的disintegrin (如triflavin)是否可以預防或減少血小板減少症。在本計畫中，使用放射線^{51}Cr標定血小板追蹤致當引起敗血症時，循環中的血小板其在各個組織器官的分布情形；同時利用穿透式及掃描式電子顯微鏡分析在敗血症發生時，血小板在組織器官及在受損血管內膜的附著情形，並觀察在投與triflavin後上述這些狀況改變的情形。

由本研究結果顯示，triflavin可明顯的抑制敗血症所引起的血小板減少現象；而其抑制的作用機轉可能為: (1) trifalvin抑制血小板凝集反應，進而抑制thromboxaneA_2的合成。(2) Triflavin可抑制血小板附著到血管內皮下基質蛋白，因此能使附著到受傷血管內皮下層蛋白的血小板及堆積在肝臟組織中的血小板明顯減少，而使循環中的血小板回復正常。

關鍵詞: 血小板，血小板凝集抑制劑，血栓素。

Abstract

Thrombocytopenia frequently occurs early in the course of gram-negative bacterial infection. Triflavin, an Arg-Gly-Asp-containing disintegrin, has been suggested to interfere with the interaction of fibrinogen with the glycoprotein (GP) IIb/IIIa complex. The present study was undertaken to determine whether triflavin could prevent thrombocytopenia in lipopolysaccharide (LPS)-treated rats. In this study, ^{51}Cr-labeled platelets were used to assess blood and tissue platelet accumulation after LPS challenge. In histological examinations and platelet adhesion assay, triflavin markedly inhibited the adhesion of platelets to subendothelial matrices in vivo and in vitro. These results indicate that triflavin effectively prevents thrombocytopenia, possibly through the following two mechanisms: (1) Triflavin markedly inhibits platelet aggregation, resulting in decreased thromboxane A_2 formation. (2) It inhibits the adhesion of platelets to subendothelial matrices, thereby leading to a reversal in the distribution of platelets in blood and liver in LPS-treated rats.

Key words: platelets, platelet aggregation inhibitors, thromboxane A_2.

二、計畫緣由與目的

Circulatory shock from gram-negative bacterial sepsis produces a spectrum of pathophysiological alterations including cardiopulmonary, renal, hematological, and metabolic dysfunction leading to vascular collapse (1). The most pronounced clinical manifestation of septic shock is disseminated intravascular coagulation (DIC). DIC is characterized by microvascular thrombosis, thrombocytopenia, and stimulation of fibrinolysis. Thrombocytopenia frequently occurs early in the course of septicemia without overt evidence of DIC in both adults and children (2). The mechanism responsible for the development of thrombocytopenia is not fully understood. There are a number of possible ways in which bacterial infections could cause platelet consumption (3): (a) by immune mechanisms; (b) by initiating DIC with thrombin-induced platelet consumption; (c) by directly aggregating platelets independently of thrombin action; (d) by producing vessel damage, which results in platelet interaction with subendothelial structures; and (e) by hypersplenism secondary to the bacterial infection. There is an investigation which has suggested possible roles for the participation of leukocytes in bacterial-induced thrombocytopenia (4).

Recently, many disintegrin antiplatelet peptides have been reported (5). These peptides all contain RGD and bind with high affinity to integrins, a family of adhesion receptors on the cell surface. The integrins comprise a superfamily of transmembrane receptors that participate in cell-cell and cell-substrata interactions (6). Triflavin is a disintegrin purified from *Trimeresurus flavoviridis* snake venom (7, 8). Its primary structure consists of 70 amino acid residues including 12 cysteines with an RGD sequence at position 49-51 (8). We previously reported that triflavin inhibits platelet aggregation by interfering with the interaction of fibrinogen with the glycoprotein (GP) IIb/IIIa complex ($\alpha_{IIb}\beta_3$ integrin) (9).

The present study was designed to determine the effect of triflavin on the development of lipopolysaccharide-induced thrombocytopenia in rats during LPS-induced septic shock, and to compare the relative activity of triflavin to that of the RGD-synthetic peptide GRGDS.

三、結果與討論

Effect of Triflavin on LPS-induced Thrombocytopenia in Rats

The administration of LPS (4 mg/kg, i.v. bolus) for 4 hours produced a significant reduction in both radiolabeled platelets in blood (Table 1) and blood total platelet concentrations (data not shown). Less than 1% of injected radioactivity was detected in plasma, and less than 0.002% of the total injected radioactivity was detected in the bile from either normal saline- or LPS-treated animals (data not shown). These findings are consistent with previous studies showing that treatment of rats with LPS results in thrombocytopenia (10). On the other hand, ^{51}Cr-labeled platelets markedly accumulated in the liver within 4 hours after LPS administration (Table 1). However, LPS did not significantly alter ^{51}Cr-labeled platelet accumulation in the spleen, kidneys, or lungs as compared with normal saline-treated rats (Table 1). Pretreatment with triflavin (500 μg/kg) prior to LPS administration resulted in a significant increase in the number of radiolabeled platelets in the blood, and markedly attenuated LPS-induced hepatic platelet accumulation (Table 1). The distribution of radiolabeled platelets in the spleen and kidney was not altered by pretreatment with triflavin after LPS challenge. Additionally, we found that pretreatment with triflavin followed by addition of LPS significantly reduced platelet accumulation in the lungs compared with the LPS-treated group (Table 1). However, pretreatment with GRGDS (20 mg/kg) did not significantly prevent the alteration of platelet accumulation in various organ

tissues after LPS challenge compared with that of LPS-treated rats (Table 1). These results indicate that triflavin but not GRGDS effectively reverses the platelet accumulation induced by LPS.

Effect of Triflavin on TxB_2 Concentrations in LPS-treated Rats

Plasma TxB_2 concentrations had increased about 5-fold at 1 hour after starting the injection of LPS (4 mg/kg) and were still elevated, although less markedly, at 2 hours and 4 hours (Fig. 1). Triflavin (500 μg/kg) but not GRGDS (20 mg/kg) markedly suppressed the elevation of plasma TxB_2 concentration by about 30% during the 4-hour period (Fig. 1). This result indicates that triflavin was more effective than GRGDS at inhibiting the increase of TxB_2 concentration in LPS-treated rats.

Effect of Triflavin on LPS-induced Alteration of 5-HT Concentrations in Organ Tissues

In this study, we measured the amount of 5-HT in organ tissues by using 5-HT EIA kits instead of conventional isotope-labeled or HPLC assays. The 5-HT content of various tissues (including blood) in normal saline- and LPS (4 mg/kg)-treated rats is shown in Fig. 2. In LPS-treated rats, 5-HT levels were significantly lower (9±2 nmol/g vs. 25±5 nmol/g) in the blood and higher (9±1 nmol/g vs. 3±0.4 nmol/g) in the liver as compared with normal saline-treated rats; however, there was no significant difference in 5-HT content in the lungs, kidneys, and spleen between normal saline- and LPS-treated rats (Fig. 2). Triflavin significantly reversed the 5-HT content in the blood and liver as compared with LPS-treated rats. In addition, GRGDS showed no significant effect (Fig. 2). These results imply that triflavin, but not GRGDS, effectively prevented the alteration of 5-HT levels in the organ tissues of LPS-treated rats.

Transmission Electron Microscopy of Liver Sections in Normal Saline- and LPS-treated Rats

In the livers of normal saline-treated rats, Kupffer cells were evenly distributed in the lumen of the sinusoid formed by intact endothelium (Fig. 3A). A notable feature of the livers removed 4 hours after LPS administration was the presence of numerous platelets, located in the Disse spaces (Fig. 3 B-C) between hepatocytes and endothelial cells. The endothelium appeared to be absent or severely damaged after LPS challenge. The platelets seen within the liver still retained granules and microtubules, indicating that these cells had not undergone degranulation. Figure 3A shows an absence of platelets in the sinusoidal spaces and lack of interaction between platelets and Kupffer cells in unchallenged liver tissue. In contrast, the majority of platelets in the sinusoidal spaces of the liver in LPS-treated rats were surrounded by well-developed microvilli of hepatocytes, and there was marked interaction between platelets and cell processes of endothelial cells (Fig. 3B) or Kupffer cells (Fig. 3C). Furthermore, many polymorphonuclear neutrophils were observed in the sinusoidal spaces, however, there was no identifiable attachment of the platelets to the neutrophils in LPS-treated livers (data not shown). Pretreatment with triflavin (500 μg/kg) obviously attenuated the LPS-induced hepatic platelet accumulation both in sinusoidal spaces and Disse spaces (Fig. 3D). This result is also reflected in Table 1, which shows that pretreatment with a similar dose of triflavin resulted in a decrease of platelet accumulation in liver of LPS-treated rats.

Scanning Electron Microscopy of Aortic Vessels in LPS-treated Rats

Figure 4A is a scanning electron micrograph of aortic endothelium in the control group rats. The LPS-treated aortic endothelium exhibited severe damage with platelets accumulating to the subendothelium (Fig. 4B). The normal discoid shape of the platelets had changed to irregular spheres, and the extension of pseudopods was

observed. On the other hand, pretreatment of triflavin (500 μg/kg) markedly reduced the accumulation of platelets to the subendothelium in the LPS-treated aorta tissue, however, triflavin did not significantly prevent the endothelial damage induced by LPS (Fig. 4C). In contrast, GRGDS (20 mg/kg) did not significantly inhibit the accumulation of platelets to damaged endothelium (data not shown).

In conclusion, the most important finding in this study is that triflavin effectively prevents thrombocytopenia in LPS-treated rats. The inhibitory property of triflavin may involve the following two mechanisms: (1) Triflavin markedly inhibits platelet activation induced by LPS, resulting in decreased TxA_2 formation from platelets, and a subsequent decrease of the TxA_2 level in plasma. (2) Triflavin inhibits the adhesion of platelets to subendothelium, thereby preventing the alteration of platelet accumulation in blood and liver in LPS-treated rats, and subsequently lowering the 5-HT level in the liver and increasing the 5-HT level in the blood.

四、計畫成果自評

This study completely corresponds to the original project, and it will be submitted to publish in international journal. These results demonstrate the usefulness of triflavin in the prevention of LPS-induced thrombocytopenia in a rat septicemia model. A combination of Arg-Gly-Asp-containing disintegrins with other therapeutic agents (i.e., platelet activating factor antagonists, antibiotics) may represent a new approach in the treatment of septicemia.

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TABLE 1. Effect of RGD-Containing Peptides on Platelet Distribution in Organ Tissues

Tissue	SAL+SAL	SAL+LPS	Triflavin+LPS	GRGDS+LPS
Blood	4.5±0.3	1.8±0.4†	3.8±0.5§	2.1±0.2†
Liver	11.5±0.8	37.2±5.3†	16.4±1.8*§	35.6±4.9†
Spleen	10.3±1.9	13.5±2.1	11.8±1.4	12.6±1.7
Kidney	1.8±0.4	2.4±0.4	1.6±0.2	1.9±0.3
Lung	15.7±2.0	20.3±2.6	14.1±1.2‡	18.7±1.9

Rats were pretreated with triflavin (500 μg/kg), GRGDS (20 mg/kg), or normal saline (SAL) 15 minutes before the administration of LPS (4 mg/kg IV bolus) as described in Methods. Data are presented as a percentage of injected radioactivity in blood (per milliliter) or entire organ (mean±SEM), n=8 per group. *$P<0.05$; †$P<0.001$, significant difference compared with resting group (SAL+SAL). ‡$P<0.05$; §$P<0.01$, significant difference compared with LPS-treated group (SAL+LPS).

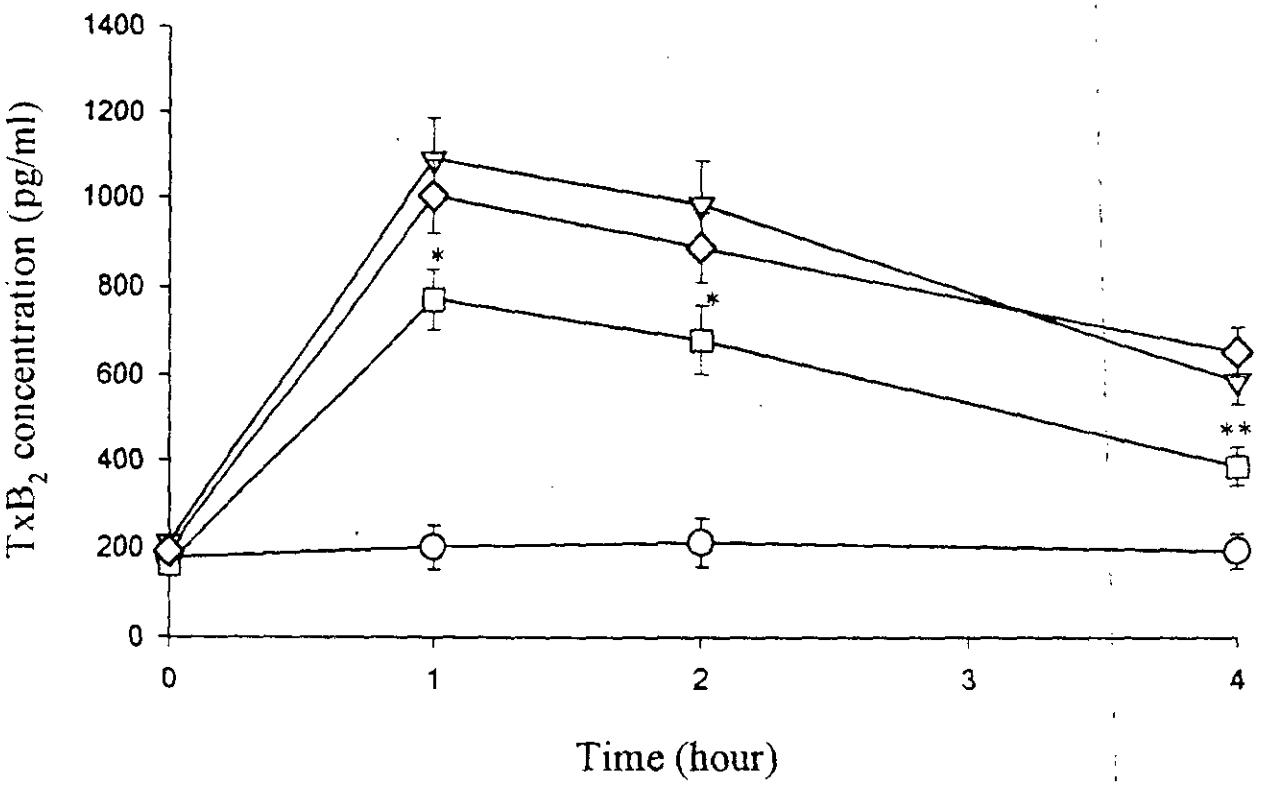

Figure 1. Effect of RGD-containing peptides on the release of TxB_2 from plasma in LPS-treated rats at various times after the start of LPS injection. The rats received normal saline (○), LPS alone (4 mg/kg, IV bolus; ▽), or pretreatment with triflavin (500 μg/kg; □) or GRGDS (20 mg/kg, ◇)followed by addition of LPS. Data are presented as mean±SEM (n=8). *$P<0.05$; **$P<0.01$, significant difference compared with LPS-treated rats.

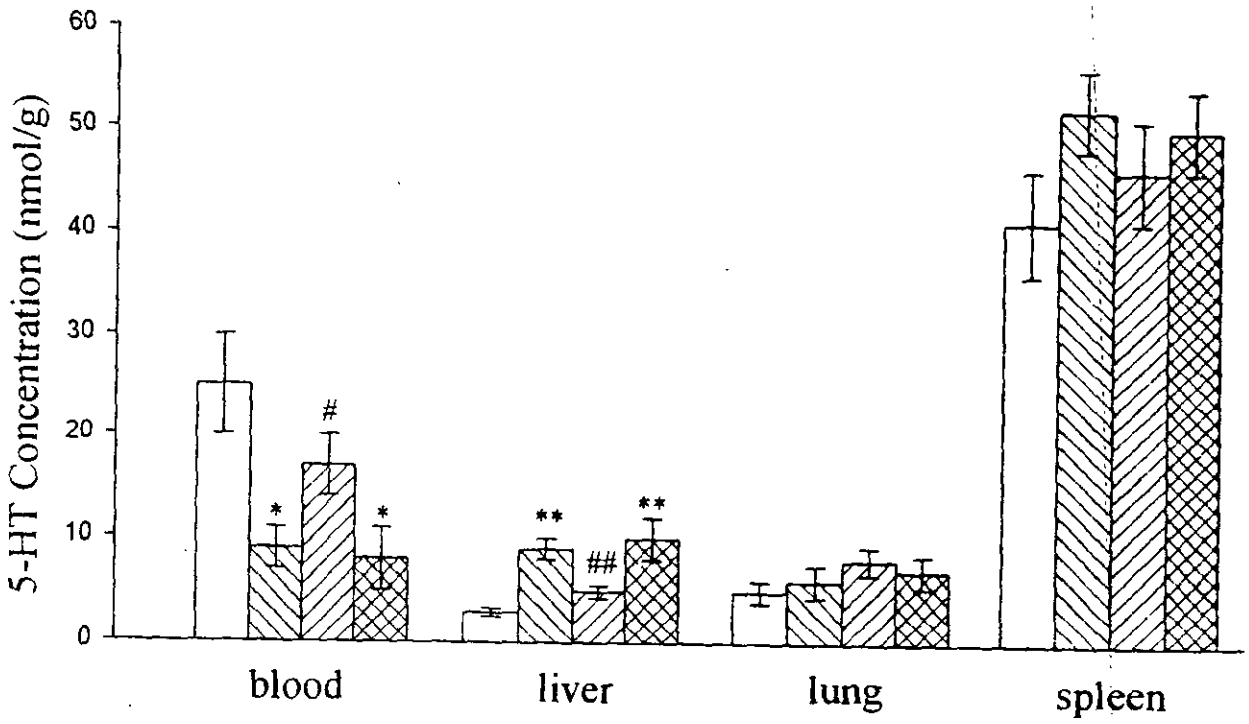

Figure 2. Effect of RGD-containing peptides on the accumulation of 5-HT in the blood and organ tissues 4 hours after the start of LPS injection in rats. The rats received normal saline (□), LPS alone (4 mg/kg IV bolus; ▨), or pretreatment with triflavin (500 μg/kg; ▨) or GRGDS (20 mg/kg; ▩) followed by addition of LPS. Data are presented as mean±SEM (n=8) *$P<0.01$; **$P<0.001$, significant difference compared with normal saline–treated rats. #$P<0.05$; ##$P<0.01$, significant difference compared with LPS-treated rats.

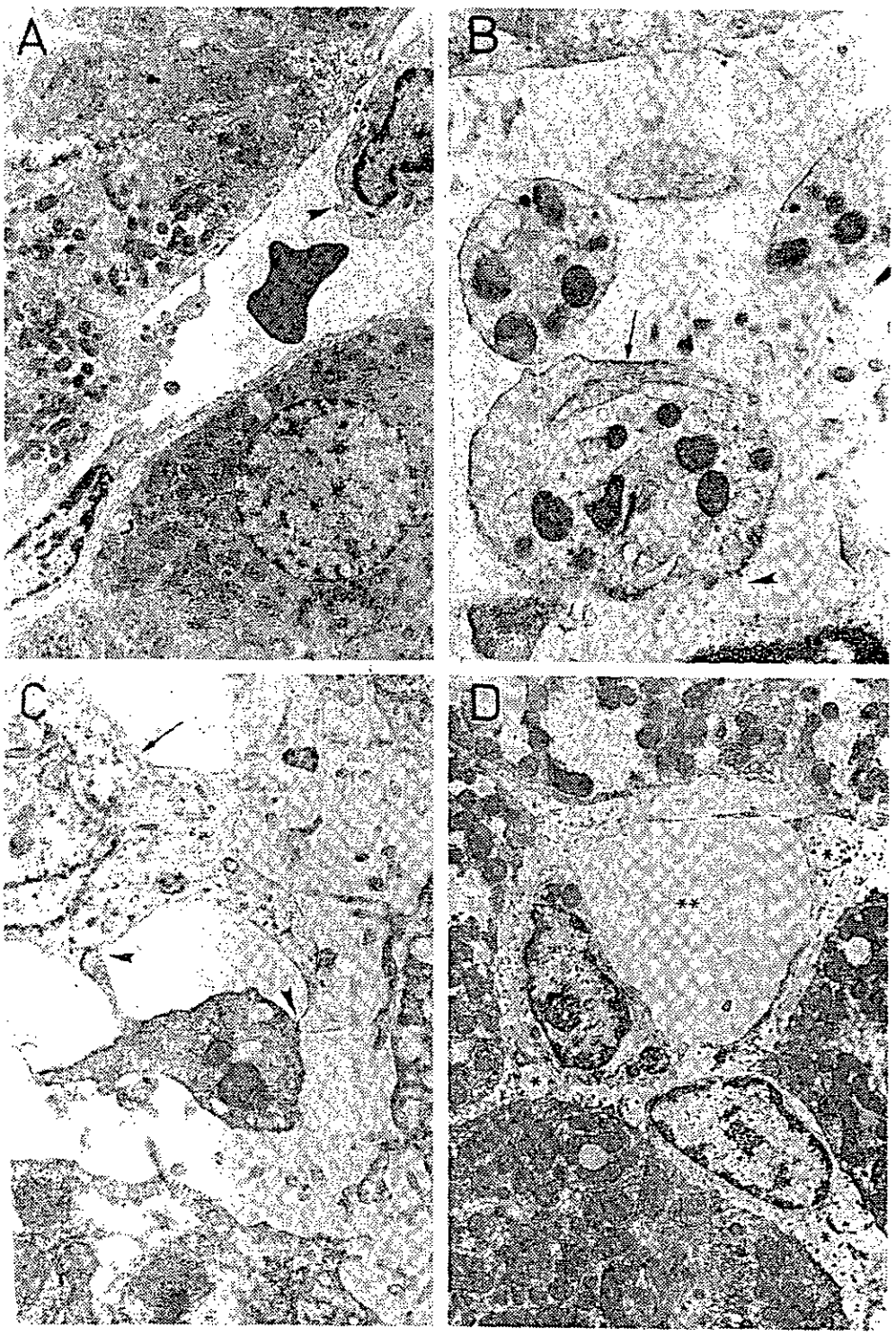

Figure 3. Transmission electron micrographs of liver sections in normal saline-treated (A), LPS-treated (4 mg/kg IV bolus) (B and C), and pretreatment with triflavin (500 μg/kg) followed by addition of LPS (D) in rats. A, Kupffer cell (arrowhead) is seen in the hepatic sinusoidal spaces of normal liver (magnification ×3750). B, Undegranulation of platelets (arrow) interacts with the cell processes of an endothelial cell (arrowhead) in the Disse spaces (magnification ×20 250). C Platelet (arrowhead) is localized in a Disse space and interacts with the cell processes of a Kupffer cell (arrow) (magnification ×20 800). D, No platelets are seen in either the sinusoidal spaces (**) or Disse spaces (*) (magnification ×5250).

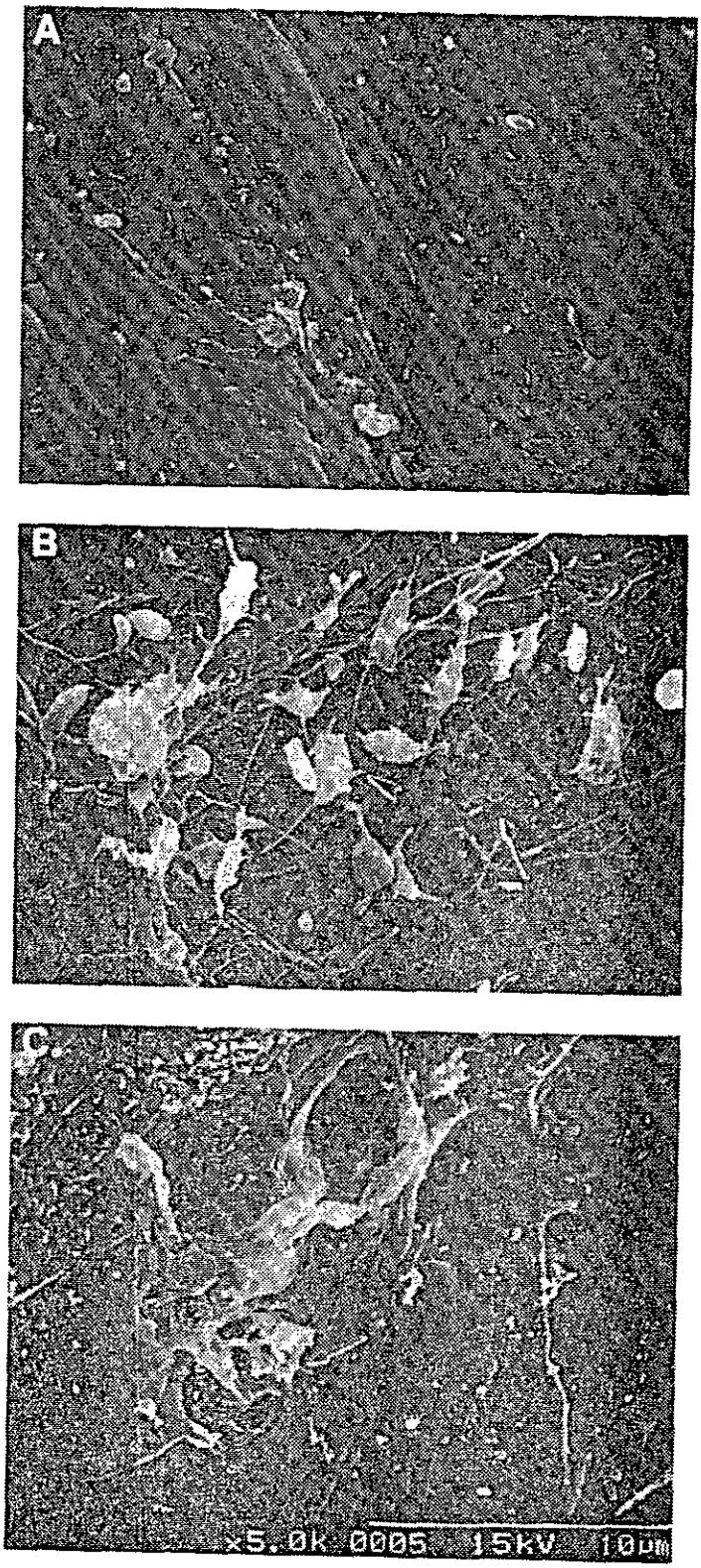

Figure 4. Effect of triflavin on scanning electron micrograph of endothelium in normal saline–treated and LPS-treated rat thoracic aorta. Rats received (A) normal saline (control), (B) LPS (4 mg/kg IV bolus) alone, or (C) pretreatment with triflavin (500 µg/kg) followed by addition of LPS. Arrow indicates damaged endothelium; arrowhead indicates aggregating platelets. All photographs are in the same field and are a representative example of 5 similar experiments.