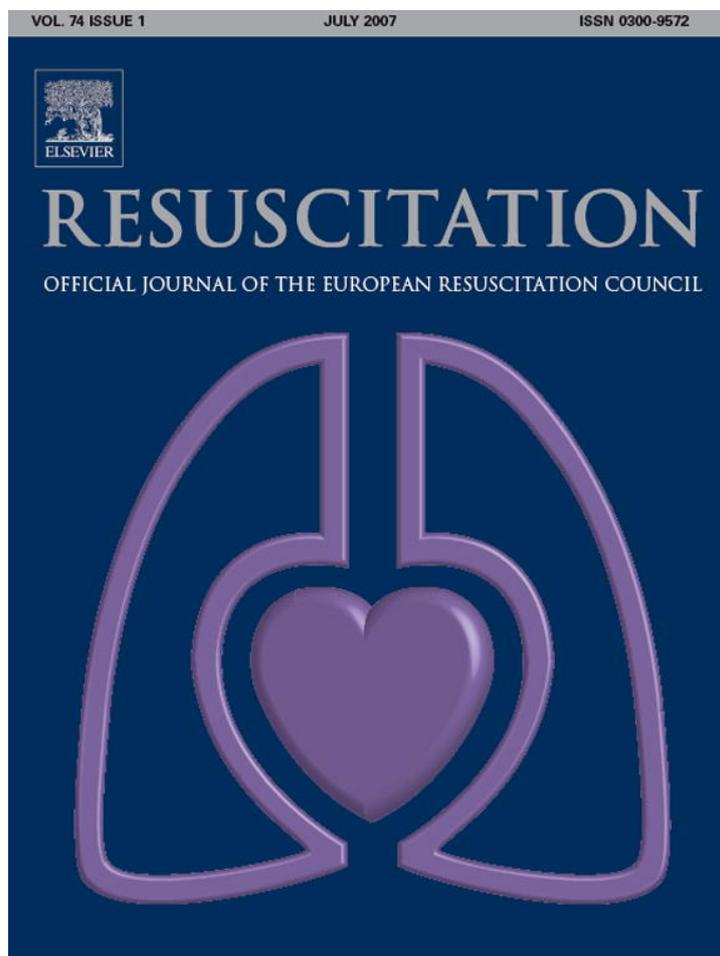


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EXPERIMENTAL PAPER

Fluvastatin ameliorates endotoxin induced multiple organ failure in conscious rats[☆]

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KEYWORDS

Septic shock;
Fluvastatin;
Hypotension;
Nitric oxide

Summary

Objectives: Sepsis is a severe inflammatory disorder that may lead to multiple organ failure. Lipopolysaccharide (LPS) is associated with Gram-negative sepsis and can activate monocytes and macrophages to release pro-inflammatory mediators such as tumor necrosis factor- α (TNF- α), nitric oxide (NO) and anti-inflammatory mediator such as interleukin-10 (IL-10). In this present study, we used fluvastatin, a HMG-CoA reductase inhibitor, to study its effects upon LPS-induced endotoxic shock in conscious rats.

Methods: The experiments were designed that rats received an intravenous injection of 1 mg/kg fluvastatin followed 10 min later, by an intravenous injection of 10 mg/kg *Klebsiella pneumoniae* LPS, the latter inducing endotoxic shock amongst conscious rats. Subsequently, the levels of certain biochemical variables and cytokines in serum were then measured during the ensuing 48-h period following sepsis. These included total cholesterol (TCH), triglyceride (TG), blood urea nitrogen (BUN), creatinine (Cre), creatine phosphokinase (CPK), lactic dehydrogenase (LDH), aspartate transferase (GOT), alanine transferase (GPT), tumor necrosis factor- α , interleukin-10 and nitric oxide.

Results: LPS significantly increased blood TG, BUN, Cre, LDH, CPK, GOT, GPT, TNF- α , IL-10 and NO levels but decreased the blood TCH level. Pretreatment of test

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rats with fluvastatin decreased blood levels of certain markers of organ injury, suppressed the release of TNF- α and increased IL-10, and NO levels following LPS treatment. Fluvastatin did not affect the blood TCH and TG level subsequent to the development of sepsis.

Conclusions: Pre-treatment with fluvastatin suppresses the release of plasma TNF- α , increases plasma IL-10, and NO production, and decreases the levels of markers of organ injury associated with endotoxin shock, so ameliorating LPS-induced organ damage amongst conscious rats.

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Introduction

Sepsis is often a major clinical problem associated with a high level of mortality.¹ Lipopolysaccharides (LPS) from the outer membrane of Gram-negative bacteria are strongly associated with septic shock.² LPS activates the inflammatory cells and increases the production of pro-inflammatory cytokines, including tumor necrosis factor- α (TNF- α), and nitric oxide (NO) which, in turn, leads to organ damage.³ Interleukin-10 (IL-10) is a pleiotropic cytokine produced by both T cells and macrophages and possesses both anti-inflammatory and immunosuppressive properties which may become active following sepsis.⁴ The relative severity of sepsis is related to the effective balance between pro-inflammatory and anti-inflammatory states after sepsis.⁵

HMG-CoA reductase inhibitors (statins) have been shown to exhibit important immunomodulatory effects independently of any lipid-lowering effect that it may elicit.⁶ These pleiotropic effects of statins have been demonstrated to include anti-inflammatory actions, improvement of endothelial and microvascular function, and modulation of endothelial nitric oxide synthase (eNOS).^{7,8} To the best of our knowledge, the relevant literature does not appear to contain any study focusing on the effects of fluvastatin upon LPS-induced shock and TNF- α , NO and/or IL-10 production following LPS administration to conscious rats. In the present study, we used fluvastatin to investigate its effects on the LPS-induced organ damage (kidney, heart and liver) and the relationship between pro-inflammatory cytokines (TNF- α , NO), and anti-inflammatory cytokine (IL-10) in cases of endotoxin shock in conscious rats.

Materials and methods

Preparation of animals

Thirty-two male Wistar-Kyoto rats weighing 280–300 g were purchased from the National

Animal Center. Rats were housed in our animal centre under a controlled environment at a temperature of $22 \pm 1^\circ\text{C}$ with a 12-h light:12-h dark cycle. Food and water were provided ad libitum. The experimental protocol was approved by the Animal Usage Regulation Committee of Tzu Chi Hospital. The animals were anaesthetised by ether inhalation for about 10 min. During the period of anaesthesia, a femoral artery was cannulated for blood samples. A femoral vein was catheterised for the intravenous administration of drugs. The operation was completed within 15 min, and the section wound was as small as possible (less than 0.5cm^2). After the operation, the animal was placed in a conscious rat metabolic cage (Shing-shieying Instruments, Hualien, Taiwan). The rat awakened soon after the operation as described previously.⁹

Endotoxin shock

Endotoxin shock was induced by a slow intravenous infusion of 10 mg/kg of *Klebsiella pneumoniae* LPS (Sigma Chemical, St. Louis, MO, USA) in 1 ml normal saline over 20 min. The infusion began 12 h after the cannulation procedure. The agent was dissolved in sterile saline immediately before use. All invasive procedures were operated under aseptic conditions. After endotoxin administration, the animals were observed continuously for 48 h.

Experimental design

Animals were randomly divided into four groups. In the Vehicle group ($n=8$), rats were given 1 ml an intravenous infusion of vitamin K over 10 min and then an intravenous infusion of 1 ml normal saline over 20 min. Rats in the LPS group ($n=8$) were pretreated with intravenous 1 ml vitamin K over 10 min, and then received intravenous LPS. In the Fluvastatin + LPS group ($n=8$), rats received intravenous 1 mg/kg fluvastatin (Novartis Pharmaceuticals, Cambridge, MA, USA) in 1 ml vitamin K over 10 min and then received intravenous LPS. In the Fluvastatin group ($n=8$), rats received intra-

venous 1 mg/kg fluvastatin in 1 ml vitamin K over 10 min and then received an intravenous infusion of 1 ml normal saline over 20 min. Rats were sacrificed by decapitation 48 h after LPS administration.

Blood sample analyses

Blood samples (0.5 ml) for measurements of total cholesterol (TCH), triglyceride (TG), blood urea nitrogen (BUN), creatinine (Cre), creatine phosphokinase (CPK), lactic dehydrogenase (LDH), aspartate transferase (GOT), alanine transferase (GPT), tumor necrosis factor- α , interleukin-10 and nitric oxide were taken at 60 min before LPS and 1, 3, 6, 9, 12, 18, 24, 48 h after LPS administration. Blood samples were immediately centrifuged at $3000 \times g$ for 10 min. The plasma was decanted and separated in two parts; one part of plasma was stored at $4^{\circ}C$ for biochemical examinations within 1 h after collection. Plasma levels of TCH, TG, BUN, Cre, CPK, LDH, GOT and GPT were measured with an autoanalyser (Vitros 750, Johnson & Johnson, NY, USA) for evaluating various biochemical data. Another part was stored at $-80^{\circ}C$ for later analysis of TNF- α , IL-10 and NO concentration.

TNF- α , IL-10 and NO measured by ELISA

TNF- α , IL-10 and NO concentrations in blood samples were measured separately by antibody enzyme-linked immunosorbent assay (ELISA) with the commercial antibody pair, the recombinant standard and the biotin-streptavidin-peroxidase detection system (Endogen, Rockford, IL, USA) as described previously.⁹ Blood samples were collected in serum-separated tubes. All reagents, samples and working standards were brought to room temperature and prepared according to the manufacturer's directions. Quantification of the reactions were determined by the optical density using an automated ELISA reader (Sunrise, Tecan Co., Grödingen, Austria) at 450/540 nm wavelengths.

Histological examination

Kidney, heart and liver were removed immediately after sacrifice. These tissue specimens were fixed overnight in 4% buffered formaldehyde, processed by standard methods, and stained for haematoxylin and eosin (H and E). One observer performed the analysis of tissues in a blind fashion. Renal tubular injury was scored by estimating the percentage of tubules in the cortex or the outer

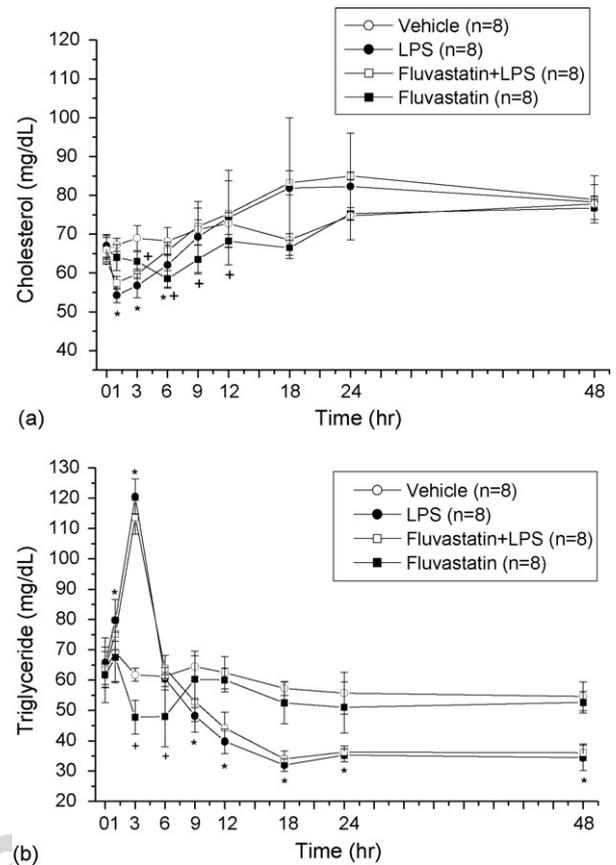


Figure 1 Change in serum total cholesterol (TCH) (a) and triglyceride (TG) (b) after endotoxic shock in conscious rats. * $p < 0.05$ for the LPS group compared with the Vehicle group. † $p < 0.05$ for the Fluvastatin group compared with the Vehicle group.

medulla that showed epithelial necrosis or had luminal necrotic debris and tubular dilation, as follows: 0, none; 1, <5%; 2, 5–25%; 3, 25–75% and 4, >75%.¹⁰ Heart injury was scored by estimating the percentage of myocardial lesions consisting of oedema, myofibrillar degeneration, myofibrillar lysis and hypercontraction band formation. Lesions were graded on a scale of 0–4 as follows: 0, no change; 1, minimal; 2, mild; 3, moderate and 4, marked, according to the degree and extent of the changes described above.¹¹ The severity of liver injury in the sections was evaluated as follows: 0, minimal or no evidence of injury; 1, mild injury consisting of cytoplasmic vacuolation and focal nuclear pyknosis; 2, moderate to severe injury with extensive nuclear pyknosis, cytoplasmic hypereosinophilia, and loss of intercellular borders and 3, severe necrosis with disintegration of hepatic cords, hemorrhage and neutrophil infiltration.¹² All evaluations were made on five fields per section and five sections of kidney, heart and liver.

Statistical analysis

Data were expressed as means \pm S.E.M. The significance of differences in the measured values between groups was analyzed using a two-way analysis of variance (ANOVA) for repeated measurements followed by a Fisher's protected *t*-test. The significance in the measured values within groups was analyzed with one-way ANOVA for repeated measurements followed by a Fisher's protected *t*-test. A *p*-value less than 0.05 was considered statistically significant.

Results

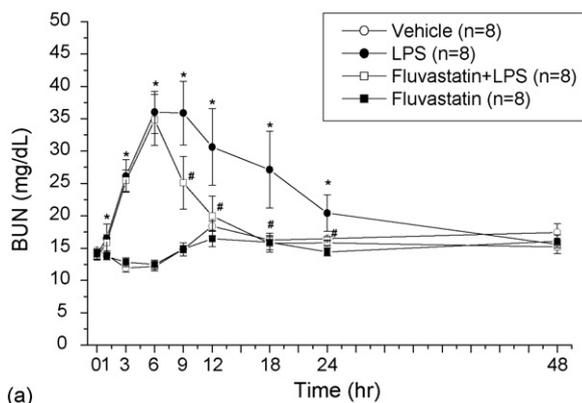
Effects of fluvastatin on serum total cholesterol and triglyceride

The values of serum TCH decreased at 1, 3 and 6 h after LPS injection compared with Vehicle group

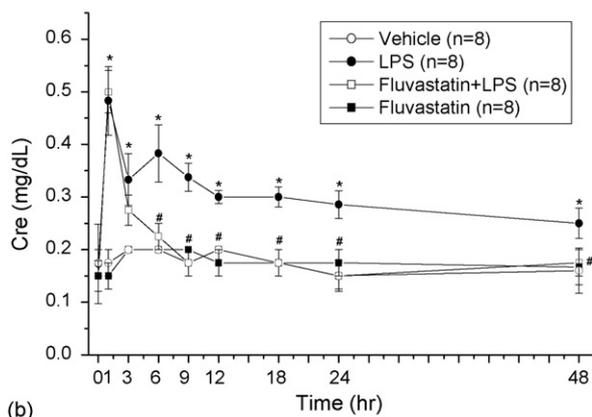
(Figure 1a). Fluvastatin alone decreased serum TCH at 3, 6, 9 and 12 h compared with the Vehicle group (Figure 1a). Compared with the LPS group, pre-treatment with fluvastatin did not affect serum TCH after endotoxin. Serum TG increased at 1 and 3 h and then decreased at 9, 12, 18, 24 and 48 h after LPS injection compared with Vehicle group (Figure 1b). Fluvastatin alone decreased serum TG at 3 and 6 h compared with the Vehicle group (Figure 1b). Compared with the LPS group, pre-treatment with fluvastatin did not affect serum TG after LPS injection.

Effects of fluvastatin on serum blood urea nitrogen and creatinine

LPS increased blood BUN to a peak at 9 h (Figure 2a). Thereafter, it declined at 12 h and returned to a level close to that in the Vehicle group at 48 h. Fluvastatin significantly affected the blood BUN at 9,

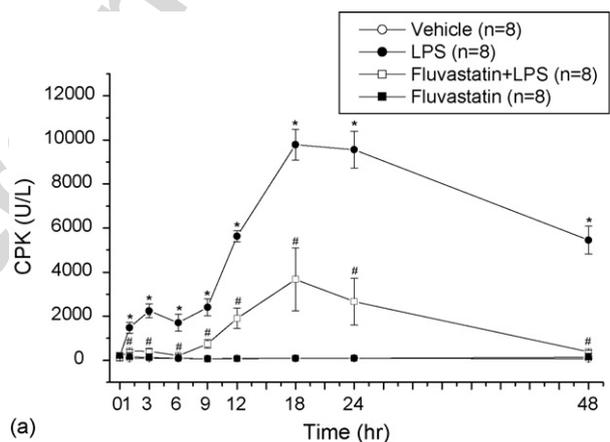


(a)

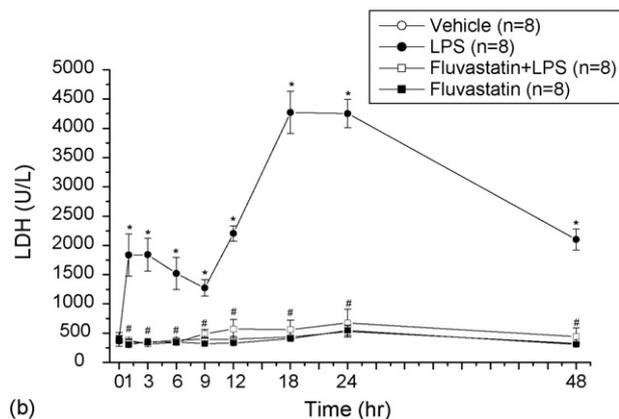


(b)

Figure 2 Change in serum blood urea nitrogen (BUN) (a) and creatinine (Cre) (b) after endotoxic shock in conscious rats. **p* < 0.05 for the LPS group compared with the Vehicle group. #*p* < 0.05 for the Fluvastatin + LPS group compared with LPS group.



(a)



(b)

Figure 3 Change in serum creatine phosphokinase (CPK) (a) and lactic dehydrogenase (LDH) (b) after endotoxic shock in conscious rats. **p* < 0.05 for the LPS group compared with the Vehicle group. #*p* < 0.05 for the Fluvastatin + LPS group compared with LPS group.

12, 18 and 24h after LPS (Figure 2a). The blood Cre increased after LPS administration at 1, 3, 6, 9, 12, 18, 24 and 48 h (Figure 2b). The peak value of blood Cre occurred at 1 h after giving LPS. Compared with the LPS group, fluvastatin decreased the blood Cre at 6, 9, 12, 18, 24 and 48h after LPS (Figure 2b).

Effects of fluvastatin on serum creatine phosphokinase and lactic dehydrogenase

The values of blood CPK reached to a peak at 18h after LPS injection (Figure 3a) and continued to increase above the values in the Vehicle group. Compared with the LPS group, pre-treatment with fluvastatin decreased the blood CPK at 1, 3, 6, 9, 12, 18, 24 and 48 h (Figure 3a). The values of blood LDH reached to a peak at 18h after LPS administration (Figure 3b). Compared with the LPS group, fluvastatin decreased the

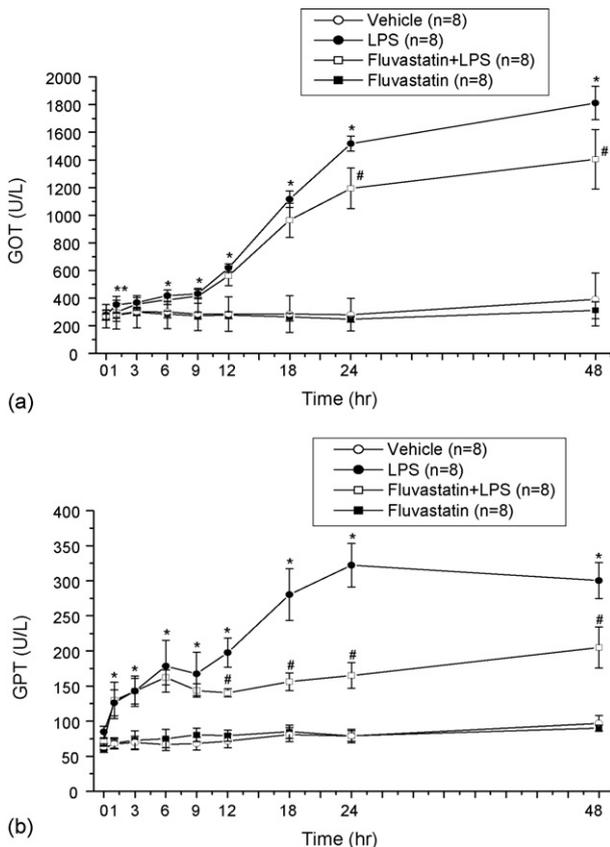


Figure 4 Change in serum aspartate transferase (GOT) (a) and alanine transferase (GPT) (b) after endotoxic shock in conscious rats. **p* < 0.05 for the LPS group compared with the Vehicle group. #*p* < 0.05 for the Fluvastatin + LPS group compared with LPS group.

blood LDH at 1, 3, 6, 9, 12, 18, 24 and 48h (Figure 3b).

Effects of fluvastatin on serum aspartate transferase and alanine transferase

GOT increased to a peak at 48h after LPS (Figure 4a). Compared with the LPS group, pre-treatment with fluvastatin decreased the plasma GOT at 24 and 48 h (Figure 4a). Blood GPT reached a peak at 24h and remained high throughout the whole time course after LPS (Figure 4b). Compared with the LPS group, fluvastatin decreased the blood GPT at 12, 18, 24 and 48 h (Figure 4b).

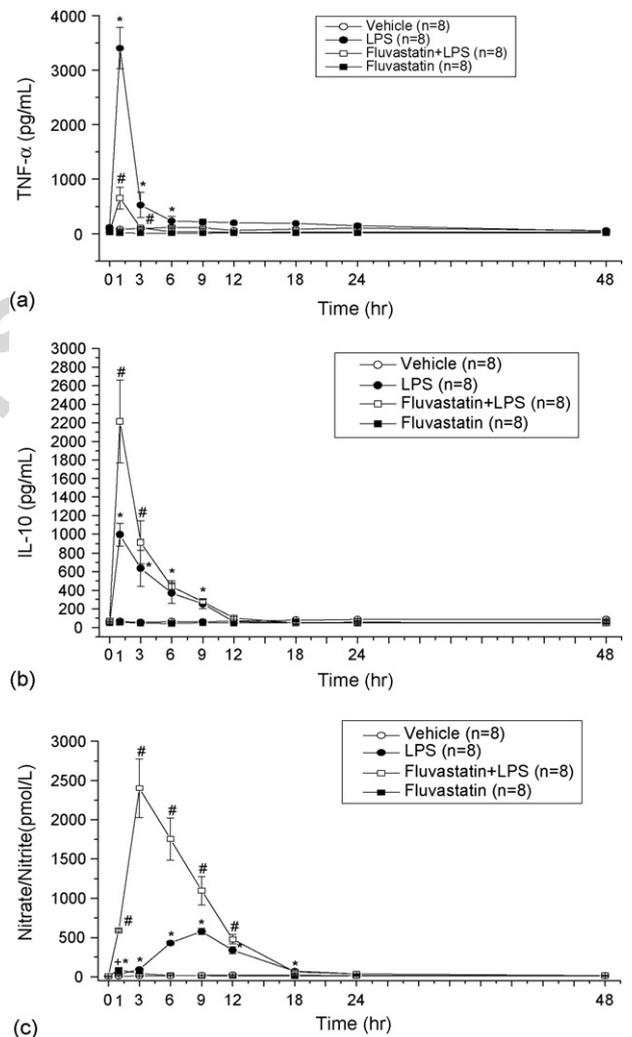


Figure 5 Change in plasma tumor necrosis factor-α (TNF-α) (a), interleukin-10 (IL-10) (b) and nitric oxide (NO) (c) after endotoxic shock in conscious rats. **p* < 0.05 for the LPS group compared with the Vehicle group. #*p* < 0.05 for the Fluvastatin + LPS group compared with LPS group.

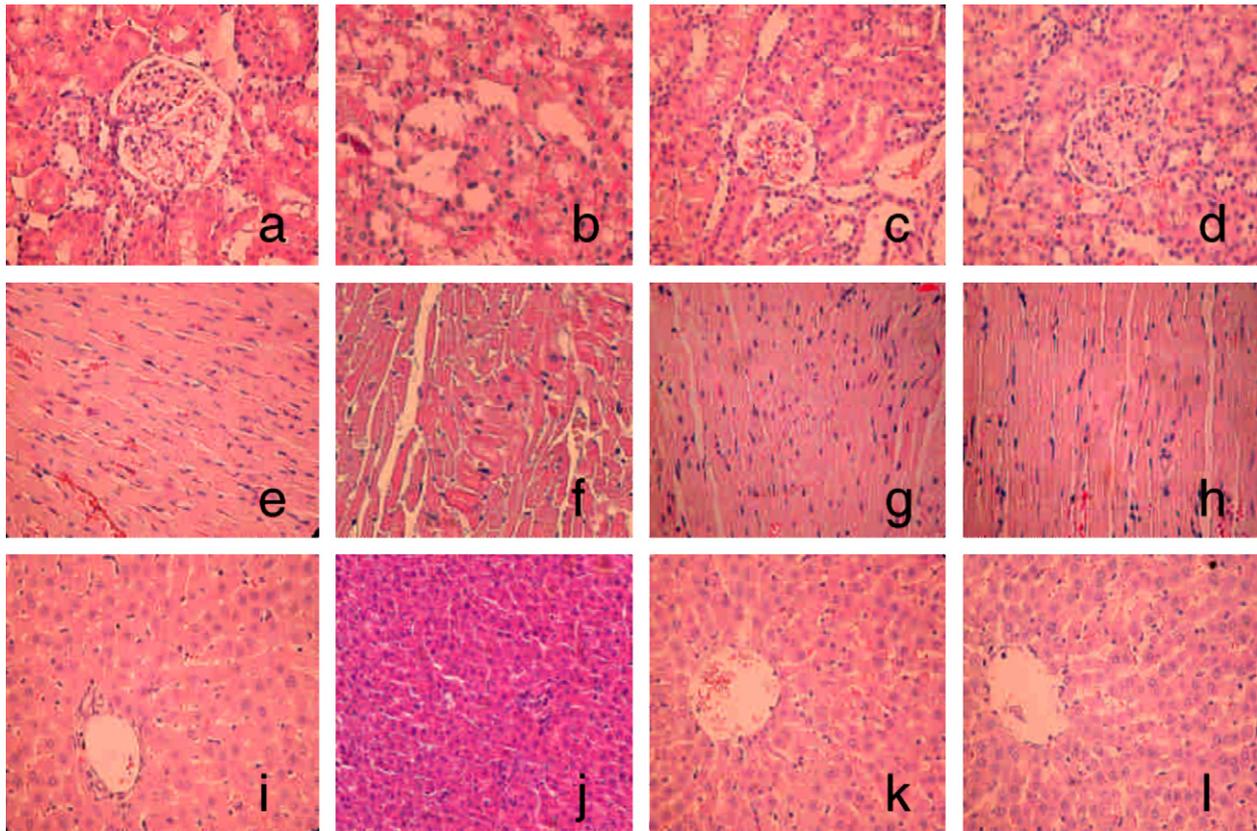


Figure 6 Pre-treatment with fluvastatin affects the histopathologic changes in the kidney, heart and liver. Histologic sections from Vehicle group (a, e, i), LPS group (b, f, j), Fluvastatin group (c, g, k) and Fluvastatin+LPS group (d, h, l), stained with haematoxylin and eosin (magnification $\times 400$). The photomicrography a–d are kidney sections; e–h are heart sections and i–l are liver sections.

Effects of fluvastatin on plasma levels of tumor necrosis factor- α , interleukin-10 and nitric oxide

LPS greatly elevated the plasma TNF- α at 1 h (Figure 5a). Then, the level returned to the pre-LPS value at 9 h. Pre-treatment with fluvastatin significantly decreased the plasma TNF- α after LPS at 1 and 3 h (Figure 5a). LPS increased the plasma IL-10 at 1, 3, 6 and 9 h (Figure 5b). The value returned to the pre-LPS value at 12 h. Fluvastatin increased the plasma IL-10 at 1 and 3 h (Figure 5b). LPS significantly increased the plasma NO at 1 h and reached a peak at 9 h (Figure 5c). Then, the level returned to the pre-LPS value at 24 h. Pre-treatment of fluvastatin significantly increased the plasma NO after LPS at 3, 6, 9 and 12 h (Figure 5c).

Effects of fluvastatin on histopathology of kidney, heart and liver

Histopathologic analysis of the H and E-stained tissue sections from various groups revealed mild

leukocyte infiltration, swelling of the tubular cells, nuclear and brush border loss in the kidney (Figure 6b), leukocyte infiltration with myofibrillar lysis in the heart (Figure 6f), and leukocyte infiltration with local abscess in the liver in LPS group (Figure 6j). Compared with the Vehicle group, fluvastatin alone did not produce significantly histopathological changes in the kidney, heart and liver. Pre-treatment of fluvastatin significantly abolished the histopathological changes in the kidney, heart and liver after LPS (Figure 6d–l). The injury score of kidney, heart and liver was significantly decreased by Fluvastatin+LPS group compared with LPS group ($\#p < 0.05$; Figure 7a–c).

Discussion

The important finding of this study was that pre-treatment of test rats with fluvastatin ameliorates LPS-induced organ damage (kidney, heart and liver) accompanied by decreasing plasma TNF- α and increasing plasma NO and IL-10

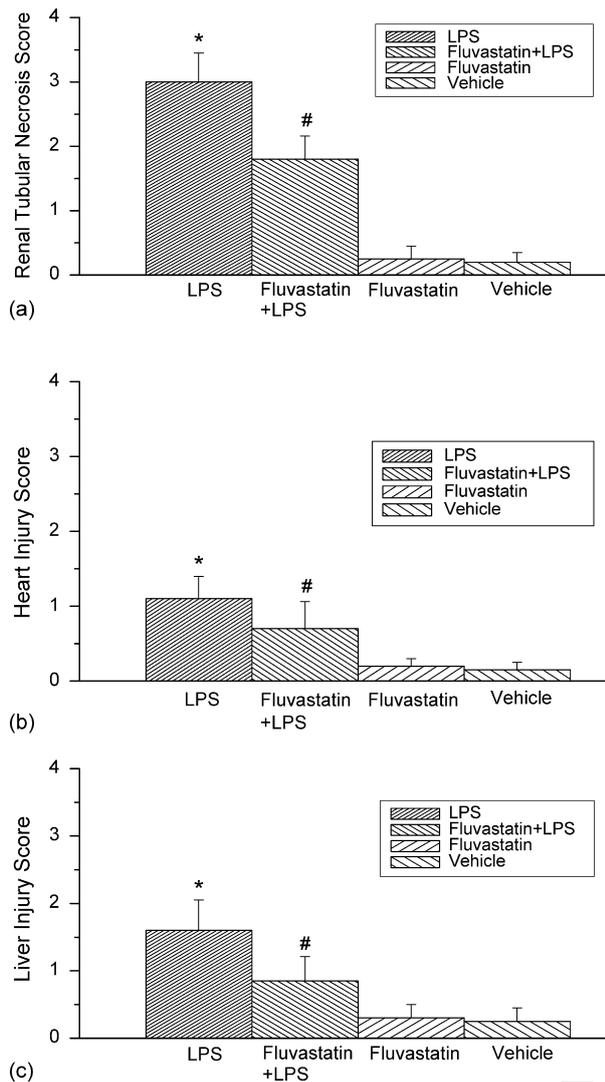


Figure 7 Histopathologic injury score in kidney (a), heart (b) and liver (c) after endotoxic shock in conscious rats. * $p < 0.05$ for the LPS group compared with the Vehicle group. # $p < 0.05$ for the Fluvastatin +LPS group compared with LPS group.

levels subsequent to sepsis amongst conscious rats.

Hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) have often been reported to be potent cholesterol-lowering drugs.⁸ In addition to their cholesterol-lowering properties, statins also are able to exert a pleiotropic effects that includes improvement of endothelial function, increased NO bioavailability, provision of antioxidant properties, stabilization of atherosclerotic plaques, regulation of progenitor cells, inhibition of inflammatory responses and enhancement of certain immunomodulatory actions.¹³ Prophylactic therapies for potential sepsis sufferers with certain statins may reduce the rate of development of

sepsis amongst humans subsequent to certain bacterial infection.^{14,15} Cerivastatin has been reported to improve survival of mice with LPS-induced sepsis, and simvastatin has been reported to improve survival and reduce acute renal injury following caecal ligation and puncture (CLP)-induced sepsis amongst test mice.^{16,17} Post-treatment of test mice with fluvastatin also appears to prolong survival following CLP-induced sepsis in mice.¹⁸ Our results also show that treatment of conscious rats with fluvastatin reduced the LPS-induced the level of plasma GOT, GPT, BUN, Cre, CPK and LDH elevation independent of any lipid-lowering effects and this statin also alleviated most of the histopathological changes arising in the kidney, heart and liver following LPS administration to conscious rats and the development of sepsis.

In response to endotoxemia, the organism provokes release of TNF- α and NO into surrounding tissues, thereby causing tissue damage and organ failure.³ Further, fluvastatin attenuates the leukocyte-to-endothelial cell adhesion response to the presence of platelet-activating factor and leukotriene B4 in hypercholesterolemic rats.¹⁹ Fluvastatin also reduces macrophage accumulation within carotid lesions in rabbits and reduces the level of interleukin-6 in vitro, within human vascular smooth muscle cells.^{20,21} In our study, fluvastatin effectively reduced LPS induced plasma TNF- α elevation effectively. These benefits of fluvastatin are possibly due to an anti-inflammatory effect as is the case for a number of other statins.^{16,17}

Following the development of sepsis, the host attempts to counterbalance the exacerbated pro-inflammatory response by increasing the production of anti-inflammatory cytokines such as IL-10.³ Administration of IL-10 prior to the development of sepsis by test individuals may have a beneficial effect upon the overall outcome of sepsis by reducing the normal exaggerated pro-inflammatory cytokine production following sepsis.⁴ Our results reveal that fluvastatin increases plasma IL-10 production when given after the development of sepsis in conscious rats and may feature therapeutic benefits in the treatment of LPS-induced shock.

Sepsis leads to endothelial dysfunction, which, in part, may be related to reduced eNOS expression and increased inducible nitric oxide synthase (iNOS) expression.²² Further, inhibition of eNOS activity by non-selective NOS inhibitors can exacerbate endothelial dysfunction and further impair microvascular homeostasis, a situation which may be reversed by the presence of selective iNOS inhibitors.²³ Statins have been demonstrated to restore eNOS production by upregulating eNOS

protein expression and blocking the formation of reactive oxygen species.²⁴ Fluvastatin has been shown previously to inhibit iNOS gene expression within macrophages *in vitro*.²⁵ Our results reveal that fluvastatin increases plasma NO production after the development of sepsis in conscious rats, and the effects may be due to its ability to contribute to the restoration of eNOS production. These results appear to differ from those of certain other studies in which cerivastatin and simvastatin were shown to inhibit NO production following sepsis.^{16,26} The effects of NO upon the outcome of sepsis may be dependent on the actual experimental conditions such as the drug, dose, timing, methods and individual's conscious state. Clearly further investigations relating to the possible interaction between fluvastatin and eNOS after the development of sepsis are required.

A number of earlier clinical studies have addressed the effects of statins on the primary prevention of atherosclerosis and ischaemic heart disease.^{27–29} Therapy with statins before the development of sepsis reduced the mortality rate amongst human sepsis sufferers, such therapy having been demonstrated to play a role in the primary prevention of sepsis.^{14,15} Our study has revealed that pre-treatment of conscious rats with fluvastatin suppresses the release of a variety of inflammation cytokines and increases anti-inflammatory cytokine levels following the development of sepsis for test rats. Further, from our preliminary results, it would appear that a number of large clinical randomised trials are needed to investigate whether pre-sepsis treatment with fluvastatin is able to elicit primary (sepsis) prevention for patients who would otherwise be likely to suffer from sepsis.

Conclusions

In this study, pre-treatment with fluvastatin suppresses the release of plasma TNF- α and increases plasma NO and IL-10 in endotoxin shock. These beneficial effects protect the kidney, heart and liver from the LPS-induced damage in conscious rats.

Conflict of interest

None.

Acknowledgements

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Author's personal