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Beneficial Effects of Enalapril on Chlorhexidine Digluconate-Induced Liver Peritoneal Fibrosis in Rats

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Abstract

Peritoneal fibrosis (PF) is a recognized complication of long-term peritoneal dialysis (PD) and can lead to ultrafiltration failure. The present study was designed to investigate the protective effects of enalapril on chlorhexidine digluconate-induced liver PF by decreasing transforming growth factor-β1 (TGF-β1) production in rats. PF was induced in Sprague-Dawley rats by daily administration of 0.5 ml 0.1% chlorhexidine digluconate in normal saline *via* PD tube for one week. Rats received daily intravenous injections of low dose enalapril (1 mg/kg), or high dose enalapril (2.5 mg/kg), for one week. After 7 days, conventional 4.25% Dianeal (30 ml) was administered *via* a PD catheter with a dwell time of 4 h and assessment of peritoneal function. At the end of dialysis, the rats were sacrificed and liver peritoneum was harvested for microscopic examination and immunohistochemistry. There was no significant difference in mean arterial pressure and heart rate between groups. After 4 h of PD, the $D_4/P_{4\ urea}$ level was reduced, the D_4/D_0 glucose level, serum and the dialysate TGF-β1 level was increased, the liver peritoneum was markedly thicker, and the expression of TGF-β1, alpha-smooth muscle actin (α-SMA), fibronectin, collagen and vascular endothelial growth factor (VEGF) were elevated in the PF group compared with the vehicle group. High dose of enalapril decreased the serum and dialysate TGF-β1 levels, decreased the thickness of the liver peritoneum, and decreased the expression of TGF-β1, α-SMA, fibronectin, collagen and VEGF-positive cells in the liver peritoneum. Low dose of enalapril did not protect against chlorhexidine digluconate-induced PF in the rat. Enalapril protected against chlorhexidine digluconate-induced PF in rats by decreasing TGF-β1 production.

Key Words: chlorhexidine digluconate, peritoneal fibrosis, enalapril

Introduction

Peritoneal fibrosis (PF) is invariably observed in patients undergoing long-term peritoneal dialysis (PD). The condition is thought to occur in response to a variety of insults including bioincompatible dialysates, peritonitis, uremia and chronic inflammation (14, 26, 28). Activation of the renin–angiotensin system may have an important role in PF (14, 26). Local angiotensin II generating systems are also noted in human peritoneal mesothelial cells (15). A variety of local and systemic stimuli induce reactivation of the local peritoneal angiotensin II, which in turn initiates the production of transforming growth factor-β1 (TGF-β1) and contributes to extracellular matrix (ECM) accumulation and induces PF (14, 26).

The renin-angiotensin hormonal cascade begins with the biosynthesis of renin, normally secreted in

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response to underperfusion of the kidney, cleaves the decapeptide angiotensin I from angiotensinogen, and angiotensin I is converted to angiotensin II by the angiotensin-converting enzyme (2). Inhibition of the angiotensin-converting enzyme blocks the conversion of angiotensin I to angiotensin II (17). Enalapril is an antihypertensive drug of the class of angiotensin-converting enzyme inhibitors (ACEI). Studies noted administration of enalapril decreased serum TGF-β1 in diabetic rats (7), decreased glomerular TGF-β1 mRNA and protein expression in anti-Thy1 model of glomerulonephritis (16, 27), decreased renal TGF-β1 mRNA and protein expression in 5/6 nephrectomy rats (13), and reduced liver fibrogenesis induced by bile-duct ligation by decreasing liver tissue TGF-β1 in rats (25). Enalapril may be beneficial to decrease TGF-β1 production in PF. In this study, we evaluated the effects of enalapril on chlorhexidine digluconate-induced liver PF in rats.

Materials and Methods

Preparation of Animals and Peritoneal Tube Insertion

Thirty-two male Sprague-Dawley rats weighing 280-300 grams were purchased from the National Animal Center (Taipei, Taiwan) and housed in the university Animal Center in a controlled environment at 22 ± 1°C with a 12 h light/dark cycle. Food and water were given *ad libitum*. The experimental protocol was approved by the Animal Care and Use Committee of Tzu Chi University. Rats were anesthetized with ether inhalation for about 30 min. During the period of anesthesia, fur over the abdominal wall and right inguinal area were closely shaved. A polyethylene catheter (PE-240; PD catheter), length about 30 cm, was inserted about 4 cm into the peritoneal cavity through a midline incision below the xiphoid process. The catheter was then tunneled subcutaneously to the right inguinal area. A polyethylene catheter (PE-50) was inserted into the right femoral artery to collect blood samples and was connected to a pressure transducer (Gould Instruments, Cleveland, OH, USA) to record arterial pressure (AP) and heart rate (HR) on a polygraph recorder (Power Lab, AD Instruments, Mountain View, CA, USA). Another PE-50 catheter was inserted into the femoral vein for intravenous administration of drugs. All procedures were performed under sterile conditions. Sterilized stainless steel covers were used to cover the PD catheters, femoral artery PE catheters and femoral vein PE catheters to prevent the rats from biting and dislocating the catheters. After the operation, animals were placed in a conscious rat metabolic cage (Mike Biological Technologies, Hualien, Taiwan). Rats awakened soon after the operation and peritoneal sclerosis were induced 24 h later with the rats in a conscious state (10).

Peritoneal Fibrosis

Peritoneal fibrosis (PF) was induced by daily administration of 0.5 ml 0.1% chlorhexidine digluconate (Sigma-Aldrich, St. Louis, MO, USA) in normal saline solution *via* a PD catheter and later 1 ml of normal saline was administrated after chlorhexidine digluconate injection *via* PD catheter (10).

Experimental Design

Rats were randomly divided into four groups. The vehicle group (n = 8) received 0.5 ml normal saline and later 1 ml of normal saline daily *via* a PD catheter and 0.5 ml daily intravenously *via* the femoral vein for one week. The PF group (n = 8) received 0.5 ml 0.1% chlorhexidine digluconate and later 1 ml of normal saline daily and intravenously 0.5 ml normal saline *via* the femoral vein daily for one week. The low dose enalapril group (n = 8) received 0.5 ml 0.1% chlorhexidine digluconate and later 1 ml of normal saline daily and intravenously 1 mg/kg enalapril (Merck Sharp & Dohme, Whitehouse Station, NJ, USA) in 0.5 ml normal saline *via* the femoral vein daily for one week. The high dose enalapril group (n = 8) received 0.5 ml 0.1% chlorhexidine digluconate and later 1 ml of normal saline daily and intravenously 2.5 mg/kg enalapril in 0.5 ml normal saline *via* the femoral vein daily for one week (4).

Peritoneal Dialysis

At the end of PF (7 days), the rats were given 30 ml of conventional 4.25% glucose-containing peritoneal fluid (Dianeal; Baxter Healthcare SA, Singapore Branch, Singapore) *via* PD catheter to the peritoneal cavity, with a dwell time of 4 h. After 4 h of PD, animals were sacrificed for pathological examination (10).

Blood and Dialysate Fluid Sample Analysis

Blood and dialysate fluid samples (0.5 ml) were collected for measurement of glucose at 0 and 4 h after initiation of PD. Within 1 h of collection, these samples were centrifuged at 3,000 *g* for 10 min prior to the subsequent biochemical analysis. The serum was decanted and separated into two parts; one part was stored at 4°C within an hour after collection for biochemical analysis. Serum and dialysate fluid levels of glucose and urea were measured with an autoanalyzer (COBAS Integra C111, Roche Diagnostics, Basel, Switzerland) to obtain various biochemical data (10). The other part of the serum collected at

4 h after PD was stored at -80°C for later measurement of TGF-β1 concentrations.

Serum and Dialysate TGF-β1 Measurement by ELISA

At 4 h of PD, TGF-β1 concentrations in the blood and dialysate samples were measured separately by antibody enzyme-linked immunosorbent assay (ELISA) with commercial antibody pairs, recombinant standards, and a biotin-streptavidin-peroxidase detection system (Assay Designs, Ann Arbor, MI, USA) as described previously (10). All reagents, samples and working standards were brought to room temperature and prepared according to the manufacturers' directions. Reactions were quantified by optical density using an automated ELISA reader (Sunrise, Tecan Co., Grödingen, Austria) at 450/540 nm wavelength.

Peritoneal Solutes Transport Analysis

Peritoneal solute transport was calculated from the dialysate concentration relative to its concentration in the initial infused dialysis solution (D_4/D_0 glucose) for glucose, and the dialysate-to-plasma concentration ratio ($D_4/P_{4\ urea}$) at 4 h for urea (10).

Histological Assessment

The liver peritoneum were fixed overnight in 4% buffered formaldehyde, processed by standard methods, and stained with hematoxylin and eosin (H and E). Liver peritoneal surfaces were evaluated by morphometry and immunohistochemistry. Thickening of the liver peritoneum was defined from the liver surface to the peritoneal cavity. Quantification of the liver peritoneum was performed on paraffin-embedded tissue sections and was counted digitally using a 200 × objective lens *via* a computer imaging analysis system (Image-Pro Plus 4.5, Media Cybernetics, Bethesda, MD, USA) as described previously (10, 20). Briefly, the thickness of the liver peritoneum was measured at 10 points in each rat.

Immunohistochemistry

For immunohistochemistry (IHC) of liver peritoneums, serial 4-μm sections were deparaffinized, rehydrated and incubated with different mouse monoclonal antibodies at 4°C overnight according to the manufacturer's directions. Antigen retrieval was used for transforming growth factor-beta 1 (TGF-β1), alpha-smooth muscle actin (α-SMA), fibronectin, type-I collagen (collagen) and vascular endothelial growth factor (VEGF). Dilutions were 1 in 100 for TGF-β1 (Abcam, Cambridge, MA, USA), fibronectin, collagen (Rockland, PA, USA), VEGF and α-SMA (Bio SB, Santa Barbara, CA, USA). After incubation, tissue sections were covered with biotinylated goat anti-mouse polyvalent secondary antibody and incubated at room temperature for 10 min. Sections were then washed and the slides were incubated in peroxidase conjugated streptavidin-biotin complex (Dako, Copenhagen, Denmark) for 10 min. The areas where there were positive cells for TGF-β1, α-SMA, fibronectin, collagen and VEGF were evaluated from a 0.01 mm^2 region (200× magnification field; 0.1 mm width × 0.1 mm length) of four fields, and were counted digitally *via* a computer imaging analysis system (Image-Pro Plus 4.5, Media Cybernetics, Bethesda, MD, USA). Results are reported as the percentage of positive cells/mm^2 of peritoneal tissue (10, 20). All scoring was performed in a blinded manner on coded slides.

Statistical Analysis

Data are expressed as means ± SDs. Statistical comparisons between groups were made by repeated measures of two-way ANOVA followed by a *post hoc* test (Bonferroni's method). Histological scores were analyzed by the Kruskal-Wallis test followed by the Mann-Whitney U test. $P < 0.05$ was considered statistically significant.

Results

Mean Arterial Pressure (MAP), Heart Rate (HR) and Peritoneal Function

There was no significant difference in MAP and HR between groups (Figs. 1A and 1B). The D_4/D_0 glucose level was significantly higher, and $D_4/P_{4\ urea}$ level was significantly lower in the PF group than in the vehicle group ($P < 0.05$; Fig. 2, A and B). The D_4/D_0 glucose level was significantly lower, and $D_4/P_{4\ urea}$ level was significantly higher in the high dose enalapril group than in the PF group ($P < 0.05$; Fig. 2, A and B). Moreover, The D_4/D_0 glucose level was significantly lower in the high dose enalapril group than in the low dose enalapril group ($P < 0.05$; Fig. 2A). The D_4/D_0 glucose level and $D_4/P_{4\ urea}$ level was no statistically significant in the low dose enalapril group compared with the PF group (Fig. 2, A and B).

Serum and Dialysate TGF-β1 Level

PF greatly elevated the serum and dialysate TGF-β1 levels compared with the vehicle group (Fig. 3A, and B). The serum and dialysate TGF-β1 levels were significantly lower for the high dose enalapril group compared to the PF group ($P < 0.05$; Fig. 3, A and B). Compared with the low dose enalapril

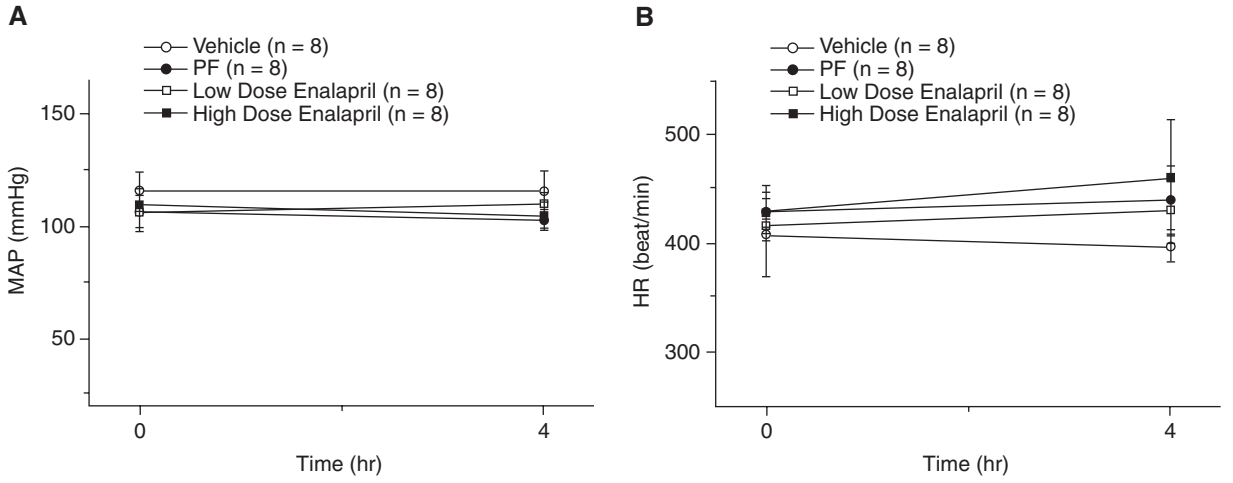

Fig. 1. Change in mean artery pressure (MAP) (A) and heart rate (HR) (B) during chlorhexidine digluconate-induced peritoneal fibrosis in rats. There was no significant difference between MAP and HR groups.

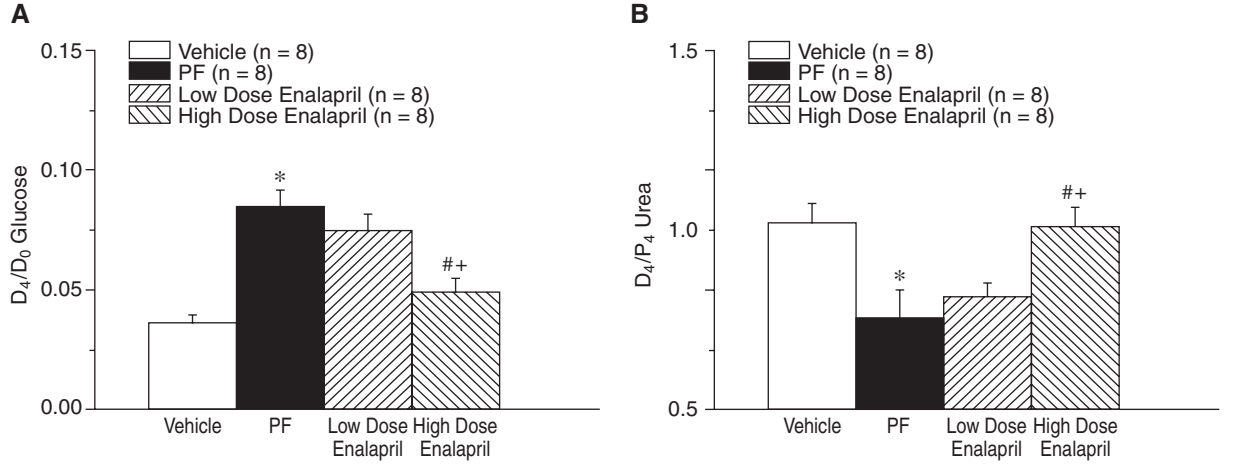

Fig. 2. The D_4/D_0 glucose level (A) and the $D_4/P_{4\ urea}$ level (B) after chlorhexidine digluconate-induced peritoneal fibrosis in rats. $^{*}P < 0.05$ for the PF group compared with the vehicle group. $^{\#}P < 0.05$ for the high dose enalapril group compared with the PF group. $^{+}P < 0.05$ for the high dose enalapril group compared with the low dose enalapril group.

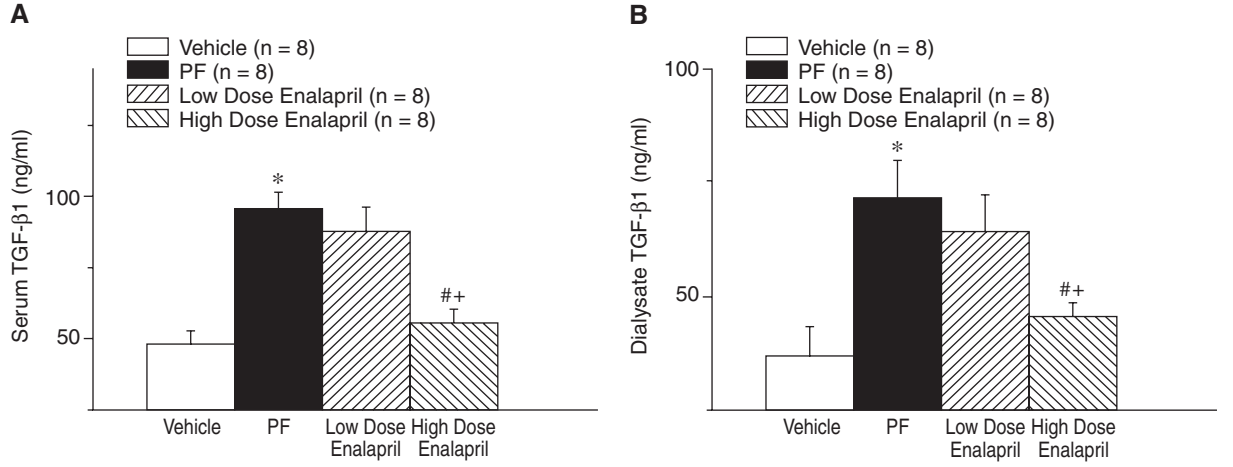

Fig. 3. Serum (A) and dialysate (B) transforming growth factor-beta 1 (TGF-β1) levels after chlorhexidine digluconate-induced peritoneal fibrosis in rats. $^{*}P < 0.05$ for the PF group compared with the vehicle group. $^{\#}P < 0.05$ for the high dose enalapril group compared with the PF group. $^{+}P < 0.05$ for the high dose enalapril group compared with the low dose enalapril group.

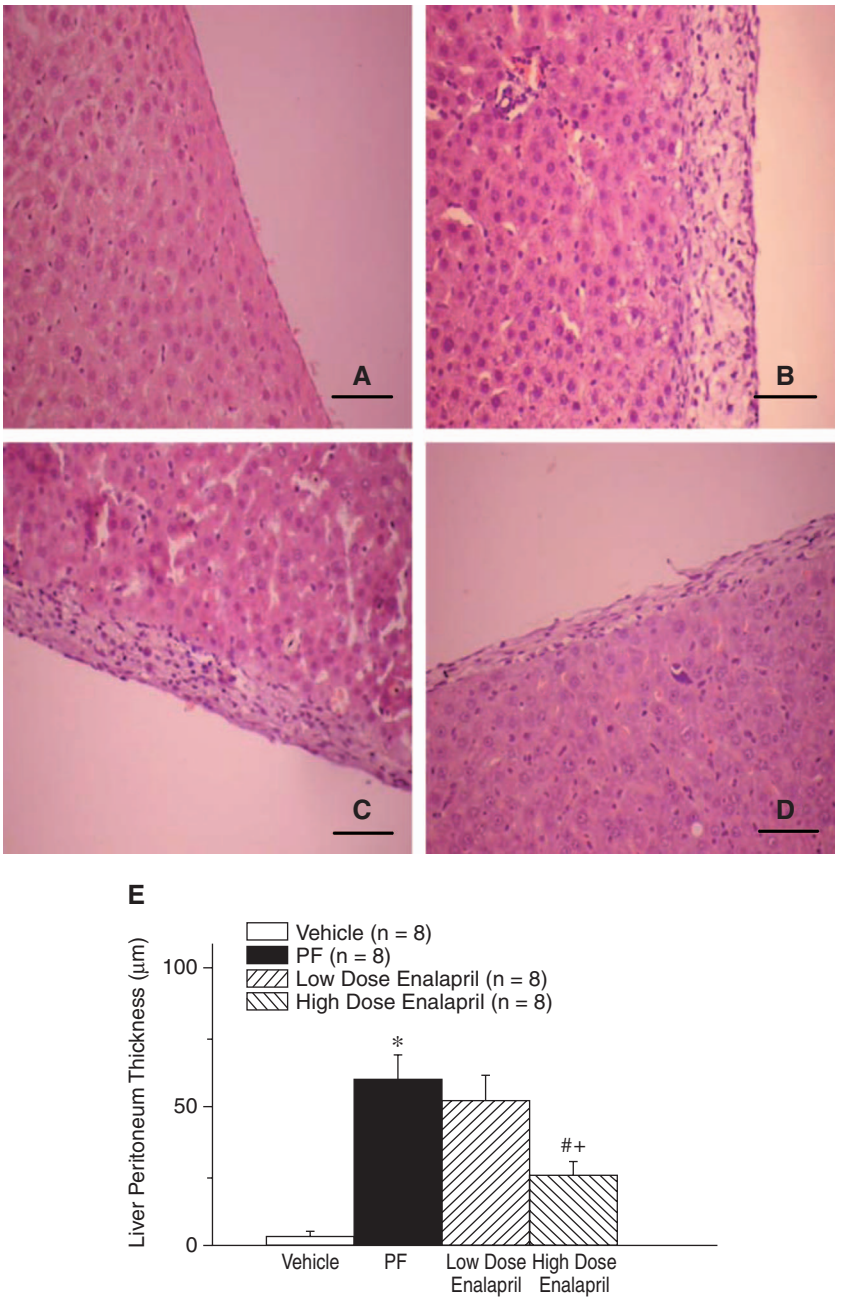

Fig. 4. Hematoxylin and eosin staining of liver peritoneum. Histologic sections from the vehicle group (A), peritoneal fibrosis (PF) group (B), low-dose enalapril group (C) and high-dose enalapril group (D) (magnification × 200). Thickness of liver peritoneum after chlorhexidine digluconate-induced PF in rats (E). $^{*}P < 0.05$ for the PF group compared with the vehicle group. $^{\#}P < 0.05$ for the high dose enalapril group compared with the PF group. $^{+}P < 0.05$ for the high dose enalapril group compared with the low dose enalapril group. Scale bar = 50 μm.

group, the serum and dialysate TGF-β1 levels were lower in the high dose enalapril group ($P < 0.05$; Fig. 3, A and B). The serum and dialysate TGF-β1 levels were not statistically significant in the low dose enalapril group compared with the PF group (Fig. 3, A and B).

Histopathology of Liver Peritoneum

Daily administration of chlorhexidine digluconate increased the thickness of the liver peritoneum (Fig. 4B). The PF group had thicker liver peritoneum than the vehicle group ($P < 0.05$; Fig. 4E). The thickness of the liver peritoneum was significant lower for the high dose enalapril group compared with the PF group ($P < 0.05$; Fig. 4E). Compared with the low dose enalapril group, the thickness of liver

peritoneum was lower in the high dose enalapril group ($P < 0.05$; Fig. 4E). The thickness of the liver peritoneum was not statistically significant in the low dose enalapril group compared with the PF group (Fig. 4E).

Immunohistochemistry of Liver Peritoneum

Compared with the vehicle group, rats in the PF group had more cells that were positive for TGF-β1, α-SMA, fibronectin, collagen and VEGF (Figs. 5B, 5F, 5J, 5N and 5R). Cells positive for TGF-β1, α-SMA, fibronectin, collagen and VEGF were significantly lower for the high dose enalapril group compared with the PF group ($P < 0.05$; Fig. 6, A to E). Compared with the low dose enalapril group, cells positive for TGF-β1, α-SMA, fibronectin, collagen and VEGF were lower in the high dose enalapril group ($P < 0.05$; Fig. 6, A to E). Cells positive for TGF-β1, α-SMA, fibronectin, collagen and VEGF were not statistically significant in the low dose enalapril group compared with the PF group (Figs. 6A to 6E).

Discussion

This study found that intravenous administration of 2.5 mg/kg/day enalapril decreased the chlorhexidine digluconate-induced thickness of the liver peritoneum by decreasing the serum and dialysate TGF-β1 level, and also significantly decreased the expression of TGF-β1, α-SMA, fibronectin, collagen and VEGF in liver peritoneum in rats.

The most commonly used agent to create a PF rat model was by intraperitoneal injection of 0.1% chlorhexidine gluconate in 15% ethanol (23). However, this technique is cumbersome and requires 15% ethanol as the control group. Ethanol decreases collagen production, and increases the production of TGF-β1 and the levels of matrix degrading enzymes in a murine excisional wound model (19). Acetaldehydes, the first metabolite of ethanol, can upregulate the transcription of collagen I directly as well as indirectly by upregulating the synthesis of TGF-β1 (18). Hence, ethanol may affect the production of TGF-β1, collagen and matrix degrading enzymes. Another problem is that the administration of intraperitoneal chlorhexidine gluconate in rats may unintentionally result in injection into abdominal organs, which could have misleading effects on the results. Our model is different in that chlorhexidine digluconate was used as a chemical irritant to induce PF and ethanol was not used as the control solution. The daily administrated chlorhexidine digluconate was *via* PD catheter and daily intraperitoneal injection was not used to avoid mistaken administration into abdominal organs (10).

PF is characterized by the activation of peritoneal resident cells, accumulation and deposition of excess matrix proteins within the interstitium, and neoangiogenesis and vasculopathy of the peritoneal microvasculature (9, 28). Cultured human peritoneal mesothelial cells were shown to constitutively express angiotensinogen, angiotensin converting enzyme, the angiotensin II receptors as well as TGF-β and fibronectin (15). High glucose concentration, low pH, peritonitis and the presence of glucose degradation products stimulate the peritoneal mesothelial cell to activate the renin-angiotensin system and produce TGF-β1 leading to peritoneal membrane neoangionesis, fibrosis and progressive PF (14). TGF-β1 is a profibrotic cytokine and overexpression of TGF-β1 further increases α-SMA and VEGF, and promotes PF (8, 12). Enalapril has been found to decrease serum TGF-β1 in diabetic rats (7), decrease glomerular TGF-β1 mRNA and protein expression in anti-Thy1 model of glomerulonephritis (16, 27), decrease renal TGF-β1 mRNA and protein expression in 5/6 nephrectomy rats (13), and to reduce liver fibrogenesis induced by bile-duct ligation by decreasing liver tissue TGF-β1 in rats (25). Our study also noted that high dose of enalapril decreased serum and dialysate TGF-β1 levels after chlorhexidine digluconate-induced liver PF in rats.

Angiotensin II is known to promote fibrosis and inflammation in various tissues and ACEI has been shown to attenuate those effects. Angiotensin II, acting both directly as well as indirectly *via* TGF-β1 production, has been shown to be an important mediator of epithelial-to-mesenchymal transition (5). The induction of the process of epithelial-to-mesenchymal transition of peritoneal mesothelial cells by TGF-β1 can lead to PF (1). Pharmacologic inhibition of angiotensin II attenuated the epithelial-to-mesenchymal transition and reduced renal fibrosis (11). Enalapril is an ACEI and it blocks the conversion of angiotensin I to angiotensin II (25). Enalapril decreased glomerular fibronectin and collagen I IHC staining in the anti-Thy1 model of glomerulonephritis (27), decreased α-SMA in renal interstitium in hyperoxaluric rats (24) and attenuated the overproduction of VEGF due to tumor necrosis factor alpha (TNF-α) and interleukin-1alpha (IL-1α) stimuli (21). Intraperitoneal enalapril administration protected liver peritoneal thickness and partially preserved the peritoneal function by inhibiting the TGF-β1 overexpression induced by 3.86% glucose PD solution in rats on chronic PD (6). Our study noted that intravenous administration of 2.5 mg/kg/day enalapril ameliorated chlorhexidine digluconate-induced liver peritoneum thickness and significantly decreased the expression of TGF-β1, α-SMA, fibronectin, collagen and VEGF in liver peritoneum in rats and also noted

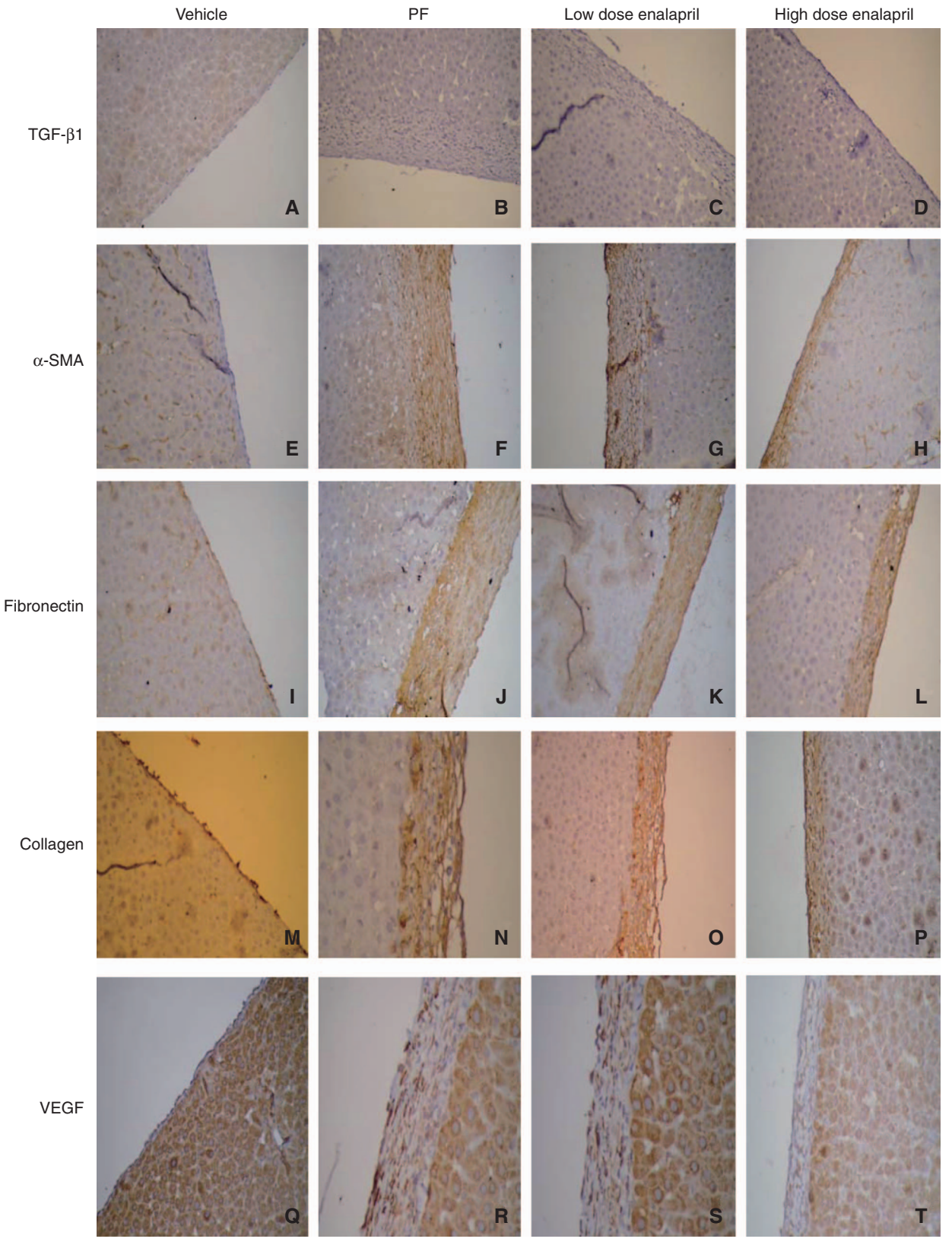

Fig. 5. Immunohistochemical (IHC) staining for transforming growth factor-beta 1 (TGF-β1; panels A, B, C, D), alpha-smooth muscle actin (α-SMA; E, F, G, H), fibronectin (I, J, K, L), collagen (M, N, O, P) and enalapril endothelial growth factor (VEGF; Q, R, S, T) in the liver peritoneum. Histologic sections from the vehicle group (A, E, I, M, Q), PF group (B, F, J, N, R), low-dose enalapril group (C, G, K, O, S) and high-dose valsartan group (D, H, L, P, T) (magnification × 200) after chlorhexidine digluconate-induced peritoneal fibrosis in rats.

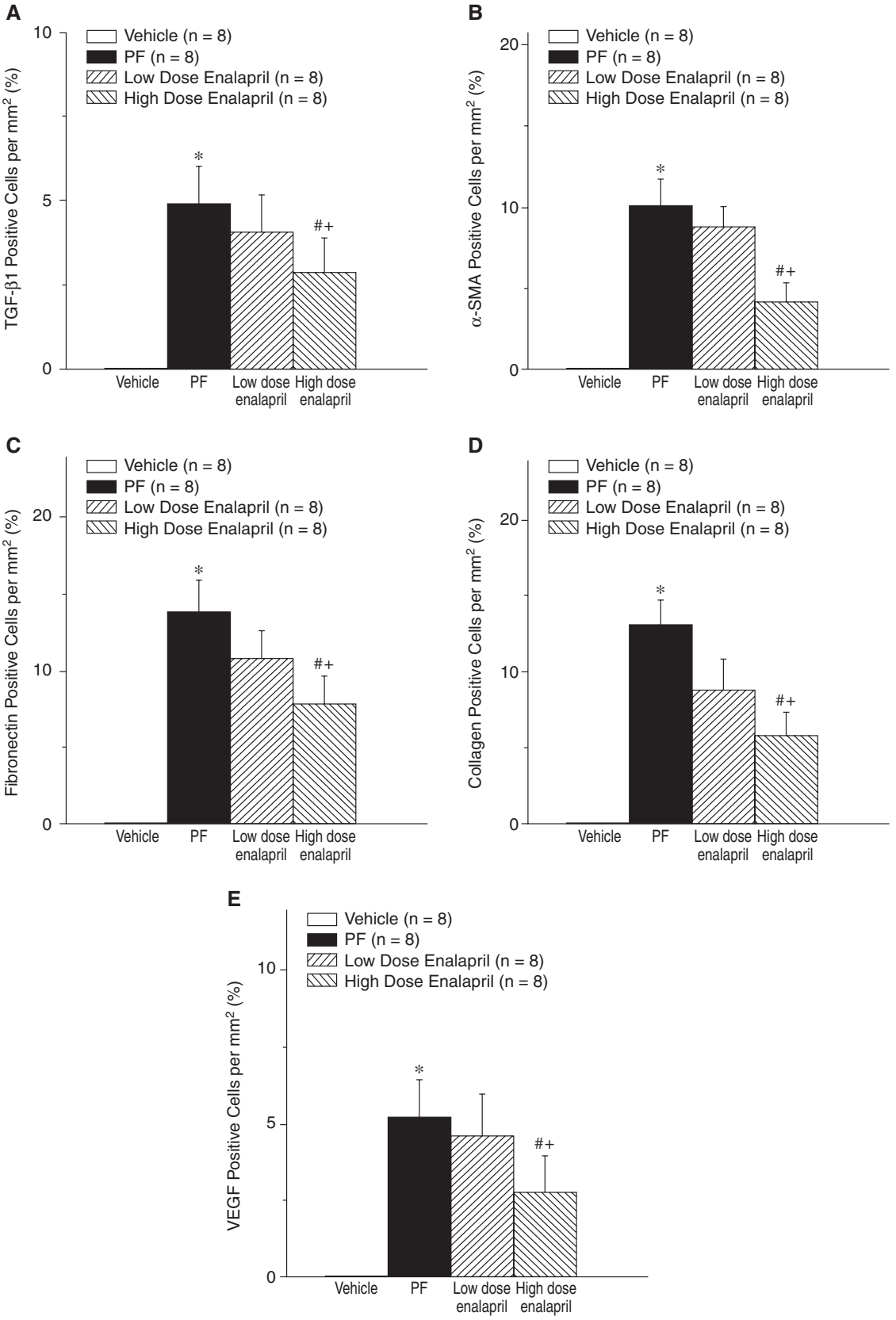

Fig. 6. Percentage of TGF-β1-positive cell score (A), α-SMA-positive cell score (B), fibronectin-positive cell score (C), collagen-positive cell score (D) and VEGF-positive cell score (E) after chlorhexidine digluconate-induced peritoneal fibrosis in rats. $^{*}P < 0.05$ for the PF group compared with the vehicle group. $^{\#}P < 0.05$ for the high dose enalapril group compared with the PF group. $^{+}P < 0.05$ for the high dose enalapril group compared with the low dose enalapril group.

the D_4/D_0 glucose level that was significantly lower, and $D_4/P_{4\ urea}$ level was significantly higher in the high dose enalapril group than in the PF group. Our results suggest the need for clinical randomized trials to investigate whether treatment with enalapril can elicit significant protective effects for PD patients who suffer from PF.

Limitations of the present study include the fact that it was an animal study of short duration. The clinical presentation of encapsulating peritoneal sclerosis (EPS) is a rare but serious complication of PD patients (22). EPS is felt to be a separate entity that occurs in a minority of patients with underlying peritoneal membrane fibrosis- the etiology is unclear but is thought to be due to a possible "second hit" that occurs to the peritoneal membrane exposed to chronic PD (3). In this study, by stating that PF occurs after one week of chlorhexidine digluconate and that PF is the same as clinical presentation of EPS is incorrect. Additional studies will be required to ascertain whether the enalapril may have more protective effects from EPS.

In conclusion, intravenous administration of 2.5 mg/kg/day enalapril ameliorated chlorhexidine digluconate-induced PF by decreasing serum and dialysate TGF-β1 levels and significantly decreased the expression of TGF-β1, α-SMA, fibronectin, collagen and VEGF in liver peritoneum in rats.

Acknowledgments

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