$1,25(OH)_2D_3$ treatment attenuates high glucose-induced peritoneal epithelial to mesenchymal transition in mice

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Abstract. It has been previously demonstrated that $1,25(OH)_2D_3$ prevents the progression of epithelial to mesenchymal transition (EMT). However, it remains unclear whether $1,25(OH)_2D_3$ has a role in peritoneal EMT stimulated by high glucose (HG) peritoneal dialysis fluid (PDF). The present study was performed to investigate the role of $1,25(OH)_2D_3$ in the progression of EMT in the peritoneal mesothelium. A total of 35 male Kunming mice were randomly assigned into seven groups. In the control group, no diasylate or saline was infused. In the saline group, the mice were intraperitoneally injected with saline every day for 4 weeks. In the vitamin D group, the mice were subjected to intraperitoneal injections of 1 or 5 μg/kg of $1,25(OH)_2D_3$ once weekly (every Monday) for 4 weeks. The peritoneal dialysis (PD) group were intraperitoneally injected with a conventional 4.25% PDF daily for 4 weeks. The vitamin D+PD group were intraperitoneally injected with 4.25% PDF daily and co-treated with 1 μg/kg or 5 μg/kg $1,25(OH)_2D_3$ once weekly, for 4 weeks. The peritoneal morphology and thickness were assessed by hematoxylin and eosin and Masson's trichrome staining. The peritoneal protein level of EMT markers (α-smooth muscle actin, fibronectin and E-cadherin), vitamin D receptor (VDR), B cell lymphoma-2 (Bcl-2), Bcl-2-associated X protein, transforming growth factor (TGF)-β and Smad3 were evaluated by western blot analysis or immunohistochemical staining. Furthermore, apoptosis was assessed using a Caspase-3 activity assay. The results demonstrated that after 4 weeks of intraperitoneal injections in mice, HG-PDF decreased the expression of VDR, promoted EMT and apoptosis, and increased the thickness of the peritoneal membrane. However, $1,25(OH)_2D_3$ treatment attenuated HG-induced EMT and apoptosis, and decreased peritoneal thickness, which may partially occur through inhibition of transforming growth factor TGF-β/Smad pathways via $1,25(OH)_2D_3$ binding to VDR. The present study demonstrated that $1,25(OH)_2D_3$ attenuated HG-induced EMT and apoptosis in the peritoneal mesothelium through TGF-β/Smad pathways. $1,25(OH)_2D_3$ treatment in conjunction with HG dialysate may provide an improved solution to the peritoneal injury in the process of PD.

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Abbreviations: EMT, epithelial to mesenchymal transition; PDF, peritoneal dialysis fluid; HG, high glucose; VDR, vitamin D receptor; PD, peritoneal dialysis; ESRD, end-stage renal disease; FN, fibronectin

Key words: $1,25(OH)_2D_3$, peritoneal dialysis, epithelial to mesenchymal transition, transforming growth factor-β/Smad

Introduction

Peritoneal dialysis (PD) has become one of the most important renal replacement therapies for patients with end-stage renal disease (ESRD) (1-4). Long-term PD is limited due to recurrent peritonitis, inadequate dialysis and peritoneal fibrosis (5-8). High concentrations of glucose in dialysate, which damages the structure and function of the peritoneal membrane, is considered to be one of the most important factors leading to peritoneal fibrosis and ultrafiltration failure (9). Epithelial to mesenchymal transition (EMT) has an important role in peritoneal membrane fibrosis and dysfunction. EMT is characterized by the loss of normal epithelial cell features, including cell polarity and adhesion due to reduced expression of epithelial makers such as E-cadherin, and gaining features of mesenchymal cells, including invasion and migration, which are associated with increases in mesenchymal markers such as α-smooth muscle actin (α-SMA) and fibronectin (FN). High glucose (HG) was previously reported to accelerate EMT mediated by inflammation in peritoneal mesothelial cells and the kidney (10,11). Preventing EMT may mitigate peritoneal fibrosis and preserve mesothelial cells during PD. It was also demonstrated that HG induces apoptosis in peritoneal mesothelial cells (12). The apoptosis of peritoneal mesothelial cells may also induce the progression and development of peritoneal fibrosis during long-term PD, which eventually leads to ultrafiltration failure (13,14).

An increasing amount of evidence indicates a role for $1,25(OH)_2D_3$ and its analogues in the regulation of cell immunomodulation, proliferation and differentiation (15-17).

Several studies have also demonstrated the protective effect of vitamin D on peritoneal fibrosis (18-22). In addition, the effects of vitamin Don EMT have been previously investigated. For example, vitamin D was reported to ameliorate cancer cell EMT (23) and inhibit migration, invasion and EMT induced by TGF-β in human airway epithelial cells (24). $1,25(OH)_2D_3$ regulates calcium, phosphate and bone metabolism by binding to the vitamin D receptor (VDR). The VDR forms a heterodimer with the retinoid X receptor, which subsequently regulates the expression of genes in the nucleus. Our previous research demonstrated that $1,25(OH)_2D_3$ exhibited a modulatory effect on apoptosis (25). However, the effect of $1,25(OH)_2D_3$ on HG-induced EMT and apoptosis in the peritoneal mesothelium and the underlying molecular mechanism remain to be established.

The present study aimed to investigate whether $1,25(OH)_2D_3$ protects the peritoneal mesothelium from HG-induced EMT and apoptosis, and to identify the molecular mechanism.

Materials and methods

Animals and experimental treatments. All the animals and experimental procedures were approved by the Experimental Animals Ethics Committee of China Medical University (Shenyang, China). Kunming male mice (n=35; age, 8-12 weeks; weight, 28-30 g) were obtained from the Department of Laboratory Animals, China Medical University, and housed in a room with controlled temperature (22˚C) and humidity (60-65%) on a 12-h light/dark cycle. Food and water were provided *ad libitum* throughout the experiment and mice were given one week to acclimate to their new environment, The mice were randomly assigned into the following seven groups (n=5 per group): Control group, no dialysate or saline was infused; saline group, mice received 50 ml/kg saline intraperitoneal injection everyday for 4 weeks; low dose vitamin D group, the mice were subjected to intraperitoneal injections of 1 μg/kg $1,25(OH)_2D_3$ (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) once weekly (every Monday) for 4 weeks; high dose vitamin D group, the mice were subjected to intraperitoneal injections of 5 μg/kg $1,25(OH)_2D_3$ once weekly (every Monday) for 4 weeks; PD group were intraperitoneally injected with 50 ml/kg conventional 4.25% peritoneal dialysis fluid (PDF; Baxter Healthcare Co., Ltd., Guangzhou, China) daily for 4 weeks; PD +low dose vitamin D group, mice were intraperitoneally injected with 50 ml/kg conventional 4. 25% PDF daily, and intraperitoneal injections of 1 μg/kg $1,25(OH)_2D_3$ once weekly (every Monday) for 4 weeks; and PD + high dose vitamin D group, mice were intraperitoneally injected with 50 ml/kg conventional 4. 25% PDF daily, and subjected to intraperitoneal injections of 5 μg/kg $1,25(OH)_2D_3$ once weekly (every Monday) for 4 weeks. At the end of the experimental period (4 weeks), the mice were starved for 12-13 h and sacrificed, parietal peritoneum was used for morphometric and histological analyses, and the visceral peritoneum was used for western blot analysis.

Histology and immunohistochemical (IHC) analyses of the peritoneum. The parietal peritoneum was fixed overnight with PBS (pH 7. 2) containing 4% paraformaldehyde at 4˚C, impregnated and embedded in paraffin wax. Samples were cut into 4-μm sections. Tissue sections were stained with hematoxylin (room temperature for 20 min) and eosin (room temperature for 3 sec) (H&E staining) to examine the peritoneal morphology. The collagen thickness in the parietal peritoneum was measured in tissue sections by using Masson's trichrome stain (room temperature for 10 min). The collagen thickness of the parietal peritoneum, including the mesothelium and submesothelial tissue, was measured. Each tissue section was measured at ten random locations by two blinded observers.

Following deparaffinization, tissues were hydrated with graded alcohol and blocked with non-immune goat serum, (Fuzhou Maixin Biotech Co., Ltd., Fuzhou, China) at 37˚C for 15 min. Antigen retrieval was performed using 0. 01 M citrate buffer (pH 6. 0) at 100˚C for 2 min, followed by washing in PBS. Tissues were incubated with an α-SMA primary antibody (1:200; ab32575; Abcam, Cambridge, UK) at 37˚C for 2 h. An Elivision™ *Super* horseradish peroxidase (HRP) IHC kit (KIT-9922; Fuzhou Maixin Biotech Co., Ltd) was used as a ready-to-use secondary antibody; sections were incubated at 37˚C for 1 h. Positive binding was detected using diaminobenzidine staining. Counterstaining with hematoxylin was performed at room temperature for 10 min. Antigens were visualized using a fluorescence microscope (Nikon Corporation, Tokyo, Japan) at a magnification of x400.

Western blot analysis. Mouse visceral peritoneum was lysed in radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China), sonicated three times for 10 sec each time, and protein was quantified with the BCA Protein assay kit (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Protein (50 μg) from visceral peritoneum lysates was loaded and separated by 10% SDS-PAGE, and was then transferred onto nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). Blots were incubated with bovine serum albumin solution (10%; Sigma-Aldrich; Merck KGaA) at 37˚C for 1 h to limit non-specific antibody binding. The blots were incubated overnight at 4˚C with primary antibodies against E-cadherin (1:1,000; sc-7870; Santa Cruz Biotechnology, Inc.), α-SMA (1:1,000; ab32575; Abcam), FN (1:1,000; sc-9068; Santa Cruz Biotechnology, Inc.), VDR (1:1,000; ab109234; Abcam), Bcl-2 (1:1,000; cat. no. 3498; Cell Signaling Technology, Inc., Danvers, MA, USA), Bax (1:1,000; cat. no. 2772; Cell Signaling Technology, Inc), TGF-β (1:1,000; sc-146; Santa Cruz Biotechnology, Inc.), Smad family member 3 (Smad3; 1:1,000; cat. no. 9523; Cell Signaling Technology, Inc.), phosphorylated-Smad3 (1:1,000; cat. no. 9520; Cell Signaling Technology, Inc.) or β-actin (1:1,000; sc-130656; Santa Cruz Biotechnology, Inc.). This was followed by incubation at room temperature for 2 h with the HRP-conjugated secondary antibody (1:10,000; sc-2004; Santa Cruz Biotechnology, Inc.). The blots were developed using an ECL Western Blotting Substrate kit (32109; Pierce; Thermo Fisher Scientific, Inc.) and the images were captured with a G:BOX EF Chemi HR16 system (Syngene, Frederick, MD USA). ImageJ software 1.6.0 (National Institutes of Health, Bethesda, Maryland, USA) was used to measure the band densities and the densitometric intensity of each band was normalized against β-actin expression.

Caspase-3 activity assay. A Caspase-3 Activity assay kit was used to detect the activity of caspase-3 (Beyotime Institute of Biotechnology). Protein concentrations of tissue lysates were

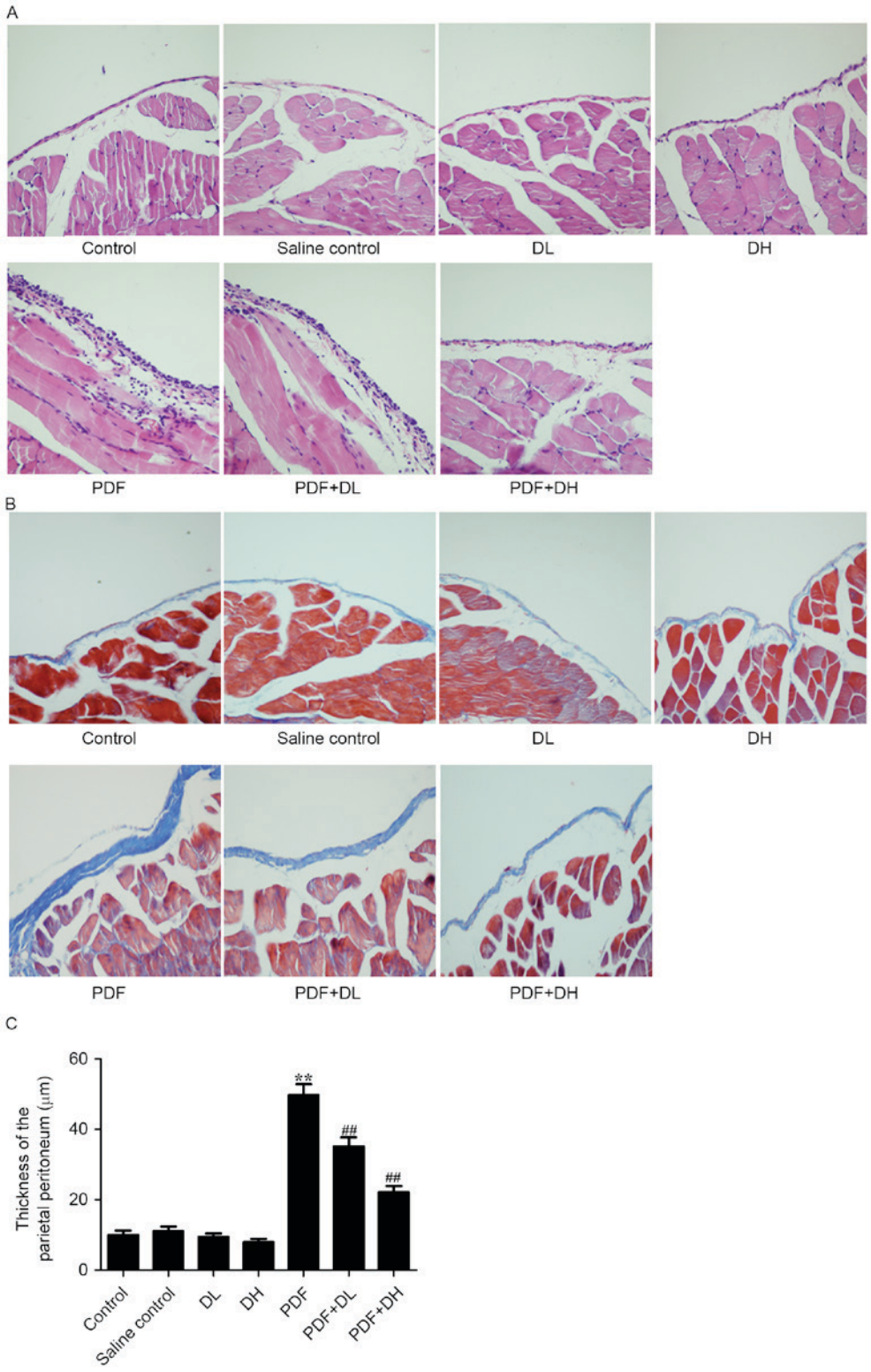

Figure 1. Effects of $1,25(OH)_2D_3$ on high glucose PDF-induced morphological changes and collagen thickness in the peritoneal mesothelium. Representative images of (A) hematoxylin and eosin and (B) Masson's trichrome-stained parietal peritoneum mesothelium tissue sections. Magnification, x400. (C) Collagen thickness was measured in tissue sections following Masson's trichrome staining. **P<0. 01 vs. control; $^{\#\#}$P<0. 01 vs. PDF. PDF, peritoneal dialysis fluid; DL, vitamin D low dose [1 μg/kg $1,25(OH_2D_3]$; DH, vitamin D high dose [5 μg/kg $1,25(OH)_2D_3$].

measured by the Bradford assay kit (Beyotime Institute of Biotechnology). Protein-normalized supernatants (10 μl) were mixed with 10 μl Ac-DEVD-pNA (2 mM) in assay buffer. Caspase-3 activity was determined by spectrophotometry (NanoDrop Technologies; Thermo Fisher Scientific, Inc.).

Statistical analysis. Data were analyzed using SPSS 18 (SPSS, Inc., Chicago, IL, USA). Results are presented as the mean ± standard error of the mean. One way analysis of variance was used for comparisons among groups. P<0.05 was considered to indicate a statistically significant difference.

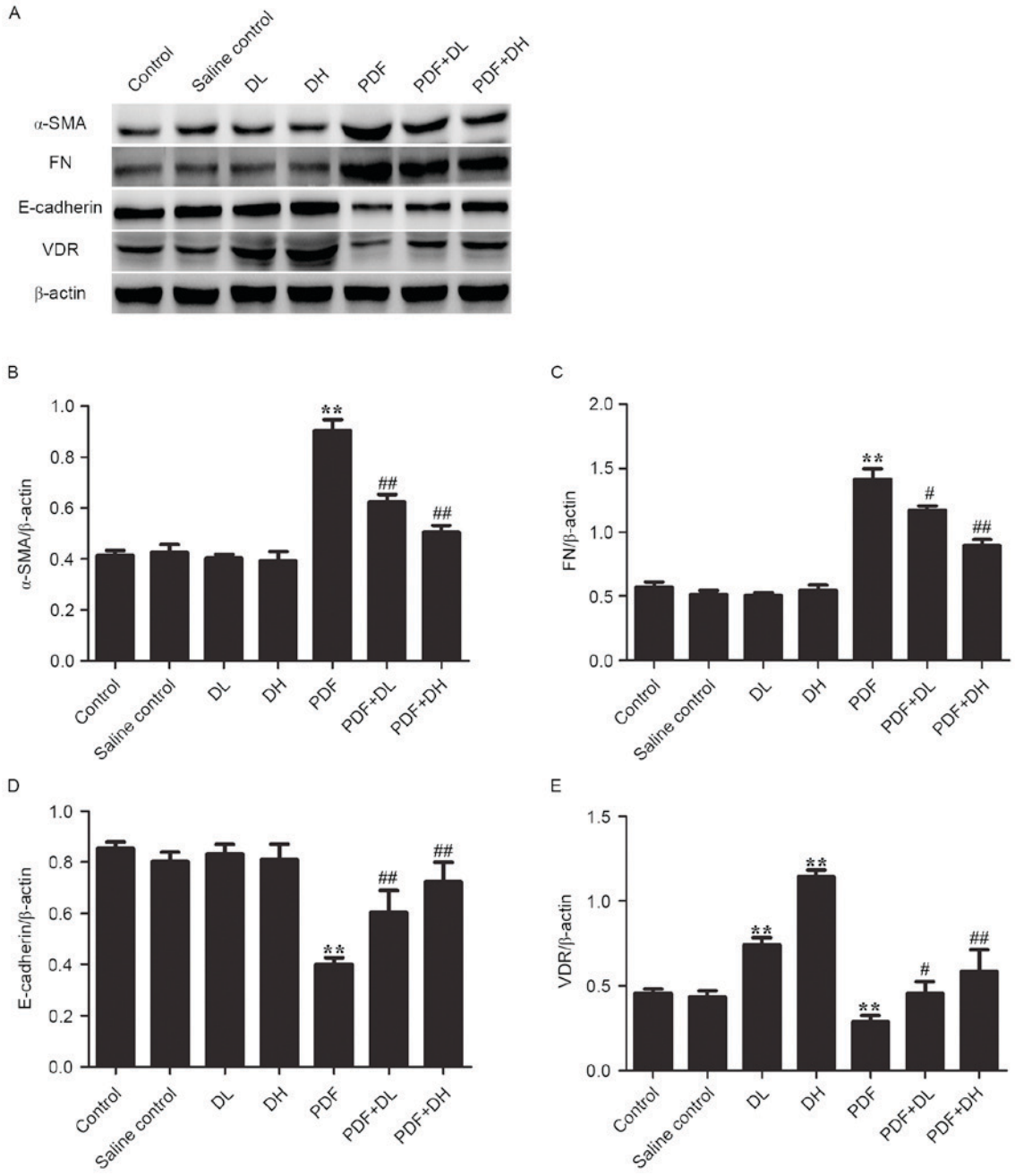

Figure 2. Effects of 1,25(OH)$_2$D$_3$ on the protein expression of epithelial to mesenchymal transition biomarkers and VDR in high glucose PDF-treated peritoneal mesothelium. (A) Representative western blot bands for each group. Relative protein expression of (B) α-SMA, (C) FN, (D) E-cadherin and (E) VDR was calculated and normalized to the loading control. Corresponding protein levels were assessed using densitometry and are presented as relative intensities. Each value represents the mean + standard error of the mean, n=5. **P<0. 01 vs. control; $^{\#}$P<0. 05 and $^{\#\#}$P<0. 01 vs. PDF. VDR, vitamin D receptor; PDF, peritoneal dialysis fluid; α-SMA, α-smooth muscle actin; FN, fibronectin; DL, vitamin D low dose [1 μg/kg 1,25(OH)$_2$D$_3$]; DH, vitamin D high dose [5 μg/kg 1,25(OH)$_2$D$_3$].

Results

Effects of 1,25(OH)$_2$D$_3$ on HG PDF-induced morphological changes in the peritoneal mesothelium. H&E staining demonstrated that the normal peritoneal mesothelium was covered by a single layer of flat peritoneal mesothelial cells. When exposed to HG PDF, H&E and Masson's trichrome staining demonstrated that the mesothelial cells became round, cylindrical and cells shed, fibers were exposed and inflammatory cells infiltrated the interstitium. However, 5 μg/kg 1,25(OH)$_2$D$_3$ co-treatment once weekly observably reduced the changes in cell morphology induced by HGPDF (Fig. 1A and B). Masson's trichrome stain also demonstrated that, after 4 weeks of PDF treatment in mice, the thickness of the peritoneal membrane in the PDF group was significantly increased compared with the control group (Fig. 1C). 1,25(OH)$_2$D$_3$ co-treatment (5 μg/kg) significantly decreased peritoneal thickness compared with the PDF group (Fig. 1C).

Treatment with 1, 25 (OH) $_2$D$_3$ decreases HG-induced EMT in the peritoneal mesothelium. Exposure of the peritoneal mesothelium to HGPDF for 4 weeks significantly

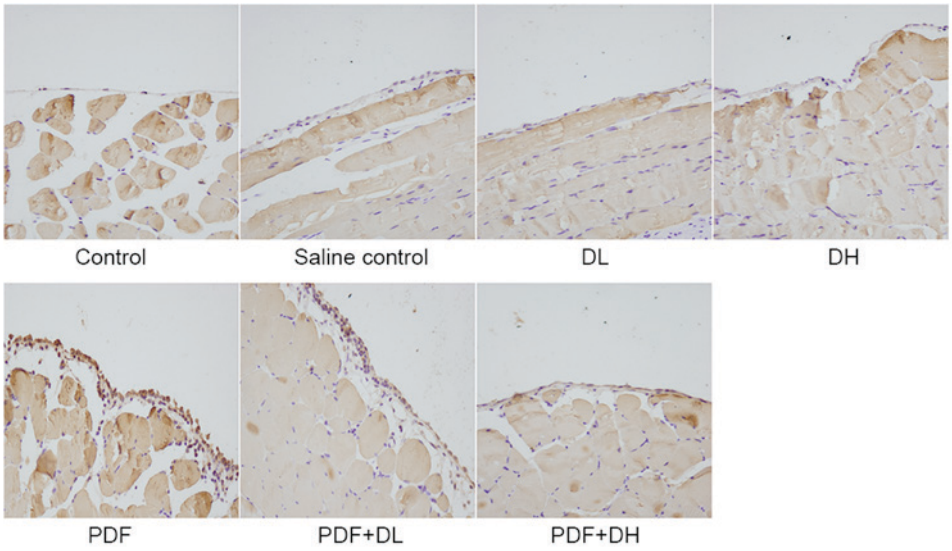

Figure 3. Immunohistochemical staining for α-SMA in the peritoneum of seven groups of mice. The intensity of a-SMA staining was higher in PDF mice compared with the control group, and 1,25(OH)$_2$D$_3$ reversed these changes. Magnification, x400. α-SMA, α-smooth muscle actin; PDF, peritoneal dialysis fluid; DL, vitamin D low dose [1 μg/kg 1,25(OH)$_2$D$_3$]; DH, vitamin D high dose [5 μg/kg 1,25(OH)$_2$D$_3$].

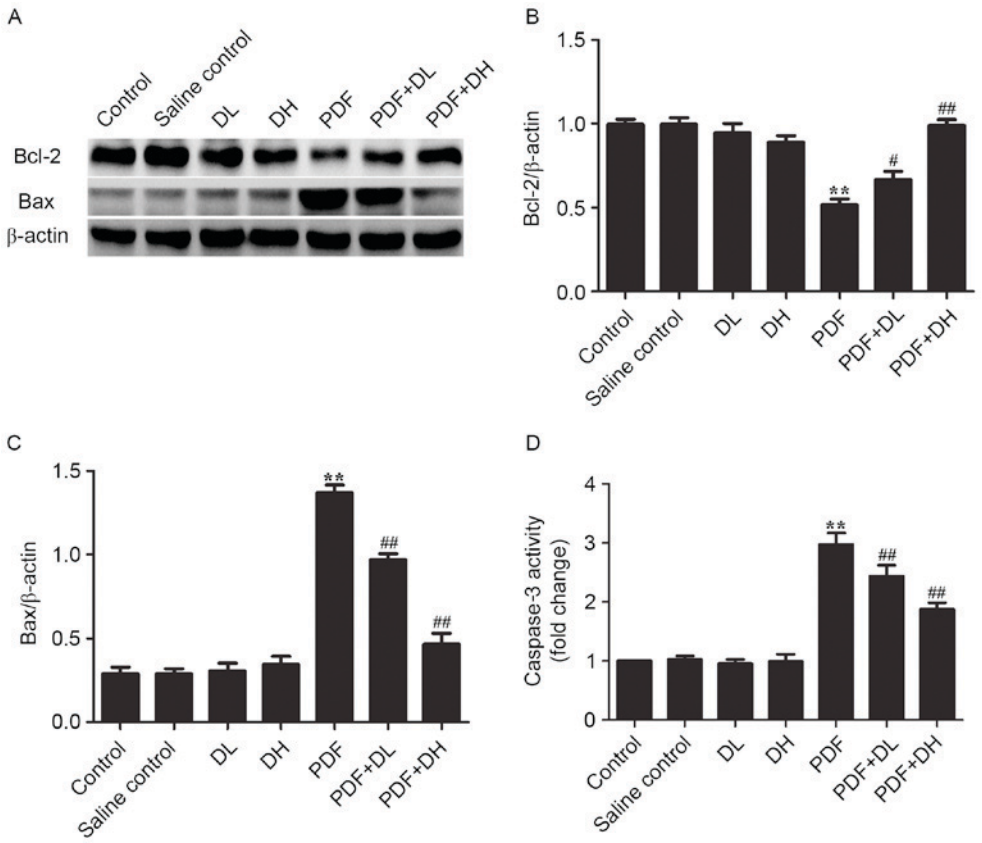

Figure 4. Effects of 1,25(OH)$_2$D$_3$ on high glucose PDF-induced apoptosis in the peritoneal mesothelium. (A) Representative western blot bands for each group. The protein expression of (B) Bcl-2 and (C) Bax was assessed using densitometry and are presented as relative intensities. Relative expression of Bax and Bcl-2 was calculated and normalized to the loading control. (D) Caspase-3 activity was measured using caspase-3 activity kit and a spectrophotometer. Each value represents the mean + standard error of the mean, n=5. **P<0. 01 vs. control; $^{\#}$P<0. 05 and $^{\#\#}$P<0. 01 vs. PDF. PDF, peritoneal dialysis fluid; Bax, Bcl-2-associated X; DL, vitamin D low dose [1 μg/kg 1,25(OH)$_2$D$_3$]; DH, vitamin D high dose [5 μg/kg 1,25(OH)$_2$D$_3$].

decreased the protein expression of the epithelial cell marker E-cadherin, and significantly increased the expression levels of the mesenchymal markers α-SMA and FN (Fig. 2A-D), compared with the control group. 1,25(OH)$_2$D$_3$ treatment (1 and 5 μg/kg) significantly increased the protein expression of the epithelial cell marker and decreased expression of mesenchymal markers, compared with the mice treated with PDF alone (Fig. 2A-D).

The results also demonstrated that the expression of VDR protein was significantly lower in mice treated with 4.25% PDF compared with the control group (P<0.01), and these changes were partially attenuated by 1 and 5 μg/kg 1,25(OH)$_2$D$_3$ treatment (P<0.05; Fig. 2E).

Furthermore, immunohistochemical staining of the parietal peritoneum also revealed that the expression of the EMT marker α-SMA was visibly higher in mice treated with 4. 25% PDF compared with the control group, and 1,25(OH)$_2$D$_3$ treatment attenuatedα-SMA expression in PDF mice (Fig. 3).

Treatment with 1,25(OH)$_2$D$_3$ decreases HG-induced apoptosis in the peritoneal mesothelium. Exposure of the peritoneal mesothelium to HGPDF for 4 weeks increased the protein expression of Bcl-2-associated X (Bax) and decreased the protein expression of Bcl-2 significantly compared with the control group (Fig. 4A-C), and the activity of caspase-3 was significantly increased compared with the control group (Fig. 4D), which indicates that HGPDF may induce peritoneal mesothelium apoptosis. However, 1,25(OH)$_2$D$_3$ treatment (1 and 5 μg/kg) increased the expression of Bcl-2, and decreased the expression of Bax and the activity of caspase-3 compared with the mice treated with PDF alone.

Effects of 1,25(OH)$_2$D$_3$ on the TGF-β/Smad signaling pathway. Western blot analysis demonstrated that exposure to HGPDF for 4 weeks significantly increased the protein expression of TGF-β and the phosphorylation of Smad3 compared with the control group (Fig. 5). 1,25(OH)$_2$D$_3$ treatment (1 and 5 μg/kg) significantly decreased the expression of TGF-β and the phosphorylation of Smad3 compared with the PDF group.

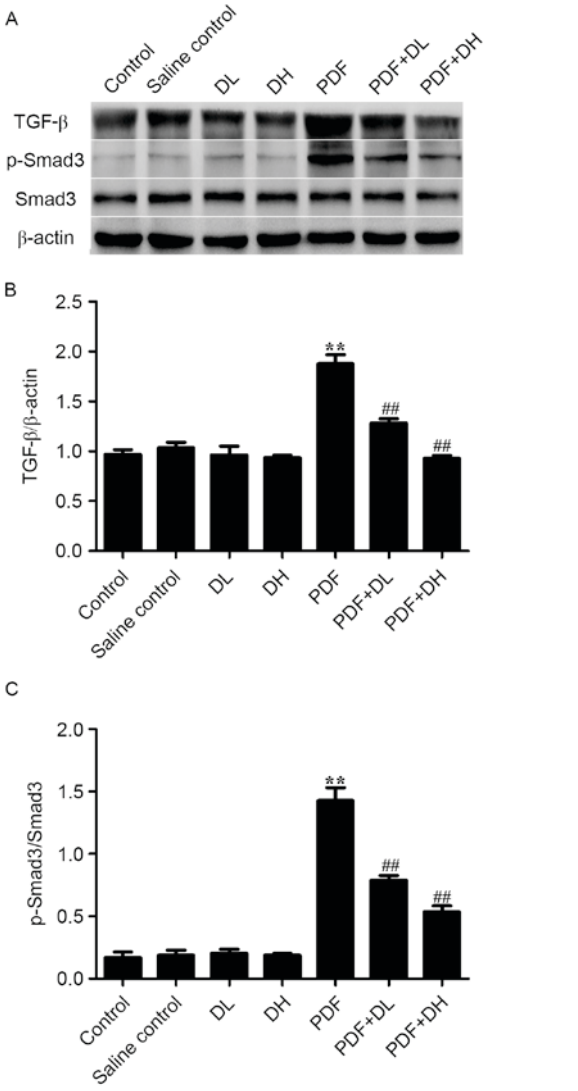

Figure 5. Effects of 1,25(OH)$_2$D$_3$ on the TGF-β/Smad pathway in high glucose PDF-treated peritoneal mesothelium. (A) Representative western blot bands for each group. The protein expression of (B) TGF-β and (C) p-Smad3 were quantified using densitometry and are presented as relative intensities. Each value represents the mean ± standard error of the mean, n=5. **P<0. 01 vs. control; $^{\#\#}$P<0. 01 vs. PDF. TGF, transforming growth factor; PDF, peritoneal dialysis fluid; Smad3, Smad family member 3; p-Smad3, phosphorylated-Smad3; DL, vitamin D low dose [1 μg/kg 1,25(OH)$_2$D$_3$]; DH, vitamin D high dose [5 μg/kg 1,25(OH)$_2$D$_3$].

Discussion

An earlier study identified that PD accounted for >10% of all forms of renal replacement therapy worldwide in ESRD (26), however, this has reached 23% in Asia (27). During PD, the peritoneal mesothelium is usually exposed to hyperglycemic, hyperosmotic and acidic dialysis solutions, which may induce peritoneal fibrosis and eventual lead to PD failure. In addition, HG itself may induce a proinflammatory and profibrotic reaction (28). EMT has a central role in the alterations of the peritoneal mesothelium that lead to fibrosis and peritoneal failure associated with PD (29).

Accumulating evidence indicates that 1,25(OH)$_2$D$_3$ may affect organ EMT and fibrosis (30-32). A previous study indicated that active vitamin D effectively increased VDR expression and inhibited EMT in a mouse unilateral ureteral obstruction model (33). In addition, 1,25(OH)$_2$D$_3$ was reported to prevent the progression of pancreatic and lung cancer by inhibiting EMT (34,35). Furthermore, EMT was demonstrated to occur in human peritoneal mesothelial cells due to the recurrent use of HG PDF, and has been associated with peritoneal function decline and peritoneal fibrosis (36,37). Therefore, we hypothesized that 1,25(OH)$_2$D$_3$ may have an effect on peritoneal fibrosis via inhibition of EMT. The present study employed HG PDF to reproduce the injury of peritoneal EMT *in vivo*, and subsequently investigated the effect of 1,25(OH)$_2$D$_3$ on peritoneal EMT. The results demonstrated that PDF increased peritoneal thickness, decreased VDR expression, altered peritoneal morphology and the expression of certain EMT markers, exhibiting reduced E-cadherin levels and increases in α-SMA and FN expression. The results of the present study indicated that 1,25(OH)$_2$D$_3$ has an important role in protecting against EMT and attenuating peritoneal thickness in PD mice by binding to the VDR.

Apoptosis is important in the maintenance of normal homeostasis, however, changes in the physiological rate of apoptosis may result lead to disease (38). It was previously demonstrated that HG induced apoptosis in peritoneal mesothelial cells (12). This effect on the peritoneal homeostasis may lead to failure of PD (13,14). The current study demonstrated that PDF induced apoptosis, with western blot analysis demonstrated a decrease in theBcl-2/Bax ratio and an increase incapase-3 activity. 1,25(OH)$_2$D$_3$ was also observed to have an important role in protecting against apoptosis in PD mice.

TGF-β is a key cytokine that is involved in EMT in peritoneal mesothelial cells (39). TGF-β exerts its functions primarily through activation of Smad-dependent signaling pathways (40). The involvement of the TGF-β/Smad pathway in EMT and apoptosis has been investigated extensively. A previous study demonstrated that vitamin D attenuated renal tubular cell injury by suppressing EMT and inflammation process via inhibition of the TGF-β/Smad, β-catenin and nuclear factor-κB signaling pathways (41). The present study demonstrated that $1,25(OH)_2D_3$ decreased peritoneal thickness, and attenuated EMT and apoptosis *in vivo*, and this may occur via the TGF-β/Smad signaling pathway.

In conclusion, the current study demonstrated that $1,25(OH)_2D_3$ may attenuate HGPDF-induced EMT and apoptosis via the TGF-β/Smad signaling pathway in the peritoneal mesothelium of mice. $1,25(OH)_2D_3$ treatment in conjunction with HG dialysate may provide an improved solution to the EMT-mediated fibrosis and peritoneal injury in the development of PD.

Acknowledgements

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