

Original Article

Effects of 1,25(OH)₂D₃ on proliferation and apoptosis of human glomerular mesangial cells

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Abstract: Objective: To investigate the effect of 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] on cell proliferation and apoptosis of human glomerular mesangial cells. Methods: Human glomerular mesangial cells were cultured for 48 h and randomly divided into four groups, normal control group (N group), proliferation group (EGF group), vitamin D₃ intervention group (VD₃ group), proliferation intervention group (EGF+VD₃ group). Flow cytometry was used to detect the cell cycle and apoptosis rate, and Western blot was used to detect the PCNA and Caspases-3 expression. The effect of 1,25(OH)₂D₃ on human mesangial cell proliferation was detected by CCK-8. Results: Compared with the control group, G1 phase cells in EGF group were significantly reduced, S, G₂/M phase cells were increased, and the higher PI and PCNA expression levels were increased; G1 phase cells in VD₃ group were increased, S, G₂/M phase cells were decreased, and the lower PI and PCNA expression levels were reduced. Compared with EGF group, G1 phase cells in EGF+VD₃ group were increased, S, G₂/M phase cells were decreased, and the lower PI and PCNA expression levels were significantly reduced. Compared with normal control group, the apoptosis rate in EGF group was significantly lower, and the Caspase-3 expression level was reduced; the apoptosis rate in VD₃ group was significantly higher, and Caspase-3 expression was significantly increased. Compared with EGF group, the apoptotic rate of mesangial cells in EGF+VD₃ group was significantly increased, and the Caspase-3 expression level was increased, with significant difference. Conclusion: 1,25(OH)₂D₃ inhibited the proliferation of mesangial cells and induced their apoptosis by blocking the cell cycle, inhibiting expression of PCNA, and upregulating Caspase-3 expression.

Keywords: Mesangial cells, 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], proliferating cell nuclear antigen (PCNA), Caspase-3, epidermal growth factor (EGF)

Introduction

Mesangial cells (MCs) are important intrinsic glomerular cells, with the maintenance of glomerular ultrafiltration function. Studies show that primary glomerulonephritis is the leading cause of ESRD [1], and mesangial proliferative glomerulonephritis has the highest incidence among various glomerulonephritis [2]. Glomerular sclerosis is the most common chronic kidney disease (CKD), which is characterized by excessive proliferation of MC and extracellular matrix (ECM) accumulation. Research on the MC proliferation-related factors, which can reverse the glomerular injury, is very important [3]. In the repair process of inflammation in kidney disease, apoptosis is able to eliminate the excessive proliferation of MCs [4]. PCNA (proliferating cell nuclear antigen) is the established standard of cell proliferation [5]. Caspases-3

is the key enzyme in the process of apoptosis [6]. 1,25-dihydroxy vitamin D₃ [1,25(OH)₂D₃] is a biologically active form of vitamin D₃, which has many new biological effects, including inhibition of cell proliferation and induction of cell apoptosis. Studies have shown that 1,25(OH)₂D₃ significantly inhibits rat MCs proliferation and induces apoptosis [7, 8]. Mesangial cells can produce HB-EGF, which binds with mesangial cell surface EGF receptor to regulate its various functions by autocrine and paracrine pathways [9]. It is possible to take EGF effects on mesangial cells to promote human mesangial cell proliferation in vitro microenvironment. Experiments have confirmed that in Thy-1 nephritis models of rats, 1,25(OH)₂D₃ can inhibit MC proliferation and cell cycle, and induce MC apoptosis to restore normal glomerular structure [10]. This study examined the effects of 1,25(OH)₂D₃ and EGF combined with 1,25(OH)₂D₃ on human

MC proliferation, apoptosis and PCNA and Caspase-3 expression, and the acting mechanisms were further explored, which can provide a theoretical basis for the extensive application of 1,25(OH)₂D₃ in clinical practice.

Materials and methods

Materials

Human mesangial cells (4200) were purchased from Xiangya Medical Center Laboratory. Reagents: epidermal growth factor (EGF) (US Peprtech) and 1,25(OH)₂D₃, phosphate buffer, propidium iodide PI (Sigma, USA); AnnexinV/PI double staining apoptosis kit (US Biovision Inc.), trypsin and low glucose DMEM, fetal bovine serum (US Gibco Inc.), mouse anti-human β -actin monoclonal antibody, rabbit anti-human PCNA monoclonal antibody, rabbit anti-human Caspase-3 monoclonal antibody (Cell Signaling Inc.); goat anti-rabbit IgG secondary antibody, goat anti-mouse IgG secondary antibody (Beijing Golden Bridge biotechnology Co., Ltd. in shirt).

Instruments and equipment: Flow cytometer (German Partec Inc.), inverted phase contrast microscope (Olympus IX71, Japan), ordinary optical microscope (Olympus, Japan).

Cell culture and packet

MCs were cultured by serum DMEM complete medium containing 10% fetal bovine for 48 h, and randomly divided into four groups, normal control group (N group, containing 5% fetal bovine serum DMEM medium), proliferation group (EGF group, plus 10 μ g/L of EGF), intervention group (VD group, plus 10⁻⁸ mol/L 1,25(OH)₂D₃), and proliferation intervention group (EGF+VD group, plus 10 μ g/L EGF and 10⁻⁸ mol/L 1,25(OH)₂D₃).

Cell cycle was detected by flow cytometry

The cells at logarithmic phase (2 \times 10⁵/mL) were seeded in 6-well plates at 2 mL/well. After cultured cells were digested with trypsin and collected, they were divided into experimental groups according to the methods (75% ice ethanol and armed uniform were added, followed by fixation at -20°C overnight). The cells were centrifuged and washed with PBS once, followed by addition of 500 μ L PBS to the resuspended cells. RNA enzyme was added until a final concentration of 10 μ g/ μ L. After 30 min,

PI staining was performed, and the final concentration became 50 mg/L. The solution was stored in the dark at 37°C. After 30 min, flow cytometry was carried out, and the proliferative index $[PI=(S+G_2M)/(G1+S+G_2M)]$ was calculated. The experiment was repeated three times.

Apoptosis was detected by flow cytometry

After the cells at logarithmic phase were counted, they were seeded in a 6-well plate. The cells were divided into experimental groups according to the culture methods. They were collected after washed twice with PBS (centrifugation at 2000 rpm for 5 min), and 500 μ L of 1 \times buffer was added to each tube, followed by 5 μ L Annexin V and 10 μ L PI. Chemical reaction occurred in the dark at room temperature for 10 minutes. Apoptosis in each group was detected by flow cytometry, and the images were acquired. The experiment was repeated for three times, and the rate of apoptosis was calculated.

Western blot detected the expression of PCNA and Caspase-3

The cells at logarithmic phase were taken after digested by trypsin. They were counted and seeded in disposable flasks. They were then cultured in serum-free DMEM medium for 24 h until cells were attached and synchronized at G0 phase. The cells were collected and lysed, followed by high-speed cryogenic centrifugation for 25 min, with the supernatant extracted and boiled for 5~10 min to denature the proteins. The samples underwent electrophoresis, which were transferred into the PVDF membrane. The PVDF was placed in the first antibody for incubation at 4°C overnight, and TBST was used for washing for 3 \times 10 min. The samples were then incubated with the secondary antibody at room temperature for 1 h, followed by washing using TBST for 3 \times 10 min. ECL luminescent reagent was added and exposed in the darkroom.

CCK-8 method was used to detect the proliferation of mesangial cells

The cells at logarithmic phase were selected, and the cell concentration was adjusted to 1 \times 10⁴/ml. They were seeded in a 96-well plate, and cultured for 24 h until the cells were completely adherent. With serum-free DMEM culture medium, the cells were synchronized for 24 h. After the experimental drug intervention

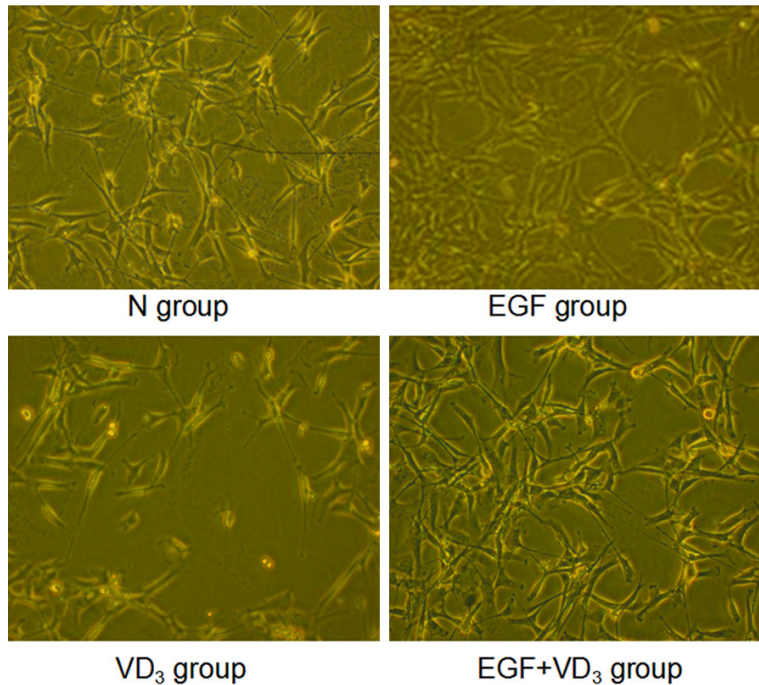


Figure 1. Changes in cell morphology of mesangial cells after 48 h drugs intervened ($\times 200$).

Table 1. In each group was detected by flow cytometry mesangial cell cycle ($n=10$, $\bar{x} \pm s$)

Group	Phases of the cell cycle (%)			PI (%)
	G1 Phase	S Phase	G ₂ /M Phase	
N group	74.38 \pm 1.63	19.75 \pm 1.27	5.08 \pm 1.87	25.02 \pm 1.11
VD group	80.89 \pm 1.47 ^a	14.54 \pm 1.11 ^a	4.42 \pm 2.00 ^a	18.98 \pm 1.58 ^a
EGF group	64.71 \pm 1.70 ^a	26.73 \pm 1.48 ^a	7.44 \pm 2.26 ^a	34.52 \pm 1.97 ^a
EGF+VD group	73.88 \pm 1.37 ^b	20.58 \pm 1.72 ^b	6.08 \pm 1.89 ^b	26.48 \pm 2.12 ^b
<i>F</i>	184.350*	124.391*	4.283*	135.424*

Note: * $P<0.05$; ^a: compared with N group, ^b: compared with EGF group, $P<0.05$.

was added, the cells were incubated for 48 h. 4 h before the end of the intervention, 10 μ l CCK-8 solution was added into each hole, and the cells were cultured for 4 h. The absorbance of each well (A450) was measured at 450 nm wavelength using a microplate reader. The inhibition of cell proliferation (inhibition rate, IR) was calculated according to the formula $IR = (\text{control group A value} - \text{the experimental group A value}) / (\text{control group A value}) \times 100\%$.

Statistical methods

SPSS 17.0 statistical software was applied to process data, and measurement data were expressed as mean \pm standard deviation ($\bar{x} \pm s$). The comparison among multiple groups was

carried out using ANOVA, while LSD method was employed for pairwise comparison. $P<0.05$ was considered statistically significant.

Results

MC morphology and growth

Under the microscope, cells in N group were bright, fusiform, irregular radiated, and dendritic, with densely overlapping reticular cell cytoplasm and clearer cytoplasm. EGF group had a large number of cells, but no significant changes were found in morphology compared with the N control group; the VD₃ group showed a certain degree of apoptosis, and the cells became small with nuclear shrinkage and reduced cell number, compared with the N group; cell morphology of EGF+VD₃ group was roughly the same, but cell number was increased slightly compared with N group (**Figure 1**).

Impact of 1,25(OH)₂D₃ on the cell cycle of MCs

Compared with N group, the cells at G1 phase of EGF group were significantly reduced, but those at S, G₂/M phase were increased, with

higher PI; the cells at G1 phase in VD₃ group were increased, but those at S, G₂/M phase were decreased, with lower PI, and the differences were statistically significant ($P<0.05$); cell cycle phase of EGF+VD₃ group and N group was not statistically significant. Compared with EGF group, cells at G1 phase of EGF+VD₃ group were increased, while those at S, G₂/M phase were decreased, with lower PI, and the difference was statistically significant ($P<0.05$, **Table 1**, **Figure 2**).

Impact of 1,25(OH)₂D₃ on the apoptosis rate of MCs

Compared with N group, the apoptosis rate of EGF group was significantly reduced, and that

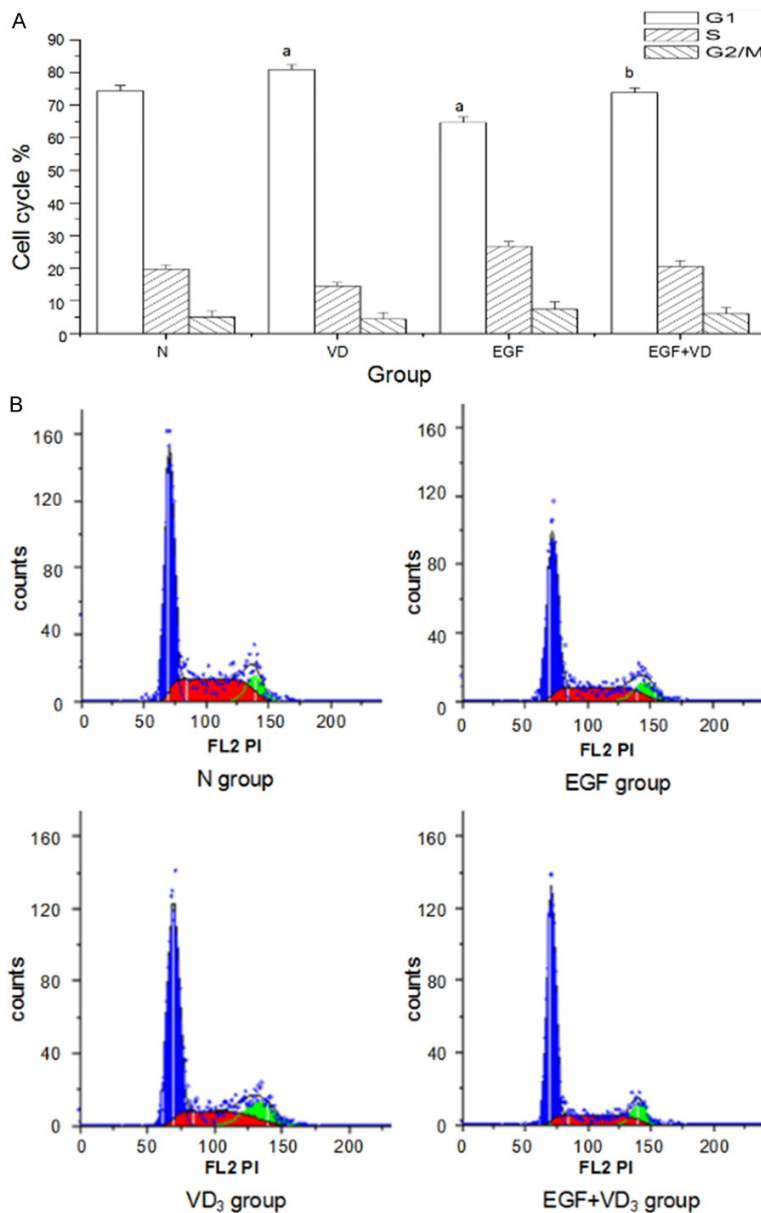


Figure 2. A: Flow cytometry detect the cell cycle of each group; B: Flow cytometry detect the cell cycle of each group.

Table 2. Flow cytometry detect the apoptosis rate of cells of each group (n=10, %, $\bar{x} \pm s$)

Group	Apoptosis rate (%)
N group	3.107±0.989
EGF group	0.563±0.276 ^a
VD group	6.395±0.767 ^a
EGF+VD group	3.449±0.959 ^b
F	133.598 [*]

Note: ^a: compared with N group, ^b: compared with EGF group, ^{*}P<0.05; ^{*}P<0.01.

of VD₃ group was significantly higher ($P<0.01$). The apoptosis rate of EGF+VD₃ group had no significant difference with N group. Compared with the EGF group, the apoptosis rate of EGF+VD₃ group was significantly increased ($P<0.01$, Table 2 and Figure 3).

The expression of PCNA detected by Western blot

Compared with N group, VD₃ group showed lighter and thinner protein bands, with significantly reduced expression of PCNA. However, EGF group had crude and deeper protein, with significant increase in the expression of PCNA. Compared with EGF group, EGF+VD₃ group showed lighter and thinner protein bands, with significantly decreased expression of PCNA, but close to N group (Figure 4).

The expression of Caspase-3 detected by Western blot

Compared with N group, Caspase-3 expression in EGF group was significantly reduced, and Caspase-3 expression levels were significantly higher in VD group, while the expression in EGF+VD group was similar to N group. Compared with the EGF group, Caspase-3 expression levels in EGF+VD group were significantly higher (Figure 5).

The proliferation of mesangial cells detected by CCK-8 method

Compared with the control group, the IR inhibition rate in EGF group was -23.60%, which significantly promoted the proliferation of MCs ($P<0.01$); the IR inhibition rate in VD group was 12.58%, which significantly inhibited the proliferation of MCs ($P<0.01$); EGF+VD group had no significant proliferation of MCs ($P>0.05$). Compared with EGF group, EGF+VD group had

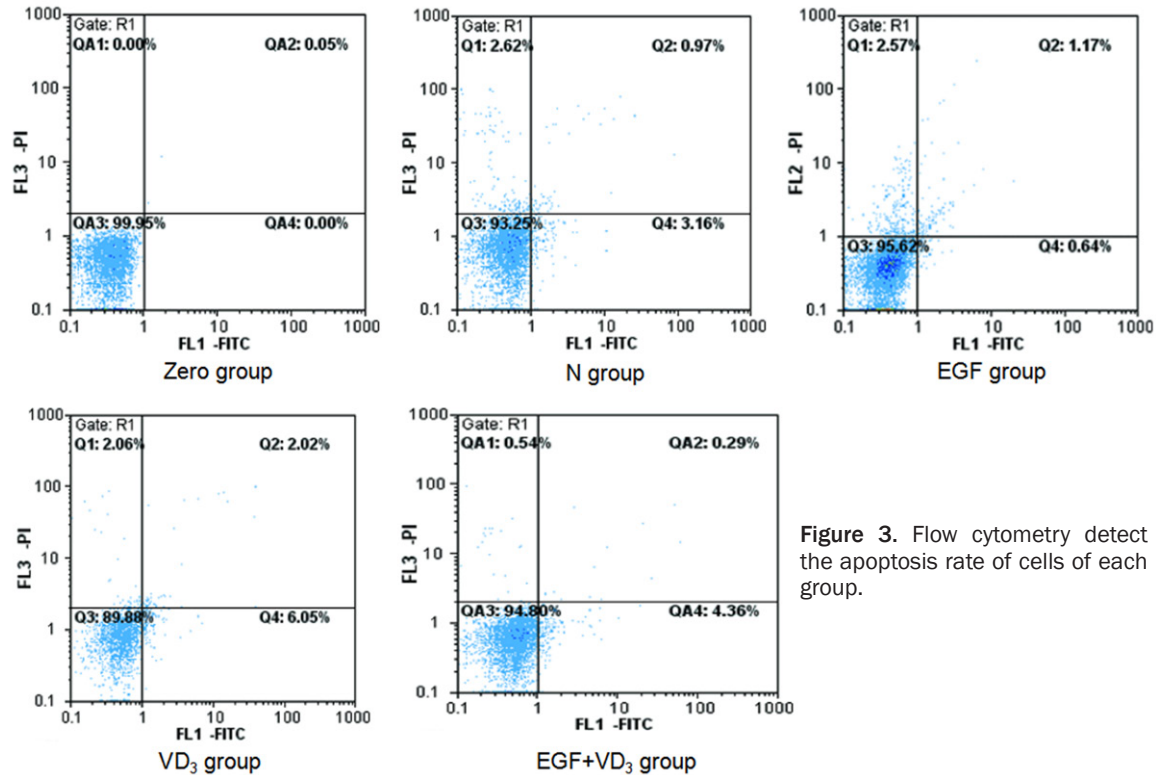


Figure 3. Flow cytometry detect the apoptosis rate of cells of each group.

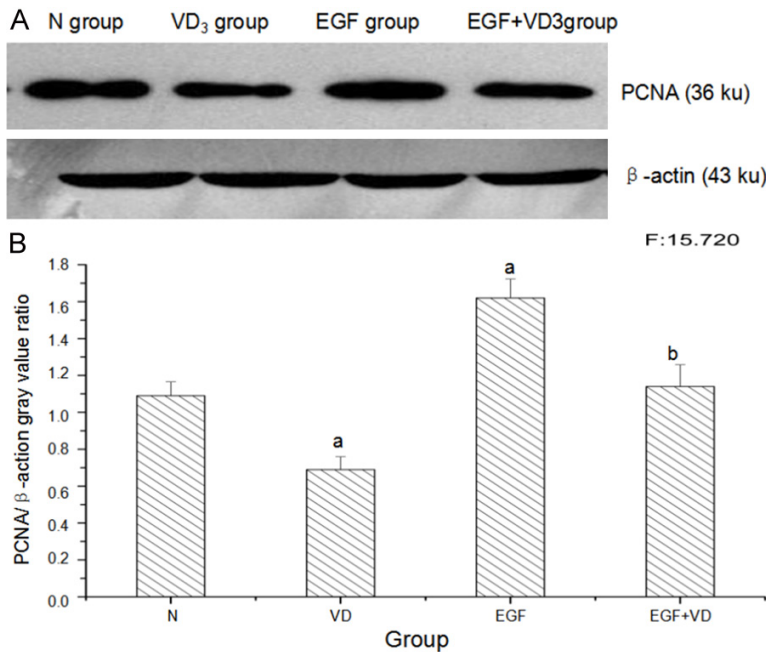


Figure 4. A: Western-blot detection of cases in each experimental group mesangial cells PCNA expression; B: Mesangial cells in each group situation PCNA/β-actin gray value comparisons.

proliferation of MCs, which had a significantly inhibitory effect ($P < 0.01$, Table 3, Figure 6).

significantly, and the number of S, G₂/M phase cells was decreased significantly, suggesting

Discussion

1,25(OH)₂D₃ is the biologically active form of vitamin D₃. In addition to the classic calcium and phosphorus regulation, but it also arrests cell cycle, inhibits cell proliferation and induces apoptosis. All these effects are getting more and more attention [11, 12]. 1,25(OH)₂D₃ can reduce proteinuria, inflammatory cells, inhibit MC proliferation and accelerate the important role of glomerular injury repair during the treatment of rats with nephritis. Our previous study found that active vitamin D₃ can inhibit proliferation of cultured rat mesangial cells and induce apoptosis of MC [13]. The assay results of MC cell cycle showed G1 phase cells were increased

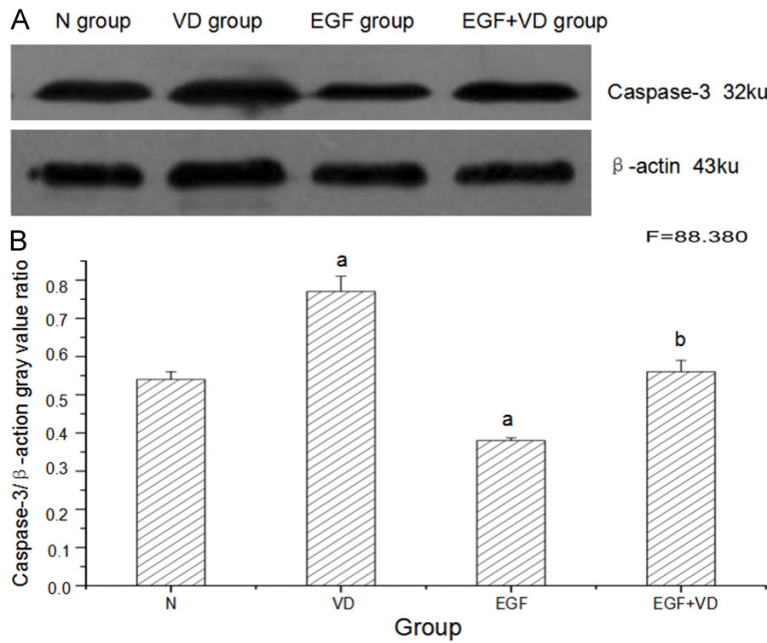


Figure 5. A: Western-blot detection of cases in each experimental group mesangial cells Caspase-3 expression; B: Mesangial cells in each group situation Caspase-3/β-actin gray value comparisons.

Table 3. CCK-8 in each group after the intervention 48h human mesangial cell proliferation ($\bar{x} \pm s$)

Group	A ₄₅₀ ($\bar{x} \pm s$)	IR (%)
N group	0.8381±0.0568	0.00
EGF group	1.0359±0.0452 ^a	-23.60
VD ₃ group	0.7327±0.0365 ^a	12.58
EGF+VD ₃ group	0.8471±0.0285 ^b	-1.07
F	128.974	
P	<0.01	

Note: ^a: compared with N group, $P < 0.01$; ^b: compared with EGF group, $P < 0.01$.

that 1,25(OH)₂D₃ can block cell cycle at G1 phase, thus inhibiting proliferation of MCs. Moreover, flow cytometry results showed that compared with N group, the cell count which expressed the MC apoptosis rate was increased after 1,25(OH)₂D₃ intervention for 48 h, indicating that 1,25(OH)₂D₃ can induce apoptosis of human MCs, consistent with results of previous studies.

PCNA is an established method to study cell proliferative activity, while it is found to have regulatory effects on cell cycle and DNA replication in a variety of cells [14]. Studies have found

that after 1,25(OH)₂D₃ intervention in a rat model of renal failure, PCNA can be used to detect proliferation of MCs. When the cells were stimulated by apoptosis signal, Caspases activated and induced apoptosis, and both inside and outside apoptosis pathways increased expression of Caspase-3 [15]. 1,25(OH)₂D₃ can induce Caspase-3 activation, while apoptosis is related to decrease of protein kinase B (Akt) phosphorylation levels, whereas the MC apoptotic pathway depends on the activation of Caspase-3 [16, 17]. In this study, results of Western blot analysis of PCNA, Caspase-3 expression levels showed PCNA expression of VD group was significantly lower than the normal level, and the Caspase-3 expres-

sion level was significantly higher than the normal level, suggesting that 1,25(OH)₂D₃ inhibited cell proliferation by suppressing the expression of PCNA in human MCs and simultaneously upregulating the expression of Caspase-3 to induce apoptosis.

EGF can reduce apoptosis and promote cell proliferation [18]. Research has shown that aldosterone stimulates MC proliferation via activating EGFR-dependent PI3K/Akt signaling pathway [19]. Therefore, in this study, EGF-induced MC proliferation microenvironment was established, and it was observed that 1,25(OH)₂D₃ can inhibit proliferation and promote apoptosis of MCs under the condition that MC proliferation was induced. The results showed compared with EGF group, G1 phase cells in EGF+VD group were significantly increased, and the number of cells at S, G₂/M phase was significantly reduced, while the MC apoptosis rate was significantly higher. Therefore, 1,25(OH)₂D₃ can inhibit MC proliferation under the role of EGF in promoting apoptosis. We also found that compared to EGF group, EGF+VD group had a decreased expression level of PCNA and an increased expression level of Caspase-3, indicating that 1,25-(OH)₂D₃ may suppress MC proliferation by inhibiting the

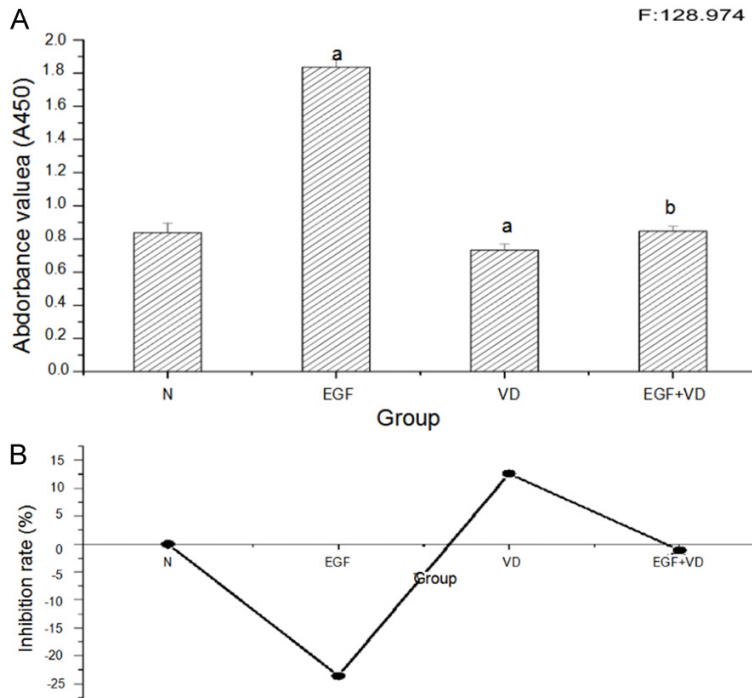


Figure 6. A: Each group after the intervention 48 h detected by CCK-8 on cell proliferation; B: Compare inhibition rate in each group after the intervention 48 h.

expression of PCNA under the role of EGF, whereas EGF may inhibit apoptosis of MCs by down-regulating the expression of Caspase-3. Furthermore, $1,25(\text{OH})_2\text{D}_3$ can inhibit proliferation of MCs.

In summary, $1,25(\text{OH})_2\text{D}_3$ can inhibit the proliferation of human MCs by arresting cell cycle and inducing apoptosis; $1,25(\text{OH})_2\text{D}_3$ upregulates the expression of Caspase-3 to inhibit the proliferation of human MCs and induce their apoptosis by inhibiting the expression of PCNA in human MCs; $1,25(\text{OH})_2\text{D}_3$ can inhibit proliferation of human MCs under the role of EGF. But at present, the specific mechanism of $1,25(\text{OH})_2\text{D}_3$ affecting MCs and whether PI3K/Akt signaling pathway is involved remain to be confirmed by further studies.

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Disclosure of conflict of interest

None.

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