Food Science and Technology
ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)
DOI: https://doi.org/10.1590/fst.77221

DHA-promoted repair of human corneal epithelial cells in high-glucose environment

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Abstract

Aim: The purpose of this study was to evaluate DHA's effect and relative mechanisms in diabetic keratopathy (DK) treatment by vitro study. Methods and materials: Using high glucose to make DK cell model; measuring cell apoptosis by flow cytometry and Hoechst 33342 staining; evaluating cell migration abilities by wound healing assay; relative gene and proteins including PEDF, VEGF, Caspase-3, Caspase-9 and NF-κB(p65) expression by RT-qPCR and WB assay; And evaluatign PDEF and p-NF-κB(p65) protein expression by immunofluorescence detection. Results: After high glucose treatment, TKE-2 cell apoptosis was significantly increased and cell migration ability was significantly depressed with PEDF, Caspase-3, Caspase-9 and NF-κB(p65) gene and protein significantly increasing and VEGF expression significantly depressing ($P < 0.001$, respectively); with DHA supplement, TKE-2 cell biological activities were significantly improved with relative gene and proteins significantly changed; However, with DHA and PEDF supplement, DHA's treatment was disappeared with PEDF, Caspase-3, Caspase-9 and NF-κB(p65) gene and protein significantly increasing and VEGF expression significantly depressing ($P < 0.001$, respectively). Conclusion: DHA had effects to improved DK by regulation PEDF pathway in vitro study.

Keywords: DK; DHA; PEDE; TKE-2.

Practical Application: DNA could improve repair of human corneal epithelial cells.

1 Introduction

Diabetes mellitus (DM) is one the most common systemic diseases. China is the country with the most diabetes patients around the world, and the incidence is gradually increasing (Yang et al., 2010). Recently, studies have shown that corneal lesions may occur in about 47% to 64% of diabetes patients. At present, there are few studies on diabetic keratopathy (DK), which was first proposed by Schultz in 1981 and has clinical features including significantly increased corneal tactile sensation, significantly reduced corneal sensitivity, delayed in repair of corneal epithelial injury, repeated exfoliation after healing of the corneal epithelium, and corneal ulcer with neuroimaging disorder (Schultz et al., 1981; Schwartz, 1974; Xu & Yu, 2011; Saghizadeh et al., 2010). Therefore, how to improve the delayed repair of corneal epithelial injury in a high-glucose environment has become one of the hot topics in ophthalmology research.

Docosahexaenoic acid (DHA) has anti-inflammatory effects, but its mechanism remains unclear. Studies have shown that DHA can produce anti-inflammatory effects by inhibiting TLR2/3/4 and TNF-α [6]. Research on the therapeutic effect of DHA on DK is currently relatively limited. In this study, a high concentration of glucose (25 mmol/L) was used to stimulate mouse corneal epithelial progenitor cells (TKE2) to establish an in vitro DK cell model. Different concentrations of DHA (50, 100, and 200 mg/L) were used to intervene in the cells of model group, so as to observe the biological activity of TKE2 cells in each group and to further explore the mechanism of DHA.

2 Materials and methods

2.1 Materials

Corneal epithelial progenitor cells (TKE2) (ATCC); high-glucose DMEM (Giboco); trypsin 0.25% (NanJing KeyGen Biotech); flow cytometry kit (NanJing KeyGen Biotech); Hoechst 33258 staining solution (Beyotime Institute of Biotechnology); PEDF, VEGF, Caspase-3, Caspase-9, and p-NF-κB (p65) antibodies (Abcam); PEDF, VEGF, Caspase-3, Caspase-9, and NF-κB (p65) primer sequence (NanJing KeyGen Biotech).

2.2 Cell culture

After resuscitation, TKE2 cells were resuspended in complete medium (high-glucose DMEM+10% FBS) and placed in an incubator with 5% carbon dioxide at 37 °C. When the cells had grown to more than 80% adherently, subculture was conducted: The cells in the logarithmic growth phase were digested to prepare a single cell suspension, which was then inoculated in the culture plate at 1×104 / well and cultured in a cell incubator with 0.5% CO2 at 37 °C. After the cells were subcultured for one to two passages, they were subcultured to a total of 10 culture dishes of 35mm in diameter following the above method. The human corneal epithelial cells were stimulated with high-glucose concentration (25 mmol/ L glucose in DMEM complete medium) to establish the Model group.

Received 23 Aug., 2021
Accepted 05 Sept., 2021
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2.3 Transfection method

The TKE2 cells were inoculated in a 6-well plate and incubated in an incubator with 5% CO_2 at 37 °C, and then the cells were plated; 2 h before transfection, the cells were transferred to a serum-free DMEM medium; 100 μL serum-free opti-DMEM with 10 μL of PEDF (synthesized by NanJing KeyGen Biotech) was diluted, mixed gently with a pipette tip, and set aside for 5 min at room temperature for later use. Lipofectamine™ 2000 was gently mixed before use, and then 5 μL of Lipofectamine™ 2000 was diluted in 100 μL of opti-DMEM and let stand for 5 min at room temperature. Lipofectamine™ 2000 and PEDF were mixed gently, mixed well, and placed for 3 min to form a transfection complex. Then 200 μL of the mixed solution was added to each well and placed in an incubator for 6 h. The mixed solution was transferred to a conventional medium and cultured in an incubator with 5% CO_2 at 37 °C for 48 h, then the cells were used for the next experiment.

2.4 Cell processing

NC group: TKE2 cells were cultured with conventional DMEM complete medium; Model group cells: DHA-L group: 50 μM of DHA was added on the basis of Model group; DHA-M group: 100 μM of DHA was added on the basis of Model group; DHA-H group: 200 μM of DHA was added on the basis of Model group; DHA+PEDF group: after transfection of PEDF into the TKE2 cells, 200 μM of DHA was added on the basis of Model group.

2.5 Cell apoptosis by flow cytometry

Cells of each group were collected and centrifuged at 1000 g/min for 5 min, and the supernatant was removed. The pellet was resuspended in 500 μL of buffer, added with 5 μL of Annexin V- fluorescein isothiocyanate and 10 μL of propidium iodide (PI), incubated at room temperature for 5 min in the dark, and tested on a flow cytometer.

2.6 Hoechst 33342 staining

After the cells in each group were treated for 48 h, the suspending cells were collected by centrifugation, and the adherent cells were digested with trypsin. Cell smears were prepared from a single cell suspension and fixed in 4% paraformaldehyde at 4 °C for 10 min. After FBS wash, Hoechst 33342 (1 μg/mL) staining solution was added for 10 min. After the slides were washed with distilled water, excess liquid was removed with filter paper, mounted with the mounting medium and observed with a fluorescence microscope. More than 10 random fields were observed, and the number of apoptotic cells was counted.

2.7 RT-qPCR assay

After the cells of each group were treated for 48 h, RNA was extracted according to the instructions of the RTIzol reagent, and the OD_{260}/OD_{280} of the RNA solution was detected by the nucleic acid protein analyzer. The reverse transcription reaction was conducted according to the instructions of the cDNA synthesis kit, and the PCR reaction solution was configured according to the instructions of the Taq enzyme mixture. The amplification program was set up for primer sequence as shown in Table 1, and PCR amplification reaction was conducted. PCR reaction conditions: 50 °C for 2 min, 95 °C for 10 min (1 cycle); 95 °C for 5 s, 65 °C for 1 minute, 75 °C for 20 s (45 cycles); and 75 °C for 5 min (1 cycle). Each real-time PCR was repeated 3 times, GAPDH was used as an internal reference, and $2^{-\Delta\Delta Ct}$ was used to calculate the relative expression of the targeted gene.

2.8 Wound healing detection

Cells of each group were collected after corresponding treatment for 48 h, and 5×10^5 cells were inoculated in a 6-well plate. After the cells were plated with the bottom of the petri dish, a ruler was used to guide the pipette tip to be perpendicular to the petri dish without tilting. Then the marked cells were washed off with PBS for three times, added into the serum-free medium, and placed and cultured in an incubator with 5% CO_2 at 37 °C. The cells were sampled and photographed at 0, 24, and 48 h.

2.9 Western Blotting (WB)

The total protein of cells was extracted, then electrophoresis was conducted on the denatured sample after boiling to separate the protein. After that the protein was transferred to the PVDF membrane via the membrane transfer procedure, and 5% skim milk was used as a blocking solution for 1 hour. The PVDF membrane was completely covered with primary antibody diluent containing 1:1000 of PEDF, VEGF, Caspase-3, Caspase-9, p-NF-κB (p65), and GAPDH antibodies and incubated overnight at 4 °C. The membrane was removed and washed with TBST solution for 3×10 min, and the secondary antibody was incubated for two hours at room temperature and rinsed with TBST solution for 3×10 min; chemiluminescent reagent was added to develop imaging. The expression of the target protein was denoted by the ratio of the gray value of the target protein band to that of the GAPDH band.

Table 1. The primer sequence.

Gene Name	F:(5'-3')	R:(5'-3')
PEDF	TGTGCAGGCTTAGAGGGACT	GTTCACGGGGCTTAGAGGGACT
VEGF	TTCAGAGCGGAGAAAGCATT	GAGGAGGCTCCTTCCTGC
Caspase-3	CAGACAGTGGAACTGACGAT	TTTCAGCATGGCGCAAAGTG
Caspase-9	CTCGGTCTGGTACACTATGTCGATG	GGTTAACCCGGGTAAGAATGTGCA
NF-κB(p65)	TGCTGTGCGGCTCTGCTTCC	AGGCTGGGGTCTGCGTAGGG
GAPDH	GGTGAAGGTCGGTGTGAACG	GCTCCTGGAAGATGGTGATGG

2.10 Immunofluorescence detection

The cells of each group after treatment for 48 h were collected. The slides were dipped and rinsed in PBS three times, 3 min each time. After the PBS solution was absorbed, goat serum was dripped on the slide, which was then blocked for 30 min at room temperature. Primary antibody of PEDF or p-NF-κB (p65) (1:1000) was added to the corresponding slide, which was incubated overnight in a humid chamber at 4 °C. The slides were washed with PBST three times, 3 min each time. After the PBST was completely absorbed, the secondary antibody (1:2000) was added dropwise and incubated in a humid chamber at 37 °C for 1 hour. After the secondary antibody was discarded, the slides were rinsed with PBST three times, 5 min each time. After DAPI was added dropwise, the slides were incubated in the dark for 5 minute and rinsed with PBST 4 times, 5 min each time. Finally, the samples were observed and photographed under a fluorescence microscope, and Image pro plus 6.0 software was used for fluorescence quantitative analysis.

2.11 Statistical analysis

GraphPad Prism 6.0 software was used for graphing. All data were expressed as mean ± standard deviation (Mean±SD). SPSS 17.0 software was used for statistical analysis. Data comparison between groups was performed by analysis of variance. Pairwise comparison between means was performed by t-test. $P < 0.05$ was considered statistically significant.

3 Results

3.1 DHA-improved repair of high-glucose damages to TKE2 cells

The result of flow cytometry (Figure 1) showed that compared with the NC group, the apoptosis rate and the number of cells with nuclear damage were significantly increased in the high-glucose-stimulated Model group ($P < 0.001$, respectively, Figures 1A, B). After DHA intervention, compared with the Model group, the apoptosis rate and the number of cells with nuclear damage were significantly reduced in the DHA groups ($P < 0.05$, respectively, Figures 1A, B), and there were significant differences between the DHA groups ($P < 0.05$, respectively, Figures 1A, B).

3.2 Effects of DHA on the recovery of TKE2 cells in high-glucose environment

The result of wound healing test showed that compared with the NC group, the migration ability of TKE2 cells in the Model group in the high-glucose environment was significantly inhibited ($P < 0.001$, Figure 2). To explore the effects of DHA on the recovery of TKE2 cells in a high-glucose environment, wound healing method was used to treat TKE2 cells in a high-glucose environment after intervention with various concentrations of DHA. Compared with the Model group, the invasion ability of TKE2 cells was significantly restored ($P < 0.05$, respectively, Figure 2), and there were also significant differences between the DHA intervention groups ($P < 0.05$, respectively, Figure 2).

3.3 DHA affects relative genes expression

The result of RT-qPCR showed that compared with the NC group, the expression levels of PEDF, Casapase-3, Caspase-9, and NF-κB (p65) genes were significantly increased ($P < 0.001$, respectively, Figure 3), while that of VEGF gene was significantly reduced in the Model group ($P < 0.001$, Figure 3). After DHA intervention, compared with the Model group, the expression levels of PEDF, Casapase-3, Caspase-9, and NF-κB (p65) genes were significantly reduced ($P < 0.05$, respectively, Figure 3), while that of VEGF gene was significantly increased in the DHA intervention groups ($P < 0.05$, respectively, Figure 3). Moreover, among the DHA intervention groups, there were significant differences in the expressions of VEGF, PEDF, Casapase-3, Caspase-9, and NF-κB (p65) genes ($P < 0.05$, respectively, Figure 3).

3.4 DHA affects relative protein expression

Western blotting (WB) result showed that compared with the NC group, the expression levels of PEDF, Casapase-3, Caspase-9, and NF-κB (p65) proteins were significantly increased ($P < 0.001$, respectively, Figure 4), while that of VEGF gene was significantly reduced in the Model group ($P < 0.001$, Figure 3). After DHA intervention, compared with the Model group, the expression levels of PEDF, Casapase-3, Caspase-9, and p-NF-κB (p65) proteins were significantly reduced ($P < 0.05$, respectively, Figure 4), while that of VEGF protein was significantly increased in the DHA intervention groups ($P < 0.05$, respectively, Figure 4). Moreover, among the DHA intervention groups, there were significant differences in the expressions of VEGF, PEDF, Casapase-3, Caspase-9, and p-NF-κB (p65) proteins ($P < 0.05$, respectively, Figure 4).

3.5 DHA affects PEDF and p-NF-κB(p65) protein fluorescent expression

Cell immunofluorescence showed that compared with the NC group, the expression levels of PEDF and p-NF-κB (p65) proteins were significantly increased in the Model group ($P < 0.001$, respectively, Figures 5A, B). After DHA intervention, compared with the Model group, the expression levels of both PEDF and p-NF-κB (p65) proteins were significantly reduced in the DHA intervention group ($P < 0.001$, respectively, Figures 5A, B). In terms of the expression levels of PEDF and p-NF-κB (p65) proteins, there were significant differences between the DHA intervention groups ($P < 0.05$, respectively, Figures 5A, B).

3.6 Apoptosis and nuclear damage of TKE2 cells in each group

The results of flow cytometry and Hoechst 33342 staining showed that compared with the NC group, the apoptosis rate and the number of cells with nuclear damage were both significantly increased in the Model group ($P < 0.001$, respectively, Figure 6A, B). After DHA intervention, compared with the Model group, the apoptosis rate and the number of cells with nuclear damage were both significantly reduced in the DHA group ($P < 0.001$, respectively, Figure 6A, B). However, after transfection of PEDF into cells, compared with the DHA group, the apoptosis rate and the number of cells with nuclear damage

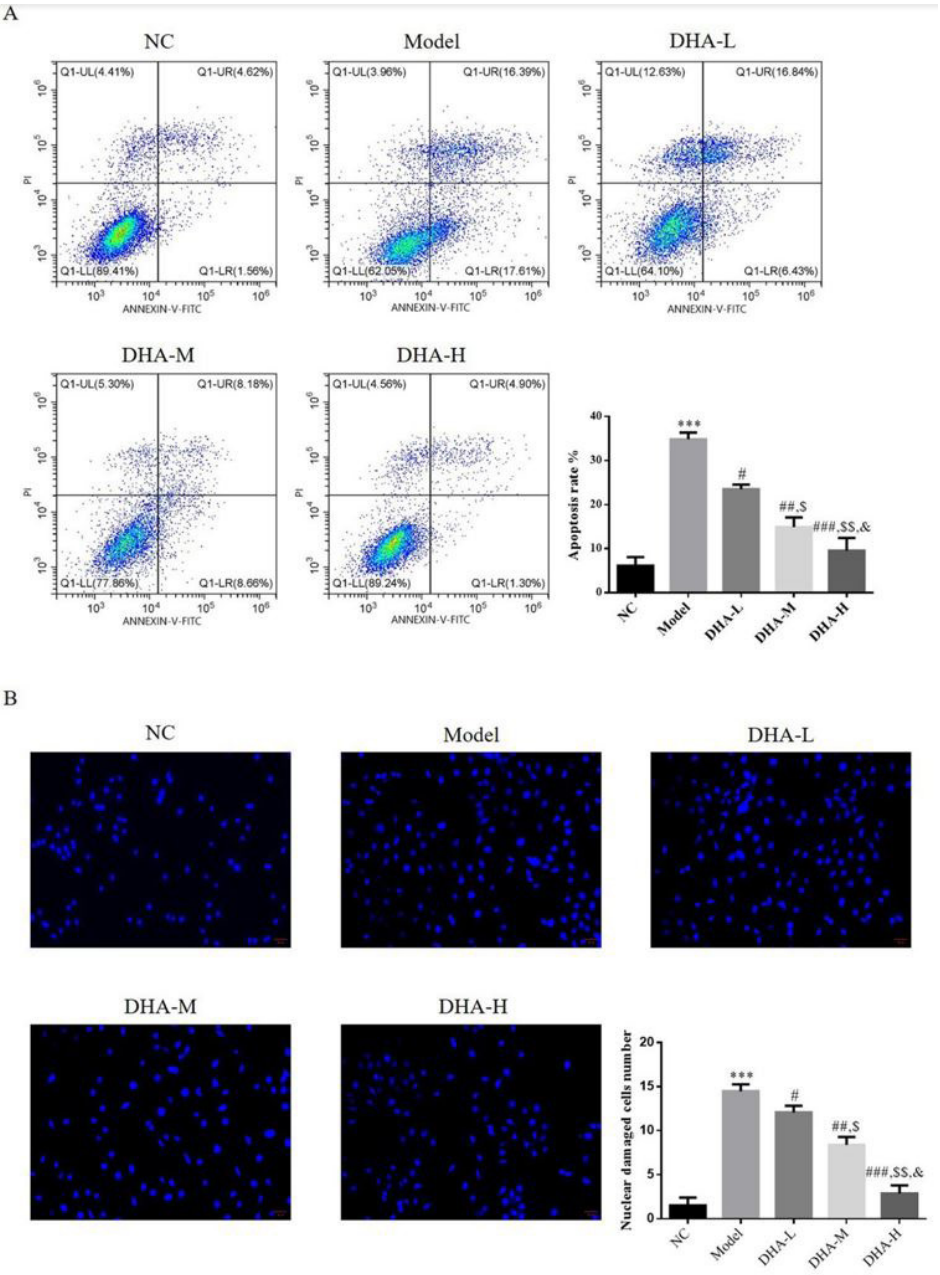

Figure 1. DHA-improved repair of high-glucose damages to TKE2 cells. NC group: TKE2 cells were cultured with conventional DMEM complete medium; Model group cells: DHA-L group: 50 μM of DHA was added on the basis of Model group; DHA-M group: 100 μM of DHA was added on the basis of Model group; DHA-H group: 200 μM of DHA was added on the basis of Model group. A. The cell apoptosis rate in different groups by flow cytometer. B. Nuclear damaged cell number in different cell groups (100×). ***: $P < 0.001$, compared with NC group; #: $P < 0.05$, ##: $P < 0.01$, ###: $P < 0.001$, compared with Model group; $: $P < 0.05$, $$: $P < 0.01$, compared with DHA-L; &: $P < 0.05$, compared with DHA-M.

were both significantly increased in DHA+PEDF group ($P < 0.001$, respectively, Figure 6A, B).

3.7 The role of PDEF in DHA treatment

The result of wound healing showed that compared with the NC group, the wound healing rates at 24 h and 48 h were both significantly reduced in the Model group ($P < 0.001$, respectively, Figure 7). 在After DHA intervention, compared with the Model group, the wound healing rates at 24 h and 48 h were both significantly increased in the DHA group ($P < 0.001$, respectively, Figure 7). However, after PDEF was transfected into the cells at the same time, compared with the DHA group, the wound healing rates at 24 h and 48 h were both significantly reduced in the DHA+PEDF group ($P < 0.001$, respectively, Figure 7).

3.8 Related gene expression

RT-qPCR showed that compared with the NC group, the expression levels of PEDF, Caspase-3, Casapase-9, and NF-κB

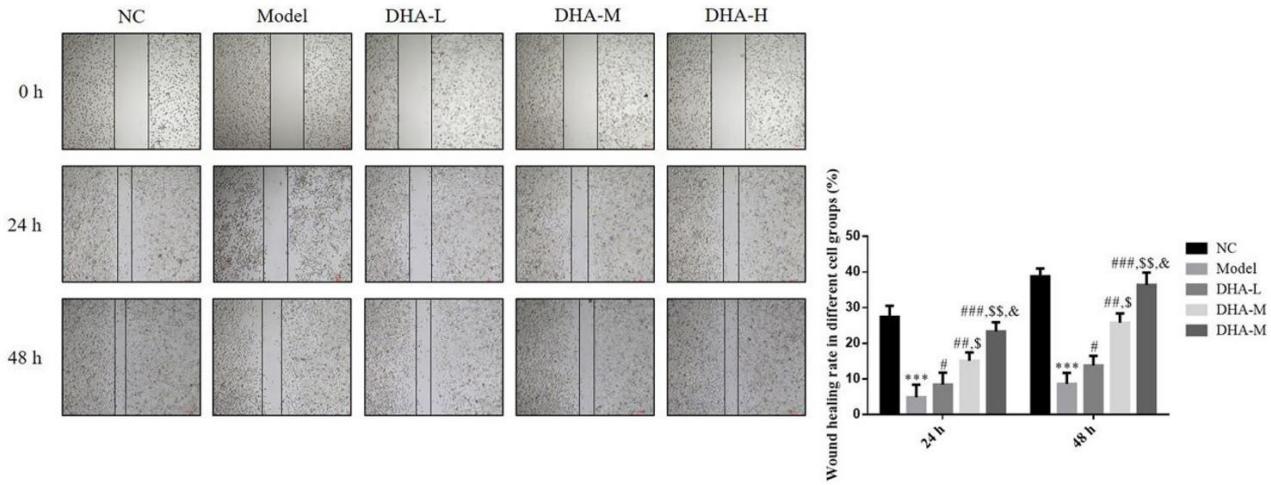

Figure 2. Effects of DHA on the recovery of TKE2 cells in high-glucose environment. NC group: TKE2 cells were cultured with conventional DMEM complete medium; Model group cells: DHA-L group: 50 μM of DHA was added on the basis of Model group; DHA-M group: 100 μM of DHA was added on the basis of Model group; DHA-H group: 200 μM of DHA was added on the basis of Model group. ***: P < 0.001, compared with NC group; #: P < 0.05, ##: P < 0.01, ###: P < 0.001, compared with Model group; $: P < 0.05, $$: P < 0.01, compared with DHA-L; &: P < 0.05, compared with DHA-M.

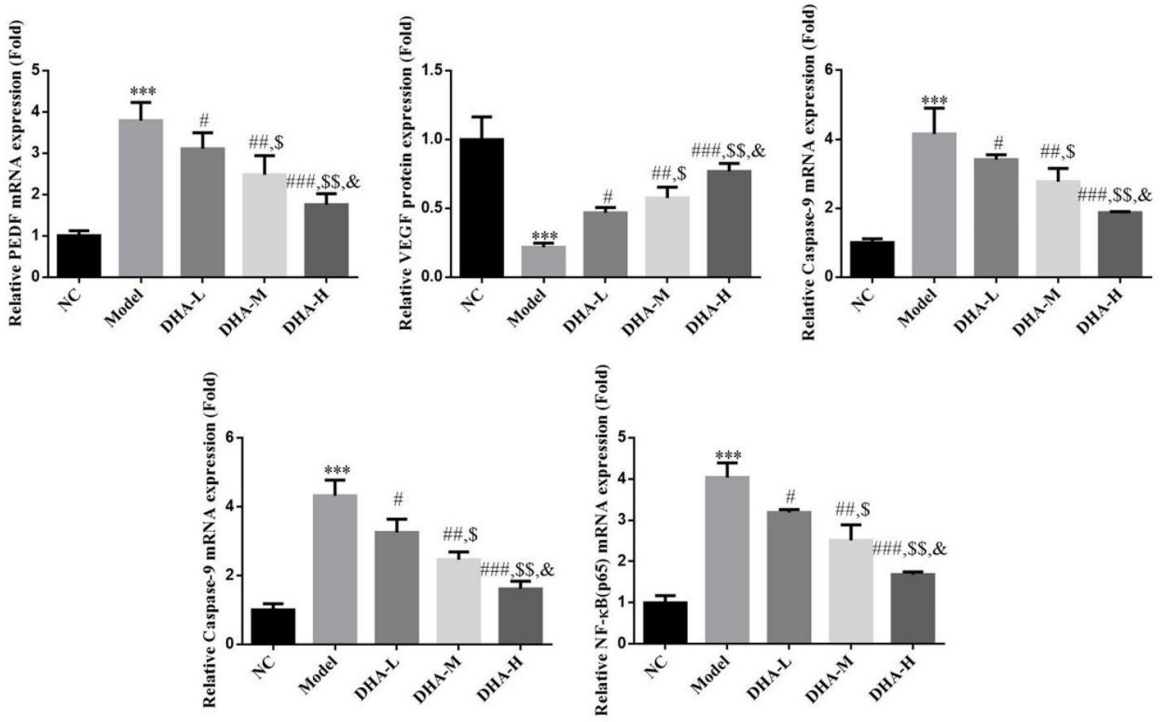

Figure 3. DHA affects relative gene expression. NC group: TKE2 cells were cultured with conventional DMEM complete medium; Model group cells: DHA-L group: 50 μM of DHA was added on the basis of Model group; DHA-M group: 100 μM of DHA was added on the basis of Model group; DHA-H group: 200 μM of DHA was added on the basis of Model group. ***: P < 0.001, compared with NC group; #: P < 0.05, ##: P < 0.01, ###: P < 0.001, compared with Model group; $: P < 0.05, $$: P < 0.01, compared with DHA-L; &: P < 0.05, compared with DHA-M.

(p65) genes were significantly increased, while that of VEGF gene was significantly reduced in the Model group (P < 0.001, Figure 8). After DHA intervention, compared with the Model group, the expression levels of PEDF, Caspase-3, Casapase-9, and NF-κB (p65) genes were significantly reduced, while that of VEGF gene was significantly increased in the DHA group (P < 0.001, Figure 8). After PEDF was transfected into the cells at the same time, compared with the DHA group, the

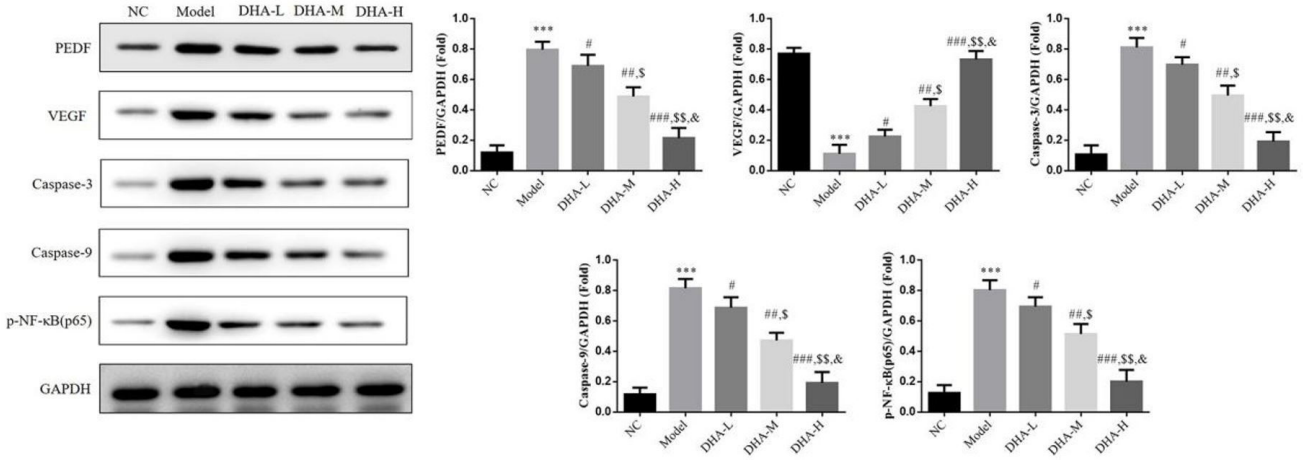

Figure 4. DHA affects relative protein expression. NC group: TKE2 cells were cultured with conventional DMEM complete medium; Model group cells: DHA-L group: 50 μM of DHA was added on the basis of Model group; DHA-M group: 100 μM of DHA was added on the basis of Model group; DHA-H group: 200 μM of DHA was added on the basis of Model group. ***: P < 0.001, compared with NC group; #: P < 0.05, ##: P < 0.01, ###: P < 0.001, compared with Model group; $: P < 0.05, $$: P < 0.01, compared with DHA-L; &: P < 0.05, compared with DHA-M.

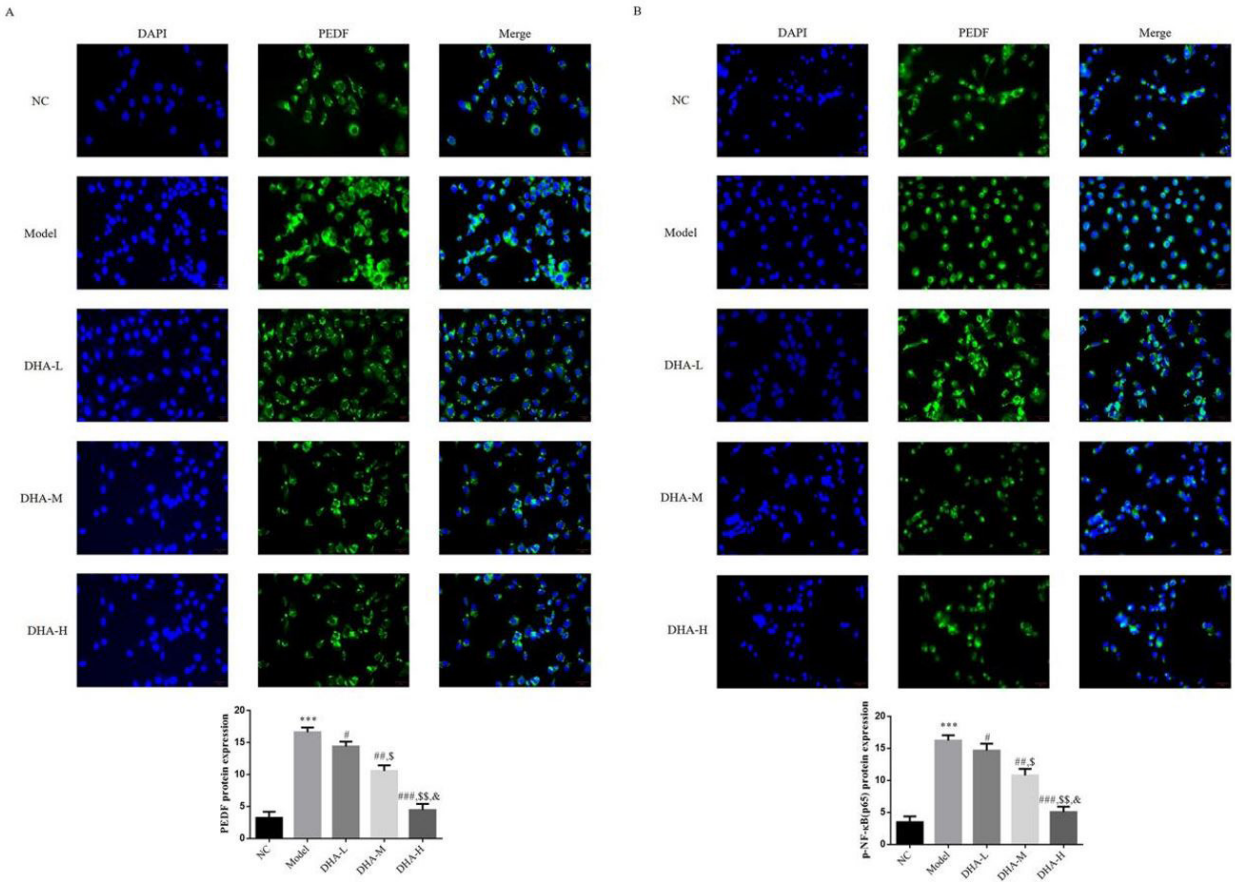

Figure 5. DHA affects PEDF and p-NF-κB(p65) protein expression. NC group: TKE2 cells were cultured with conventional DMEM complete medium; Model group cells: DHA-L group: 50 μM of DHA was added on the basis of Model group; DHA-M group: 100 μM of DHA was added on the basis of Model group; DHA-H group: 200 μM of DHA was added on the basis of Model group. A. PEDF protein expression (200×). B. p-NF-κB(p65) protein expression (200×). ***: P < 0.001, compared with NC group; #: P < 0.05, ##: P < 0.01, ###: P < 0.001, compared with Model group; $: P < 0.05, $$: P < 0.01, compared with DHA-L; &: P < 0.05, compared with DHA-M.

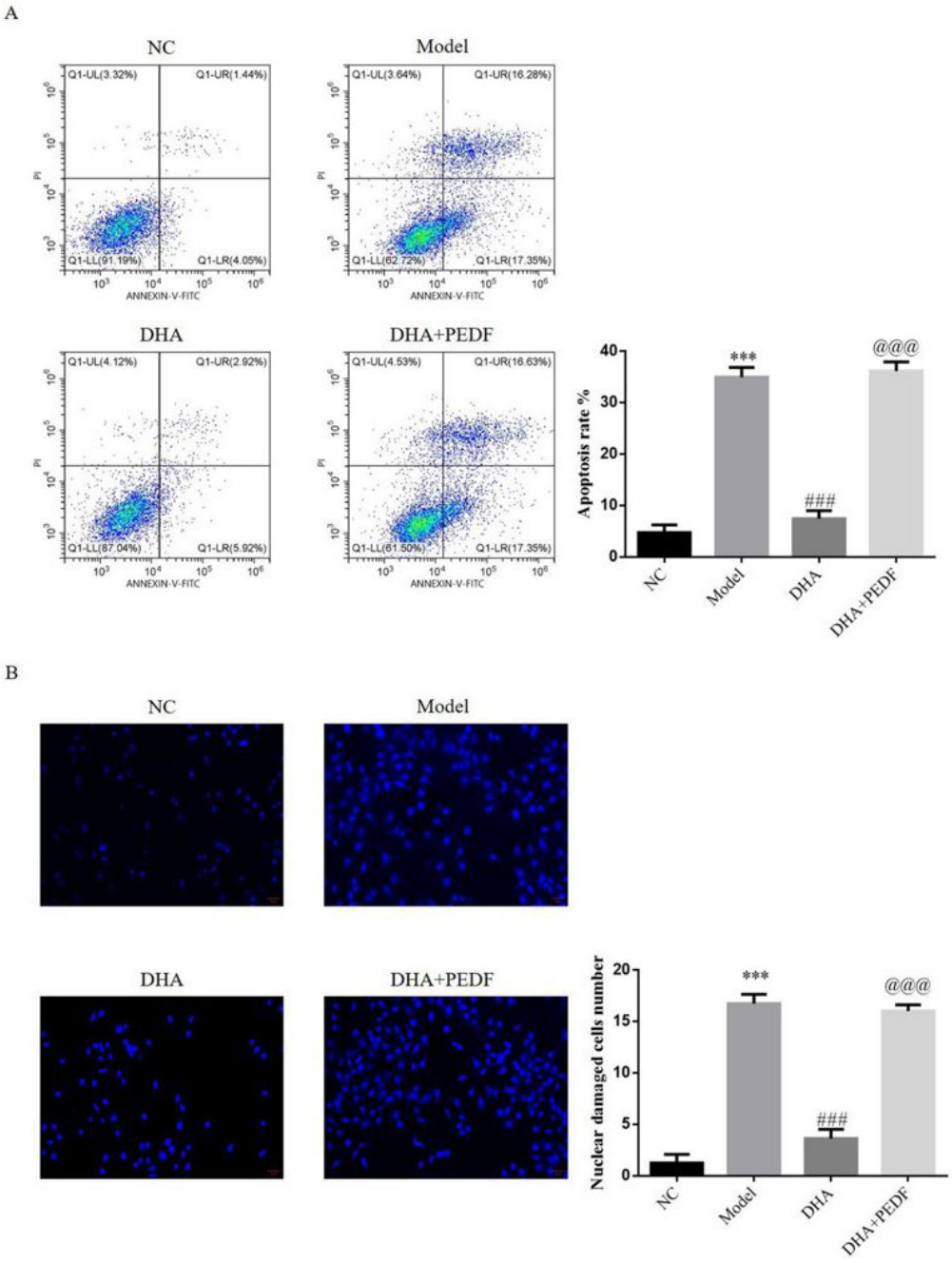

Figure 6. Apoptosis and nuclear damage of TKE2 cells in each group. NC group: TKE2 cells were cultured with conventional DMEM complete medium; Model group cells: DHA-L group: 50 μM of DHA was added on the basis of Model group; DHA: 200 μM of DHA was added on the basis of Model group; DHA+PEDF group: after transfection of PEDF into the TKE2 cells, 200 μM of DHA was added on the basis of Model group. A. Apoptosis rate. B. Nuclear damaged cells number. ***: $P < 0.001$, compared with NC group; ###: $P < 0.001$, compared with Model group; @@@: $P < 0.001$, compared with DHA group.

expression levels of PEDF, Caspase-3, Casapase-9, and NF-κB (p65) genes were significantly increased, while that of VEGF gene was significantly reduced in the DHA+PEDF group ($P < 0.001$, Figure 8).

3.9 Related protein expression

The result of WB showed that compared with the NC group, the expression levels of PEDF, Caspase-3, Casapase-9, and NF-κB (p65) proteins were significantly increased, while that of VEGF protein was significantly reduced in the Model group ($P < 0.001$, Figure 9). After DHA intervention, compared with the Model group, the expression levels of PEDF, Caspase-3, Casapase-9, and NF-κB (p65) proteins were significantly reduced, while that of VEGF protein was significantly increased in the DHA group ($P < 0.001$, Figure 9). After PEDF was transfected into the cells at the same time, compared with the DHA group, the expression levels of PEDF, Caspase-3, Casapase-9, and NF-κB

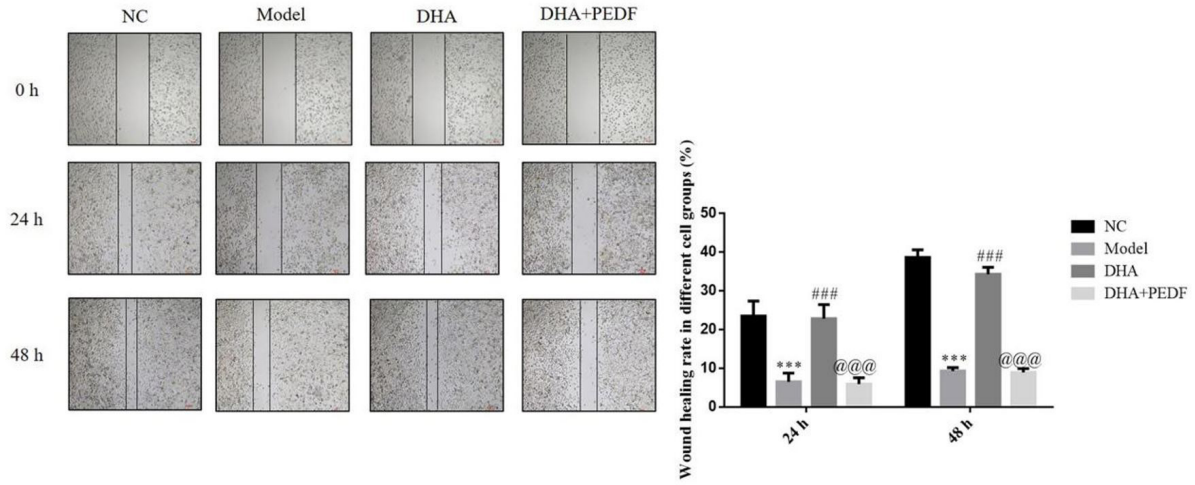

Figure 7. Wound healing rate in different cell groups by Wound healing assay (100×). NC group: TKE2 cells were cultured with conventional DMEM complete medium; Model group cells: DHA-L group: 50 μM of DHA was added on the basis of Model group; DHA: 200 μM of DHA was added on the basis of Model group; DHA+PEDF group: after transfection of PEDF into the TKE2 cells, 200 μM of DHA was added on the basis of Model group. ***: $P < 0.001$, compared with NC group; ###: $P < 0.001$, compared with Model group; @@@: $P < 0.001$, compared with DHA group.

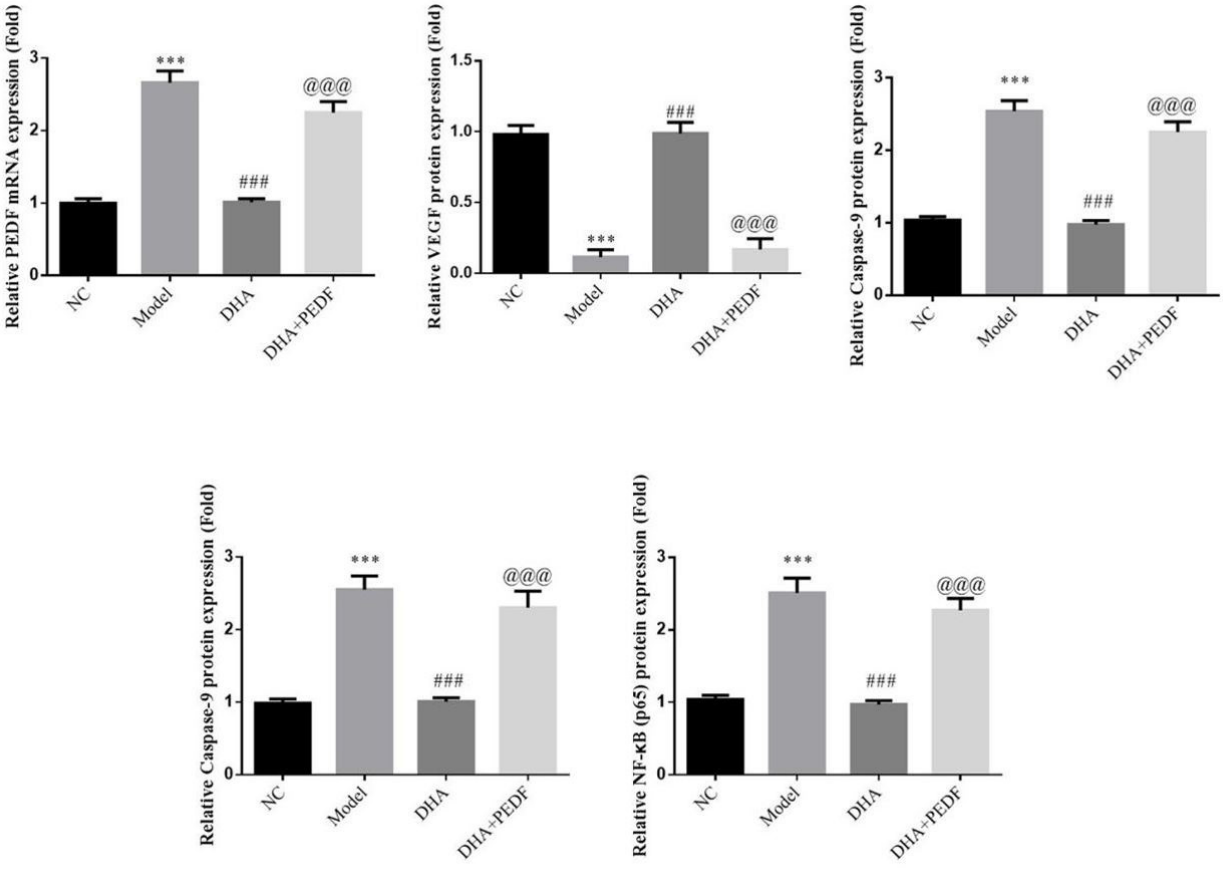

Figure 8. Relative gene expression in different cell groups. NC group: TKE2 cells were cultured with conventional DMEM complete medium; Model group cells: DHA-L group: 50 μM of DHA was added on the basis of Model group; DHA: 200 μM of DHA was added on the basis of Model group; DHA+PEDF group: after transfection of PEDF into the TKE2 cells, 200 μM of DHA was added on the basis of Model group. ***: $P < 0.001$, compared with NC group; ###: $P < 0.001$, compared with Model group; @@@: $P < 0.001$, compared with DHA group.

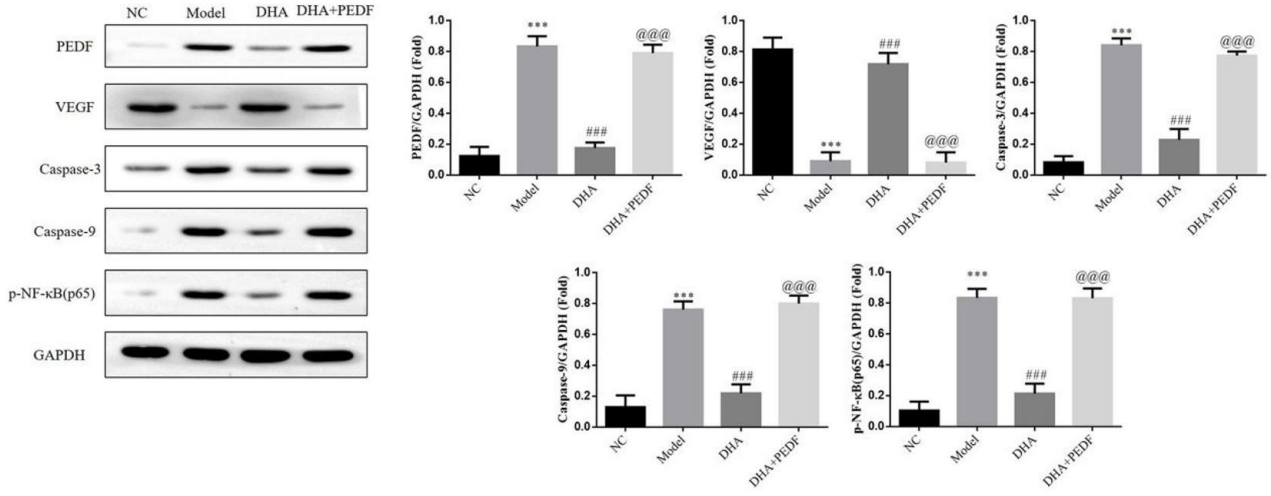

Figure 9. Relative protein expression in different cell groups. NC group: TKE2 cells were cultured with conventional DMEM complete medium; Model group cells: DHA-L group: 50 μM of DHA was added on the basis of Model group; DHA: 200 μM of DHA was added on the basis of Model group; DHA+PEDF group: after transfection of PEDF into the TKE2 cells, 200 μM of DHA was added on the basis of Model group. ***: $P < 0.001$, compared with NC group; ###: $P < 0.001$, compared with Model group; @@@: $P < 0.001$, compared with DHA group.

(p65) proteins were significantly increased, while that of VEGF protein was significantly reduced in the DHA+PEDF group ($P < 0.001$, Figure 9).

3.10 Immunofluorescence detection of PEDF and p-NF-κB (p65) proteins expressions

Immunofluorescence showed that compared with the NC group, the expression levels of PEDF and p-NF-κB (p65) proteins were significantly increased in the Model group ($P < 0.001$, Figure 10A, B). After DHA intervention, compared with the Model group, the expression levels of PEDF and p-NF-κB (p65) proteins were significantly reduced in the DHA group ($P < 0.001$, Figure 10A, B). After PEDF was transfected into the cells at the same time, compared with the DHA group, the expression levels of PEDF and p-NF-κB (p65) proteins were significantly increased in the DHA+PEDF group ($P < 0.001$, Figure 10A, B).

4 Discussion

The repair of corneal injury is a complex process, which is closely related to multiple factors. Studies have shown that the end products of advanced glycosylation can decelerate the repair of corneal injury through the formation of reactive oxygen species (Shi et al., 2013); smoking can decelerate the repair of corneal epithelial injury in human (Jetton et al., 2014); a too high concentration of blood calcium can decelerate the repair of corneal injury in ovariectomized rats (McDermott et al., 2003). The healing of corneal wound is a necessary clinical step to achieve the optimal recovery of vision after various eye surgeries and injuries, such as ocular trauma caused by chemical eye burns. Since the corneal epithelium serves as the first barrier against the invasion of external pathogens, corneal damage can cause vision loss. In severe cases, the cornea may even fall off and perforate, leading to blindness. Accelerating the repair of corneal injury can minimize the occurrence of such adverse events. Therefore, research on the drug mechanism that effectively promotes the repair of corneal epithelial injury can provide theoretical basis and research methods for clinical treatment.

DHA has anti-inflammatory effects, but its mechanism of action remains unclear. DHA reduces the inflammation, fibrosis, and oxidative stress of non-alcoholic fatty liver by inhibiting the fatty acid desaturase 1 (FADS1) (Younce & Kolattukudy, 2012; Depner et al., 2013). Relevant studies (Oh et al., 2010; Kirk et al., 2008) have confirmed that DHA could improve the symptoms of diseases and damages to the body by inhibiting inflammation. In this study, the results confirmed that the use of DHA could effectively improve the slow repair of corneal epithelial cells in a high-glucose environment. In order to further explore the mechanism of DHA on corneal epithelial cells, the related genes and proteins were tested.

Pigment epithelial-derived factor (PEDF) belongs to the non-protease inhibitor of the serine protease inhibitor family and has many functions such as inhibiting vascular proliferation, neurotrophic protection and anti-tumor (Facco et al., 2019; Filleur et al., 2009; He et al., 2015). In this study, it was found that in a high-glucose environment, the expression level of PEDF was significantly increased in the corneal epithelium. After DHA intervention, with the restored activity of corneal epithelial cell TKE2, the expression levels of PEDF genes and proteins were reduced. However, after PEDF was transfected into the TKE2 cells, the therapeutic effect of DHA tended to diminish. On this basis, it could be inferred that the therapeutic effect of DHA might be closely related to the expression of PEDF.

The activated NF-κB (p65) as a downstream protein of PEDF plays an important role in various diseases (Ren et al., 2017; Zhou et al., 2013; Zhu et al., 2012). Studies (Ali et al., 2020; Hassanein et al., 2021) have shown that Caspases, especially Caspase-3, are regulated by NF-κB (p65) and plays a key role in the execution of apoptosis. When apoptosis occurs, after being

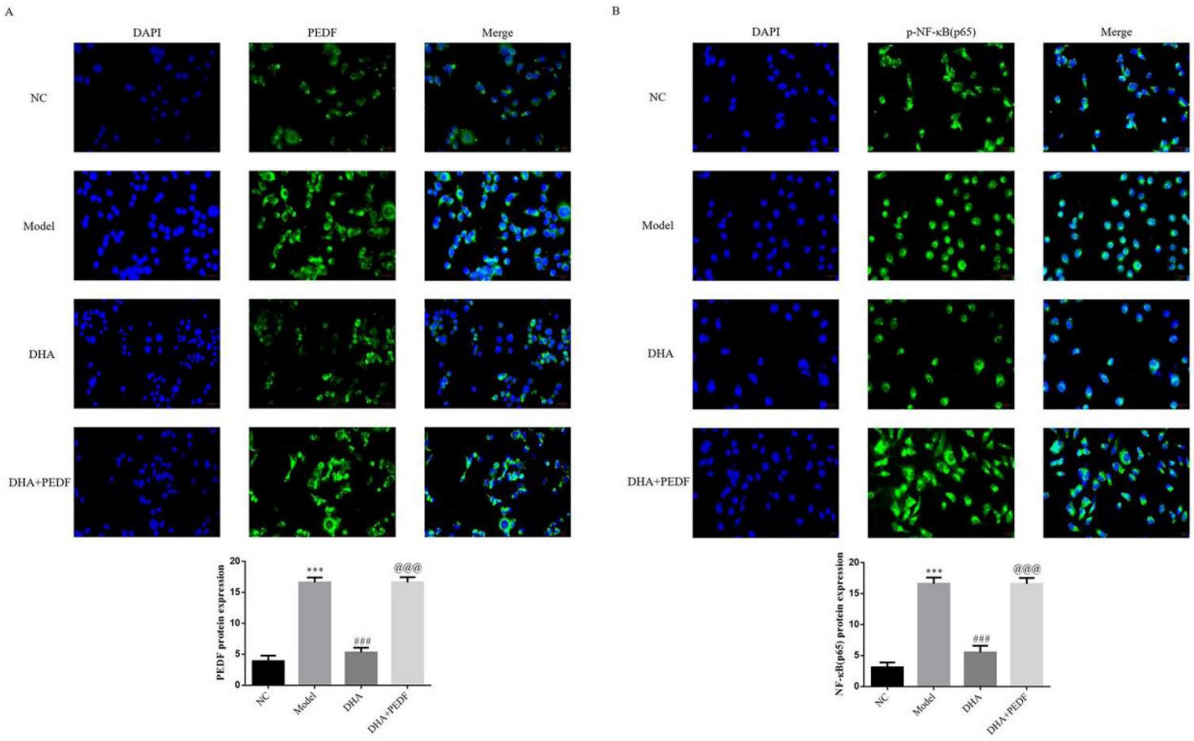

Figure 10. PEDF and p-NF-κB(p65) protein expression in different groups. NC group: TKE2 cells were cultured with conventional DMEM complete medium; Model group cells: DHA-L group: 50 μM of DHA was added on the basis of Model group; DHA: 200 μM of DHA was added on the basis of Model group; DHA+PEDF group: after transfection of PEDF into the TKE2 cells, 200 μM of DHA was added on the basis of Model group. A. PEDF protein expression (200×). B. p-NF-κB(p65) protein expression (200×). ***: $P < 0.001$, compared with NC group; ###: $P < 0.001$, compared with Model group; @@@: $P < 0.001$, compared with DHA group.

stimulated by the apoptotic signals, the effector Caspases are crucial to the progress of the apoptotic program, but the activation of this process requires the initiator Caspases to cut their precursors. Among them, as an effector Caspase, Caspases-3 needs to be activated by the upstream Caspases-9 (Wang et al., 2020). The results of this study showed that after DHA intervention, the apoptosis rate of corneal epithelial cell TKE2 was significantly improved in a high-glucose environment. The underlying mechanism might be related to the inhibited expressions of Caspase-9 and Caspase-3. VEGF is closely related to angiogenesis and cell migration (22. Melincovici et al., 2018) and is regulated by NF-κB (p65) (Lee et al., 2017). Relevant findings showed that the activation of NF-κB (p65) could inhibit the expression of VEGF (Li et al., 2020). This study proved that after DHA intervention, the expression level of VEGF was significantly increased in TEK2 cells; however, after PDEF was transfected into the cells, the improvement function of DHA was lost. Therefore, it could be inferred that how DHA improves the migration ability of TKE2 in a high-glucose environment might be related to the increased expression of VEGF.

In conclusion, DHA could effectively enhance the biological activity of TKE2 cells to cell apoptosis in a high-glucose environment. However, after transfection of PDEF into TKE2 cells, the therapeutic effect of DHA disappeared, so it was speculated that its mechanism might be closely related to the regulation of PDEF expression.

Funding

This study was supported by applied medicine research project of Hefei Health Commission (hwk2019zd011).

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