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# Melatonin inhibits the inflammation and apoptosis in rats with diabetic retinopathy *via* MAPK pathway

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**Abstract. – OBJECTIVE:** To investigate the effect of melatonin on diabetic retinopathy rats through the mitogen-activated protein kinase (MAPK) pathway.

**MATERIALS AND METHODS:** A total of 48 Sprague Dawley (SD) rats were randomly divided into normal group (n=12), model group (n=12), melatonin group (n=12), and inhibitor group (n=12). The rats in normal group received no treatment. Those in model group, mel[illegible] group, and inhibitor group were prepa[illegible] to models of diabetic retinopathy and intr[illegible]i-toneally injected with normal saline, melato[illegible] and SB 203580, respectively. After 7 days of [illegible]tervention, the materials were [illegible]n. The e[illegible]pressions of B-cell lympho[illegible]l-2) and Bcl-2-associated X protein[illegible]) we[illegible]etected through immunohistoche[illegible]try. We[illegible]rn blotting was employed to de[illegible]ine th[illegible]pression levels of p3[illegible]MA[illegible]phory[illegible]d (p)-p38 MAPK, and [illegible]steiny[illegible]tate specific proteinase-3 (C[illegible]ase-3). Th[illegible]senger ribonucleic acid [illegible]expression[illegible] of Bax and Bcl-2 we[illegible]ea[illegible]l *via* quanti[illegible]ve Polymerase Chain [illegible]eactio[illegible]CR). Enzyme-linked immunos[illegible]nt assay (E[illegible]) was performed to dete[illegible]e levels of seru[illegible]erleukin-1β (IL-1β) a[illegible]-18. Th[illegible]apoptosis w[illegible]s determined by ter[illegible] deox[illegible]eotidyl transferase-mediated deo[illegible]lin[illegible]phosp[illegible]te-biotin nick end-labeling[illegible]).

**RESU[illegible]** ased[illegible] immunohistochemistry, grou[illegible]onin group, and inhibitor gro[illegible]exhibite[illegible]gnificantly increased positive exp[illegible]sion of Bax but notably decreased positi[illegible] of Bcl-2 in comparison with normal group ([illegible]<0.05). Compared with those in [illegible]odel group, the positive expression of Bax was [illegible]ly reduced, while the positive expression of B[illegible] as overtly raised in melatonin group and inhi[illegible]tor group ($p<0.05$). The results of Western blotting showed that there was no difference in the protein expression of p38 MAPK among all groups ($p>0.05$). C[illegible]ed with normal group, the o[illegible]ree group[illegible] remarkably elevated [illegible] expressions [illegible] p-p38 MAPK and C[illegible]ase-3 ($p<0.05$). The protein expressions of [illegible]8 MAPK an[illegible]aspase-3 in melatonin group [illegible]inhibitor gro[illegible]were significantly lower than t[illegible] in model [illegible]up decreased ($p<0.05$). QP-CR[illegible] reve[illegible] that the mRNA expression of Bax [illegible]ly lower in normal group than that in the other three groups, while the mRNA expression of Bcl-2 was significantly higher[illegible]rmal group than that in the other three [illegible] ($p<0.05$). Compared with model group, melatonin group, and inhibitory group showed clearly declined mRNA expression level of Bax and notably increased mRNA expression level of Bcl-2 ($p<0.05$). TUNEL results revealed that the apoptosis rate was remarkably elevated in the other three groups compared with that in normal group ($p<0.05$). In comparison with model group, melatonin group and inhibitor group exhibited significantly reduced apoptosis rate ($p<0.05$).

**CONCLUSIONS:** Melatonin inhibits the inflammation and apoptosis in rats with diabetic retinopathy by repressing the MAPK pathway.

*Key Words:*
Diabetic retinopathy, P38 MAPK, MAPK signaling pathway, Inflammation, Apoptosis.

## Introduction

Diabetes, one of the most common endocrine diseases in clinical practice, often leads to many complications, including diabetic nephropathy, diabetic retinopathy, and diabetic foot[1,2]. These complications, especially diabetic retinopathy, severely affect the health of humans. Diabetic retinopathy refers to retinal destruction and optic

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nerve cell damage due to long-term hyperglycemia and is characterized by symptoms such as visual loss or even blindness. This leads to the loss of labor capacity of patients, has a severe influence on life health and quality of life of patients, and aggravates the economic burden of the patient family and society[3,4].

With the in-depth study of diabetic retinopathy, researchers have discovered that inflammation and apoptosis are important pathological processes of diabetic retinopathy, which often lead to apoptosis and necrosis of optic nerve cells, thus affecting repair after retinal injury and resulting in visual loss or even blindness in patients[5,6]. Therefore, how to regulate the inflammation and apoptosis in diabetic retinopathy becomes a new direction and a new thought for the treatment of diabetic retinopathy. Mitogen-activated protein kinase (MAPK) signaling pathway, one of the important cells signaling pathways, exerts an important regulatory role in pathological processes, including cell proliferation, apoptosis, necrosis, and inflammation. Under an injured environment, the intracellular MAPK signaling pathway is activated by the release of massive cyto[illegible] and inflammatory factors, leading to the tion of various downstream regulatory [illegible]rs and effector molecules to induce inflamma and apoptosis[7,8]. Melatonin is one of the impo ant hormones in the body and h proved have good anti-inflammatory poptotic effects; however, the mecha remai unclear.

Therefore, this work ai explo ts of melatonin on the inf mi a popu rats with diabetic re pathy the MAPK pathway. Also, it ther clarif relevant mechanisms, p new ideas e treatment of diabe etino in clinical practice.

## Mate als and Methods

### Lab r nimal d Grouping

A to 8 Spr Dawley (SD) rats (half and f es) weighing (220±20) g we urchas the Shanghai SLAC Laboratory Animal C., Ltd. [license number: SCXK (S ia) 2014-0003] and randomly di- ed into normal group (n=12), model group =12), melatonin group (n=12), and inhibitor g (n=12) using a random number table. This res ch was approved by the Animal Ethics Committee of the Dalian Medical University Animal Center.

### Laboratory Reagents and Instruments

Streptozotocin (Sigma-Aldrich, St. Louis, MO, USA), p38 MAPK inhibitor SB 203580 (MCE, Monmouth Junction, NJ, USA), prim bodies including anti-B-cell lympho (Bcl- antibody, anti-Bcl-2-associated X tein (Bax) antibody, anti-cysteinyl aspartat cific proteinase-3 (Caspase-3) antibody, anti MAPK antibody, and anti-phospho ed (p)-p PK antibody (Abcam, Cam ge, MA, USA zyme-linked immun bent y (ELI ) kit (Boster, Wuhan a), Q quantitative polymerase chain act K), SY reen Master Mix kit HiSc O R perMix for qPCR ( A wiper) me, Nanjing, Chin deoxynuc dyl transferase-media deo line triphosphate-biotin nick end labeling (T ) apoptosis detection kit Aldrich, St. L MO, USA), optical m scope (Leica DMI 4000B/DFC425C; Wetz Germany), orescence qPCR instrument 7500; Appl Biosystems, Foster City, CA, U and Imag image analysis system, and Ima analysis system (Bio-Rad; Hercules, C A).

### ing

S ozotocin solution (1%) was prepared and injected into the rats *via* the abdominal cavity at a dose of 60 mg/kg. After 3 days, the tail vein blood was collected from the rats to detect blood glucose and the blood glucose >16.7 mmol/L suggested successful modeling.

### Processing of Rats in Each Group

The rats in normal group were raised normally and underwent no treatment. Those in model group were prepared into diabetic retinopathy models, given the same amount of normal saline *via* an intraperitoneal injection and killed. In melatonin group, the rats were prepared into models of diabetic retinopathy and intraperitoneally injected with melatonin at the dose of 10 mg/kg·d. The rats in inhibitor group were prepared into diabetic retinopathy models and received intraperitoneal injection of inhibitor SB 203580 at 100 mg/kg·d. Then, (1 h later) melatonin at 10 mg/kg·d. The intervention was performed for successive 7 days. Next, materials were collected.

### Collection of Materials

Materials were taken after 7 days of intervention. Blood samples were collected from abdominal aorta of all rats after successful

anesthesia and centrifuged. Subsequently, the serum was collected for ELISA. 6 rats in each group were subjected to perfusion fixation with paraformaldehyde and retinal tissues were taken and fixed in 4% paraformaldehyde solution at 4°C for 48 h. Next, paraffin tissue sections were prepared for immunohistochemistry and TUNEL detection. As to the remaining 6 rats, retinal tissues were taken directly and placed in Eppendorf (EP) tubes (Eppendorf, Hamburg, Germany) for Western blotting and qPCR detection.

### Immunohistochemistry

Paraffin-embedded tissues were prepared into sections (5 μm in thickness), placed in warm (42°C) water for spreading, scraping and baking, and prepared into paraffin tissue sections. Then, the paraffin tissue sections were sequentially immersed in xylene solution and gradient alcohol for conventional deparaffinization. Thereafter, the above sections were soaked in a citric acid buffer and heated in a microwave for 3 times (3 min of heating + 5 min of simmer/time) for fully antigen retrieval. After tha[illegible] sections were rinsed, and endogenous [illegible]dase blocker was dropwise added to the [illegible]mens for 10 min of blocking, rinsed, and a[illegible] dropwise with goat serum for blocking for [illegible] min. Next, the goat serum bl[illegible] solutio[illegible] was removed, and the spe[illegible]re added with primary anti-Bc[illegible]antibo[illegible](1:200), primary Bax antibody [illegible]0), an[illegible] in a refrigerator at 4°C [illegible] day, the specimens [illegible] rins[illegible] added with the secondary a[illegible]body solutio[illegible] 10 min of incubation. [illegible]at, the spe[illegible]s were thoroughly w[illegible]ed a[illegible]cted with streptomyces anti-bi[illegible]n-peroxida[illegible]lution for 10 min, followed [illegible] color develop[illegible] with dropwise additi[illegible]f diam[illegible]nobenzidine (DAB), counterstai[illegible] of nu[illegible] with hematoxylin, mounting, and [illegible].

### Western Blotting

The retinal tissues cryopreserved were added with lysis solution, subjected to ice-bath for 1 h, and centrifuged at 14,000 g for 10 mi[illegible] centrifuge. Next, protein quantificat[illegible]was ca[illegible]ried out *via* the bicinchoninic acid [illegible]A) method (Pierce, Rockford, IL, USA). Th[illegible]eins were detected using a microplat[illegible] reade[illegible]obtain optical density and stand[illegible]curves, [illegible] or[illegible] which protein concentra[illegible]in tissues wa[illegible] calculated. Thereafter[illegible]e prote[illegible]were de[illegible]tured and separated [illegible]h so[illegible]n dode[illegible]yl sulphate-polyacrylam[illegible]e g[illegible]ophore[illegible](SDS-PAGE), while t[illegible]osition[illegible]a Ma[illegible]protein was observed[illegible] reaction wa[illegible]a when the Marker pr[illegible]ed the bott[illegible]of the glass plate in a[illegible]traight[illegible] After that, the proteins were transferred onto[illegible]vinylidene difluoride (PV[illegible]embranes (M[illegible]ore, Billerica, MA, U[illegible]) and blocked with blocking solution for [illegible]h. Subsequ[illegible]y, primary anti-p38 MAPK [illegible]body (1:100[illegible] primary anti-p-p38 MAPK a[illegible]dy (1:100[illegible] primary Caspase-3 antibody (1:1[illegible]ndary antibody (1:1000) were sequent[illegible]added, followed by rinsing and developed in a dark place. Lastly, the membrane [illegible]ed with a chemiluminescent reagent for 1 [illegible]eaction for full development.

### QPCR Assay

Total ribonucleic acids (RNAs) were extracted and reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using the reverse transcription kit. The reaction system was 20 μL. The reaction conditions: reaction at 51°C for 2 min, pre-denaturation at 96°C for 10 min, denaturation at 96°C for 10 s, and annealing at 60°C for 30 s, for 40 cycles. With glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference, the relative expression level of related messenger RNAs (mRNAs) was calculated. The primer sequences are shown in Table I.

Table I. Primer[illegible]es.

| [illegible]ame | Primer sequence |
|---|---|
| [illegible]ax | Forward primer: 5'-TCCACCAAGAAGCTGAGCGAG-3'<br>Reverse primer: 5'-GTCCAGCCCATGATGGTTCT-3' |
| [illegible]2 | Forward primer: 5'-CCTCGTGCTGTCGGACCCATA-3'<br>Reverse primer: 5'-CAGGCTTGTGCTCTGCTTGTGA-3' |
| GADPH | Forward primer: 5'-ACGGCAAGTTCAACGGCACAG-3'<br>Reverse primer: 5'-GAAGACGCCAGTAGACTCCACGAC-3' |

### Detection of Apoptosis by TUNEL

Apoptosis in brain tissues was detected in accordance with the instructions of the TUNEL apoptosis detection kit.

### ELISA

Abdominal aorta blood was collected and centrifuged, and the serum was taken. The ELISA kit was used for the following procedures: sample loading, addition of standard, biotinylated antibody working solution, enzyme conjugate working solution, plate washing, and determination at 450 nm using the microplate reader according to the instructions.

### Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was utilized for statistical analysis. Enumeration data were expressed as mean ± standard deviation. Comparison between multiple groups was done using One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). The *t*-test was used for the data in line wit... mal distribution and homogeneity of va... corrected *t*-test for the data in line with n... al distribution and heterogeneity of variance, ... non-parametric test for the data not in line with normal distribution and homogeneity of variance. Rank sum test was employed for ranked data, and chi-square test was adopte... meration data.

## Results

### Results of Immunohi...chemistry

As shown in Figur... dark b... a sugge... positive expression... orma... oup, t... positive expression ... Ba... wer, a... at of Bcl-2 was grea... Model g... had ... e positive expressi... f Bax and B... statistical results (Fi... howed that ... pared with normal g... p, the ... three groups exhibited clearly elevated aver... ptical density of Bax pos... pression and ... rkably reduced the n... optical density of Bcl-2 positive expres-..., showing s... tically significant differenc-... ($p<0.05$). C... ared with those in model g... the mea... tical density of Bax positive exp... vertly decreased, while the mean o... density of Bcl-2 positive expres-...ion was evidently raised in melatonin group ...ibitor group, and the differences were ...ally significant ($p<0.05$). There were no

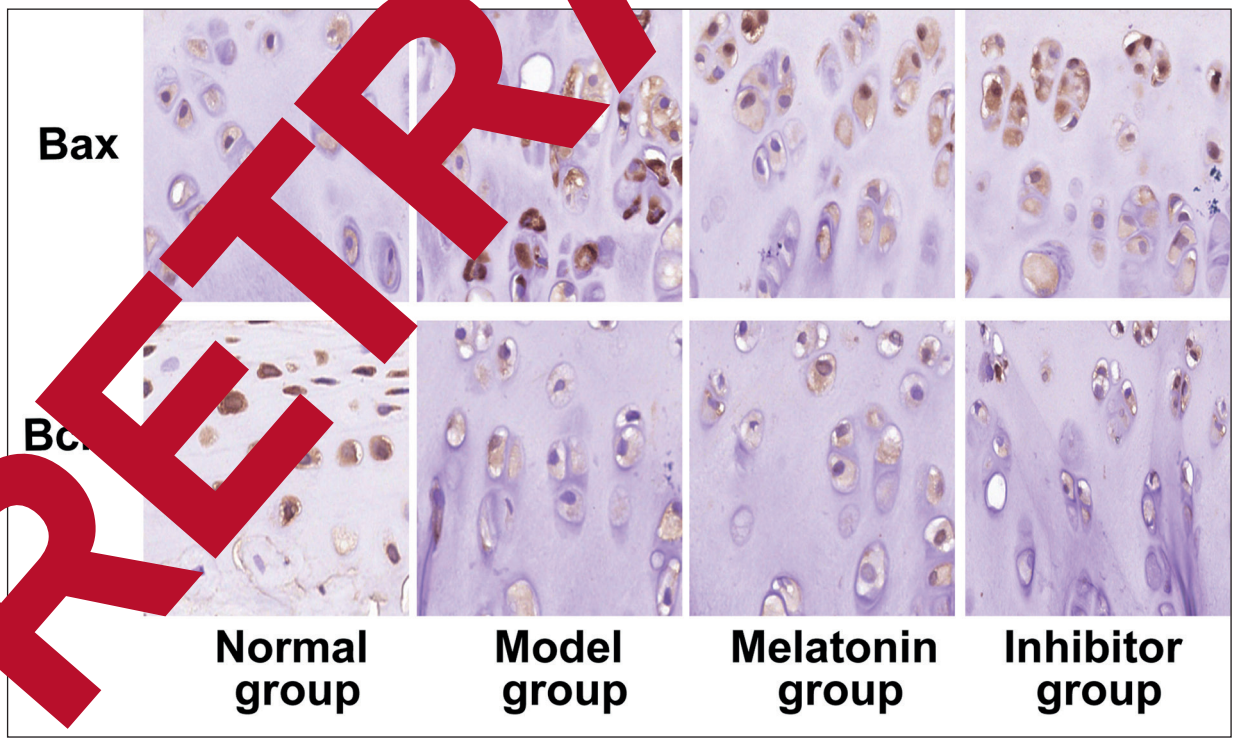


**Figure 1.** Results of immunohistochemistry (×200).

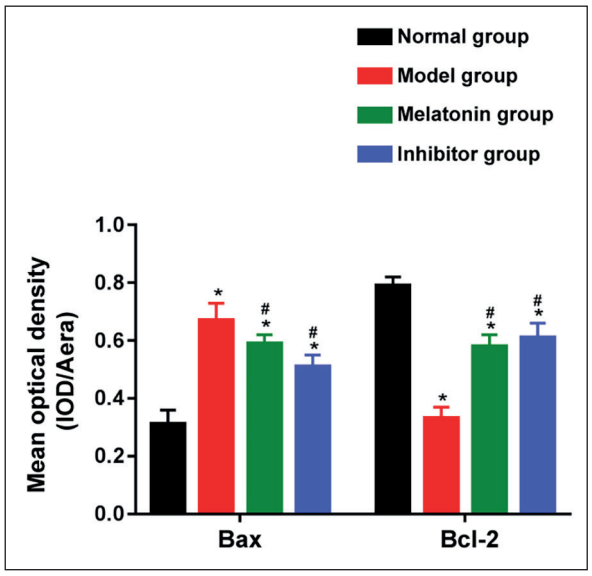


**Figure 2.** Mean optical density of positive expression. Note: $^{*}p<0.05$ *vs.* normal group, $^{\#}p<0.05$ *vs.* model group.

significant differences in the mean optical density of Bax positive expression and that of Bcl-2 positive expression between melatonin group and inhibitor group ($p>0.05$).

### *Protein Expressions of p38 MAPK and p-p38 MAPK Detected via Western Blotting*

According to Figure 3, a[illegible] ount of p38 MAPK protein was ex[illegible]ed in [illegible] group, and fewer protein expre[illegible]s of p[illegible]APK and Caspase-3 were [illegible]el[illegible]al g[illegible], while more protein [illegible]ession[illegible]-p38 MAPK and Caspase-3 w[illegible]ound in mo[illegible]roup. The results of statis[illegible]lysis (Figure [illegible]evealed that the relat[illegible] prote[illegible]pression level of p38 MAPK w[illegible]not signifi[illegible] different among

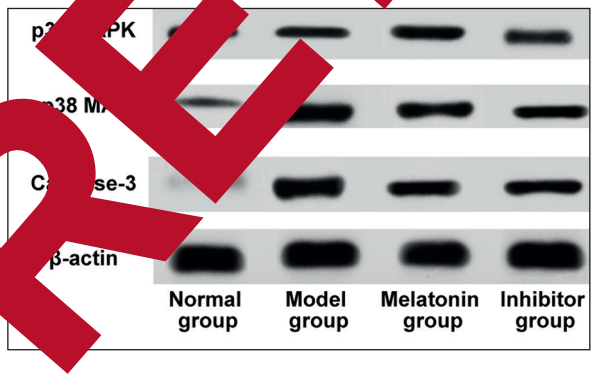


**Figure 3.** Relevant protein expression detected through Western blotting.

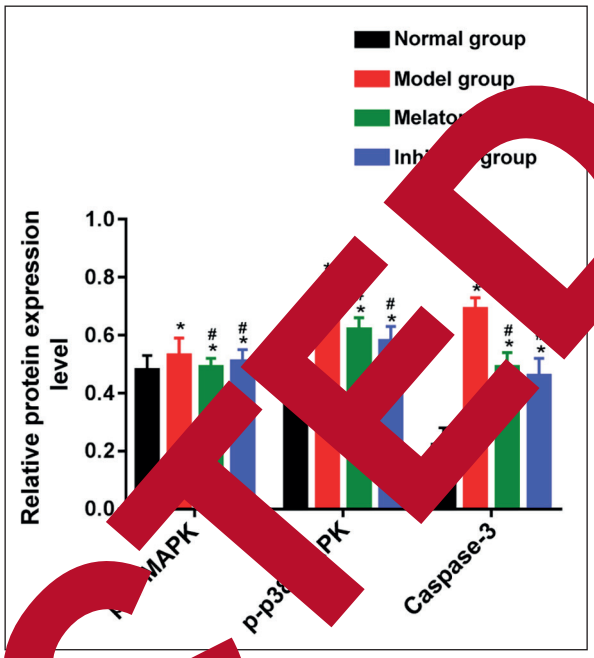


**Figure 4.** Relative [illegible]ein expression levels in each group. [illegible] $^{*}p<0.05$ *vs.* no[illegible] group, $^{\#}p<0.05$ *vs.* model group.

[illegible]ach group ($p>0.05$), while the relative pro[illegible]ressions of p-p38 MAPK and Caspase-3 [illegible]rkedly higher in the other three groups than those in normal group, and the differences were of statistical significance ($p<0.05$). Compared with model group, melatonin group and inhibitor group displayed notably declined relative protein expression levels of p-p38 MAPK and Caspase-3, showing statistically significant differences ($p<0.05$). No statistically significant differences were detected in the relative protein expression levels of p-p38 MAPK and Caspase-3 between melatonin group and inhibitor group ($p>0.05$).

### *Related MRNA Expressions Detected by QPCR*

As shown in Figure 5, the relative mRNA expression level of Bax was significantly higher in the other three groups than in normal group, while the relative mRNA expression level of Bcl-2 was distinctly lower than that in normal group, displaying statistically significant differences ($p<0.05$). Compared with those in model group, the relative mRNA expression level of Bax was overtly decreased, while the relative mRNA expression level of Bcl-2 was markedly elevated in melatonin group and inhibitor group, and the differences were statistically significant ($p<0.05$).

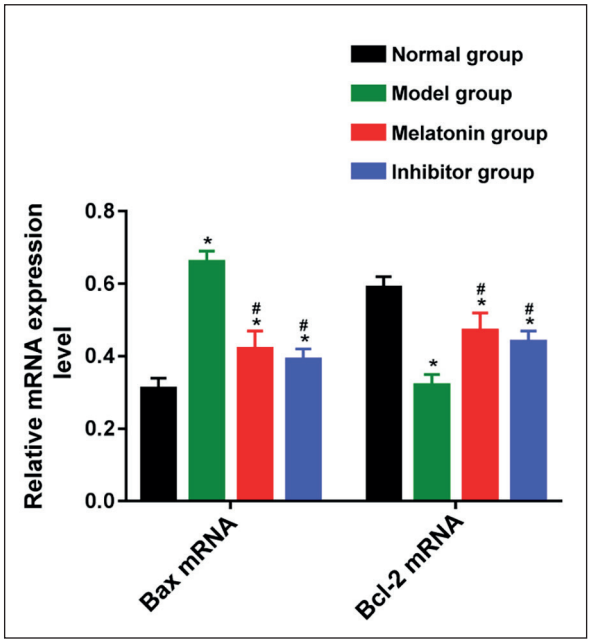


**Figure 5.** Relative mRNA expression levels in each group. Note: $^{*}p<0.05$ *vs.* normal group, $^{\#}p<0.05$ *vs.* model group.

The relative mRNA expression levels of B Bcl-2 exhibited no statistically significa f- ferences between melatonin group and inhi group ($p>0.05$).

### *Apoptosis Determined V*

The apoptosis rate was ntly ra d in the other three groups comp with th group and the differe es nificant ($p<0.05$). s sig tly lower in melatonin group inhibitor g than that in model grou statistica nificant differences. T wa ifference in the apoptosis rate b veen melat roup and inhibitor group ( 5) (Figure 6).

### *Res of SA*

As s n Figu , compared with normal the groups had significantly inc ed the nt of interleukin-1β (IL-1β) and -18, while the differences were of statistic ($p<0.05$). Compared with that in del group, the content of IL-1β and IL-18 was rtly decreased in melatonin group and inhibi- up, with statistically significant differences ($p$ 5). No difference was found in the content of IL-1β and IL-18 between melatonin group and inhibitor group ($p>0.05$).

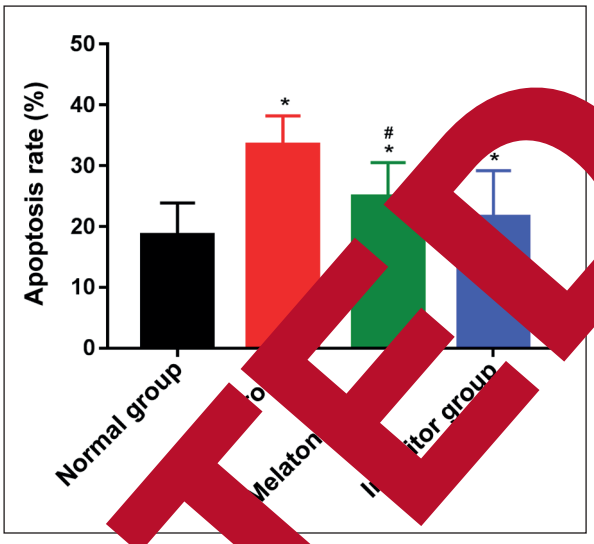


**Figure** optosis rate group. Note: $^{*}p<0.05$ *vs.* nor , $^{\#}p<0.05$ *vs.* mo up.

## scussion

pathy is one of the common compli of diabetes, whose incidence rate increases with that of diabetes. In particular, idence rate of diabetes displays a signifi- pward tendency in recent years with the changes in people's lifestyles, increases in work stress, and environmental changes, leading to a distinctly raised incidence rate of diabetic retinopathy[9,10]. Diabetic retinopathy affects the reti-

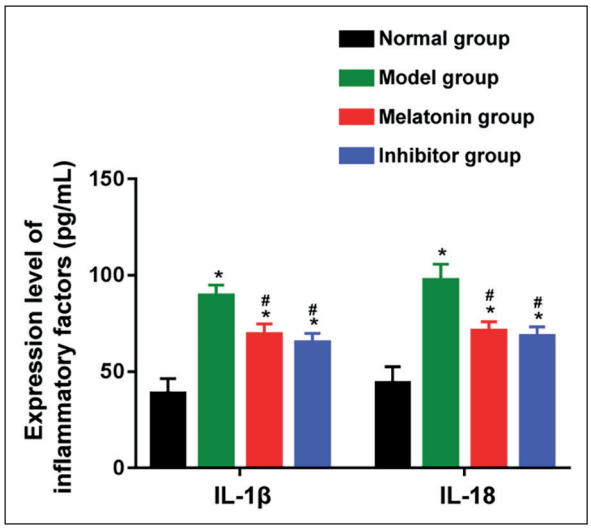


**Figure 7.** Expression level of inflammatory factors in each group. Note: $^{*}p<0.05$ *vs.* normal group, $^{\#}p<0.05$ *vs.* model group.

na and optic nerve cells, often leading to visual loss, blurred vision, or even blindness in patients. Therefore, diabetic retinopathy is recognized as one of the important blinding diseases. Current reports on diabetic retinopathy have manifested that the pathogenesis of diabetic retinopathy is very complex and remains unclear. However, it is clear that inflammation and apoptosis are one of the important pathological responses in diabetic retinopathy, which are able to further exacerbate the damage to the retina and the apoptosis and necrosis of the optic nerve cells. Also, they are not conducive to the repair of diabetic retinopathy. Currently, it is believed that as an important cell signal transduction pathway, MAPK is closely related to the inflammation and apoptosis in diabetic retinopathy rats by regulating the apoptosis, autophagy, inflammation and other related pathological reactions[11,12]. The MAPK signaling pathway, an important signal transduction pathway, includes p38 MAPK, JNK, and ERK, among which it is known that p38 MAPK, a MAPK, has close correlations with stress responses and inflammatory reactions and is considered to participate in many physiological and pathologic[illegible] sponses of the body. Also, it can be closely[illegible] to the pathogenesis of many diseases[13,14]. p38 MAPK is capable of participating in the d[illegible] opment and progression of inflammation in c[illegible] of injury, which can aggravate [illegible]flammatio[illegible] after injury to form a vicio[illegible] exacerbate the disease. After the i[illegible]y, a la[illegible] number of inflammatory factors [illegible]leased [illegible] tissues and cells. Thes[illegible] ly work on p38 MA[illegible]o acti[illegible]d phosphorylate p38 MAPK [illegible]m p-p38 M[illegible][15,16]. P-p38 MAPK enters [illegible]us to modu[illegible]multiple downstream [illegible]aling [illegible]ways, the release of inflammat[illegible] factors su[illegible] IL-1β and IL-18, and the [illegible]ressions of Ba[illegible] Bcl-2 closely relate[illegible] apopto[illegible], thereby af[illegible]cting the expressio[illegible] the ap[illegible]sis effector protein Caspase-3 and [illegible]g inflam[illegible]ation and apoptosis[17,18]. Reports [illegible]ve rev[illegible]d that the activation of MAP[illegible]pe[illegible]ally blocked by its potent in[illegible]ors, th[illegible]king the MAPK pathway to effe[illegible]ely allevi[illegible]e inflammatory responses after i[illegible]

[illegible]n this work, it was discovered that the expres[illegible]n of Bax (a pro-apoptotic gene) was markedly [illegible]ulated in the retina of rats with diabetic reti[illegible]pathy, while that of Bcl-2 (an apoptosis suppressor gene) was evidently lowered, and the protein expression level of Caspase-3 (an apoptosis effector protein) was significantly increased. Massive cells were apoptotic, indicating that severe apoptosis occurs in the retina of rats with diabetic retinopathy. Apoptosis is o[illegible] important pathological reactions in [illegible]etic re[illegible]inopathy. Besides, the serum con[illegible] of inflammatory factors IL-1β and IL-18 [illegible]levated in diabetic retinopathy rats, sug[illegible]esting [illegible]here is remarkable inflammation. [illegible]r treati[illegible]with melatonin, the serum co[illegible]t of IL-1β and declined clearly, the e[illegible]ssion [illegible]e pro-apoptotic gene Bax and t[illegible]otein [illegible]pressio[illegible] of the apoptosis effecto[illegible]rot[illegible]ase-3 [illegible] significantly lowe[illegible] the ex[illegible]n o[illegible]-2 was evidently rai[illegible]he apoptosi[illegible]ammation were atten[illegible]ts with diab[illegible] retinopathy, implying [illegible] mela[illegible] has good anti-inflammatory and anti-apoptoti[illegible]cts. To further investigat[illegible]chanisms of a[illegible] the key substances i[illegible] MAPK pathway were detected. Also, it revealed tha[illegible]e protein expression of p-p38 [illegible]PK in the re[illegible] of rat models of diabetic retinopathy was si[illegible]ficantly increased, indicating that [illegible]K signaling pathway is activated. This [illegible]e one of the possible causes of the inflammation and apoptosis in the retina of rat [illegible]f diabetic retinopathy. Melatonin overtly [illegible]d[illegible] the protein expression of p-p38 MAPK and had similar effects to those of p38 MAPK inhibitor, implying that melatonin effectively suppresses the activation of the p38 MAPK pathway in the retina of rat models of diabetic retinopathy.

## Conclusions

We showed that melatonin represses the inflammation and apoptosis in rats with diabetic retinopathy by inhibiting the MAPK pathway.

### Conflict of Interest

The Authors declare that they have no conflict of interests.

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