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Correlations of the severity of diabetic retinopathy with EPO, Caspase-3 expression and oxidative stress

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Abstract. – OBJECTIVE: To investigate the relationships of the severity of diabetic retinopathy with erythropoietin (EPO), Caspase-3 expression, and oxidative stress.

PATIENTS AND METHODS: A total of 20 patients with non-proliferative diabetic retinopathy hospitalized from January 2017 to January 2018 were enrolled as observation group 1, 20 patients with proliferative diabetic retinopathy were chosen as observation group 2, and 20 patients with idiopathic macular hole were selected as control group. After admission, patients received all necessary examinations and underwent vitrectomy during which vitreous and retinal tissues were taken, and venous blood was collected. Then, the content of EPO, Caspase-3, nitric oxide (NO), and malondialdehyde (MDA) was detected through enzyme-linked immunosorbent assay (ELISA), the messenger ribonucleic acid (mRNA) levels of EPO, Caspase-3, NO, and MDA were measured *via* quantitative Polymerase Chain Reaction (qPCR), and the severity of diabetic retinopathy was evaluated by diabetic retinopathy grading score.

RESULTS: Observation group 1 and 2 had significantly decreased the content of EPO ($p<0.05$) and overtly increased Caspase-3, NO, and MDA content ($p<0.05$) in comparison with control group. Compared with those in observation group 1, the EPO content was clearly lowered in observation group 2 ($p<0.05$), and the content of Caspase-3, NO, and MDA was evidently elevated ($p<0.05$). The diabetic retinopathy grading score was remarkably lower in control group than that in both observation group 1 and observation group 2 (p $p<0.05$), and it was significantly enhanced in observation group 2 compared with that in observation group 1 ($p<0.05$). Correlation analysis showed that the EPO content was negatively correlated with the severity of diabetic retinopathy, while the content of Caspase-3, NO, and MDA was positively related to the severity of diabetic retinopathy.

CONCLUSIONS: The severity of diabetic retinopathy has a negative association with EPO and positive correlations with Caspase-3, NO, and MDA content.

Key Words:
Diabetic retinopathy, EPO, Caspase-3, Oxidative stress response, Correlation.

Introduction

Diabetic retinopathy is a common complication of diabetes mellitus in clinical practice at present. Besides, the incidence rate of diabetic retinopathy is increasing with the development of society, improvement of people's living standards, and aging of population[1,2]. According to statistics, diabetic retinopathy has become one of the major factors leading to blindness in young and middle-aged people, resulting in the loss of capacity of young and middle-aged people who play important roles in the society to work, and is deemed to be one of the four diseases causing blindness in human beings, bringing a heavy economic burden on the family and society[3,4]. Current studies have manifested that the major pathological responses of diabetic retinopathy include vascular endothelial injury, pericyte decrease, progressive thickening of vascular basement membrane, microaneurysm formation, and neovascularization, resulting in severe damage to the retinal vascular endothelial function.

Erythropoietin (EPO) is an important cytokine playing an important role in regulating the production of red blood cells. EPO has various important physiological functions[5] including anti-oxidation, apoptosis inhibition, and nerve protection. Therefore, some scholars guess that the abnormal expression of EPO is closely correlated with the onset and progress of diabetic retinopathy. Apoptosis is an important mode of cell death and considered to participate in the development and progress of diabetic retinopathy. Furthermore, Caspase-3, an important protein regulating apoptosis[6], is thought to be an important pathway for the cascade of apoptosis proteases and be able to

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participate in the pathological process of diabetic retinopathy by regulating apoptosis. Moreover, oxidative stress response, an important pathological process, is capable of participating in various secondary pathological responses like apoptosis and inflammation, and the resulting pathological products including malondialdehyde (MDA) and nitric oxide (NO) may lead to damage of tissue cells and are deemed to be involved in the pathological responses of diabetic retinopathy.

However, the associations of the severity of diabetic retinopathy with EPO, Caspase-3, and oxidative stress response remain unclear. We aim to find out the relationships of the severity of diabetic retinopathy with EPO, Caspase-3, and oxidative stress response, providing guidance for further study of the pathological responses of diabetic retinopathy.

Patients and Methods

Patients

A total of 20 patients with non-proliferative diabetic retinopathy who were hospitalized from January 2017 to January 2018 were enrolled as observation group 1, 20 patients with proliferative diabetic retinopathy as observation group 2 and 20 patients with idiopathic macular hole as control group. All the three groups of patients received an elective vitrectomy. There were 8 males and 12 females aged (53.22±4.69) years old in observation group 1, 10 males and 10 females aged (51.37±5.19) years old in observation group 2, and 9 males and 11 females aged (54.42±6.11) years old in control group. All patients signed the informed consent and agreed to participate in this study. This investigation was approved by the Ethics Committee of Fuxing Hospital (Beijing, China).

Inclusion Criteria

Inclusion criteria for proliferative diabetic retinopathy: patients (1) with retinopathy caused by type 2 diabetes mellitus, (2) with repeated vitreous hemorrhage, with formed pre-retinal organization shown on color ultrasound images and receiving no drug therapy, and (3) with formed proliferative membrane or tractional detachment of retina at eye fundus based on examination of eyes.

Reagents and Instruments

AceQ quantitative Polymerase Chain Reaction (qPCR) SYBR Green Master Mix kits (Vazyme Biotech Co., Ltd., Nanjing, China), HiScript II Q RT SμperMix for qPCR (+gDNA wiper) kits (Vazyme Biotech Co., Ltd., Nanjing, China), TUNEL apoptosis assay kits (Sigma-Aldrich, St. Louis, MO, USA) and fluorescence qPCR instruments (ABI 7500, Foster City, CA, USA).

Research Methods

After patients who met the inclusion criteria in this study were admitted to the hospital, all necessary examinations were carried out, venous blood was collected, and vitrectomy was performed under anesthesia. The vitreous body (0.5 mL) was sampled using a vitreous tip and stored in a centrifuge tube. During surgery, pre-retinal proliferative membrane tissues were taken from patients in observation group 1 and 2, and inner limiting membrane tissues were collected from patients in control group. Next, tissues collected were stored in centrifuge tubes at -80°C.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was conducted to detect the content of EPO, Caspase-3, NO, and MDA in tissue samples. The specific procedures: venous blood collected was centrifuged, and the supernatant was taken. Then, the standard substance was diluted and loaded, and the blank control well and well for samples to be tested, as well as three duplicate wells, were also set. Next, samples were subjected to reaction at 37°C for 30 min. Thereafter, the washing solution was diluted 30 times with distilled water for later use. After washing, an enzyme standard reagent was added for reaction, followed by washing. After that, a color developing agent was added to each well for the color development, followed by the addition of stop buffer to stop the reaction. Lastly, a microplate reader was used for measurement at 450 nm.

QPCR Assay

Firstly, total ribonucleic acid (RNA) was extracted from tissue samples and then reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using a 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. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference, and the relative expression level of related messenger RNA (mRNA) was calculated. The primer sequences are shown in Table I.

Table I. Primer sequences.

Organization	Primer sequence
EPO	Upstream primer: 5'TAGGAATTCATGGGCTGCACGGACTCT 3' Downstream primer: 5'TACGGCGGCCGCGTCGTCGTCCTGGGTGA 3'
Caspase-3	Upstream primer: 5'TGGAACAAATGGACCTGTTGACC 3' Downstream primer: 5'AGGACTCAAATTCTGTTGCCACC 3'
NO	Upstream primer: 5'GAG-CTTCTACCTCAAGCTATC 3' Downstream primer: 5'CCTGATGTTGCCATTGTTGGT 3'
MDA	Upstream primer: 5'TTCTTTGAGTTCGGTGGGGTC 3' Downstream primer: 5'TGCATATTTGTTTGGGGCAGG 3'
GAPDH	Upstream primer: 5'ACGGCAAGTTCAACGGCACAG 3' Downstream primer: 5'GAAGACGCCAGTAGACTCCACGAC 3

Diabetic Retinopathy Grading Score

After mydriasis of all patients, field and fundus images were taken and read, and grading was performed based on the above field and fundus images. Criteria for grading: grade 1: 1-4 points, grade 2: 5-8 points, grade 3: 9-12 points, and grade 4: 15-20 points.

Statistical Analysis

In this study, the Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. The measurement data were expressed as mean ± standard deviation. The *t*-test was utilized for data with normal distribution and homogeneity of variance, corrected *t*-test was employed for those with normal distribution and heterogeneity of variance, and non-parametric test was performed for those without normal distribution and homogeneity of variance. The rank sum test was applied for ranked data. For enumeration data, the chi-square test was employed. The Pearson analysis was used for correlation analysis. p-values < 0.05 were considered statistically significant.

Results

Diabetic Retinopathy Grading Score in Each Group

According to Figure 1, the diabetic retinopathy grading score was (0.56±0.03) in control group, (10.21±2.23) in observation group 1, and (18.66±3.28) in observation group 2. It can be seen that the score was significantly increased in observation group 1 and 2 compared with that in control group, showing statistically significant differences (p<0.05), and it was significantly higher in observation group 2 than that in observation group 1, and the difference was statistically significant (p<0.05).

ELISA

Compared with those in control group, the EPO content was decreased evidently in observation group 1 and 2, while the content of Caspase-3, MDA, and NO was overtly increased, displaying statistically significant differences (p<0.05). In comparison with observation group 1, observation group 2 had significantly declined EPO content and remarkably elevated content of Caspase-3, MDA, and NO, and differences were statistically significant (p<0.05) (Figure 2).

QPCR Assay

As shown in Figure 3, observation group 1 and 2 had significantly decreased mRNA level of EPO and evidently elevated mRNA levels of Caspase-3, MDA, and NO in comparison with control group, and the differences were statistically significant (p<0.05). Compared with those in observation group 1, the mRNA level of EPO

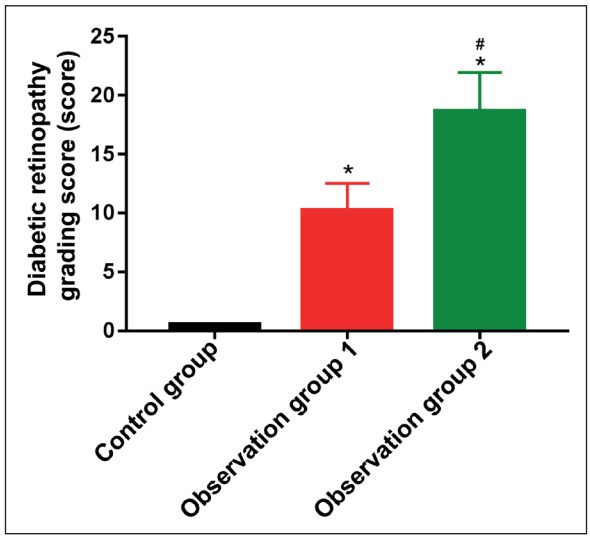

Figure 1. Diabetic retinopathy grading score in each group.

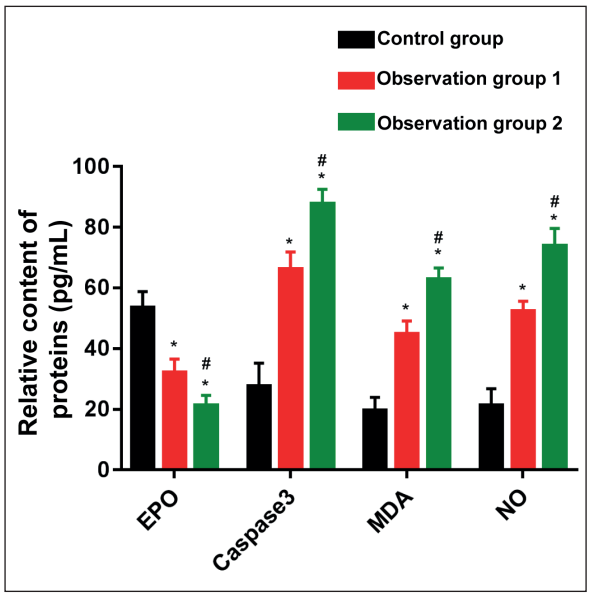

Figure 2. Relative content of proteins detected via ELISA.

was significantly reduced in observation group 2, and the mRNA levels of Caspase-3, MDA, and NO were distinctly increased, showing statistically significant differences ($p<0.05$).

Correlation Analysis

Based on calculation, the diabetic retinopathy grading score was negatively correlated with EPO ($r=-0.612$, $p=0.03$) (Figure 4) and positively related to Caspase-3 ($r=0.766$, $p=0.001$) (Figure 5), MDA ($r=0.812$, $p=0.001$) (Figure 6), and NO ($r=0.672$, $p=0.001$) (Figure 7).

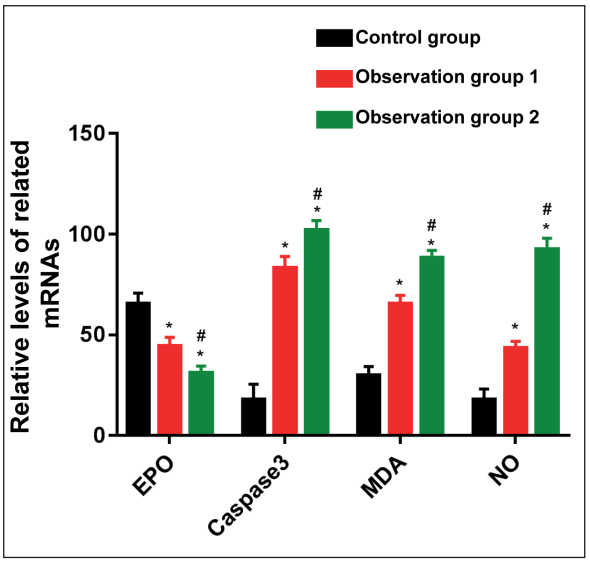

Figure 3. Relative levels of related mRNAs.

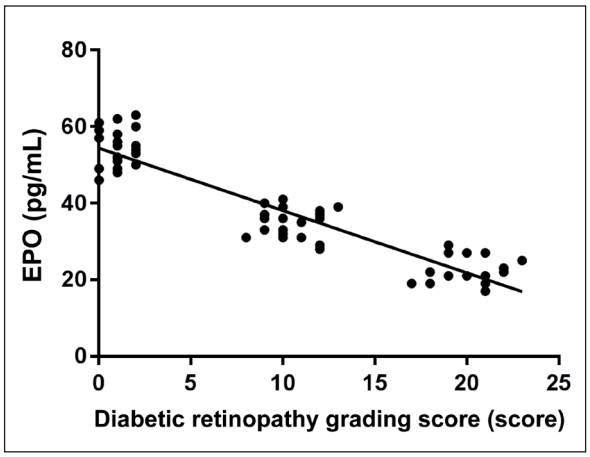

Figure 4. Relationship between EPO and diabetic retinopathy grading score.

Discussion

Diabetic retinopathy, a major complication of diabetes mellitus, causes a great or even an irreversible damage to the visual acuity of patients, which is one of the important causes of blindness in patients. In particular, with the development of society and the improvement of human living standards, diabetic retinopathy is more and more common with the increasing incidence rate of diabetes mellitus, eventually leading to blindness and loss of ability of most patients with diabetes mellitus to work[7,8]. Besides, diabetic retinopathy is a microvascular disease caused by diabetes mellitus and capable of inducing retinal vascular endothelial dysfunction and neovascularization in the retina, eventually pulling the retina, causing retinal detachment and resulting in decreased visual acuity or even blindness. Apoptosis

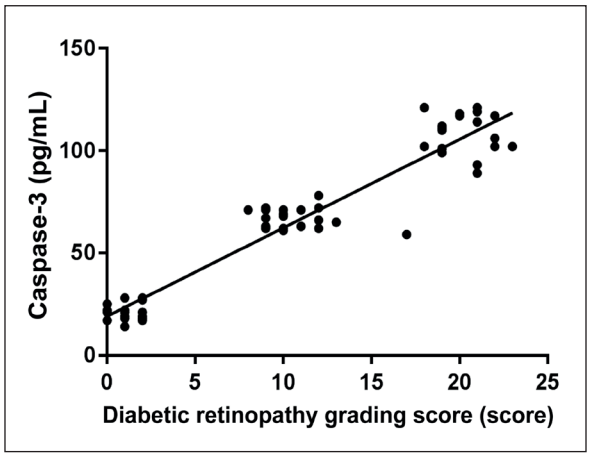

Figure 5. Relation between Caspase-3 and diabetic retinopathy grading score.

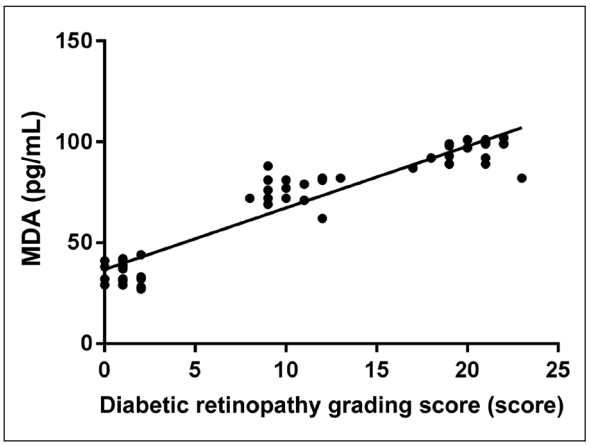

Figure 6. Correlation between MDA and diabetic retinopathy grading score.

and oxidative stress response are important pathological responses and involved in the onset of diabetic retinopathy, and they play important regulatory roles in the severity of diabetic retinopathy. Apoptosis of retinal cells (especially capillary endothelial cells and their surrounding cells) is considered to be one of the important pathological responses at the early stage of diabetic retinopathy. Moreover, apoptosis is one of the leading causes of retinal neurodegeneration and nerve tissue injury[9,10]. In this research, we found that the expression of Caspase-3 that plays a key regulatory role in apoptosis was abnormal in retinal tissues of patients with diabetic retinopathy, which was abnormally high in tissues of patients with both non-proliferative diabetic retinopathy and proliferative diabetic retinopathy. At the same time, further analysis confirmed that the expression level of Caspase-3 in tissues of patients

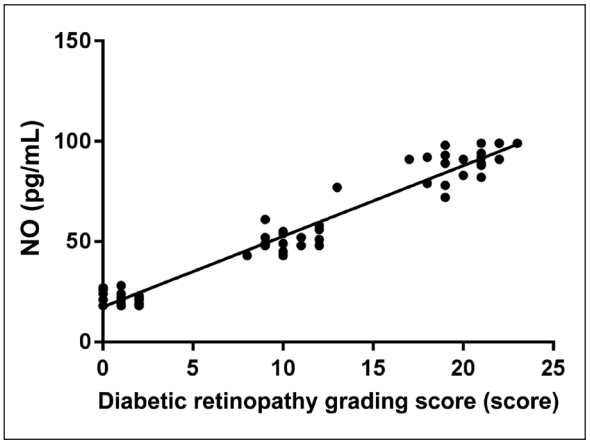

Figure 7. Association between NO and diabetic retinopathy grading score.

with proliferative diabetic retinopathy was markedly higher than that of those with non-proliferative diabetic retinopathy, indicating that the abnormally high expression level of Caspase-3 is positively correlated with the severity of diabetic retinopathy, namely, the higher the abnormally high expression level of Caspase-3 in tissues of patients with diabetic retinopathy, the severer the diabetic retinopathy.

Oxidative stress response is the most common physiological and pathological response in human body, which occurs in case that the body is damaged or stimulated by external factors and is able to trigger various harmful pathological responses such as production of NO and SOD, break the dynamic equilibrium between normal oxidation system and antioxidant system in the body and thus lead to further damage to tissues[11,12]. Reports have manifested that oxidative stress response can act on retinal tissues through harmful pathological products. On the one hand, it leads to ischemia and hypoxia of retinal microvessels and thus results in thrombosis, giving rise to apoptosis and necrosis of retinal cells due to ischemia and hypoxia[13,14]. On the other hand, the pathological products of oxidative stress response can directly act on retinal cells, cause abnormal expression of Caspase-3 protein in retinal cells and induce apoptosis, mediating programmed death of retinal cells[15,16]. Hence, in this study, it was discovered that the pathological products of oxidative stress response [NO and superoxide dismutase (SOD)] were abnormally expressed in tissues of patients with diabetic retinopathy, the content of NO and SOD was evidently higher in patients with proliferative diabetic retinopathy than that in patients with non-proliferative diabetic retinopathy, and it was positively correlated with the severity of diabetic retinopathy. Meanwhile, the abnormally high expression level of Caspase-3 was related to the abnormally high expression levels of pathological products of oxidative stress response (NO and SOD). The abnormally high expression levels of NO and SOD were able to lead to the abnormally high expression level of Caspase-3, which was closely correlated with apoptosis.

EPO is mainly secreted by and expressed in liver and kidney tissues. Scholars[17,18] have suggested that EPO is often produced by stimulation of factors such as hypoxia, and has a good regulatory effect on pathological responses including inflammation, oxidative stress response, apoptosis, and nervous system repair. Furthermore, EPO exerts various important functions such as anti-in-

flammation, anti-oxidation, and anti-apoptosis through many cell signaling pathways including MAPK and JAK/STAT signaling pathways[19,20]. The results of this work showed that the EPO expression was inhibited and abnormally low in tissues of patients with diabetic retinopathy, which may be one of the possible causes of abnormally excessive apoptosis and abnormally excessive oxidative stress response in diabetic retinopathy. At the same time, the EPO content in patients with proliferative diabetic retinopathy was overtly lower than that in patients with non-proliferative diabetic retinopathy, and the correlation analysis revealed that the EPO content had a negative correlation with the severity of diabetic retinopathy, i.e., the lower the EPO content, the severer the diabetic retinopathy. In conclusion, this study indicates that the severity of diabetic retinopathy is negatively related to EPO expression, and positively associated with Caspase-3 expression and oxidative stress response.

Conclusions

This study indicates that the severity of diabetic retinopathy is negatively related to EPO expression, and positively associated with Caspase-3 expression and oxidative stress response.

Conflict of Interests

The authors declare that they have no conflict of interest.

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