Original Research Article

Neuroprotective effects of etanercept on diabetic retinopathy via regulation of the TNF-α/NF-κB signaling pathway

Jiajian Guo[1], Qingqing Yu[2], Genlan Yang[1], Wei Wei[3], Pengjun Tang[1], Ying Zheng[2], Fusheng Zhang[1], Qisheng Liu[1], Yujie Wang[4]*

[1]Venus Eye Hospital, Ganzhou, [2]The 903rd Hospital of the Joint Logistics Support Force of PLA, Hangzhou, [3]Department of Ophthalmology, Armed Police Force First Mobile Corps Hospital, Dingzhou, [4]Department of Ophthalmology, Zhuji people's Hospital of Zhejiang Province, Zhuji, China

*For correspondence: **Email:** 1303572621@qq.com; **Tel:** 0575-87029855

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Abstract

Purpose: To study the influence of etanercept on diabetic retinopathy in rats via tumor necrosis factor alpha (TNF-α)/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway.
Methods: Thirty-six Sprague-Dawley (SD) rats were randomly divided into normal, model and etanercept groups. The expression of Caspase-3 was determined by immunohistochemistry, while the relative protein and mRNA expression levels of TNF-α and NF-κB were determined by Western blotting and quantitative polymerase chain reaction, respectively. Besides, the contents of TNF-α and interleukin-1 beta (IL-1β) were evaluated using enzyme-linked immunosorbent assay (ELISA), while cell apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL).
Results: Immunohistochemical studies showed that the mean optical density of tissues positive for caspase-3 in both the model and etanercept groups were significantly higher than in the normal group (p < 0.05), while the mean optical density in the etanercept group was significantly lower than that in the model group (p < 0.05). The protein expression levels of TNF-α and NF-κB in the etanercept group were significantly lower than those in the model group (p < 0.05). Furthermore, mRNA expressions of TNF-α and NF-κB declined in the etanercept group (p < 0.05); in addition, TNF-α, and IL-1β levels in the etanercept group were lower than in the model group (p < 0.05). Cell apoptosis in the etanercept group was also lower than in the model group.
Conclusion: Etanercept suppresses TNF-α/NF-κB signaling pathway thereby repressing inflammation and cell apoptosis in diabetic retinopathy rats. Therefore, etenercept's neuroprotective effect may potentially be useful in developing a suitable therapy for diabetic neuropathy.

Keywords: Diabetic retinopathy, Etanercept, TNF-α/NF-κB signaling pathway, Inflammation, Apoptosis

INTRODUCTION

The increase in the morbidity rate of diabetes makes diabetic retinopathy increasingly common in clinic, so that it has gradually become a severe microvascular complication of diabetes [1,2]. According to the statistics, diabetic retinopathy has been one of the leading causes of blindness

in patients in the United States, seriously affecting the life, health, labor capacity and quality of life of patients. Therefore, it is vital to further explore the pathogenesis of diabetic retinopathy and the relevant treatment methods for alleviating diabetic retinopathy-induced damage to patients, and improving the life, health and quality of life of patients with this disease [3-6].

In the pathogenesis of diabetic retinopathy, massive optic nerve cell apoptosis (one of the crucial pathological reactions) can cause optic nerve damage, blindness and other critical conditions. Studies have demonstrated that the pivotal tumor necrosis factor alpha (TNF-α)/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway in organisms can regulate multiple pathological processes, such as apoptosis and inflammation [7,8]. The TNF-α/NF-κB signaling pathway-mediated inflammation and cell apoptosis are now considered to play crucial roles in diabetic retinopathy.

Etanercept, efficacious inhibitor of TNF-α in clinic, binds to the receptor of TNF-α to repress the pro-inflammatory effect of TNF-α, thereby effectively inhibiting inflammation, but the specific mechanism of action therein has not yet been elucidated [9,10]. Therefore, the present study aims to explore the influence of etanercept on diabetic retinopathy in rats through the TNF-α/NF-κB signaling pathway.

EXPERIMENTAL

Animals

Thirty-six specific pathogen-free laboratory Sprague-Dawley (SD) rats aged 1 month (Shanghai SLAC Laboratory Animal Co. Ltd., certificate No. SCXK (Shanghai, China) 2013-0006) were bred in the Experimental Animal Center of Venus Eye Hospital and given normal feed and sterile filtered water daily under a 12/12 h light-dark cycle, in a temperature-controlled room (21 ± 2 °C). This study was approved by the Animal Ethics Committee of Venus Eye Hospital Animal Center (approval no. 2019-07-12). All procedures were conducted in accordance with the 'Animal Research: Reporting *in vivo* experiments guidelines 2.0' [11].

Reagents and instruments

Etanercept was purchased from Shanghai Guojian Pharmaceutical Co., Ltd. (Shanghai, China). Anti-TNF-α, anti-NF-κB, and anti-Caspase-3 primary antibodies, and secondary antibodies were purchased from Abcam (Cambridge, MA, USA). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) apoptosis assay, enzyme-linked immunosorbent assay (ELISA) kits and quantitative polymerase chain reaction (qPCR) kits were from Vazyme Biotech (Nanjing, China).

Animal grouping

A total of 36 SD rats were randomly divided into normal group (n = 12), model group (n = 12) and etanercept group (n = 12) using a random number table, and they were adaptively fed in the Experimental Animal Center for 7 days before experiments.

Establishment of diabetic retinopathy model

First, 1 % streptozotocin solution was prepared, and intraperitoneally injected into rats at a dose of 60 mg/kg, and 3 days later, venous blood was withdrawn from the tails of the rats for determination of blood glucose, with the blood glucose > 16.7 mmol/L as the sign of successful diabetic modeling.

Following successful modeling, the rats in normal group were given normal food, but not treated, while the rats in model group and etanercept group were intraperitoneally injected with an equal amount of normal saline and etanercept at 5 mg/kg, respectively. After intervention for 7 days, all the rats were sacrificed by cervical dislocation (after being anesthetized using peritoneal administration of pentobarbital sodium at a dose of 40 mg/kg).

Sampling

Upon the completion of anesthesia, the retinal tissues were directly collected from 6 rats in each group, rinsed with normal saline, and preserved in Eppendorf (EP) tubes at -80 °C for subsequent Western blotting and qPCR. The remaining 6 rats in each group were fixed through perfusion for sampling as follows: The thoracic cavity of the rats was cut open to expose the heart, and 400 mL of 4 % paraformaldehyde was perfused through the left atrial appendage. The retinal tissues were taken out, and fixed in 4 % paraformaldehyde for immunohistochemistry and TUNEL assays.

Immunohistochemistry

The pre-paraffin-embedded tissues were sliced and placed in warm water at 42 °C to expand,

they are then mounted, baked and prepared into paraffin-embedded tissue sections. The tissue sections were soaked in xylene solution and gradient ethanol successively for routine deparaffinization and hydration. Subsequently, the resulting sections were immersed in citrate buffer and subjected to complete antigen retrieval by heating repeatedly using a micro-wave oven 3 times (heating for 3 min and braising for 5 min per time). After rinsing, the tissue sections were added dropwise with endogenous peroxidase blocker, reacted for 10 min, rinsed and sealed with goat serum which was added dropwise for 20 min. With the goat serum blocking buffer discarded, the tissue sections were incubated with the anti-Caspase-3 primary antibody (1:200) in a refrigerator at 4 °C overnight. The next day, the sections were rinsed, added dropwise with the secondary antibody solution, and reacted for 10 min. After they were fully rinsed, the sections were reacted with streptavidin-peroxidase solution for 10 min, and added dropwise with DAB for color development, and cell nucleic was counterstained using hematoxylin. Finally, the sections were sealed and observed under a microscope.

Western blotting (WB)

The cryopreserved retinal tissues were added with lysis buffer, bathed on ice for 1 h and centrifuged at 14,000 g in a centrifuge for 10 min. Then, the proteins were quantified using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA), and the concentration of proteins in the tissues was calculated based on the absorbance and standard of proteins determined using a microplate reader.

Subsequently, the extracted proteins were separated using a 10 % sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and subsequently transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA), followed by being sealed with blocking buffer for 1.5 h, and incubated successively with the anti-TNF-α primary antibody (1:1,000), anti-NF-κB primary antibody (1:1,000) and secondary antibodies (1:1,000). After being rinsed, the proteins were reacted with

chemiluminescent reagent for 1 min in the dark for complete image development.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

First, the total ribonucleic acids (RNAs) were extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) from tissue specimens and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using the reverse transcription kit. Then, qPCR was performed in a 20 µL reaction system through 40 cycles of reaction at 51 °C for 2 min, pre-degeneration at 96 °C for 10 min, degeneration at 96 °C for 10 sec, and annealing at 60 °C for 30 s. Finally, the relative expression levels of the related messenger RNAs (mRNAs) were calculated by $2^{-\Delta\Delta Cq}$ method, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference. The primer sequences are detailed in Table 1.

Enzyme-linked immuno sorbent assay (ELISA)

The preserved tissues were ground and subjected to ELISA according to the instructions of the manufacturer. First, the sample and standard were separately loaded into a plate, added with biotinylated antibody working solution and then with enzyme-conjugated substance working solution. Subsequently, the plate was washed. Finally, the resulting standard and samples were detected at 450 nm in a microplate reader.

TUNEL assay

The tissues embedded in paraffin earlier were first made into 5 µm-thick sections, extended in warm water at 42 °C, mounted, baked and prepared into paraffin-embedded tissue sections. Then, these sections were routinely de-paraffinized and hydrated by immersing them successively in xylene solution and gradient ethanol. Subsequently, the sections were added dropwise with TdT reaction solution, reacted in the dark for 1 h, and incubated with deionized water added in drops for 15 min to terminate the reaction.

Table 1: Primer sequences used in PCR studies

Gene	Forward (5'-3')	Reverse (5'-3')
TNF-α	TCCACCAAGAAGCTGAGCGAG	GTCCAGCCCATGATGGTTCT
NF-κB	TCCACCAAGAAGCTGAGCGAG	GTCCAGCCCATGATGGTTCT
GAPDH	ACGGCAAGTTCAACGGCACAG	GAAGACGCCAGTAGACTCCACGAC

Afterwards, the sections were added dropwise with hydrogen peroxide to block the activity of endogenous peroxidase, then reacted with working solution added in drops for 1 h, rinsed, and added dropwise with diaminobenzidine (DAB) solution (Solarbio, Beijing, China) for color development. Finally, the rinsed sections were sealed and observed.

Statistical analysis

In the present study, statistical analysis was conducted using Statistical Package for Social Sciences (SPSS) 20.0 software (IBM, Armonk, NY, USA), and measurement data are presented as mean ± standard deviation (SD). The *t*-test were performed for data meeting normal distribution and homogeneity of variance, corrected *t*-test for those fulfilling normal distribution and heterogeneity of variance, and nonparametric test for those not conforming to normal distribution and homogeneity of variance. Ranked data and enumeration data were subjected to rank sum test and chi-square test, respectively. $P < 0.05$ indicated statistical significance.

RESULTS

Immunohistochemistry results

Tan colored samples represents positive expression, and there were fewer tissues positive for Caspase-3 in the normal group, but more in the other groups. Based on the results in Figure 1, the mean optical density of Caspase-3-positive tissues in both the model group and the etanercept group was significantly higher than that in the normal group, showing a statistically significant difference ($p < 0.05$). While the mean optical density in the etanercept group was significantly lower than that in the model group ($p < 0.05$).
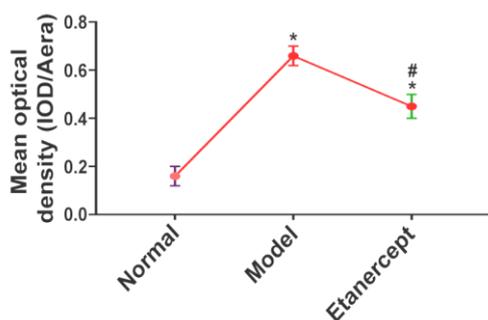

Figure 1: Mean optical density of positive tissues based on Immunohistochemistry results in each group. *$p < 0.05$ *vs.* normal group, and #$p < 0.05$ *vs.* model group

Expression of TNF-α and NF-κB proteins

The normal group showed fewer expressed TNF-α and NF-κB proteins, while there were more expressed TNF-α and NF-κB proteins in the other groups. The statistical results showed that the relative protein expression levels of TNF-α and NF-κB in the model and etanercept groups were considerably higher than those in the normal group, showing statistically significant differences ($p < 0.05$), whereas the etanercept group had significantly lower relative protein expression levels of TNF-α and NF-κB than the model group, (Figure 2) ($p < 0.05$).
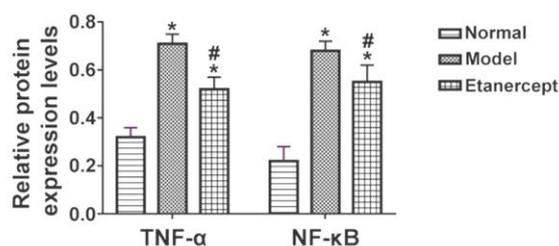

Figure 2: Expressions of proteins detected *via* WB. Relative protein expression levels in each group. *$p < 0.05$ *vs.* normal group, and #$p < 0.05$ *vs.* model group

QPCR results

As shown in Figure 3, both the model group and the etanercept group had significantly higher relative mRNA expression levels of TNF-α and NF-κB than the normal group, and the differences were statistically significant ($p < 0.05$). Moreover, the relative mRNA expression levels of TNF-α and NF-κB in the etanercept group were significantly lower than those in the model group ($p < 0.05$).
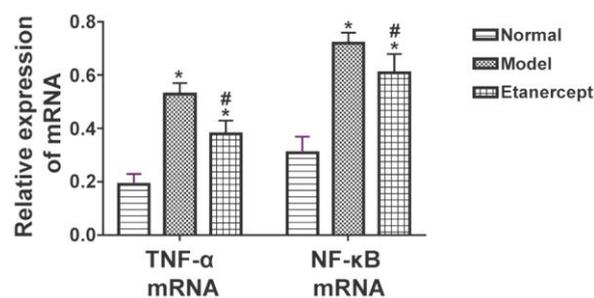

Figure 3: MRNAs determined *via* qPCR. *$p < 0.05$ *vs.* normal group, and #$p < 0.05$ *vs.* model group

TNF-α and IL-1β levels

The model and etanercept groups had significantly higher TNF-α and IL-1β content than the normal group ($p < 0.05$), whereas their content in the etanercept group was significantly

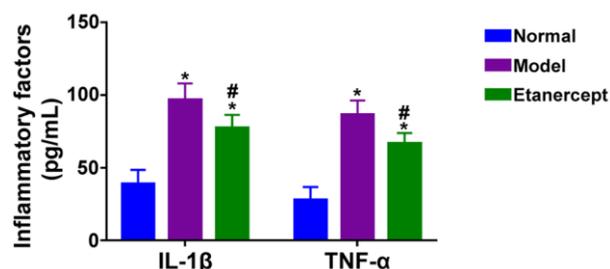lower than that in the model group ($p < 0.05$) (Figure 4).

Figure 4: Content of inflammatory factors in each group. *$p < 0.05$ *vs.* normal group, and #$p < 0.05$ *vs.* model group

Cell apoptosis

Apoptotic cells are tan in color, and there were fewer apoptotic cells in the normal group, but more in the other groups. The apoptosis of cells in the model and etanercept groups was significantly higher than that in normal group ($p < 0.05$), while the etanercept group exhibited a significantly lower cell apoptosis rate than the model group ($p < 0.05$) (Figure 5).
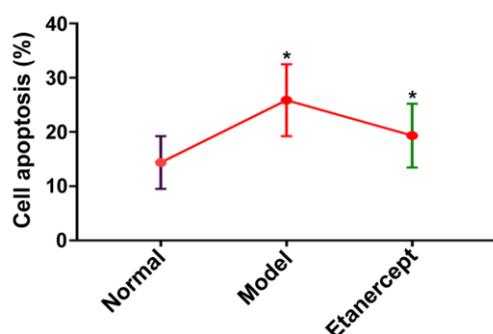

Figure 5: Cell apoptosis (detected *via* TUNEL) in each group. *$p < 0.05$ *vs.* normal group, and #$p < 0.05$ *vs.* model group

DISCUSSION

As one of the common severe complications of diabetes in clinics, diabetic retinopathy mainly damages retinal microvessels, thereby impairing retinal optic nerves and the relevant tissues. Current studies have unraveled that diabetic retinopathy has an increased morbidity rate annually with the increase in that of diabetes, which is one of the leading causes of visual disturbance and even blindness in diabetic patients [12-14]. As the pathogenesis of diabetic retinopathy continues to be researched, it has been gradually realized by researchers that the development of diabetic retinopathy is a long-term complex cascade reaction involving multiple physiological and pathological reactions. Cell apoptosis and inflammation play an important role in the pathogenesis of diabetic retinopathy. In hyperglycemia, pathological changes occurred in retinal microvessels and cause ischemia and hypoxia in the retinal tissues, resulting in tissue damage. After tissue injury, numerous signaling pathways are further activated by ischemia-hypoxia injury and release of various inflammatory factors and cytokines, thereby triggering a series of complex pathological reactions in which the TNF-α/NF-κB signaling pathway serves as a crucial player [15,16].

Moreover, ischemia-hypoxia injury and the release of various inflammatory factors and cytokines can further aggravate the expression and release of TNF-α in retinal tissue, thereby activating the TNF-α/NF-κB signaling pathway. As such, a vicious circle is formed, which further exacerbate retinal nerve cell apoptosis and retinal tissue injury, leading to visual disturbance, even blindness and other severe conditions in patients [17,18]. The results of this study further corroborated that the expression of apoptosis effector molecule Caspase-3 rose significantly and aberrantly, and was highly expressed in the retinal tissues of rats with diabetic retinopathy, thereby significantly elevating the cell apoptosis rate. The protein and mRNA expressions of the crucial molecules, TNF-α and NF-κB in the TNF-α/NF-κB signaling pathway were abnormally raised, suggesting that the TNF-α/NF-κB signaling pathway is activated and involved in the pathological reaction of diabetic retinopathy. The contents of pro-inflammatory factors TNF-α and IL-1β was significantly increased, implying that severe inflammation occurs in the retinal tissues of rats with diabetic retinopathy.

As a commonly used anti-inflammatory drug, etanercept, is a good inhibitor of TNF-α receptor, which suppresses the pro-inflammatory effect of TNF-α through the competitive binding to the receptor of TNF-α [19]. In the present study, it was found that after treatment with etanercept, the diabetic retinopathy model rats had prominently decreased Caspase-3 expression and cell apoptosis rate as well as improvement in the aberrantly high protein and mRNA expressions of TNF-α and NF-κB, suggesting the repressed TNF-α/NF-κB signaling pathway. Besides, compared in model group, the contents of TNF-α and IL-1β declined significantly, indicating that etanercept exert favorable anti-inflammatory and anti-apoptotic effects.

CONCLUSION

Etanercept inhibits NF-α/NF-κB signaling pathway thereby repressing inflammation and

cell apoptosis in diabetic retinopathy rats and exerting a neuroprotective effect. Its potential role in the clinical management of diabetic retinopathy should be therefore be further investigated.

DECLARATIONS

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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REFERENCES

1. Jaya T, Dheeba J, Singh NA. Detection of hard exudates in colour fundus images using fuzzy support vector machine-based expert system. J Digit Imaging 2015; 28(6): 761-768.
2. Fiori A, Terlizzi V, Kremer H, Gebauer J, Hammes HP, Harmsen MC, Bieback K. Mesenchymal stromal/stem cells as potential therapy in diabetic retinopathy. Immunobiol 2018; 223(12): 729-743.
3. Zhang R, Feng B. Efficacy and safety of a combination of Shuangdan mingmu capsules and laser therapy for diabetic retinopathy, and its effect on blood glucose indices and quality of life of patients. Trop J Pharm Res 2022; 21(2): 417-423 doi: 10.4314/tjpr.v21i2.27
4. Graham PS, Kaidonis G, Abhary S, Gillies MC, Daniell M, Essex RW, Chang JH, Lake SR, Pal B, Jenkins AJ, et al. Genome-wide association studies for diabetic macular edema and proliferative diabetic retinopathy. Bmc Med Genet 2018; 19(1): 71.
5. Elmasry K, Mohamed R, Sharma I, Elsherbiny NM, Liu Y, Al-Shabrawey M, Tawfik A. Epigenetic modifications in hyperhomocysteinemia: potential role in diabetic retinopathy and age-related macular degeneration. Oncotarget 2018; 9(16): 12562-12590.
6. Kong L, Zhang Z. Artemisinin ameliorates diabetic retinopathy by upregulating CASC2/miR-155/SIRT1 axis. Trop J Pharm Res 2021; 20(10): 2023-2028 doi: 10.4314/tjpr.v20i10.2
7. Veeraraghavan J, Natarajan M, Aravindan S, Herman TS, Aravindan N. Radiation-triggered tumor necrosis factor (TNF) alpha-NFkappaB cross-signaling favors survival advantage in human neuroblastoma cells. J Biol Chem 2011; 286(24): 21588-21600.
8. Altomare DA, Menges CW, Pei J, Zhang L, Skele-Stump KL, Carbone M, Kane AB, Testa JR. Activated TNF-alpha/NF-kappaB signaling via down-regulation of Fas-associated factor 1 in asbestos-induced mesotheliomas from Arf knockout mice. Proc Natl Acad Sci U S A 2009; 106(9): 3420-3425.
9. Moreland LW, Schiff MH, Baumgartner SW, Tindall EA, Fleischmann RM, Bulpitt KJ, Weaver AL, Keystone EC, Furst DE, Mease PJ, et al. Etanercept therapy in rheumatoid arthritis. A randomized, controlled trial. Ann Intern Med 1999; 130(6): 478-486.
10. Mease PJ, Goffe BS, Metz J, VanderStoep A, Finck B, Burge DJ. Etanercept in the treatment of psoriatic arthritis and psoriasis: a randomised trial. Lancet 2000; 356(9227): 385-390.
11. Percie du Sert N, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M, Browne WJ, Clark A, Cuthill IC, Dirnagl U, et al. The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. PLoS Biol 2020; 18(7): e3000410.
12. Wong TY, Klein R, Islam FM, Cotch MF, Folsom AR, Klein BE, Sharrett AR, Shea S. Diabetic retinopathy in a multi-ethnic cohort in the United States. Am J Ophthalmol 2006; 141(3): 446-455.
13. Kern TS. Contributions of inflammatory processes to the development of the early stages of diabetic retinopathy. Exp Diabetes Res 2007; 2007: 95103.
14. Joussen AM, Poulaki V, Le ML, Koizumi K, Esser C, Janicki H, Schraermeyer U, Kociok N, Fauser S, Kirchhof B, et al. A central role for inflammation in the pathogenesis of diabetic retinopathy. Faseb J 2004; 18(12): 1450-1452.

15. Klein R, Klein BE, Moss SE. The Wisconsin epidemiologic study of diabetic retinopathy: an update. Aust N Z J Ophthalmol 1990; 18(1): 19-22.

16. Fong DS, Aiello L, Gardner TW, King GL, Blankenship G, Cavallerano JD, Ferris FR, Klein R. Diabetic retinopathy. Diabetes Care 2003; 26(1): 226-229.

17. Lee SU, Sung MH, Ryu HW, Lee J, Kim HS, In HJ, Ahn KS, Lee HJ, Lee HK, Shin DH, et al. Verproside inhibits TNF-alpha-induced MUC5AC expression through suppression of the TNF-alpha/NF-kappaB pathway in human airway epithelial cells. Cytokine 2016; 77: 168-175.

18. Fu H, Li G, Liu C, Li J, Wang X, Cheng L, Liu T. Probucol prevents atrial remodeling by inhibiting oxidative stress and TNF-alpha/NF-kappaB/TGF-beta signal transduction pathway in alloxan-induced diabetic rabbits. J Cardiovasc Electrophysiol 2015; 26(2): 211-222.

19. Gonzalez-Lopez MA, Blanco R, Gonzalez-Vela MC, Fernandez-Llaca H, Rodriguez-Valverde V. Development of sarcoidosis during etanercept therapy. Arthritis Rheum 2006; 55(5): 817-820.