Effects of minocycline on expression of bcl−2, bax in early retinal neuropathy of diabetes in rats

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Abstract

• AIM: To study the effect of the minocycline (MNC) on expression of bcl-2, bax in retinal nerve cells of rat with diabetes.

• METHODS: Male SD rats were randomly divided into negative control group, model control group and MNC treated group. Diabetes model was established by intraperitoneal injection of 60 mg/kg streptozotocin (STZ). The protein expressions of bcl-2 and bax in retina were detected by immunohistochemistry method.

• RESULTS: Compared with the negative control group, bax immunoreactive neurons in retina were increased significantly (P <0.01) in model control group. However, bax immunoreactive neurons in retina in MNC treated group were significantly decreased (P < 0.01). Compared with the model control group, bcl-2 immunoreactive neurons in retina were increased significantly(P <0.01) in MNC treated group.

• CONCLUSION: MNC can obviously decreased expression of bax and increased expression of bcl-2 in retina with DR. It is one of path of inhibiting impairment on retinal nerve cells with DR.

• KEYWORDS: mincycline; bcl-2; bax; retina; diabetes

INTRODUCTION

Diabetic retinopathy (DR) is one of the most serious and common complications in diabetes and DR has become the primary reason that diabetes leads to blindness [1].DR has serious impact on the life quality of diabetes patients .Till now,some great progresses were made in the precise etiology of DR in recent years and there are limited choices of treatment for this kind of disease. DR which is classically defined as microangiopathy, but actually it is a neural degeneration of retina. There were lots of evidences showed that just after the diabetes onset and before the angio-complications, the molecule functions are variations in human being and experimental animals. The neuropathy is earlier than the angio-complications in the retina of diabetes, continues through all the time of diabetes; threaten the acuity of vision significant seriously. The accelerated apoptosis of retinal neurons has been observed in early retinal neuropathy of diabetes. The study is to preliminarily investigate the minocycline's preventive effects on apoptosis of retinal neurons by observing the intervention role of minocycline on expression of bcl-2 and bax in retinal nerve cells of rat with diabetes.

MATERIALS AND METHODS

Materials Rabbit polyclonal anti-bcl-2 antibody and rabbit polyclonal anti-bax were obtained from Santa Cruz biotechnology, Inc. Streptozotiocin (STZ) was purchased from Sigma (St. Louis, MO). Minocycline were obtained from Huabei biotechnology, Inc (Shijiazhuang, China). The SP Histostain™ -Plus Kits were obtained from ZSGB biotechnology, Inc (Beijing, China).

Methods All experiments were conducted in accordance with the rules of Association for Research in Vision and ophthalmology Resolution on the Care and Use of Laboratory Animals and approval of the local bioethics committee. The total of 30 age-matched male Sprague-Dawley rats (obtained from the experimental animal centre of the Xinjiang Medical University) were used in all experiment, weight (210±10)g. Rats were housed under a 12-hour light/dark cycle with free access to a standard rat food and water. The 30 rats were randomly divided into three groups (Each group has 10 rats and 20 eyes): Group 1 is the negative control; group 2 is the diabetes model control; group 3 is the minocycline treated group. Diabetes of group 2 and group 3 was induced by intraperitoneal injection of STZ (60mg/kg) dissolved in sodium citrate buffer, pH4.5, and

control rats received equivalent volumes of buffer alone. Food was withdrawn 16houre before the experiments. STZ-injected rats were considered diabetic when exhibiting blood glucose levels >16.7mmol/L by caudal vein within 3 days after diabetes induction. The diabetic rats were divided into diabetes model control and minocycline treated group. After 7 days,45mg/kg minocycline dissolved in physiological saline was given via intraperitoneal injection one time per day for 10 consecutive days in the rats of minocycline treated group, and an equivalent amount of 0.9% physiological saline was utilized at a same way in the animals of negative control group and model control group. Each week, rats blood glucose (nondiabetic, 4-8mmol/L; diabetic, 16.9-29mmol/L) estimated using an One Tuche glucometer (Lifescan, Inc, America).

Histopathology At 4 weeks after diabetic induction, ten rats of each group were anesthetized with 500mg/kg of 10% chloral hydrate injected intraperitoneally and sacrificed. Then enucleation of eyeball was performed after the rats were killed. The bulbus oculi were immersed in 10% paraformaldehyde. After fixation for 24 hours, the protomerite of eyeball was exorcized by cutting the globe along the equator at 0.5mm behind ora serrata and the rest of tissues were dehydrated by gradient alcohol. The samples were cut in central and embedded in paraffin with cutting surface down, cut continuously 4μm sections parallel to sagittal plane of optic nerve. Each eye prepared for 20 pathological sections and 10 randomly selected slices for staining with hematoxylin and eosin stain for light microscopy.

Immunohistochemistry With regard to antibodies and reagents, rabbit polyclonal anti-bcl-2 antibody and rabbit polyclonal anti-bax served as primary antibody, and biotinylated goat anti-mouse IgG as secondary antibody. All the steps followed the specification of the kits. Sections were processed with 0.01mmol/L phosphate buffer solution (PBS) instead of the primary antibody, which were used as negative control. The slices were dried at 60℃ for 2 hours. The sample were deparaffinized and rehydrated. Block endogenous peroxidases by soaking slides in 50μL 3% H_2O_2 deionized water for 20 minutes at room temperature, then washing 3×3 minutes with PBS. Add 50μL the goat serum albumin to each section for 20 minutes at room temperature, the redundant liquid were removed. Add 50μL the primary antibody and incubate 1 hour, then overnight at 4℃ in the refrigerator. Wash 3×3 minutes with PBS. Add 50μL the secondary antibody to each section for 20 minutes at room temperature and wash slides with PBS. Add 50μL biotin streptavidin to each section for 20 minutes at room temperature and wash slides with PBS. Add 50μL the DAB immediately to slides and wait for color change

Table 1 Effects of Minocycline on expression of bcl-2, bax in retinal nerve cells of rats with diabetic retinopathy

		(Mean±SD, n=20)
Group	bcl-2	bax
Negative control	0.1375 ±0.0032	0.1367±0.0178
Model control	0.2102 ±0.0095[b]	0.5359±0.0218[b]
Minocycline	0.3018 ±0.0109[d]	0.4475±0.0176[d]

[b]$P<0.01$ *vs* negative control group, [d]$P < 0.01$ *vs* model control group (ANOVA, SNK-*q* test)

(approximately 3 minutes). Immediately dehydrated in ethanol. Three slices were selected randomly for each eye.

At 400 times magnification, under the light microscopy, the number of immunofluorescent positive cells was counted based on those with yellow or brownish-yellow granules deposited in cytoplasm or nuclei. We selected 5 discontinued high power fields from each section to assess the expression intensity with metabcl-2/bax microgram analytical system to determine the integrated bcl-2/bax of positive cells. Immunohistochemical staining images were evaluated by analysis assay of bcl-2 integrated optical density (IOD) averages and bax IOD averages.

Statistical Analysis SPSS13.0 for windows statistical software was used in this study. All the experimental data were expressed by mean±SD. One-way analysis of variance (ANOVA), LSD-test was employed to compare the difference between groups.The P valve<0.05 was considered statistically significant.

RESULTS

Pathology Under light microscope, the structure in each layer of retina is distinct in negative control group and MNC treated group. The permutation of cells is compact and regular. The structure in each layer of retina is distinct in model control group. The arrangement of cells is loose, while the interspaces of cells are wider. Especially in inner nuclear layer and the ganglion cell layer. Cellular form in model control group is not obviously different from the normal group.

Immunohistochemistry There was weak bcl-2 expression in normal control group and expression in model control group; The expression of bcl-2 in MNC treated group was higher than that in normal control group and model control group. There is statistically significant difference (*P* <0.01): granules of yellowish-brown in the cytoplasm significantly increased; the color was deep. There was weak bax expression in normal control group and higher expression in model control group; granules of yellowish-brown in the cytoplasm significantly increased, and color is obviously deep. The expression of bax in MNC treated group was lower than that in model control group. There is statistically significant difference (*P* <0.01) as granules of yellowish-brown in the cytoplasm significantly decreased and the color is light (Table 1, Figure 1, 2).
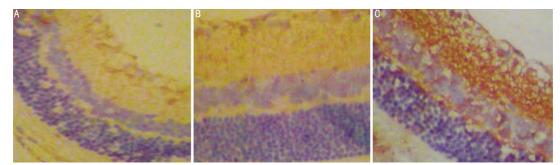
Figure 1 Expression of bcl−2 in retinal nerve cells of rats with diabetic retinopathy (×400) A: Negative control; B: Model control; C: Minocycline
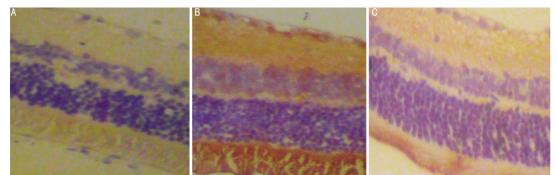

Figure 2 Expression of bax in retinal nerve cells of rats with diabetic retinopathy (×400) A: Negative control; B: Model control; C: Minocycline

DISCUSSION

DR is a series of intraocular complications induced by metabolic disorder of body sugar and lipid for long-term absolute or relative shortage of insulin secretion. It is said currently that in the early stage the retinal neurons pathological changes were happened before retinal vascular disease induced by diabetes. Diabetes metabolic disorder may first influence retinal neurons, capillary pathological changes is the results of its secondary influence. The pathological changes were closely related to nerve cell apoptosis such as retinal photoreceptor cells and ganglion cells [2]. Cell apoptosis is directly regulated by multiple genes inside the cells . Among them, the most representative is bcl-2 and bax; bcl-2 is anti-apoptosis genes, bax is apoptosis-promoting genes. The different dimer ratio of bcl-2 to bax plays key role in cell apoptosis. Research indicates that bax enhancement to bax expression in diabetes accelerated the neuron cell death and reduced axonal regeneration[3].

Minocycline is the second-generation artificial half-synthetic tetracycline drugs which has been used in the dermatologist. It makes the central nervous effect with prerequisites because of its penetration to the blood brain barrier and the security to the people. In recent years, people found that minocycline had significant nerve protective effect in central nervous disease such as brain ischemia、brain injury [4,5] and animal models to retinal diseases [6]. Minocycline has the protective functions to nerve cells in diabetic retinopathy, its mechanism is probably as follows: 1) Inhibiting cell necrosis through inhibiting proinflammatory factor release. 2) Restraining microglia activation by stopping expression of cox-2 induced by TNF in microglia. 3) Decreasing nerve cell apoptosis by inhibiting death receptor way[7] and suppressing caspase-8, -1, -9, -3 activation [3,8]. In this study, in respect of nerve cells in diabetic retinopathy, minocycline can inhibit the damage to nerve cells by increasing the protein bcl-2 and reducing the protein bax. Furthermore, this study provides a new basis of theory to minocycline's protective effect on nerve cells in diabetic retinopathy.

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