ORIGINAL ARTICLE

The oxidation role of peroxynitrite and antagonism of cholecystokinin octapeptide-8 on cataract in rats

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Abstract

Aim: To explore the damage role of peroxynitrite (ONOO−), and antagonism of cholecystokinin octapeptide-8 (CCK-8) on diabetic cataract in rats. Methods: Diabetic cataract animal model was established by peritoneal injection streptozotocine (STZ). Thirty-six rats were taken as control group, seventy two were given STZ (45mg/kg) and then divided into STZ group and CCK-8 group (peritoneal injection CCK-8). STZ-induced diabetic rats were treated with CCK-8 for 60 days. Lenses were examined with slit lamp. STZ group rats developed lens opacity at 20 days and reached high level at 60 days after STZ injection. CCK-8 group rats delayed the cataract formation in a time-dependent manner. At 20, 40, 60 days respectively, immuno-flourescent staining and Western blot analysis were used for expression of nitrotyrosine (NT, the foot print of ONOO−); RT-PCR and gene array analysis were used for expression of iNOS mRNA on lens epithelium cells (LEC). Result: There were no distinct expression of NT and iNOS mRNA in control group. But in STZ group, there were distinct expression of NT and up-regulating of iNOS mRNA as time passed by. Whereas to CCK-8 group showed weak expression of NT and down-regulating of iNOS mRNA. CONCLUSION : There were expression of NT on diabetic rat LEC, which maybe constitute the new way of oxidant stress ; CCK-8 could reverse NT damage on LEC. The results suggested that CCK-8 may be an useful therapeutic agent against diabetic cataract. The antagonizing mechanism of CCK-8 may be related to its inhibition the expression of iNOS mRNA for the production of the NO and therefore decreasing the formation of ONOO — along with direct antagonism of ONOO−.

Keywords: *Diabetic cataract; oxidation; peroxynitrite; cholecystokinin octapeptide-8.*

INTRODUCTION

Research on mechanism of cataract has made remarkable progress. The major views that are accepted today are abnormal glucose metabolic pathway and oxidation stress theories. The latter has attracted more and more interest among the ophthalmologists and is widely accepted by medical field. But traditional oxygen-free radical stress mechanism pays more attention to the role of hydrogen-peroxide (H_2O_2), nitric-oxide (NO) and superoxide-anion (O2-), while the new theory concentrates on peroxynitrite (ONOO-), a product from rapid reaction of NO and O2-, which may be an important mediator of cytotoxicity in oxidation process.[1-4] We have reported the lens oxidation by ONOO- pathway and antagonism of the puerarin.[5-9] Now we report our further experiment by which ONOO- was produced in LEC of diabetic cataract and antagonized by cholecystokinin octapeptide-8 (CCK-8).

MATERIALS AND METHODS

Animals: Pathogen-free, male, Sprague-Dawley (SD) rats (5-6 weeks old) obtained from Hebei Province Medical Animal Experimental Center (Hebei Province, China) were used in this study. All animals were treated in accordance with China law (comparable with ARVO Statement for the use of Animals in Ophthalmic and Vision Reaseach in U.S.).

Antibodies and Reagents: Monoclonal mouse anti-NT antibody, goat anti-fluorescent isothiocyanat (FITC) antibody, stroptozotocine (STZ) and CCK-8 were purchased from Sigma Company.

Groups and Animal model: Animals were divided into three groups: control group, STZ group and CCK-8 group, each containing 36 animals. SD rats in STZ group and CCK-8 group were peritoneal injected STZ (45mg/kg) respectively to establish animal model. The control group received same amount of saline. Three days after injection of STZ, CCK-8 group received CCK-8 100μg/kg per day. The blood glucose and body weight were evaluated at 20, 40 and 60 days. Animal's eyes were examined by slit lamp at same time after STZ injection for clinical signs of cataract and were graded in to 0-5 scale10 then enucleated for further study.

Immunofluorescent staining: The rats were sacrificed and the eyes were enucleated immediately on 20, 40, 60 days respectively. The lenses were dissected via posterior incision under dissecting microscope and then fixed in 70% ethanol for 24 hours. The lenses were screened with PBS until they become milky. Then, the suspension was centrifuged at 1000g r/min for 4 minutes. The sediment was mixed with PBS and centrifuged again. With indirect immuno-flourescent labeling antibody technique, NT (1:400) were added into suspension and reacted in dark for 30 minutes at room temperature. Then, goat anti mouse FITC labeling antibody was added and kept for another 30 minutes under same condition. The suspension was examined under fluorescent microscope.

Western Blot Analysis: Lens prepared as described above was homogenized and solubilized in ice cold PBS containing protease inhibitors, phenylmethylsulfonyl fluoride (1μg/ml), aprotinin (1μg/ml), leupeptin (1μg/ml), pepstatin A (1μg/ml) and EDTA (1mM). The homogenate was centrifuged at 15,000g at 4°C for 10 minutes. The protein content of the supernatants was determined by the Bradford method.[11] After sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% linear slab gel, under reducing conditions, separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane using a semidry electrophoretic transfer cell (Trans-blot; Bio-Rad, Richmond, CA). Blot was stained at room temperature with a 1:600 dilution of monoclonal mouse anti- NT antibody over night at 4°C. After washing and incubation with horseradish peroxidase-conjugated secondary antibody (1:1000 dilution), blot were developed using the enhanced chemiluminescence Western blot analysis detection systerm (ECL Plus; Amersham Pharmacia Biotech, Arlington Heights, IL).

RT-PCR Analysis: Equal amounts of the total RNA were used to detect the mRNA levels of iNOS by reverse transcription polymerase chain reaction (GeneAmp RNA-PCR kit; Applied Biosystems). Total RNA was extracted from the rat lenses in three groups, according to the kit manufacturer's specifications. The sense and anti-sense oligonucleotide primers for rat iNOS12 were synthesized by Shanghai Biological Engineering Corporation. The primer sequences are: iNOS (262bp) sense primer1: 5′-CGCCCTTCCGCAGTTCT-3′; sense primer2: 5′-TCCAGGAGGACATGCAGCAC-3′.β-actin. (420bp) sense primer1: 5′-GAGACCTTCAACACCCAGCC-3′; sense primer2: 5′-GCGGGGCATCGGAACCGCTCA-3′. And 4μg of total RNA in a total volume of 20μl (PH8.3) were for synthesizing the cDNA. RT reaction was first performed at 24°C for 10 min, then at 42°C for 15 min. The reaction mixture was heated at 99°C for 5 min, and the RT product was mixed with DNA

polymerase (AmpliTaq; Applied Biosystems) and the sense primer in a buffer containing 20mM Tris-HCL, 50mM KCl, 2.0mM MgCl2 (PH 8.3), and 50mM of each dNTP in a 100μl volume. The mixture was then amplified by PCR using 29 cycles. The thermal cycle profile used in this study was as follows: an initial denaturing at 94°C for 2 min and then 45 sec in each cycle; annealing the primer with DNA at 55°C for 45sec; and extending of the primer at 72°C for 10min. All reactions were normalized for iNOS expression. The negative controls consisted of omission of RNA template or reverse transcriptase from the reaction mixture. PCR products were analyzed on 2% agarose gel.

Gene Arrays: Lenses used in arrays were dissected free of any contaminating tissue and homogenized in trizol reagent. RNA extraction was carried out according to the manufacture's protocol. Concentration and RNA quality were assessed via spectrophotometry and formaldehyde gel electrophoresis. Amplified mRNA was then labeled with Cy3 or Cy5 (Random Primer DNA Labeling Kit). Successfully labeled control and experimental targets with 12 housekeepig genes and 12 artificial synthesize 70 mer oligo DNA which were as positive and negative control were then combined and prepared for hybridization. Array slides were obtained from Qiagen Corporation. and incubated in prehybridization for 1 h at 42°C. Targets were dried via vacuum centrifugation then resuspended in 50μl hybridization solution with added 1μl Cot 1 DNA and 1μl poly A oligonucleotide as blocking agents, heated to 95°C for 5 min and then added to the face of one slide. The printed face of the second slide of the pair was then placed face to face with the first, using the same prob. Slide pairs were then placed on a level plastic cover above some 1×SSC moistened tissue in a slide box. The slide box was sealed and placed floating in a water bath and habridized for 24-48 h at 42°C. Following hybridization, slides were washed in wash solution for 20 min and repeated for another 20min then dipped in nuclease free water, spray dried. Finally, the backs of the slides were cleaned with ddH_2O, wiped with 100% ethanol, then wiped dry and scanned by Scan Array Express Scanner (Packard Bioscience Corporation). RT-PCR array confirmations were the same as above RT-PCR analysis. Gene Pix Pro 4.0 photo soft ware (Axon Instruments Corporation) was used for clustering analysis.

Two folds higher divergence were regarded as divergence expression gene.

Statistical Analysis

Statistical analysis of the data was performed on computer (SPSS, version 10.0). The results are expressed as means ± SD. Statistical significance was determined by a one-factor analysis of variance (ANOVA) followed by the Fisher post hoc test for multiple comparisons. $P<0.001$ was considered significant.

RESULTS

Animal Model: Animals were divided into three groups: control group, STZ group and CCK-8 group, each containing 36 animals. The average initial body weight of the rats was 251.23±2.20 and the average blood glucose level before inducing diabetes was 3.48±0.19. Compared to control and CCK-8 group, there were typical diabetic symptoms in STZ group. And there were also remarkable glucose concentration and body difference among three groups during 20, 40, 60 experiment days (Table 1).

The rats' lenses were examined with slit lamp. In control group lenses were clear during whole experiment period. At 20 days, lenses were clear in CCK-8 group but developed opacity in STZ group (7 lenses in grade 2, 87% and 1 lens in grade 3, 13%); At 40 days, lenses were developed opacity in CCK-8 group (5 lenses in grade 1 62% and 3 lenses in grade 2, 38%). In STZ group (6 lenses in grade 3, 75% and 2 lenses in grade 4, 25%); At 60 days, in CCK-8 group (5 lenses in grade 1, 62% and 3 lenses in grade 0, 38%). In STZ group (2 lenses in grade 3, 25% and 6 lenses in grade 4, 75%).

Table 1 Effects of CCK-8 on blood glucose (mmol/L) and body weight (g) in diabetic cataract rats ($\bar{x}$ ±s, n=12)

Days	Control group		STZ group		CCK-8 group	
	Blood glucose	Body weight	Blood glucose	Body weight	Blood glucose	Body weight
20	3.36±0.06	323.06±2.09	20.01±0.03**†	229.07±1.38**†	10.31±0.08*	316.25±2.67*
40	3.42±0.07	383.21±2.19	22.78±0.04**†	190.98±2.63**†	9.01±0.07*	369.47±2.91*
60	3.39±0.05	460.36.±2.10	23.62±0.05**†	149.76±2.19**†	7.23±0.05*	461.09±2.76*

*P<0.05, ** P<0.001vs Control group; † P<0.01 vs.CCK-8 group.

Immunofluorescent Staining: Under fluorescent microscope, NT negative antigen showed faint-green color in nucleus and cytoplasm and NT positive antigen appeared orange-yellow color. The control group showed faint-green color in nucleus and cytoplasm. The STZ group color changed from green-yellow to orange-yellow color during the period of twenty to sixty days of experiment. Otherwise, CCK-8 group color ranged from faint-green to yellow color during the period of twenty to forty days of experiment, then to green-yellow on sixty days (Figure 1).

Western Blot Analysis: With Western blot analysis, a faint expression of NT could be seen in control group. A gradual to strong expression of NT was observed at different stages of the experiment in STZ group. But expression of NT in CCK-8 group changed from faint to gradual strong during the period of twenty to forty days of experiment, then turn to weak on sixty days (Figure 2). With computer photo-analysis, there were significant differences among three groups (P<0.001, Figure 3).

RT-PCR Analysis: There was no expression of iNOS mRNA in control group. There was distinct up-regulating of iNOS mRNA in STZ group as time passed by. But expression of iNOS mRNA in CCK-8 group appeared gradually up-regulating of iNOS mRNA during the period of 20 - 40 days of experiment, then down-regulating on 60 days (Figure 4). With computer photo-analysis, there were significant differences among the three groups (P<0.001, Figure 5).

Gene Arrays: With CCK-8 treatment, expression of iNOS mRNA appeared gradually up-regulating during the period of 20 - 40 days of experiment, then down-regulating on 60 days (Figure 6).

DISCUSSION

In order to disclose the mechanism of oxidation stress in the process of cataract formation and offer the new theory and experiment data for prevention and treatment cataract, we established diabetic cataract animal model of rats[13-18] to study the pathogenesis of ONOO- during the formation of diabetic rat cataract and the antagonism of CCK-8.

Using immuno-flurescent staining, Western blot technique, we verified that there were ONOO- produced during the formation of diabetic cataract. As described before, one of the probable pathogenesis for diabetic cataract currently is oxygen free radical route. Our study provides an important supplement to the oxygen free radical damage view i.e. stressing the effect of ONOO－during the formation of cataract. LEC is the most active metabolite part in lens. Restoring condition of the lens mainly depends on the antioxidant enzyme produced in LEC. The capability of this enzyme to clear off free radicals could be damaged by ONOO- and •OH as well as other related substances. This process leads to the formation of cataract at last.

Using RT-PCR, Gene array technique, we further verified that iNOS might contribute to the oxidation stress by helping to develop many more powerful oxidative agents such as ONOO-. As we all know, inducible nitric-oxide synthase (iNOS) is the major inhibition enzyme in production of NO, which is a new signal molecule with multi-regulation function.[19] Under pathological condition, up-regulating the LEC iNOS mRNA lead to over production of NO, accompanied by activation of the oxidant enzyme as well as increasing the O2-. Extra NO and O2- produce extra ONOO-, this extra ONOO- is the strong oxidant. Current information indicates that NO is formed in the lenses of human, rat and rabbit.[20] However, the changes among NO, iNOS and ONOO- during the formation process of diabetic cataract are still not clear. Our study showed that NT increasing greatly in LEC of diabetic rats which indicates that high glucose could induce the production of NT. Due to high blood glucose, excess (over dose) non-enzyme-glucose reaction took place in blood plasma and tissue protein. Therefore, the structure and function of protein changed and turned into abnormal condition to develop hemoglobin-associated-crystals-glucose (HbA_1C). Increasing of HbA_1C enhanced the combination between hemoglobin and oxygen and decreased their departure, which leads to lack of oxygen in plasma and tissue so as to increasing free radical in those places; this is one of the main causes of chronic diabetic complications. On one hand, CCK-8 alleviated the oxidation of LEC and other tissues induced by ONOO- and strengthened the role of eliminating free radical system in diabetic rat body, which would decrease the production of HbA_1C and free radical. On the other hand, CCK-8 could inhibit the expression of iNOS mRNA, therefore decreased the formation of ONOO-. The above two way alleviated the lens oxidation triggered by ONOO-. The express of minor NT in control group was the proof of the physiological existence of ONOO-. Using same concentration of exogenous NO and ONOO- reacted with oxen pulmonary endothelium, only ONOO- could induce cell apoptosis.[21] ONOO- affected cell's oxidant and restore condition, ionized channel, proteintase, nitro-protein, inhibiting the respiratory of mitochondria, leading to cell apoptosis and non- cell apoptosis mediated by mitochondria.[22-24] During the retinas ischemia and re-perfusion stage, there were apparently increasing levels of NO and ONOO-.[25] This supported evidence of ONOO- being produced in the eyes. Our study found that ONOO- induced apoptosis of LEC. Therefore, we deduce that iNOS induced over production of NO, later combined with O2- to form oxidant of ONOO- as well as others which constitute the new way of oxidant stress.[26]

New research suggests that CCK-8 is not only a stomach-intestine hormone but also a nerve peptide, distributing in stomach, intestine and central nerve system and has many physiological functions.[27] Kuntz E, et al[28] reported that CCK-8 could improve blood glucose concentrations in type 1 diabetic rats correlated with an increase in beta cell mass. Former studies in our department revealed that CCK-8 played a protective role in rat lungs. Then, can CCK-8 also contribute the same ability to LEC. Our study found that the damage role ONOO- and iNOS could be antagonized by CCK-8. The results indicated that CCK-8 delays the development of lens opacities and may be a useful therapeutic agent against diabetic cataract. The pathogenesis of which may be related to its anti-oxidant role, anti-iNOS-NO system, which causes reduction of lens oxidation.[29,30] We found during the period of 20-40 days, the effect of CCK-8 was not distinctly, while after 40 days its effect was outstanding. The reason for this maybe related to the concentration of CCK-8 had reached to a certain level.

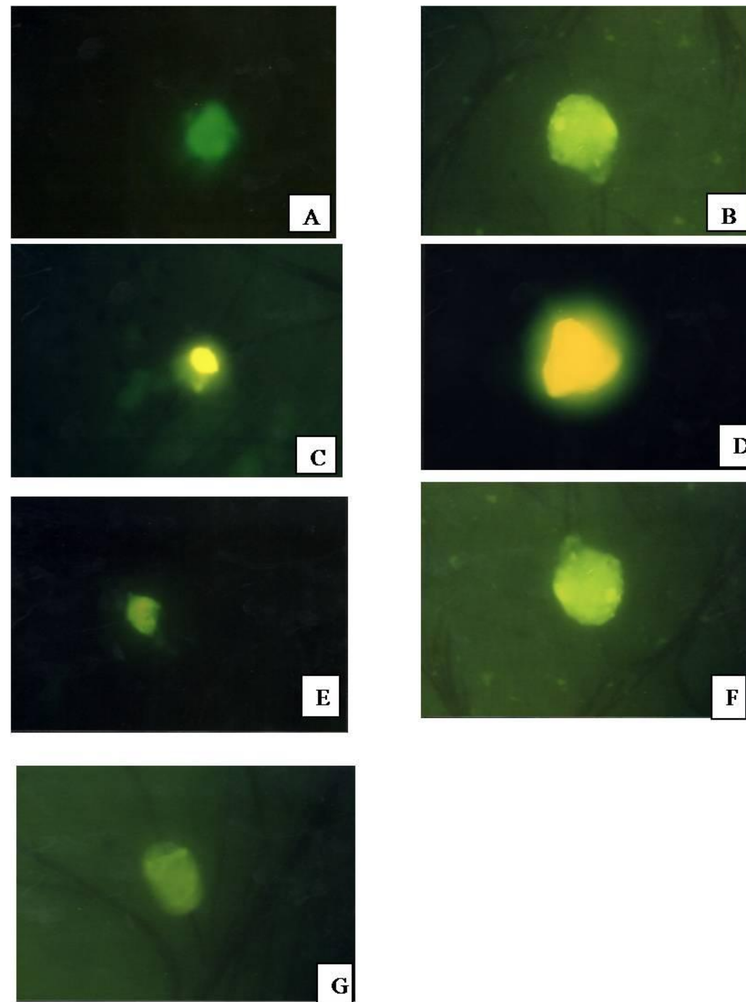

Figure 1 Appearance of LEC under fluorescent microscope; A. Control group; B, C , D 20, 40, 60 days in STZ group; E , F, G 20, 40, 60 days in CCK-8 group.

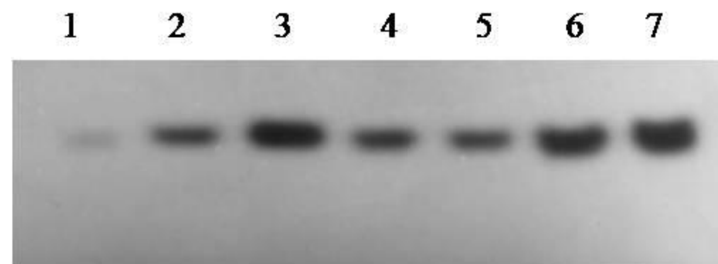

Figure 2 Detection of NT protein expression in lens of diabetic cataract with Western blot analysis. Lane 1: Control group 1; Lane 2-4: CCK-8group at 20, 40, 60 days respectively; Lane 5-7: STZ group at 20, 40, 60 days respectively.

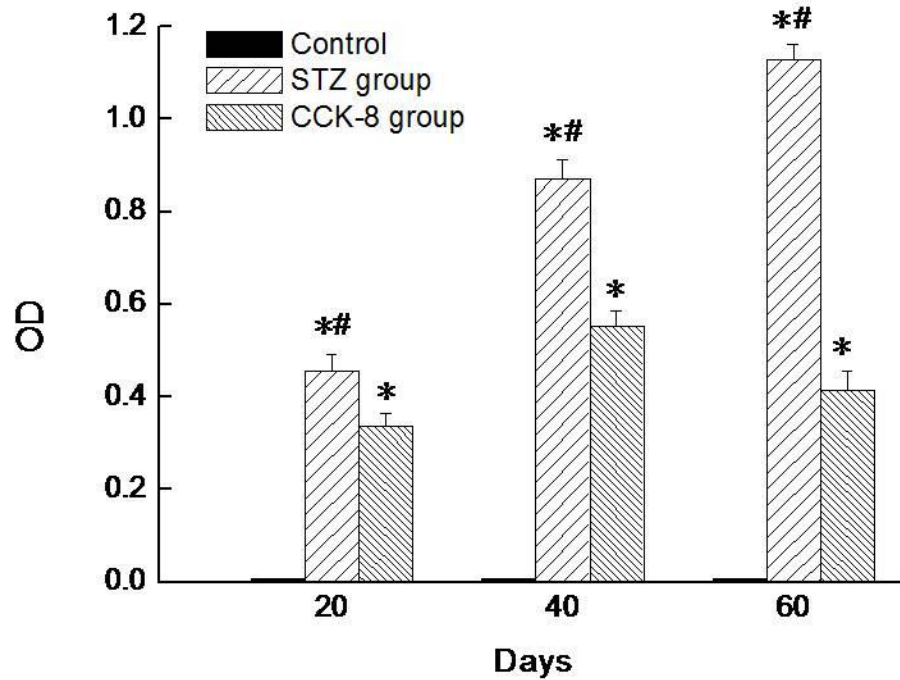

Figure 3 Detection of NT protein in diabetic rat cataract lens with Western blot analysis. * P＜0.01 vs. Control group; # P<0.05 vs CCK-8 group .

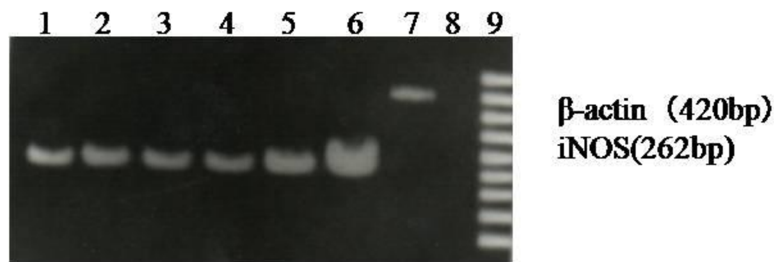

Figure 4 RT-PCR detection of iNOS mRNA expression in lens of diabetic cataract with CCK-8.
Lane 1-3: CCK-8 group at 20,40,60 days respectively; Lane 4-6: STZ group at 20,40,60 days respectively; Lane 7：β-actin； Lane 8：Control group; Lane 9: DNA Marker.

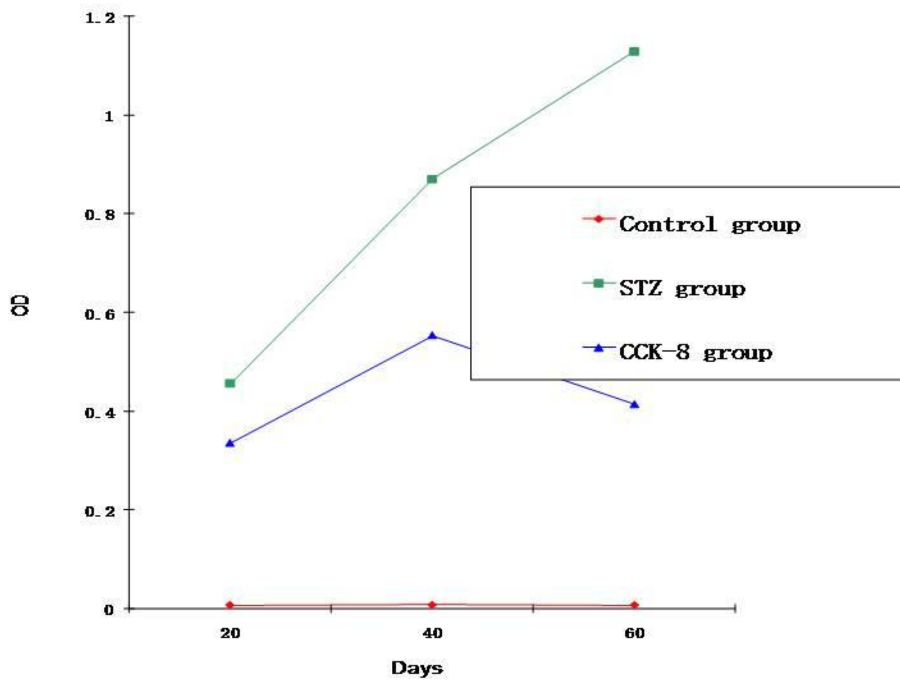

Figure 5 Detection of iNOS mRNA expression in diabetic rat cataract lens with RT-PCR method. * P<0.01 vs. Control group; # P<0.05 vs. CCK-8 group.

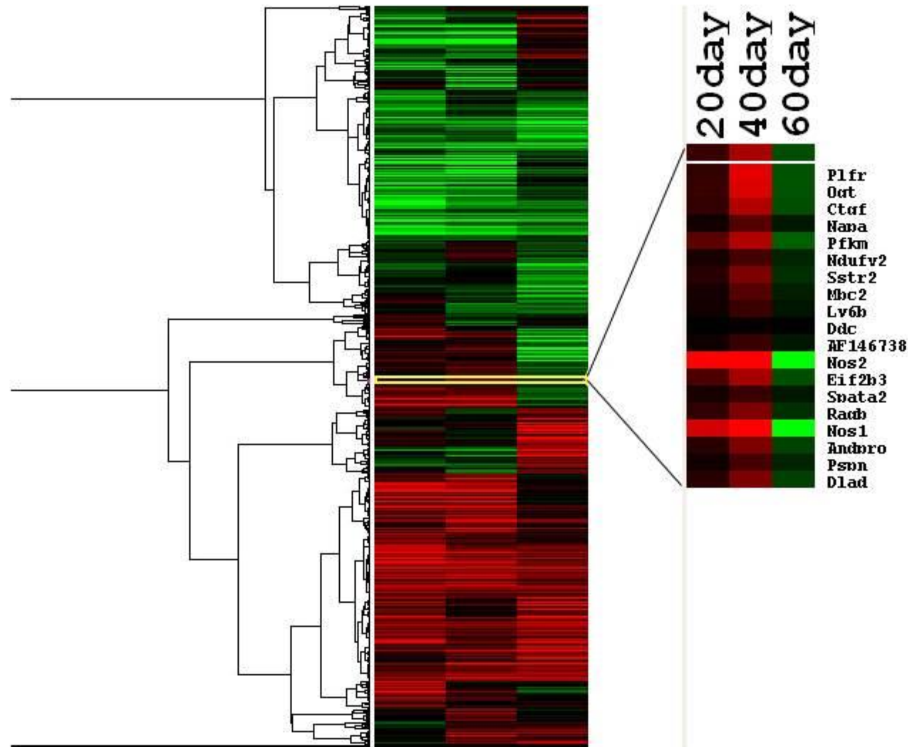

Figure 6 Gene array expression. As time passed by, CCK-8 group showed down-regulating iNOS mRNA. Red color showed up-regulating gene; Green color showed down-regulating gene; Black color showed no changes.

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