# Effects of calcium dobesilate on Nrf2, Keap1 and HO-1 in the lenses of D-galactose-induced cataracts in rats

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Abstract. This study investigated the effects of calcium dobesilate on Nrf2, Keap1 and HO-1 in the lenses of D-galactose-induced cataracts in rats. Thirty Sprague-Dawley rats were randomly divided into three groups: a blank control group, a model control group and a model administration group. A normal diet was given to the rats in the blank control group and the rats with D-galactose-induced cataracts of the model control group. Calcium dobesilate was also given to the rats with D-galactose-induced cataracts of the model administration group. A slit lamp microscope was used to check the degree of lens opacity. RT-PCR and western blot analysis were used to detect the mRNA and protein expression of Nrf2, Keap1 and HO-1 in the lenses of the three groups. There was a significant difference in the degree of lens opacity among the three groups (P<0.05). The model control group was the most turbid of the three groups, followed by the model administration group. Moreover, the mRNA and protein expression of Nrf2, Keap1 and HO-1 in the lenses of the three groups were also significantly different (P<0.05). The mRNA levels of Nrf2 and HO-1 were the highest in the model control group, followed by the model administration group, and were the lowest in the blank control group. However, the mRNA expression level of Keap1 among the three groups had an opposite trend. In conclusion, calcium dobesilate can effectively increase the levels of Nrf2 and HO-1 in the lenses of diabetic cataract rats and inhibit the level of Keap1. Therefore, the therapeutic effect of calcium dobesilate against cataracts is related to the improvement of the Nrf2-Keap1 signaling pathway.

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# Introduction

Diabetic cataracts are an eye disease featured by visual deficits and blindness. While diabetes itself is a common medical condition, the elevated blood glucose levels that patients are prone to are a major cause of complications, such as cataracts (1). Disease data indicate a high incidence rate of cataracts and a rapid disease progression, making advanced diabetic patients susceptible to the threat of blindness (2). Diabetic cataracts are similar to senile cataracts in regards to the abnormal metabolism within the lens, but the specific biomolecular mechanism of their pathogenesis is not clear. There are presently three popular theories on its pathogenesis (3), of which oxidative stress is the most recognized by scholars. The theory holds that there are a variety of antioxidant enzyme systems in a normal functioning lens, including the well-documented superoxide dismutase, glutathione peroxidase and others (4,5). On the one hand, the high glucose environment in diabetic patients can promote increased production of reactive oxygen species. On the other hand, the regulation of key antioxidant factors can be impaired in diabetics, leading to an imbalance between oxidation and reduction and resulting in oxidative damage within the lens. It has been confirmed that Nrf2 is a key factor in regulating the balance of oxidation in vivo, through mediation of the synthesis and expression of a series of downstream antioxidants via the Nrf2/Keap1 signaling pathway (6).

Calcium dobesilate is a vascular protective agent that is effective against microvascular circulatory disorders caused by a variety of diseases, through improving the biosynthesis of collagen in the basement membrane by inhibiting the high permeability caused by vasoactive substances (histamine, serotonin and bradykinin). Studies have shown that calcium dobesilate has a certain controlling effect on cataracts (7). Clinical research on calcium dobesilate for the treatment of diabetic cataract has been reported, but its focus was on therapeutic effects rather than the underlying mechanisms. The aim of this study was to investigate the effects of calcium dobesilate on the expression of Nrf2, Keap1 and HO-1 in rats with D-galactose-induced cataracts, in order to explore the mechanisms of calcium dobesilate on the treatment of diabetic cataracts.

### Materials and methods

A LYL-I slit lamp micrometer was purchased from Suzhou QILE Electronic Technology Co., Ltd. (Jiansu, China). A -80°C ultra-low temperature refrigerator was obtained from Haier Co., Ltd. (Qingdao, China). A QuantStudio<sup>®</sup> type 3 real-time fluorescence quantitative PCR system was purchased from Thermo Fisher Scientific Co., Ltd. (Waltham, MA, USA). ECL Chemiluminescence kit and PVDF Membrane were both purchased from Invitrogen (Carlsbad, CA, USA). Thirty Sprague-Dawley (SD) male rats were provided from Nanjing Qinglongshan Animal Technology Co., Ltd. (Nanjing, China). A tissue mRNA extraction kit was obtained from EMD Millipore (Darmstadt, Germany). A reverse transcription kit was purchased from Beijing Zhijie Fangyuan Technology Co., Ltd. (Beijing, China). The primary antibodies of Nrf2, Keap1, HO-1 and the secondary antibody were all purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The pairs of primers used for the study are as follows: Nrf2 (163 bp) sense, 5'-CCTTCCTCTGCTGCCATTAGT-3' and antisense, 5'-CCTTCCTCTGCTGCCATTAGT-3'; Keap1 (154 bp) sense, 5'-GGAATGCTATGACCCAGACA-3' and antisense, 5'-TGCTCAGGTAGTCCAAGTGC-3'; HO-1 (144 bp) sense, 5'-AGCATGTCCCAGGATTTGTC-3' and antisense, 5'-GTACAAGGAGGCCATCACCA-3'. All PCR primers were synthesized by Shanghai Bioengineering Co., Ltd. (Shanghai, China).

Experimental groups. The experiment lasted for 21 days. Thirty male SD rats were randomly divided into three groups: A blank control group, a model control group, and a model administration group, with 10 rats in each group. Group assignment was randomized using a random number table grouping. Rats in the blank control group were fed a normal diet with water and ordinary feed (nutrient ratio of protein:fat:carbohy drate=2:1.5:4) over the course of the experiment. Rats in the model groups were fed 12.5% D-galactose for 7 days from the beginning of the experiment and with 10% D-galactose from day 8 until the end of the experiment. The rats in the model administration group were also given calcium dobesilate daily at a dose of 150 mg/kg/day by intragastric administration from the first day of the study. The specific dosage for each rat was calculated according to body weight and the administration continued until the end of the study.

Specimen collection. The lens samples of the rats were collected for the expression analysis of Nrf2, Keapl and HO-1. Twenty-one days after the study, rats in each group were fasted for 24 h. The rats were sacrificed by intraperitoneal injection of 10% chloral hydrate and the eyeball was removed. The lens was then rapidly separated and homogenized under ice bath to make the lens homogenate, and then stored at -80°C.

Degree of lens opacity. The degree of lens opacity was examined and classified on the 21st day of the study using a slit lamp microscope. The grading criteria for glucose-induced cataracts was based on literature published by Suryanarayana *et al* (8), which graded opacity into six levels, with grade 0 showing no turbidity of the lens and grade V indicating that the lens was completely turbid.

The expression of Nrf2, Keapl and HO-1 mRNA in lens. The expression of Nrf2, Keap1 and HO-1 mRNA in lens tissue was detected by real-time quantitative PCR. The target mRNA in the rat lens homogenate was extracted using the RNA extraction kit under stringent RNase-free and sterile conditions according to the kit procedure. The RNA extraction process was kept at a low temperature to prevent RNA degradation. The purity of the extracted RNA was determined by spectrophotometer and the ratio of the absorbance at 260 nm to the absorbance at 280 nm was between 1.8 and 2.0. The required samples were stored at -80°C under sterile and RNase-free conditions. Reverse transcription was conducted using the reverse transcription kit to synthesize the first strand of cDNA in strict sterile conditions, with addition of the reagents, followed by incubation at 40°C for 45 min and then inactivation of reverse transcriptase at 90°C. Real-time quantitative PCR was used to amplify and quantitatively detect the expression of the three mRNAs in the lens samples. The mRNA expression of the blank control group was taken as a control and quantified as 1 to obtain the relative values of the other two groups. PCR amplification cycle parameters were set as follows: 55°C for 2 min, 90°C for 10 min, 95°C for 15 sec, 58°C for 30 sec and 72°C for 30 sec, repeated over 50 cycles. Amplification was performed with the internal reference group and the blank control group (DEPC water).

The expression of Nrf2, Keap1 and HO-1 protein in lens. The total protein of rat lens samples was extracted and the standard curve of protein concentration was drawn by BCA method to calculate the expression levels of protein to be measured. The protein was loaded into SDS-PAGE gel for electrophoresis and the protein was separated. After electrophoresis, the protein was transferred to the membrane and the membrane was taken out, washed and incubated with the primary antibody and then the secondary antibody. The protein bands on the membrane were detected by enhanced chemiluminescence and the gray scale was scanned by an automatic gel imaging analyzer. The relative gray value was then calculated as the expression levels of the protein.

Statistical analysis. SPSS 17.0 statistical analysis software (IBM SPSS, Armonk, NY, USA) was used to evaluate the differences of the indices before and after treatment. The F-test was used for comparison between groups and the rank data were analyzed by the rank sum test. P<0.05 was considered to indicate a statistically significant difference.

# Results

Degree of lens opacity. A comparison of grading levels of lens opacity at 21 days for all three groups of rats is shown in Table I. All the lenses in the blank control group rats were clarified and clear, with no turbidity, and the degree of lens opacity for all was graded as 0. In the model control group, the lenses in rats showed white turbidity, with no boundary between the core and the cortex of the lens, and the degree of lens opacity for most rats was graded as V, with a minority of the rats graded as IV. The lenses of rats in the model administration group showed turbidity, but the degree was significantly lighter than that of

Table I. Comparison of the opacity of lens samples between the 3 groups (n=60).

Groups	No. of eyes	0	Ι	II	III	IV	V
Blank control	20	20	0	0	0	0	0
Model control	20	0	0	0	0	6	14
Model administration	20	0	0	2	6	8	4
F-value		23.870 <sup>a</sup>					
P-value		< 0.001					

<sup>&</sup>lt;sup>a</sup>Rank sum test results indicate a statistically significant difference in ranking between the groups.

Table II. Comparison of expression of Nrf2, Keap1 and HO-1 mRNA in lens samples among the 3 groups (n=30).

Groups	n	Nrf2	Keap1	HO-1
Blank control	10	1	1	1
Model control	10	$0.36 \pm 0.25$	$0.26 \pm 0.17$	0.41±0.15
Model administration	10	$0.79 \pm 0.23$	0.68±0.13	0.87±0.09
F-value		10.808a	13.415 <sup>a</sup>	12.874a
P-value		< 0.001	< 0.001	< 0.001

<sup>&</sup>lt;sup>a</sup>F-test results indicate a statistically significant difference between the groups.

the model control group. The rats in the model administration group had mild white opacities in the lens cortex, but the core was transparent. According to the rank sum test, there was a significant difference in the degree of lens opacity among the three groups (P<0.05).

Expression of Nrf2, Keap1 and HO-1 mRNA in lens samples. The mRNA expression levels of Nrf2, Keap1 and HO-1 in the rat lens samples are shown in Table II. The mRNA expression levels of Nrf2, Keap1 and HO-1 in the lenses of the three groups was significantly different (P<0.05). The mRNA expression levels of Nrf2 and HO-1 was the highest in the model control group, followed by the model administration group, and the lowest in the blank control group. The expression levels of Keap1 mRNA were the lowest in the model control group, followed by the model administration group, and the highest in the blank control group.

The expression of Nrf2, Keap1 and HO-1 protein in the lens. The protein expression levels of Nrf2, Keap1 and HO-1 in the lenses of rats are shown in Fig. 1 and Table III. The protein expression levels of Nrf2, Keap1 and HO-1 in the lens samples among the three groups was significantly different (P<0.05). The protein expression levels of Nrf2 and HO-1 was the highest in the model control group, followed by the model administration group, and the lowest in the blank control group. The protein expression level of Keap1 was the highest in the model control group, followed by the model administration group, and the lowest in the blank control group, and the lowest in the blank control group.

Table III. Comparison of expression of Nrf2, Keap1 and HO-1 protein in lens samples among the blank control and model administration groups (n=20).

Groups	n	Nrf2	Keap1	НО-1	
Blank control	10	0.79±0.13	1.46±0.17	0.27±0.04	
Model administration	10	$0.43 \pm 0.06$	$1.94 \pm 0.14$	0.12±0.03	
F-value		15.325a	17.436a	16.301a	
P-value		< 0.001	< 0.001	< 0.001	

<sup>&</sup>lt;sup>a</sup>F-test results indicate a statistically significant difference between the groups.

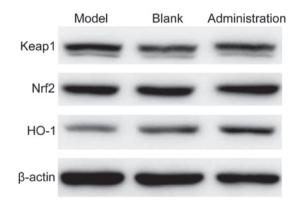


Figure 1. Expression of Keap1, Nrf2 and HO-1 protein in lens samples. Images show the relative expression levels of each protein from lens samples between the three groups (blank control, model control and model administration) as detected by western blot analysis.

# Discussion

Diabetic cataracts are a complication of diabetes that are caused by persistently elevated blood glucose levels and can result in a loss of vision and blindness. While there are similarities in the etiology of diabetic and senile cataracts, such as the abnormal metabolism of the lens, the specific biomolecular mechanism behind its pathogenesis has not been elucidated. Data have shown that the incidence of diabetic cataracts in both types of diabetes is close to 15%. Given the increase in diabetes prevalence and the duration of the disease, this figure is likely to rise (9). The progression of diabetic cataracts is fast and poses a serious threat for blindness among advanced diabetic patients. At present, the management of diabetic cataracts is still dominated by prevention and early control.

Previous clinical perspectives held that as long as control of blood glucose levels is maintained, diabetic cataracts can be effectively prevented. However, in practice, the truth has proven to be not so simple. More and more studies have suggested that controlling blood glucose levels within acceptable target ranges for diabetic treatment is not enough to control the progression of diabetic cataract pathology (10). These studies have shown that high blood glucose is only an enabling factor, rather than the fundamental causative factor. Currently, the biomolecular mechanisms of the pathogenesis of diabetic cataracts are still under study, but increasing evidence suggests that oxidative stress in the body plays a fundamental role in the disease (11).

Therefore, three evaluation indices of Nrf2, Keap1 and HO-1 were identified as molecular targets in this study. Nrf2 is a key cytokine that regulates the expression of antioxidant substances in vivo. Keap1 is the key protein that regulates the expression of Nrf2. HO-1, along with the other glutathione peroxidase, quinone oxidoreductase, constitute the Nrf2-Keap1 signaling pathway downstream of the effector protein (12). The normal expression of Nrf2 is critical for maintaining oxidant-antioxidant balance in the body and the Nrf2-Keap1 signaling pathway also plays an important role in many diseases, including atherosclerosis, diabetes, heart disease and cataracts. Further study on the Nrf2-Keap1 signaling pathway may provide a new approach to the treatment of these diseases (13).

Nrf2 is the most active transcriptional regulator in the leucine zipper transcription factor family and its biological activity is mainly exerted through an inhibitory control mechanism against Keap1. In a healthy state, Nrf2 is mainly present in the cytoplasm and binds to the Keapl protein, while being anchored to the cytoskeletal protein. If Nrf2 is not anchored in the cytoplasm, free Nrf2 protein is degraded by the ubiquitination-dependent protein; therefore, the expression levels of Nrf2 protein is usually low. However, when the cells are exposed to a hyperoxic environment, the conformation of Keap1 protein changes, preventing it from binding to Nrf2. Nrf2 is then released into the nucleus, expressed extensively, and promotes the expression of downstream antioxidant HO-1, imparting resistance to oxidative damage (14). Research has further shown that the expression levels of Nrf2, Keap1 and HO-1 are all decreased in the lenses of cataract patients, which indicates that the mechanism of the signaling pathway for antioxidant protection is damaged. On the one hand, oxidative stress in lens epithelial cells of cataract patients produces a large amount of reactive oxygen species, leading to abnormal aggregation of lens protein and damaging the antioxidant function of Nrf2-Keapl. On the other hand, the expression of Keapl protein in lens epithelial cells of cataract patients is abnormally increased, decreasing the Nrf2 content by the negative regulatory mechanism. Nrf2 content is further decreased in the disease state by the expression of protease and its degradation of Nrf2. Due to the decrease in Nrf2 expression, the expression of downstream HO-1 protein is also decreased and the body enters a highly oxidative state which further aggravates the cataract.

Calcium dobesilate is used as a traditional therapeutic drug for diabetic retinopathy, and the pharmacological effects include reducing capillary permeability and decreasing blood pressure (15). In this study, the expression levels of Nrf2, Keap1 and HO-1 in the model administration group were closer to the blank control group than the model control group. This suggests that calcium dobesilate may be able to achieve therapeutic effects by adjusting the oxidant-antioxidant balance of the lens. The degree of lens opacity in the model administration group was better than in the model control group, which may be the result of inhibition of inflammatory substances by calcium dobesilate. Most inflammatory substances are vasoactive substances, and their presence in sufficient concentrations can cause increased vascular permeability. Calcium dobesilate acts by inhibiting the secretion of these substances to achieve the effect of reducing vascular permeability.

In conclusion, our study found that calcium dobesilate can effectively increase the levels of Nrf2 and HO-1 in the lens of diabetic cataracts in rats and inhibit the levels of Keap1. These results help to define the underlying mechanism for its effects on cataracts through the improvement of Nrf2-Keap1 signaling pathway and stabilization of oxidant-antioxidant balance in the lens. Altogether, this study indicates the therapeutic potential for calcium dobesilate against the development and progression of diabetic cataracts.

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