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Original Research Article

Aryl piperazine suppresses cataractogenesis in a diabetic rat model via Nrf2/HO-1 signaling pathway

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Abstract

Purpose: *To investigate the inhibitory potential of aryl piperazine on cataract formation in diabetic rats, and the underlying mechanism of action.*
Methods: *Sprague-Dawley rats (7-week-old) were divided into 7 groups: control rats, untreated diabetic rats, and five aryl piperazine treatment groups given the drug at doses of 1, 2, 5, 10 and 15 mg/kg. Diabetes was induced in the rats via injection of streptozocin (STZ) in citrate buffer at a dose of 65 mg/kg. An ophthalmoscope was used to evaluate cataract formation, while GlucoLeader was used for the measurement of blood glucose level.*
Results: *Aryl piperazine treatment significantly reduced blood glucose level from 6 to 12 weeks following administration of STZ, and prevented cataractogenesis in the diabetic rats ($p < 0.05$). Cataract score in the diabetic rats was also significantly decreased by aryl piperazine ($p < 0.01$). Aryl piperazine exposure reversed STZ-mediated decreases in antioxidant capacity (AOC) and glutathione (GSH) levels, enhanced glutathione peroxidase (GPX) activity and decreased malondialdehyde (MDA) levels in diabetic rats ($p < 0.05$). It suppressed the expressions of vascular endothelial growth factor (VEGF) and interleukin (IL)-1β in retinal tissues, while it upregulated the expressions of nuclear factor erythroid 2-related factor 2 (NRF2) and heme oxygenase (HO-1).*
Conclusion: *Aryl piperazine suppresses cataractogenesis and reduces cataract score in diabetic rats by targeting oxidative stress. Moreover, in retinal tissues of diabetic rats, aryl piperazine activates Nrf2/mHO-1 signaling pathway. Thus, aryl piperazine has a potential for use in the prevention of cataracts.*

Keywords: *Cataract, Hyperglycemia, Diabetic retinopathy, Oxidative stress, Glutathione*

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INTRODUCTION

Cataracts and many disorders such as kidney failure and cardiovascular diseases are associated with hyperglycemia [1]. Investigations have demonstrated much higher incidence of cataracts in diabetic patients than in healthy people [1]. Dysfunction in microcirculation leads to altered microvasculature, followed by changes in morphology of arteries and veins [2]. Impairment of ocular microcirculation is a major characteristic feature of diabetic retinopathy [2]. Altered morphology of vessels causes development of vascular lesions in retinal tissues

[2]. Increases in diabetic cataract cases worldwide are associated with increases in the population of diabetic patients [3].

Some risk factors for cataractogenesis are oxidative stress, exposure to smoke and aging [4]. Cataract formation is also observed in people exposed to ionizing and ultraviolet-B radiations [4]. Deficiency of reduced glutathione (GSH) in the lens results in impairment of antioxidative processes, resulting in development of diabetic cataracts [5]. Moreover, dietary deficiencies of vitamin A, C, E and selenium lead to cataract formation [5]. Adequate levels of the antioxidant glutathione (GSH) in the lens prevent damage caused by UV-radiations and reactive oxygen species (ROS) [6]. Catalase is an enzymatic antioxidant which protects the lens from hydrogen peroxide (H_2O_2)-induced damage by converting it to water [6]. Since the transparency of the lens is very important for reception of information by the eyes, cataracts leads to visual impairment. Oxidative stress is a major predisposing factor for cataract formation. In human and animal models, GSH and CAT levels serve as markers of oxidative stress [7]. Excessive release of pro-oxidant factors into the vascular system has been detected in cataract patients [7]. Cataractogenesis and many other disorders are associated with imbalance between ROS and antioxidant species [8].

Heterocyclic compounds are vital components of several pharmacologically important compounds and drugs. Similar to many other heterocyclic moieties, aryl piperazine scaffold is present in many pharmacologically important structures which act as anti-hypertensive and antipsychotic drugs [9]. The present study investigated the anti-cataractogenic potential of aryl piperazine in diabetic rats, and the mechanism involved.

EXPERIMENTAL

Animals

Ninety Sprague-Dawley rats (7-week-old; mean weight = 210 g) were provided by the Animal Center of Shanghai University, China. The rats were relocated to the animal house and kept in individual cages under regulated temperature of 23 ± 1 ˚C and humidity of 55 ± 5 % in an environment with a 12-h light/12-h dark cycle. The rats were allowed free access to water and rat chow. The animal experiments were carried out in accordance with the guidelines issued by the National Institute of Health, China [10]. Approval for the animal studies was given by the Animal Management Committee of the Qingdao Municipal Hospital (approval no. CDF236712).

Animal grouping and treatments

The rats were divided into 7 groups: control rats, untreated diabetic rats, and five aryl piperazine treatment groups given the drug at doses of 1, 2, 5, 10 and 15 mg/kg. There were 15 rats in each group. The rats were acclimatized to laboratory conditions for two weeks, after which diabetes was induced via administration of STZ (Sigma-Aldrich) at a dose of 65 mg/kg, in citrate buffer. Rats in the control group were given only citrate buffer (0.1 M) but were not administered STZ. Aryl piperazine was administered to the rats in treatment groups for 12 weeks via intraperitoneal injection. An ophthalmoscope was used for evaluation of cataract formation in the rats after 12 weeks.

Measurement of oxidative factors

The rats were sacrificed after 12 weeks under 5% isoflurane inhalation anesthesia, prior to excision of the eye lenses. The lenses were treated with 0.2M potassium phosphate buffer, pH 7.0. Lenticular levels of GPx, MDA, GSH and other oxidative factors were assayed. Prior to the various assays, the samples were preserved frozen at -80 ˚C.

Determination of oxidative stress marker levels

Antioxidant assay kit (A015-1-2, Nanjing China) was used for measurement of AOC in accordance with manufacturer's instructions. This assay measures reduction in absorbance of ABTS at 750 nm by the sample. Thiobarbituric acid kit (A003-1-2, Nanjing, China) was used for determination of MDA levels as per protocol of the manufacturer. Hydrogen peroxide assay kit (A005-1-2, Nanjing) was employed for determination of GPx activity in lens samples. The content of GSH in lens homogenates was measured spectrophotometrically.

Measurement of levels of inflammation markers

Interleukin (IL)-1β content of the rat retinal tissues was measured using ELISA kit (H002, Nanjing, Nanjing, China) as per the manual procedure. The IL- 1β content was expressed as pg of IL-1β protein per mg of retinal total protein. The VEGF expression in retinal tissues was quantified using ELISA kit (H044, Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocol. The samples were put in monoclonal antibody-coated 96-well plates, and incubation was carried out for 3 h at

37 ˚C. After washing, the samples were read at 455 nm using VER SA max microplate reader.

Western blot analysis

The tissues were lysed on treatment with radioimmunoprecipitation buffer, and the protein content of the lysate was determined using BCA method. Then, 30-µg protein samples were resolved via 10 % SDS-polyacrylamide gel electrophoresis, followed by electroblotting of the proteins onto PVDF membranes which were incubated with blocking solution (5 % dried milk) for 1 h. Thereafter, the membranes were incubated overnight at 4˚C with primary antibodies against Nrf2 (1ab137550; Abcam), HO-1 (ab13248; Abcam) and GAPDH (A0208). Washing of membranes with TBS/Tween-20 (0.05 %) was followed by 1 h incubation with horseradish peroxidase-conjugated secondary antibody. Pierce ECL substrate (Thermo Fisher Scientific, Inc.) was used for development of the signals, while ChemiDoc MP Imaging system for visualization of the protein bands.

Reverse transcription-quantitative polymerase chain reaction

Total RNA was extracted from the rat retinal tissues by treatment with TRIzol reagent (Invitrogen) according to protocol from the manufacturer. Then, 1-µg RNA samples were subjected to reverse transcription to cDNA using reverse transcriptase kit (28025013, Thermo Fisher Scientific, Inc.). The SYBR- Green PCR kit (Takara Biotechnology Co. Ltd.) was used for qPCR. The conditions used for thermocycling consisted of 94 ˚C for 8 min, 94 ˚C for 8 sec and 53 ˚C for 8 sec, followed by 70 ˚C for 28 sec. The mRNA levels of Nrf2 and HO-1 were calculated using $2^{-\Delta\Delta CT}$ method, with GAPDH as internal control.

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM) of triplicate measurements. Data comparison was made using one-way ANOVA in combination with Tukey's post *hoc* test. The GraphPad Prism software 6.0 (GraphPad Software, Inc.) was used for all statistical analyses. Differences at $p < 0.05$ were taken as statistically significant.

RESULTS

Aryl piperazine reduced blood glucose

Aryl piperazine significantly reduced level of blood glucose in the diabetic rats in a dose-dependent manner from 6 to 12-weeks following administration of STZ ($p < 0.05$; Figure 1). The decreases in blood glucose levels caused by aryl piperazine were significant ($p < 0.05$) at doses of 1, 2, 5, 10 and 15 mg/kg at 12 weeks of STZ administration.

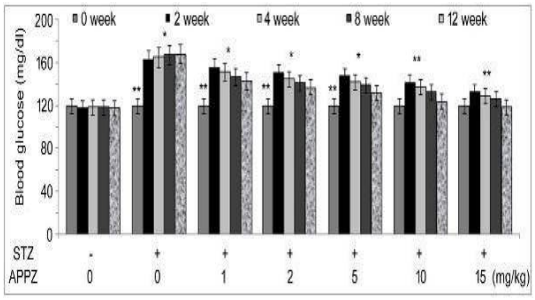

Figure 1: Effect of aryl piperazine on blood glucose level of diabetic rats. Aryl piperazine treatment of diabetic rats was followed by measurement of blood glucose levels after 2, 4, 8 and 12-weeks. *$P < 0.05$, **$p < 0.01$, vs. model group

Aryl piperazine inhibited cataract formation

Administration of STZ led to cataract development in 14 out of the 15 rats in model group (Table 1). However, aryl piperazine treatment prevented STZ-induced cataract formation in a dose-dependent manner. In the group of rats given aryl piperazine at a dose of 2 mg/kg, 11 rats developed cataracts, whereas the number of rats with cataracts decreased to 7, 4 and 0 in rat groups treated with aryl piperazine at doses of 5, 10 and 15 mg/kg, respectively. As shown in Table 2, cataract score in the diabetic rats was also significantly decreased by aryl piperazine treatment in dose- and time-dependent manners ($p < 0.01$). Aryl piperazine significantly reduced cataract score in diabetic rats at 12 weeks following STZ administration ($p < 0.05$).

Effect of aryl piperazine on levels of AOC GSH, GPx and MDA in diabetic rat lens

Levels of AOC and GSH were significantly reduced in diabetic rats ($p < 0.01$), when compared to normal rats (Figure 2). However, treatment of the diabetic rats with aryl piperazine dose-dependently reversed the STZ-mediated decreases in levels of AOC and GSH. In diabetic rats, GPx activity was lowered, whereas MDA expression was elevated significantly, when compared normal rats ($p < 0.05$). However, aryl piperazine treatment significantly promoted GPx activity and decreased MDA level in diabetic rats ($p < 0.05$).

Table 1: Effect of aryl piperazine on cataractogenesis in STZ-induced diabetic rats

Group	Total number of rats	Number of rats with cataracts
Normal	15	0
Untreated diabetic	15	14
1 mg/kg	15	13
2 mg/kg	15	11
5 mg/kg	15	7
10 mg/kg	15	4
15 mg/kg	15	0

Table 2: Effect of aryl piperazine on cataract score in STZ-induced diabetic rats

Group	Cataract score
Normal	0
Untreated diabetic	7
1 mg/kg	6
2 mg/kg	5
5 mg/kg	4
10 mg/kg	1
15 mg/kg	0

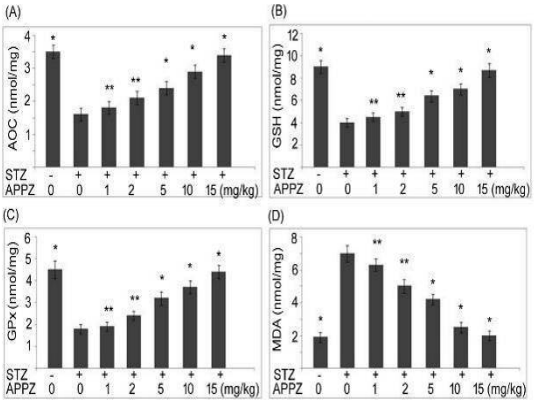

Figure 2: Effect of aryl piperazine on oxidative response in diabetic rat lens. Aryl piperazine treatment of diabetic rats was followed by determination of (A) anti-oxidant capacity, (B) level of GSH, (C) MDA and (D) activity of GPx. *P < 0.05, **p < 0.01, vs. model group

Aryl piperazine downregulated VEGF and IL-1β in diabetic rats

The expressions of VEGF and IL-1β levels were markedly elevated in retinal tissues of diabetic rats, relative to normal rats (Figure 3). However, aryl piperazine exposure at doses of 1 to 10 mg/kg led to significant downregulation of VEGF expression in retinal tissues, when compared to VEGF expression in diabetic rats (p < 0.05). Moreover, in aryl piperazine-treated diabetic rats, the STZ-induced increase in level of IL-1β was significantly reduced (p < 0.05).

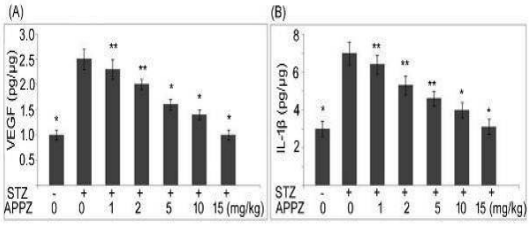

Figure 3: Effect of aryl piperazine on expression levels of VEGF and IL-1β. Aryl piperazine treatment of diabetic rats was followed by determination of (A) VEGF expression levels (B) IL- 1β expression levels. *P < 0.05, **p < 0.01, vs. model group

Aryl piperazine enhanced Nrf2/HO-1 signaling pathway in diabetic rat retinal tissues

The Nrf2 protein level was effectively suppressed in retinal tissues of diabetic rats, when compared to that in normal rat group (Figure 4). Moreover, HO-1 protein expression in diabetic rat retinal tissues was lowered, relative to that in normal rat group. However, aryl piperazine treatment of the diabetic rats at doses of 5 and 10 mg/kg caused upregulations in Nrf2 and HO-1 proteins levels. The STZ-induced downregulations of protein expressions of Nrf2 and HO-1 were reversed in diabetic rats treated with aryl piperazine. Moreover, aryl piperazine at doses of 5 and 10 mg/kg caused marked increases in mRNA levels of Nrf2 and HO-1 mRNA levels of the diabetic rats (Figure 5). The STZ-induced downregulations of mRNA expressions of Nrf2 and HO-1 were effectively reversed in diabetic rats treated with aryl piperazine at a dose of 10 mg/kg.

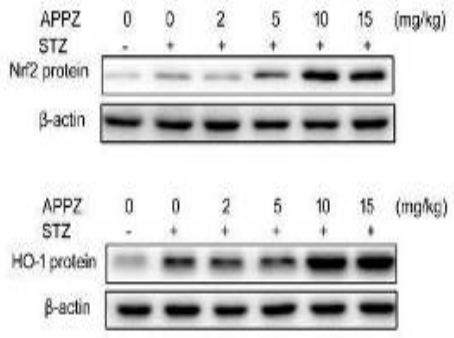

Figure 4: Effect of aryl piperazine on Nrf2/HO-1 signaling route in diabetic rats. Aryl piperazine treatment of diabetic rats at indicated doses was followed by determination of protein expressions of Nrf2 and HO-1 using western blotting.

DISCUSSION

The N-aryl piperazine scaffold is present in many pharmacologically important structures such as anti-hypertensive and antipsychotic drugs, serotonin ligand-linked molecules, calcium ion

blockers, and inhibitors of acetyl cholinesterase [9,10]. The present study investigated the cataract-inhibitory potential of aryl piperazine in diabetic rats.

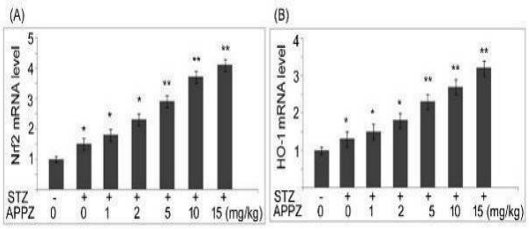

Figure 5: Effect of aryl piperazine on mRNA levels of Nrf2/HO-1. Aryl piperazine treatment of diabetic rats at indicated doses was followed by determination of (A) Nrf2 protein expression using western blotting, and (B) HO-1 mRNA level using RT-PCR. *P < 0.05, **p < 0.01, vs. model group

Initial data demonstrated that aryl piperazine reduced blood glucose level in diabetic rats in dose-depending manner. The STZ-mediated cataracts in rats was also prevented by treatment with aryl piperazine. The aryl piperazine treatment also significantly reversed STZ-induced elevation in cataract score of diabetic rats.

The involvement of oxidative stress and ROS in cataract formation has been widely demonstrated [11]. Lipids and proteins aggregate after oxidation to form insoluble mass on the lens, leading to formation of cataracts. Moreover, cytoplasmic and cell membrane perturbation induced by lipid peroxidation is associated with cataractogenesis [12]. An important indicator of cell damage associated with oxidative stress is increased level of MDA [12]. Similar to vitamins A, E and C (antioxidant vitamins), GSH is one of the oxidant-quenching factors present in the retina, and it is crucial for preventing cataract formation [13].

Cataractogenesis is facilitated by high levels of inflammatory factors present in retina, including IL- 1β and VEGF [14]. In the present study, levels of AOC and GSH were much lower in diabetic rats than in normal rats. Treatment of diabetic rats with aryl piperazine reversed STZ-mediated decreases in levels of AOC and GSH in retinal tissues. The activity of GPx was reduced, whereas MDA level was higher in diabetic rats than in normal rats. However, treatment with aryl piperazine enhanced GPx activity and suppressed MDA level in diabetic rats. The VEGF and IL-1β levels were markedly elevated in retinal tissues of diabetic rats, relative to normal rats. However, treatment with aryl piperazine suppressed VEGF expression in retinal tissues of the diabetic rats. Moreover, in aryl piperazine-treated diabetic rats, the STZ-induced increases in IL-1β levels were effectively reduced.

Polyol accumulation in the lens is also responsible for development of cataracts in diabetic patients [15]. Insulin which is required for regulation of levels of glucose and other sugars, is not present in the retinal tissues [15]. Thus, at high levels, glucose from the aqueous humor enters the lens where it is converted by aldose reductase to sorbitol which subsequently diffuses back into the aqueous humor [16]. This induces uptake of sodium ions into the aqueous humor, followed by electrolyte imbalance, swelling and cataract formation [16].

The transcription factor Nrf2r belongs to 'cap'n'collar' family, and it protects cells through the antioxidant pathway [16]. It regulates oxidative response in cells via induction of the detoxifying enzyme HO- 1 [17]. It has been reported that HO-1 inhibits lipid peroxidation, targets caspase-3 activation, and suppresses TNF-α production [18]. Activation of the Nrf2/HO-1 signaling pathway protects cells from damage induced by oxidative stress [18]. In the present study, Nrf2 mRNA level was effectively suppressed in retinal tissues of diabetic rats. The HO-1 mRNA expression in diabetic rat retinal tissues was also lower than that in normal retinal tissues. However, aryl piperazine treatment of the diabetic rats upregulated mRNA levels of Nrf2 and HO-1.

CONCLUSION

This study has demonstrated that aryl piperazine suppressed cataractogenesis and reduced cataract score in diabetic rats through targeting of oxidative stress. Moreover, in retinal tissues of diabetic rats, aryl piperazine treatment activated the Nrf2/mHO-1 signaling pathway. Thus, aryl piperazine may be useful for the prevention of cataracts in humans.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We 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 the authors. Xiu Wang and Congcong Tian performed the experimental work, carried out the literature survey, and analysed and compiled the data. Chunning Zhao designed the study and wrote the paper. All the authors read the paper thoroughly and approved it for publication.

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