Oxymatrine attenuates diabetes-associated cognitive deficits in rats

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Aim: Oxymatrine (OMT) is the major quinolizidine alkaloid extracted from the root of Sophora flavescens Ait (the Chinese herb Kushen) and exhibits diverse pharmacological actions. In this work we investigated the effects of OMT on diabetes-associated cognitive decline (DACD) in a rat model of diabetes and explored the mechanisms of action.

Methods: Male Wistar rats were injected with streptozotocin (65 mg/kg, ip) once to induce diabetes. The rats were then treated with vehicle or OMT (60 or 120 mg/kg per day, ip) for 7 weeks. Memory function was assessed using Morris water maze test. The levels of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH), NF-κB p65 unit, TNF-α, IL-1β and caspase-3 in the cerebral cortex and hippocampus were quantified.

Results: The diabetic rats exhibited markedly reduced body weight and increased plasma glucose level. The memory function of the rats assessed using Morris water maze test showed significant reduction in the percentage of time spent in the target quadrant and the number of times crossing the platform, coupled with markedly prolongation of escape latency and mean path length. Moreover, the rats showed oxidative stress (significantly increased MDA, decreased SOD and reduced GSH levels), as well as significant increases of NF-κB p65 unit, TNF-α, IL-1β and caspase-3 levels in the cerebral cortex and hippocampus. Chronic treatment with OMT dose-dependently reversed these behavioral, biochemical and molecular changes in the diabetic rats. However, the swimming speed had no significant difference among the control, diabetic and OMT-treated diabetic rats.

Conclusion: Chronic treatment with OMT alleviates diabetes-associated cognitive decline in rats, which is associated with oxidative stress, inflammation and apoptotic cascades.

Keywords: oxymatrine; quinolizidine; diabetes; diabetes-associated cognitive decline; learning memory; oxidative stress; inflammation; apoptosis; cerebral cortex; hippocampus

Introduction

Diabetes mellitus (DM) is a serious chronic metabolic disorder and can adversely affect multiple organs owing to its long-term complications. It is estimated that 171 million people suffered from DM worldwide in 2007; this figure could be more than double by 2030[1]. Emerging evidence suggests that DM might also have negative effects on the central nervous system (CNS), with cognitive impairment as the most common symptom[2,3]. A new term, “diabetes-associated cognitive decline (DACD)”, was proposed in 2006 to facilitate research in this field and to strengthen recognition of this disorder[4]. Thus, diabetes-induced cognitive impairment is a problem that is gaining increased acceptance and attention. It is essential to determine the pathophysiological changes in the generation and progression of DACD to develop potential targets for the prevention of these cognitive symptoms.

Cognitive dysfunction in diabetics appears to be caused by various factors[5]. Some investigations have revealed that diabetes-induced cognitive decline strongly correlates with cardiovascular disease, such as hypertension and cerebral vascular complications[6,7]. One report showed that diabetes-related cognitive impairment was caused by disrupted insulin signaling and glucose homeostasis in the CNS[8]. In another study hyperglycemia-associated microvascular changes in the brain triggered cognitive deficits in patients with type 1 diabetes (TIDM), and intensive insulin therapy in TIDM, leading to a durable improvement of glycemic control, reduced the risk of microvascular complications[9]. Hyperglycemia is a critical factor for the development of cognitive decline in patients with T1DM, suggesting that drugs for the alleviation of DACD
could be based on their improvement of glycemic control.

Oxidative stress is involved in the pathogenesis of diabetes. Increased oxidative stress produces serious oxidative damage in the brain under diabetic conditions\(^{[21]}\). A previous investigation determined that the excessive production of oxygen free radicals and/or antioxidant deficiency in various brain regions was associated with morphological abnormalities and memory deficits\(^{[22]}\). Endothelial oxidative stress has been shown to cause serious vascular damage\(^{[23]}\). Thus, treatment with antioxidants could protect neurons against neurodegenerative conditions. In addition, increased release of inflammatory cytokines and excessive inflammation are observed in diabetics\(^{[14]}\). Activation of the nuclear transcription factor \(\kappa B (NF-\kappa B)\) signaling pathway was shown to induce cognitive deficits\(^{[15]}\) as well as neuronal apoptosis\(^{[16]}\) in diabetics.

Oxymatrine (OMT) is the major quinolizidine alkaloid from the root of \textit{Sophora flavescens} Ait (Kushen) and exhibits diverse pharmacological actions. In both preclinical and clinical investigations, it has been confirmed to possess anti-inflammatory, anti-tumor, anti-viral, anti-oxidant and anti-apoptotic properties\(^{[17]}\). A previous report demonstrated that OMT could exert a protective effect on ischemia/reperfusion damage in liver\(^{[18]}\). In colitis induced by dextran sulfate sodium, OMT was found to ameliorate the inflammatory response via reducing the expression of NF-\(\kappa B\) in colonic mucosa\(^{[19]}\). More importantly, oral administration of OMT could significantly reduce body weight gain and blood glucose level in high fructose-fed mice, a model of fatty liver\(^{[20]}\). Therefore, it was speculated that OMT might have a protective effect on diabetes. Because DACD is the most common complication of diabetes and is closely related to inflammation, oxidative stress and apoptosis, we hypothesized that the neuroprotective effect of OMT may ameliorate the symptoms of DACD. To test this hypothesis, our experiments were designed to assess the protective effect of OMT on DACD in a rat model of diabetes.

Materials and methods

Animals
Male Wistar rats, weighing 230–250 g (obtained from the Animal Center of the Chinese Academy of Sciences, Shanghai, China), were maintained in a controlled environment (12 h:12 h day/night cycle, 50%–70% humidity, 24 °C) with free access to water and rodent chow. Great efforts were made to minimize the suffering of the animals. All experimental procedures conformed to the guidelines established by the Ministry of Health and were approved by the Animal Care Committee of Xuanwu Hospital of Capital Medical University.

Drugs and chemicals
OMT (with a purity >98%) and streptozotocin (STZ) were obtained from Sigma (St Louis, MO, USA). A glucose oxidase peroxidase diagnostic enzyme kit was purchased from India (Span Diagnostic Chemicals, India). TNF-\(\alpha\) and IL-1\(\beta\) ELISA kits were supplied by R&D Systems (USA). The NF-\(\kappa B\) p65 unit ELISA kit was obtained from Imgenex (USA). All other reagents were of analytical grade.

Induction and assessment of diabetes
Diabetes was induced in the rats by intraperitoneal injection of a single dose of 65 mg/kg STZ that was freshly dissolved in citrate buffer (pH 4.4, 0.1 mol/L). Age-matched normal rats were treated with citrate buffer only. At 48 h after the STZ injection, blood samples were collected and plasma glucose levels were measured using an enzymatic glucose oxidase peroxidase diagnostic kit. Rats with fasting plasma glucose levels higher than 250 mg/dL\(^{[21]}\) were defined as diabetic and selected for further investigation. Animals in each experiment were randomly assigned to four groups: (1) the control group (Con) \((n=10)\), including normal rats that received saline intraperitoneally (physiological saline 0.1 mL/100 g); (2) the vehicle group (DM) \((n=10)\), including diabetic rats that received saline intraperitoneally (physiological saline 0.1 mL/100 g); and (3–4) the OMT groups [DM+OMT(60) and DM+OMT(120)] \((n=10)\), including diabetic rats treated with OMT at doses of 60 and 120 mg/kg, respectively. The OMT dosage and dosing frequency were selected according to previous reports\(^{[22, 23]}\). OMT was freshly prepared by dissolving in saline and injected intraperitoneally once a day. The chemical structure of OMT is shown in Figure 1. Beginning on the third day of the experiment, the control and diabetic control groups were treated with the OMT vehicle through the seventh week.

Figure 1. The chemical structure of OMT.

After seven weeks, learning and memory functions were evaluated for 5 consecutive days in a Morris water maze. Under anesthesia induced by an intraperitoneal injection of chloral hydrate (300 mg/kg), the rats for each group were euthanized, blood samples were collected and the brains were rapidly removed. The samples were stored at -80°C until use for biochemical measurements.

Morris water maze test
The Morris water maze tests were used after seven weeks of STZ injection. The apparatus consisted of a circular water tank (90-cm inner diameter and 50-cm height) equipped with a digital pick-up camera 180 cm above the water surface to monitor the animal behavior, a platform (6 cm in diameter) and a computer program (WaterMaze) for data analysis. The tank was partially filled with tap water and the water temperature was maintained at approximately 25±2°C. Black non-toxic paint was used to render the water opaque. The rats were habituated to the water by being allowed to swim freely without
a platform present prior to performing the water maze test. The pool was placed in the center of a large room containing various visual cues and divided into four equal quadrants, N (north), S (south), E (east), and W (west). The cues remained constant throughout the study. A translucent acrylic platform was submerged approximately 1 cm below the water surface (for the navigation test) or removed from the tank (for the spatial probe test). The water maze task was used for 5 consecutive days.

**Learning test**
The animals were tested with a spatial version of the Morris water maze according to a previously described method\(^{[26]}\). In brief, the rats were subjected to 4 consecutive daily training trials for 4 d, with each trial having a time limit of 60 s and with an inter-trial interval of 30 min. For each trial, the rat swam until it climbed onto the submerged platform. After climbing onto the platform, the animals were allowed to remain on the platform for 20 s. If the rat failed to find the platform within a maximum of 60 s, it was gently placed on the platform and remained there for an equivalent amount of time. The escape latency (s) and path length (cm) to find the platform were measured in each trial and averaged over three trials for each rat. Swimming speed was analyzed by dividing the path length by the time required to find the platform.

**Memory test**
After the final escape training, each rat was administered a spatial probe test on the 5th d to assess the extent of memory\(^{[24]}\). The platform was removed from the tank and each animal was placed in the pool from the start location at the quadrant opposite the former platform quadrant. The number of times the rat crossed the former location of the platform and the time spent in the former platform quadrant were measured. The activity of SOD was determined by the method of Kono et al\(^{[26]}\). SOD was measured using a commercial kit (A001-1, Jiancheng Biotech Co). Reduced glutathione was assayed by the method of Jollow et al\(^{[27]}\). Glutathione (GSH) levels were determined with a commercial kit (A006, Jiancheng Biotech Co). Protein content was measured using Coomassie brilliant blue\(^{[28]}\).

**Biochemical assessment**
The concentration of malondialdehyde (MDA), an index of lipid peroxidation, was measured in the form of thiobarbituric acid-reactive substances at 532 nm according to a previously reported method\(^{[29]}\). MDA was determined using a commercial kit (A003-1, Jiancheng Biotech Co, Nanjing, China). Cytosolic superoxide dismutase (SOD) activity was determined by the method of Kono et al\(^{[26]}\). SOD was measured using a commercial kit (A001-1, Jiancheng Biotech Co). Reduced glutathione was assayed by the method of Jollow et al\(^{[27]}\). Glutathione (GSH) levels were determined with a commercial kit (A006, Jiancheng Biotech Co). Protein content was measured using Coomassie brilliant blue\(^{[28]}\).

**Measurement of caspase-3 activity in rat brain**
The activity of caspase-3, an executioner molecule in the apoptotic cascade, was measured by cleavage of the chromogenic caspase substrate Ac-DEVD-pNA. The amount of caspase-3 was measured spectrophotometrically at 405 nm according to the manufacturer’s instructions (R&D Systems, USA).

**Measurement of NF-κB p65 unit, TNF-α, and IL-1β levels in rat brain**
The p65 subunit may be positively correlated with the activation of the NF-κB pathway. The NF-κB p65 unit was measured using a commercial kit (Imgenex, USA) with colorimetric detection at 405 nm. TNF-α and IL-1β were quantified using commercial immunoassay kits (R&D Systems, USA) according to the manufacturer’s instructions.

**Statistical analysis**
The data are expressed as mean±SD. Statistical analysis was carried out using a one-way ANOVA followed by Dunnett’s test, with P<0.05 as the significant level.

## Results

**Effect of OMT on body weight and blood glucose levels**
There was a marked reduction in the body weights of STZ-treated rats in comparison to the age-matched control group (P<0.01), as shown in Table 1. Of note, the diabetic rats exhibited a significantly elevated (589.10±3.98 mg/dL) plasma glucose level compared to the control group (112.20±1.62 mg/dL). However, after the 7-week treatment with OMT at dose of 60 or 120 mg/kg, the blood glucose levels and body weights were both reversed in diabetic rats (P<0.01).

**Effect of OMT on diabetes-induced cognitive deficits**
Cognitive function was assessed in the Morris water maze test (7th week). The mean escape latency for the trained animals was reduced from 60 to 20 s over the course of the 20 learning days. The treatment groups showed a significant improvement in escape latency compared to the diabetic controls (DM group).

### Table 1. Effect of OMT on body weight and blood glucose levels (n=10, mean±SD) in the four groups of rats at the onset and at the end of the experiment.

| Treatment       | Onset of study | End of study | Onset of study | End of study |
|-----------------|----------------|--------------|----------------|--------------|
| Con             | 240.10±4.68    | 291.70±1.17  | 113.30±1.57    | 109.00±1.33  |
| DM              | 246.80±6.91    | 144.30±3.89  | 112.20±1.62    | 589.10±3.98  |
| DM+OMT (60)     | 238.90±4.36    | 230.50±4.28  | 108.10±1.79    | 304.90±3.57  |
| DM+OMT (120)    | 243.50±6.43    | 266.40±6.00  | 107.90±2.69    | 289.00±2.98  |

\(^{1}P<0.01\) compared with Con group. \(^{2}P<0.01\) compared with DM group. Con, control; DM, diabetes; DM+OMT (60), oxymatrine (60 mg/kg)-treated; DM+OMT (120), oxymatrine (120 mg/kg)-treated groups.
No significant difference was observed between any of the groups on the first day of testing in the Morris water maze. However, beginning with the 2nd d, the transfer latency was clearly different between diabetic (53.10±1.52 s) and control (34.60±1.43 s) rats \( (P<0.01) \). Treatment with OMT (60 or 120 mg/kg) significantly diminished the mean escape latency \( (P<0.01) \), as illustrated in Figure 2A. Diabetic rats were less able to find the platform and learn its location in the four-day training session. The poorer performance was reversed by treatment with OMT at dose of 60 or 120 mg/kg, as evidenced by the decrease in latency from the 2nd d of training \( (P<0.01) \).

The results, shown in Figure 2B, also indicated a significant increase in mean path length in the diabetic group for four consecutive days of training compared with the controls \( (P<0.01) \). However, treatment with OMT at dose of 60 or 120 mg/kg was associated with a marked reduction in this value compared to the vehicle-treated diabetic rats \( (P<0.01) \).

The probe trial of the Morris water maze test was conducted to investigate how well the animals had learned and consolidated the platform location. Animals exhibited a significant difference \( (P<0.01) \) during the 4 d of training. There was a decline in the time spent in the target quadrant in diabetic rats.

![Figure 2: Effects of OMT on (A) spatial memory acquisition phase, (B) mean path length, (C) mean percentage of time spent in the target quadrant, (D) the number of times of crossing platform and (E) swimming speed in control and diabetic rats \( (n=10, \text{mean±SD}) \). \( ^{c}P<0.01 \) compared with Con group. \( ^{f}P<0.01 \) compared with DM group. Con, control; DM, diabetes; DM+OMT (60), oxymatrine (60 mg/kg)-treated; DM+OMT (120), oxymatrine (120 mg/kg)-treated.](image-url)
compared with the control group (P<0.01), as shown in Figure 2C. Treatment with OMT (60 or 120 mg/kg) was associated with dramatically more time (P<0.01) spent in the target quadrant compared to the diabetic group. Similarly, as indicated in Figure 2D, the number of times the animals crossed the former platform location was also lower in diabetic rats (P<0.01) compared to the control group. Nevertheless, this index was significantly improved in diabetic rats after treatment with OMT (60 or 120 mg/kg) (P<0.01). As shown in Figure 2E, there was no significant difference in swimming speed among the groups across the four training days.

Effect of OMT on diabetes-induced changes in oxidative stress
Table 2 shows that STZ-induced diabetes significantly increased MDA levels in the cerebral cortex (2.05-fold) and the hippocampus (1.78-fold) compared to the age-matched control (P<0.01). However, chronic administration of OMT (60 and 120 mg/kg) prevented the increase in MDA content in the brains of diabetic rats (P<0.01). In addition, SOD activity and glutathione levels were lower in the cerebral cortex and hippocampus of diabetic animals (P<0.01) compared with the control group after 7 weeks. This reduction was significantly and dose-dependently reversed by OMT treatment in different areas in the brain of STZ-treated rats (P<0.01).

Effect of OMT on diabetes-induced changes in NF-κB p65 subunit, TNF-α and IL-1β levels
As shown in Figure 3A, the NF-κB p65 subunit was significantly elevated in the cerebral cortex (3.61-fold) and the hippocampus (3.69-fold) of diabetic rats after 7 weeks. Nevertheless, chronic treatment with OMT (60 or 120 mg/kg) significantly suppressed the elevation in different brain areas of STZ-injected rats (P<0.01). Likewise, levels of TNF-α (4.95-fold in the cerebral cortex and 5.75-fold in the hippocampus) and IL-1β (2.93-fold in the cerebral cortex and 3.01-fold in the hippocampus) were elevated in the diabetic animals after 7 weeks (Figure 3B and 3C, P<0.01). Treatment with OMT (60 or 120 mg/kg) markedly and dose-dependently inhibited TNF-α and IL-1β levels in different areas of the brain of STZ-injected rats (P<0.01).

Effect of OMT on diabetes-induced changes in caspase-3 activity
Figure 4 shows that caspase-3 levels were significantly lower in diabetic rats compared to the control group (P<0.01). Treatment with OMT (60 or 120 mg/kg) markedly and dose-dependently inhibited caspase-3 activity in different areas of the brain of STZ-injected rats (P<0.01).

Table 2. Effect of OMT on MDA, SOD, and GSH levels in the cerebral cortex and hippocampus of rats (n=10, mean±SD).

| Treatment          | MDA (nmol/mg protein) | SOD (units/mg protein) | GSH (μmol/L) |
|--------------------|------------------------|------------------------|--------------|
|                    | Cerebral cortex        | Hippocampus            | Cerebral cortex | Hippocampus | Cerebral cortex | Hippocampus |
| Con                | 1.25±0.12              | 0.88±0.09              | 8.85±0.08   | 7.62±0.08   | 32.94±0.43   | 31.94±0.61 |
| DM                 | 2.56±0.08              | 1.57±0.08              | 4.06±0.14   | 3.88±0.11   | 16.04±0.44   | 14.64±0.27 |
| DM+OMT (60)        | 2.10±0.06              | 1.34±0.06              | 7.85±0.08   | 7.62±0.08   | 28.14±0.85   | 26.94±0.67 |
| DM+OMT (120)       | 1.83±0.06              | 1.20±0.07              | 8.14±0.12   | 7.99±0.07   | 30.54±0.92   | 29.54±0.96 |

*P<0.01 compared with Con group. \(^{a}P<0.01\) compared with DM group. Con, control; DM, diabetes; DM+OMT (60), oxymatrine (60 mg/kg)-treated; DM+OMT (120), oxymatrine (120 mg/kg)-treated.
In conclusion, OMT provides beneficial effects by lowering blood glucose, improving learning and memory functions, and protecting neuronal tissue from oxidative stress. These effects may be mediated through the modulation of pro-inflammatory cytokines, the attenuation of caspase-3 activity, and the inhibition of NF-κB signaling. The protective effects of OMT suggest its potential as a therapeutic agent for the treatment of diabetes-related cognitive impairments.
reducing oxidative stress, inhibiting the TNF-α-induced NF-κB signaling pathway and diminishing caspase-3 activity in diabetic rats. These findings point toward the potential of OMT as an adjuvant therapy to conventional anti-hyperglycemic and anti-DACD regimens.

Author contribution
Jian-ping JIA contributed to the study design; Suo-bin WANG performed the research and conducted the data analysis; and Jian-ping JIA wrote the manuscript.

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