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Protective Effect of Oleuropein on Memory Impairment and Oxidative Stress in Streptozotocin-Induced Diabetes Rats via Modulation of NF-kB and Nrf-2 Pathways

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Abstract

Background & Objectives: Diabetes is the most common metabolic disease, associated with hyperglycemia and long-term complications. This study aimed to elucidate the anti-diabetic role of oleuropein (OLE) in a streptozotocin (STZ)-induced diabetic animal model.

Materials & Methods: Adult male Wistar rats (200–250 g) were randomly divided into four groups: 1) Control group, 2) STZ group: diabetic rats that received STZ (60 mg/kg), 3) OLE 50 group: diabetic rats treated with oral OLE at 50 mg/kg of body weight daily for 28 days, and 4) OLE 100 group: diabetic rats treated with oral OLE at 100 mg/kg of body weight daily for 28 days. Memory function and biochemical factors such as malondialdehyde (MDA) levels, glutathione peroxidase (GPx), and total thiol activity were evaluated in the rats' cerebral cortex and striatum tissues. Moreover, nuclear transcription factor-kappa B (NF-κB) and nuclear factor E2-related factor 2 (Nrf2) pathway activation were determined in cerebral cortex and striatum tissues by real-time polymerase chain reaction (PCR).

Results: Chronic administration of OLE ameliorated cognitive deficits and attenuated oxidative stress induced by diabetes. Additionally, OLE significantly prevented the activation of the pro-inflammatory marker NF-κB and downregulated Nrf2 expression in STZ-induced diabetic rats.

Conclusion: Our results confirm the significant protective role of OLE against STZ-induced diabetes in rats by up-regulating Nrf2 signaling and enhancing antioxidant activity.

Keywords: Diabetes, Oleuropein, Oxidative stress, Memory, Nrf-2, Rat

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Introduction

Type 2 diabetes mellitus (T2DM), an endocrine disease, is linked to neurological and systemic complications arising from chronically elevated blood glucose levels (hyperglycemia). According to the World Health Organization, over 420 million adults worldwide have diabetes. The exact causes remain unclear, but factors such as obesity, genetics, and a sedentary lifestyle are believed to contribute (1, 2). Research suggests that increased oxidative stress and altered antioxidant levels play a significant role in the pathogenesis of T2DM (3). Oxidative stress is a condition characterized by an imbalance between the generation and detoxification of reactive oxygen

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species (ROS), leading to damage in various tissues (4). In diabetes, oxidative stress is induced by both enhanced production of free radicals and a reduction in antioxidant defenses (2). Accumulating evidence indicates that oxidative stress-induced inflammation can contribute to the risk of diabetic complications (5).

The proinflammatory transcription factor, nuclear factor-kappa B (NF-κB), is known to activate the production of inflammatory cytokines (6). NF-κB and its associated signaling pathways are implicated in both normal and pathological inflammatory responses (7). Activation of NF-κB can be triggered by various stimuli, including viruses, cytokines, oxidative stress, ischemia, and reperfusion. Once stimulated, this complex translocates to the nucleus and induces the expression of over 150 genes, including tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), interleukin-17 (IL-17), chemokines, and adhesion molecules. In this context, NF-κB occupies a critical upstream position in the inflammatory cascade, regulating the production of various proinflammatory mediators (8, 9).

Interestingly, these pathways exhibit cross-talk at the transcriptional level, with protein-protein interactions or secondary messenger effects leading to mutual suppression. The nuclear factor (erythroid-derived-2) (Nrf2) pathway suppresses the activation of the NF-κB pathway by upregulating antioxidant defenses and heme oxygenase-1 (HO-1) expression. This leads to the neutralization of ROS, detoxification of toxic chemicals, and ultimately, a reduction in ROS-mediated NF-κB activation (10).

Oxidative stress-related Nrf2 activation promotes the production of cytoprotective and antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), HO-1, and NADPH quinone oxidoreductase (NQO1). It also increases the synthesis of GSH, multidrug transporters, and NADPH. Nrf2 binds to the antioxidant response element (ARE) on DNA, maintaining the body's redox balance. Growing evidence suggests a role for Nrf2 in synaptic plasticity. Nrf2 knockout mice exhibit deficits in long-term potentiation (LTP) of the perforant pathway *in vivo*. Additionally, administration of the Nrf2 activator Dl-3-n-butylphthalide to APP/PS1 transgenic mice (an Alzheimer's disease model) ameliorated synaptic plasticity deficits. Linalool, another Nrf2 activator, has been shown to reverse the decreased expression of synaptic plasticity-related proteins in an oxidative stress model of Alzheimer's disease. These studies suggest a link between impaired Nrf2 function and reduced LTP, and conversely, Nrf2 activation is associated with improved LTP (11).

Oleuropein (OLE), the main phenolic compound in olive leaves, is responsible for the bitterness of unprocessed and immature olives. This heterosidic ester of hydroxytyrosol and elenolic acid offers potential health benefits for humans (12). The therapeutic effects of OLE have been reported in various human diseases. Al-Azzawie and Alhamdani described its effectiveness in the treatment of diabetes mellitus (DM) (13). Several lines of evidence suggest that OLE's diverse pharmacological properties, particularly its potent antioxidant effects, contribute to its multifunctionality (14). Additionally, OLE exhibits significant hypotensive, hypoglycemic, antimicrobial, anti-carcinogenic, anti-inflammatory, and anticonvulsant properties (15). In the pursuit of natural agents with strong pharmacokinetic effects, research has increasingly focused on evaluating various phenolic compounds. Therefore, this study aims to investigate the efficacy of OLE against T2DM and its associated hyperglycemia, oxidative stress, and inflammation (NF-κB regulation).

Material and Methods

Animals

Forty adult male Wistar rats weighing between 200 and 250 grams were obtained from the animal house of the Animal Breeding Center at Ahvaz Jundishapur

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University of Medical Sciences. The rats were housed under standard conditions with a 12-hour light/dark cycle and controlled temperature (22 ± 2 °C). They had ad libitum access to both food and water. All procedures adhered to the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals, and the study protocol was approved by the Ethics Committee (approval number: IR.IAU.AHVAZ.REC.1401.019).

Experimental design

Animal groups: After a three-day adaptation period, animals were randomly assigned to four groups (n=10 per group):

Five rats from each group were designated for biochemical assays and five for real-time polymerase chain reaction (PCR) analysis.

Control group: Normal rats

STZ group: Diabetic rats that received a single intraperitoneal (i.p.) injection of streptozotocin (STZ) at 60 mg/kg, freshly dissolved in citrate buffer

STZ + OLE50 group: Diabetic rats that received oleuropein (OLE) orally (50 mg/kg/day; p.o.) for 28 days

STZ + OLE100 group: Diabetic rats that received oleuropein (OLE) orally (100 mg/kg/day; p.o.) for 28 days (16)

Diabetes induction

Diabetes was induced using a single intraperitoneal injection of streptozotocin (STZ) (60 mg/kg in citrate buffer solution). Three days post-injection, blood glucose levels were measured from tail vein samples using a digital glucometer. The study commenced upon confirmation of hyperglycemia. A blood glucose level exceeding 250 mg/dL was considered indicative of diabetes (17). Oleuropein, obtained from Gol Elixir Company (Pars, Mashhad, Iran), was administered at specified doses (18).

Passive Avoidance Memory Test

This test utilized a shuttle box (5500-ST, Borj Sanaat Co., Iran) comprising two compartments, light and dark, with floors covered by stainless steel wires (1-2 mm) spaced 1 cm apart. An electric current generator (75 V, 0.3 mA for 3 s) delivered mild shocks to the animals' paws in the dark chamber. For habituation, rats were placed in the shuttle box with an open guillotine door for 10 minutes, allowing free movement between compartments. Subsequently, each rat was positioned in the light chamber, and the latency to enter the dark chamber was recorded (learning). Upon entering the dark chamber, the guillotine door closed, and an electric shock was administered. After 24 hours, the latency to enter the dark chamber was measured as passive avoidance memory. This procedure was repeated for all animals across all groups (19).

Biochemical assay

Following the experiments, five rats from each group were deeply anesthetized using an overdose of ketamine and xylazine (90/10 mg/kg Alfason, Netherlands). The brains were rapidly excised, and striatum and cerebral cortex tissues were dissected on ice, washed with phosphate buffered saline, and stored at -80 °C until further analysis (20).

Malondialdehyde (MDA) measurement

Tissue MDA levels were determined spectrophotometrically using the thiobarbituric acid (TBA) (Merck Company (Darmstadt, Germany)) reagent. This method is based on the reaction between MDA and TBA at 100 °C, forming a chromogenic complex. MDA concentration was measured at 532 nm.

Measurement Thiol group

Thiol groups were evaluated using DTNB or (5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) (Merck Company (Darmstadt, Germany)) . In a test tube, 1 mL of Tris buffer (pH 6) was added to 50 µL of tissue homogenate, and its optical absorption was measured at 412 nm (A1). Subsequently, 20 µL of DTNB reagent was added, incubated at room temperature for 15 minutes, and the absorbance was measured at the same wavelength (A2). The absorbance of the control (containing buffer) was also

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measured at 412 nm (B). Thiol concentration was calculated using the equation: (A2 - A1 - B) × 1.07 / (0.05 × 13.6).

Glutathione peroxidase assay

Glutathione peroxidase activity was measured according to the instructions provided in the commercial kit (Bio Vision, Milpitas, CA, USA). Enzyme activity was defined as the amount of enzyme catalyzing the oxidation of 1 µmoL of NADPH to NADP+ per minute under the kit's specified conditions at 25 °C.

RNA extraction, cDNA synthesis, and real-time PCR

For real time-PCR (RT-PCR*),* rats were euthanized under deep anesthesia with ketamine and xylazine (90 and 10 mg/kg, i.p), and their cerebral cortices were isolated and stored at -80°C.

RNA preparation and reverse transcription: Total mRNA was isolated using the phenol-chloroform extraction protocol with a Total Extraction Kit (Pars Tous, Iran) according to the manufacturer's instructions. The RNA pellet was air-dried and resuspended in 50 µL of diethylpyrocarbonate (DEPC)-treated water. RNA concentration was determined spectrophotometrically at 230 and 260 nm using 3 µL of the total RNA solution. Complementary DNA (cDNA) was synthesized from the extracted RNA.

RT-PCR: The cDNA underwent RT-PCR using a real-time PCR Master Mix Kit (Pars Tous, Iran) under the following conditions: initial incubation at 95 °C for 5 minutes, followed by 40 amplification cycles consisting of 30 seconds at 95 °C and 30 seconds at 62 °C. β-actin (ACT-β) served as an endogenous control to minimize the effect of sample variation on the calculation of relative expression levels of target genes using the delta-delta-Ct method. The cycle threshold was used as the gene expression index. Table 1 presents the primer sequences for ACT-β, NF-κB, and Nrf-2.

Gel Electrophoresis Bands: Each band represents specific NF-κB and Nrf-2 proteins with distinct molecular weights. The bands located on the right side of the image indicate the molecular weight ladder (DNA marker 50 bp) (Figure 1).

Statistical Analysis

The results are presented as mean ± standard error of the mean (SEM). The Kolmogorov-Smirnov test was used to assess data normality. Comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. A p-value < 0.05 was considered statistically significant.

Table 1. Sequences of primers used in real-time polymerase chain reactions (22)

Gene	Primers
ACT-β	Forward: 5'- TGGAATCCTGTGGCATCCATGAAAC-3' Reverse: 5'-GCCTGACAATGACTCGACGCAAAAT-3'
NF-kB	Forward: 5'-GAAATTCCTGATCCAGACAAAAAC-3' Reverse: 5'-GATGTGTCTCCGGTAACTTCACTA-3'
Nrf-2	Forward: 5'- TCTCCTCGCTGGAAAAAGAA -3' Reverse: 5'- AATGTGCTGGCTGTGCTTTA -3'

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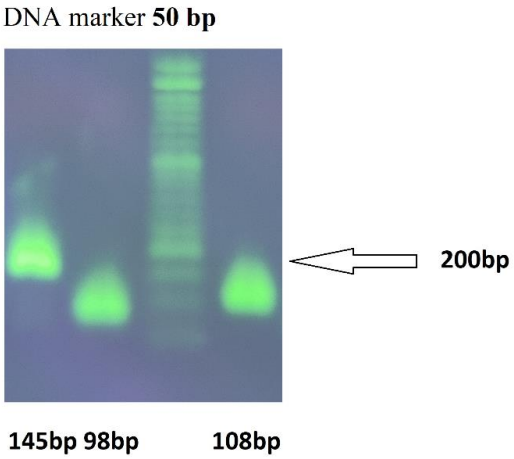

Figure 1. Gel electrophoresis of NF-kB (145 bp), Nrf-2(98 bp) , ACT-β (108bp) and DNA marker 50 bp

Results

Effects of OLE on blood glucose levels in diabetic rats

Three days following streptozotocin (STZ) injection, blood glucose levels in the experimental groups were significantly higher than those in the control group ($p < 0.001$, Figure 1). After treatment, blood glucose levels were significantly reduced in groups treated with OLE at doses of 50 and 100 mg/kg ($p < 0.001$ for both), indicating the protective effects of OLE against severe hyperglycemia in diabetic animals.

Effects of OLE on Passive avoidance task

The step-through latency (STL) was significantly reduced in DM rats compared to the control group ($p < 0.001$) (Figure 2). However, OLE treatment at doses of 50 and 100 mg/kg significantly increased STL compared to the untreated diabetic group ($p < 0.001$ for both doses).

Effects of OLE on MDA and total thiol levels in striatum and cortex tissues

The striatum and cortex tissues of the DM group exhibited significantly higher MDA levels compared to the control group ($p < 0.001$ for both). However, OLE treatment (50 and 100 mg/kg) significantly decreased MDA levels in the striatum of DM rats ($p < 0.01$ and $p < 0.001$, respectively) (Chart 3A). Similarly, MDA levels in the cortex tissue of OLE-treated DM animals (50 and 100 mg/kg) were significantly reduced compared to the DM group ($p < 0.05$ and $p < 0.001$, respectively) (Chart 3B). Furthermore, total thiol content in the DM group was significantly depleted in both striatum and cortex tissues compared to the control group ($p < 0.01$ and $p < 0.001$, respectively) (Chart 3C and 3D). In the cortex tissue of DM rats, OLE treatment at 100 mg/kg significantly increased thiol levels compared to the DM group ($p < 0.001$). However, no significant alterations in thiol levels were observed in the striatum tissue of the DM+OLE 50 and DM+OLE 100 groups compared to the DM group ($p > 0.05$).

Activity was significantly reversed by OLE treatment in the DM + OLE50 and DM + OLE100 groups in both striatum and cortex tissue compared to the DM group ($p < 0.001$ for both).

Effects of OLE on GPx activity in striatum and cortex tissues

GPx activity significantly decreased in both the striatum and cortex tissues of the DM group.

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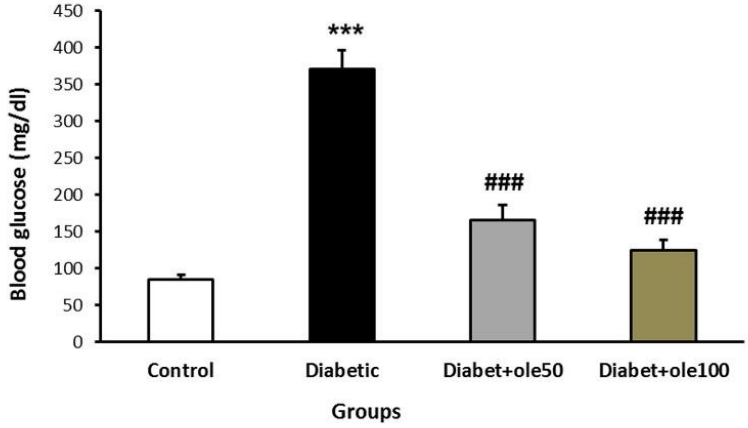

Chart 1. Effect of oleuropein (OLE) on blood glucose levels in all groups. Data are reported as Mean ± SEM. ***p < 0.001 vs. the control group, ###p < 0.001 vs. the diabetic group

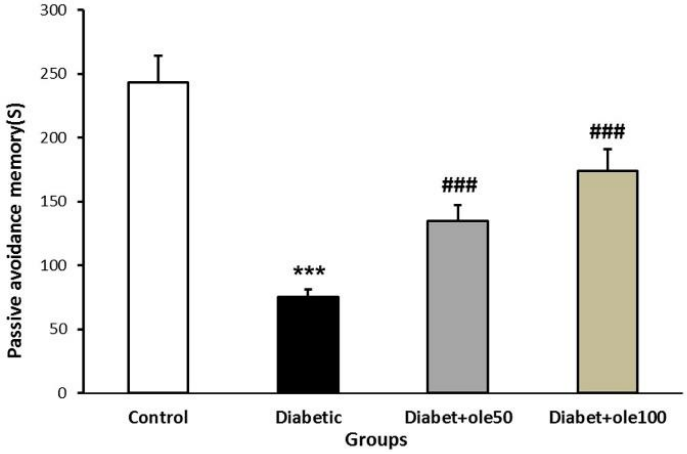

Chart 2. Passive avoidance memory of the animals. Data are reported as mean ± SEM. ***p < 0.001 vs. the control group, ###p < 0.001 vs. the diabetic group

Effects of OLE on the expression levels of Nrf-2

Nrf2 expression levels in the brain tissues of the diabetic groups were significantly reduced compared to the control group ($p < 0.001$, Figure 5). Furthermore, Nrf2 expression levels in the diabetic groups treated with OLE at doses of 50 mg/kg ($p < 0.001$) and 100 mg/kg ($p < 0.001$) were significantly increased compared to the untreated diabetic group. Notably, this increase was more pronounced in the diabetic group receiving OLE at a dose of 100 mg/kg.

Effects of OLE on the expression levels of NF-kB

The expression levels of NF-κB in the brain tissues were significantly increased in the diabetic group compared to the control group ($p < 0.001$, Figure 6). Furthermore, a significant reduction in NF-κB expression was observed in the groups receiving OLE at doses of 50 mg/kg ($p < 0.001$) and 100 mg/kg ($p < 0.01$) compared to the untreated diabetic group.

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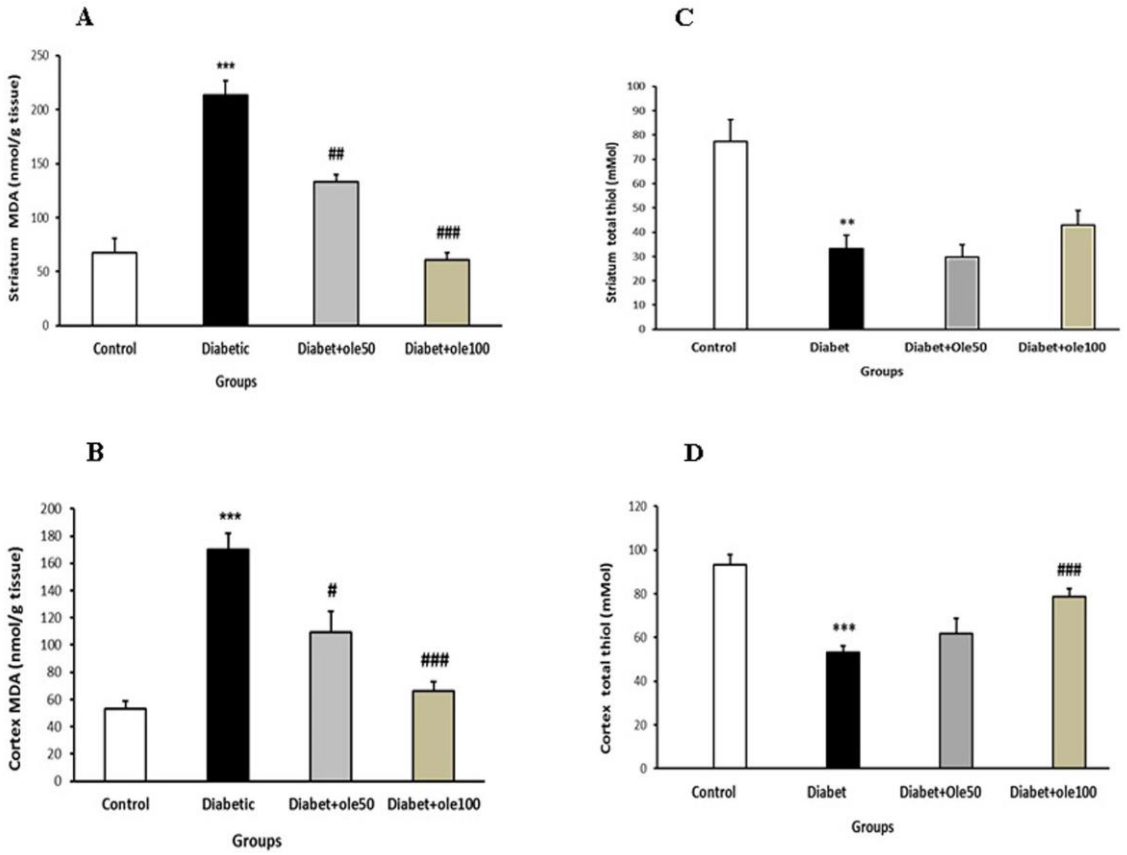

Chart 3 A-D. Effects of oleuropein (OLE) on malondialdehyde (MDA) and total thiol levels in both striatum and cortex tissues in all groups. Data are expressed as mean ± SEM. **p < 0.01 and ***p < 0.001 vs. the control group, #p < 0.05, ##p < 0.01 and ###p < 0.001 vs. the diabetic group

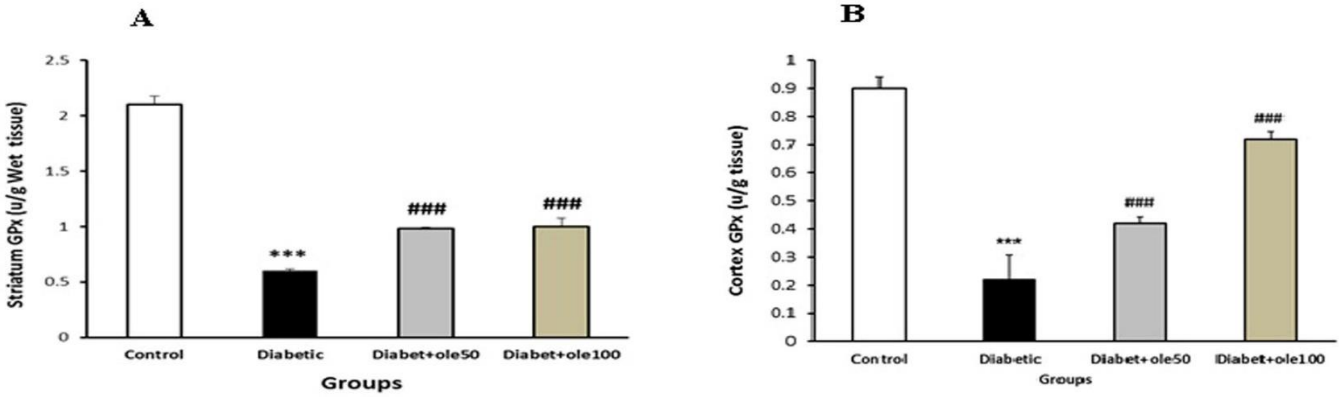

Chart 4 A-B. Effects of oleuropein (OLE) on glutathione peroxidase (GPx) activity in both striatum and cortex tissues in all groups. Data are expressed as mean ± SEM. **p < 0.01 and ***p < 0.001 vs. the control group, ###p < 0.001 vs. the diabetic group

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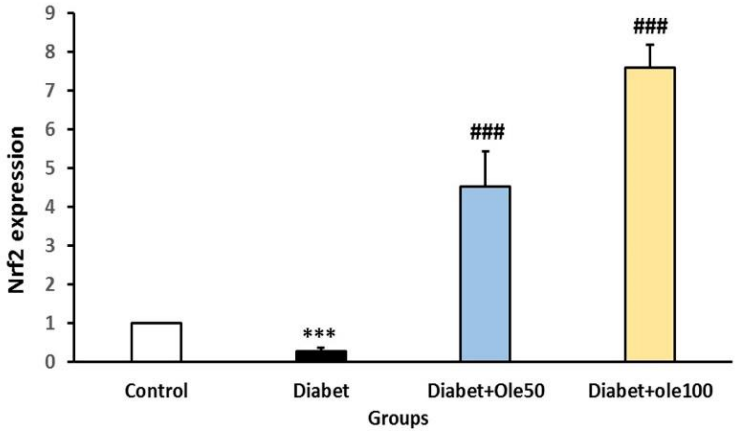

Chart 5. Effects of oleuropein (OLE) on the expression levels of Nrf-2 in all groups. Data are expressed as Mean ± SEM. ***p < 0.001 vs. the control group, ###p < 0.001 vs. the diabetic group

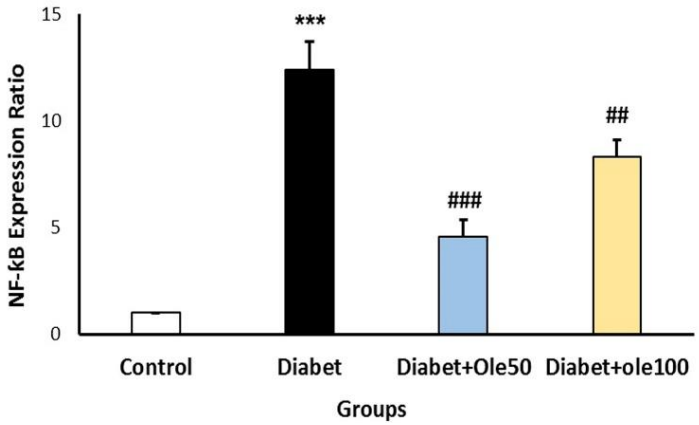

Chart 6. Effects of oleuropein (OLE) on the expression levels of NF-kB in all groups. Data are expressed as mean ± SEM. ***p < 0.001 vs. the control group, ##p < 0.01 and ###p < 0.001 vs. the DM group

Discussion

Hyperglycemia plays a crucial role in free radical generation and diminishes the antioxidant defense system, indicating a close link to oxidative stress (2, 23). In the present study, 60 mg/kg STZ was used to induce diabetes in animals, a method widely employed in experimental diabetes research (24). Our results showed that STZ significantly increased blood glucose concentrations in experimental rats, while chronic OLE treatment improved STZ-induced hyperglycemia. The hypoglycemic effects of OLE are mediated by modulating various intracellular signaling mechanisms directly associated with blood glucose regulation (25).The beneficial effects of OLE on blood glucose levels have been extensively documented in animal models of diabetes. In experimental studies, OLE significantly reduced blood glucose and markedly increased MDA levels, a marker of oxidative stress potentially contributing to diabetic complications (18, 26). Moreover, our study demonstrated that induction

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of experimental DM caused impairment in passive avoidance memory. These results are consistent with previous investigations reporting that STZ-induced diabetic rats exhibited poor performance in behavioral tasks, including decreased cognitive capacity in PAL (27). Studies have shown that cognitive deficits are a significant complication of diabetes (28). Furthermore, evidence suggests a direct relationship between neuroinflammation and cognitive impairment, with proinflammatory cytokines identified as key factors in the progression of cognitive dysfunction (29).

Our results indicated that OLE improved learning and memory impairments in STZ-induced diabetic rats in the PAL test. In agreement with our findings, experimental evidence suggests the effectiveness of OLE in enhancing cognition and memory function (30). Additionally, another study demonstrated that OLE could act as an antioxidant to improve spatial memory impairment in rats (31). In this study, we found that diabetic rats exhibited a significant decrease in striatum and cortex GPx and thiol levels following STZ injection. Consistent with our results and other reports, diabetes negatively affects antioxidant enzyme activities due to increased ROS, contributing to the development and progression of DM (32). GPx is a primary antioxidant associated with the elimination of lipid hydroperoxides. Cells are rich in GPx-1 to detoxify oxygen radicals formed within tissues (33). In the present study, OLE-treated diabetic rats exhibited an increase in GPx activity compared to those untreated diabetic animals, which aligns with other studies (34).

OLE administration at 50 and 100 mg/kg did not significantly increase total thiol levels in the treated groups. In experimental models of diabetes, OLE-rich extract significantly increased the activity of SOD, CAT, glutathione reductase (GRx), and GPx antioxidant enzymes in the kidney, liver, and erythrocytes (35). Additionally, the OLE-rich extract decreased proinflammatory cytokine secretion, enhanced IL-10 levels, and increased insulin receptor substrate-1 (IRS-1) expression in STZ-induced diabetic mice (36). OLE acts as an antioxidant by scavenging free radicals and breaking radical chains (37). However, our results showed that OLE administration had a counteractive effect on diabetes-related reductions in thiol levels and GPx activity. NF-κB is a crucial transcription factor, and its signaling pathways control the metabolism of several important cellular processes associated with inflammatory modulations (38). Many natural agents are useful in treating diabetes-related tissue damage and inflammation by inhibiting the NF-κB pathway (39). STZ administration resulted in oxidative stress and hyperglycemia, followed by inflammation, as evidenced by alterations in cytokines such as TNF-α and NF-κB (40). The Nrf2 signaling pathway regulates the antioxidant response found in the promoter area of several antioxidant/detoxifying genes, such as HO-1 (41). Under oxidative stress conditions, Nrf2 migrates to the nucleus, attaches to the antioxidant response element sequence, and induces phase II gene transcription and the subsequent cytoprotective response associated with the up-regulation of HO-1 and reduced sensitivity to oxidative stress damage. Thus, Nrf2 plays an important role in cellular defense through enhanced ROS removal (42). The protective effect of Nrf2 against renal damage through mediation of free radicals has been demonstrated in STZ-induced diabetic models (43).

In the current study, OLE administration improved Nrf2 expression levels in the brains of diabetic rats, further dampening oxidative damage. The cellular defense system against free radicals utilizes antioxidant enzymes, which are also downstream targets of Nrf2 (44). Reduction in cellular antioxidant status can lead to excessive production of free radicals and subsequently propagate lipid peroxidation (45). OLE administration restored the activities of Nrf2 downstream targets compared to diabetic rats and further limited the production of free radicals.

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Therefore, these results suggest that OLE can reduce oxidative damage in STZ-induced diabetic rats through the activation of Nrf2 downstream gene activation. We observed inhibition of NF-κB activity in OLE-treated diabetic rats, suggesting that OLE exhibits its antidiabetic activity by down-regulation of the NF-κB-mediated signaling pathway. Our results are consistent with those of Castejon et al., who reported the immunomodulatory effects of dietary OLE supplementation in pristane-induced Systemic Lupus Erythematosus (SLE) in mice through suppression of pro-inflammatory biomarkers. The activation of the Nrf2/HO-1 antioxidant pathway and the suppression of relevant signaling pathways NF-κB and mitogen-activated protein kinases may contribute to these effects (46).

Maria Tanase et al. (2022) reported that antioxidant compounds, including flavonoids, can affect pharmacological modulation. The researchers stated that increased NF-κB activity in diabetic neuropathy promotes inflammation, produces inflammatory cytokines, and causes nerve injury. Decreased Nrf2 activity leads to increased oxidative stress in neurons, resulting in the activation of poly (ADP-ribose) polymerase-mediated neuronal apoptosis, protein kinase C (PKC) activation, advance glycation end products (AGE) generation, and hyperalgesia and allodynia due to damage to sensory fibers. Nonetheless, such impaired balance can be pharmacologically modulated to attenuate various impairments in diabetic neuropathy. The pharmacological modulation of the NF-κB–Nrf2 axis through certain agents, such as plant phenolic compounds, is effective in activating Nrf2 and suppressing NF-κB (47).

Wei et al. (2022) reported that quercetin, a flavonoid compound, suppressed pyroptosis in diabetic cardiomyopathy via the Nrf2 pathway. They found that quercetin promoted Nrf2 nuclear translocation in cardiac cells of diabetic rats, increased the expression of antioxidant proteins HO-1, Glutamate-cysteine ligase (GCLC), and SOD, reduced ROS accumulation and cardiomyocyte apoptosis, and alleviated diabetes-related cardiac fibrosis (48). Nrf2 activators have been shown to improve cognition in preclinical rodent models of Alzheimer's disease (AD). Dietary supplementation with the Nrf2 activator anthocyanin improved memory in the Morris Water Maze (MWM) test in a mouse model of AD (49). The Nrf2 activator ellagic acid dose-dependently improved memory in a rat model of AD (50). Additionally, the expression levels of NF-κB in the hippocampus were increased with ellagic acid treatment. Ellagic acid treatment restored the nuclear/cytoplasmic ratio of Nrf2. Agmatine, another Nrf2 activator, improved memory in the MWM in a streptozotocin-induced AD rat model (51).

Limitations of this study

One of the limitations of the present study is the lack of access to human subjects, and another limitation is the lack of measurement of the protein of the mentioned genes, one of the reasons for which is the lack of research funding.

Conclusion

Our findings suggest that OLE attenuates memory dysfunction due to its antioxidant and anti-inflammatory effects. Therefore, OLE can be considered a promising anti-inflammatory and antioxidant agent for diabetes management. Furthermore, our data suggest that OLE administration may protect against diabetic complications by activating the Nrf2/HO-1 pathway and inhibiting the activation of the NF-κB pathway. Further studies are warranted to elucidate the molecular mechanisms underlying the antioxidant and anti-inflammatory effects of OLE in diabetes.

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Conflicts of interests

No conflicts of interest were declared by the authors.

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Ethical Considerations

All study procedures were conducted in accordance with the ethical principles outlined in the Declaration of Helsinki 2013. The study protocol was approved by the Ethics Committee (reference number: IR.IAU.AHVAZ.REC.1401.019).

Author's contributions

Conceptualization and study design: MRR, Data acquisition, Statistical analysis, and Data interpretation: ZSh, Writing – original draft: ZSh (Data analysis, writing the original draft), Review, editing, and final approval: All authors

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