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Original Research

Oleuropein can Protect the Brain Against Deleterious Effects of Bile Duct Ligation in Male Mice

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Abstract

Oleuropein (OP) is a phenolic compound in olives. OP possesses potent antioxidant activity and an extensive spectrum of other pharmacological properties, including antiviral, anti-inflammatory, and antimicrobial activities. This study investigated the effects of OP on neural injuries caused by bile duct ligation (BDL) in male mice. The mice were randomly allocated to three groups: sham, BDL, and BDL + OP. Neurobehavioral tests histological and biochemical evaluations were accomplished to assess cerebral damage. The results demonstrated that the induction of BDL led to behavioral impairments and a rise in hepatic enzymes, and OP could protect the brain against BDL-induced injuries. OP significantly

increased antioxidant enzymes. These results suggested that OP has valuable effects in the mice BDL model, probably due to its anti-inflammatory and antioxidant properties.

Keywords
Oleuropein; bile duct ligation; neuroprotection; rat

1. Introduction

Chronic liver diseases (CLD), such as alcoholic hepatotoxicity, nonalcoholic fatty liver disease, viral hepatitis, and primary biliary cholangitis, can affect the brain and cause brain dysfunction [1]. Brain dysfunction caused by liver failure and systemic portal shunting is called hepatic encephalopathy (HE), which causes neurological abnormalities, neurological and cognitive disorders, and even coma and death [1].

Various studies have shown that bile duct ligation (BDL) in animals and the consequent liver damage are associated with brain impairments, especially neurotoxicity of cerebral neurons, and can alter the level of cerebral neurotransmitters, causing cognitive and motor dysfunction [2-4]. The BDL model mimics the clinical and metabolic features of CLD and the neurological complications of HE [5]. The pathophysiological mechanism of HE is not yet well recognized, but hyperammonemia, systemic inflammation, and oxidative stress are critical factors in this disease [6]. Numerous research studies have demonstrated that ammonium plays a major and significant role in the development of HE by altering the metabolism of neurotransmitters and inducing neurotoxicity [7]. Some scientific reports have shown that hyperammonemia by BDL can cause intense activity of glial cells in the cerebellum and some areas of the hippocampus [8]. Inflammatory mechanisms activated by microglia cells affect astrocytes and lead to their dysfunction. These mechanisms also lead to astrocytic edema and cerebral edema, which are symptoms of this brain disease [9]. There is no definitive cure for this disease, and finding effective substances to prevent neuronal damage is significant. Antioxidant compounds such as gallic acid, naringenin, melatonin, and ascorbic acid have been essential in reducing brain damage caused by BDL [10-12]. Oleuropein (OP) is a flavonoid compound extracted from olive leaves and fruit, which has antioxidant and anti-inflammatory properties and can help prevent oxidative injuries [13]. Oleuropein has antimicrobial, antidiabetic, and anticancer properties [14-16]. OP has neuroprotective properties, and its use can prevent neurological disorders such as Alzheimer's, Parkinson's, stroke, depression, anxiety, and epilepsy. It seems that OP reduces the damage to neurons by decreasing the amount of inflammatory cytokines and chemokines and inactivating microglia and astrocytes [17]. Based on previous studies, this study aimed to investigate the neuroprotective effect of OP on encephalopathy caused by BDL in male mice.

2. Materials and Methods

2.1 Animals

Adult male mice (8 weeks old) were obtained from the animal house of the Neuroscience Research Center, Kerman University of Medical Science, and kept under standard status, which

included a 12-h on-off light-dark cycle. The animals had free accessibility to food and water, and the room temperature was maintained at 21 ± 2°C. The mice were randomly allocated to three groups (n = 10) (sham, BDL surgery, and BDL surgery + OP). OP (100 mg/kg) was administered intraperitoneally (i.p.) [18]—every other day for four weeks after BDL. The BDL surgery group received saline (i.p.) for four weeks. Oleuropein was dissolved in normal saline before injection [19]. The Ethics Committee of Kerman University of Medical Sciences approved the study (Ethics Code: IR.KMU.REC.1400.446).

2.2 BDL Surgery

The mice were anesthetized with a dose of 90 mg/kg ketamine and 20 mg/kg xylazine intraperitoneally. The skin of the abdomen area of the animals was shaved. An incision was made in the midline of the rectus abdominis muscles. The liver was exposed. The hepatic hilum elements were examined. The common bile duct was detected in the anterior side of the portal vein and on the right side of the hepatic artery, then both its proximal and distal ends of the bile duct were ligated with 4-0 silk, and the abdominal wall was closed [20, 21]. The common bile duct was identified and manipulated in sham-operated mice but was not ligated. The mice were maintained for four weeks after the BDL and had ad libitum access to food and water.

2.3 Biochemical Assays

The mice were euthanized, and blood samples were obtained from their hearts under deep anesthesia. Then, the blood samples were centrifuged at 3000 rpm for 15 minutes, and their serum was separated. Liver markers, including aspartate transaminase (AST), alkaline phosphatase (ALP), alanine aminotransferase (ALT), and total bilirubin were analyzed in the blood serum using a commercial Kits of Pars Azmoon (Pars Azmoon, Iran) and autoanalyzer tool (BT 1000, made in Italy).

2.4 Open Field Test

Four weeks after BDL surgery, the mice were put in the middle of the open-field device. This device, which was made of Plexiglas, had a square area [90 × 90 × 30 (H) cm], and its floor was divided into 16 squares consisting of central and peripheral squares. The animal activities were recorded for 5 minutes and analyzed using EthoVision software (ver. 7.1), a video tracking software for automating behavioral paradigms. The following parameters were recorded for each animal: total distance moved (cm), velocity (cm/s), and time spent in the central area (inner zone) and the peripheral zone (outer zone) (s) [10].

2.5 Histopathological Study

Four weeks after BDL, the mice were euthanized under deep general anesthesia. The brain tissues were removed and fixed with 10% formalin. Tissue processing was done with increasing degrees of alcohol for dehydration and clarification by xylene, then embedded in paraffin. A microtome cut coronal sections (5 µm) from the cerebral cortex (Leika RM 2145). Tissue sections were deparaffinized with xylene and hydrated with descending degrees of alcohol, and then

sections were stained with H&E and mounted with entellan [20]. The morphology of cortical neurons was examined at a magnification of 400×.

2.6 Malondialdehyde (MDA) Measurement

MDA levels were measured using the thiobarbituric acid (TBA) method. Briefly, 20 µl of the sample with 400 µl of TBA was mixed and incubated for 1 h at 95°C. 400 µl of 50 mM KH_2PO_4 buffer (pH = 6.8) was added to each sample and centrifuged for 5 minutes at 4°C. Then, the absorbance of the products was read at 530 nm [22].

2.7 Superoxide Dismutase (SOD), Glutathione Peroxidase (GPx) Activity and Total Antioxidant Capacity (TAC)

SOD activity was determined by the SOD Randox kit (Catalogue number: SD125). Briefly, 20 µl of homogenized tissue was mixed with 5 µl of H_2O. Then 170 µl of the primary reagent was added to it. After adding 25 µl of xanthine oxidase and 10 µl of H2O, its absorbance was read at 560 nm.

GPx activity was evaluated using a GPx Randox kit (Catalogue number: RS505). First, the primary reagent was prepared by mixing 8 ml of KH_2PO_4 buffer (pH 7.4), 4 ml of glutathione reductase, 2 ml of glutathione, and 2 ml of NADPH. Then, to start the reaction, 25 µl of the sample, 200 µl of the primary reagent, and 10 µl of H_2O were mixed together, and its absorbance was read at the wavelength of 340 nm.

TAC was measured using the metmyoglobin chromogen method. Briefly, 10 µl of homogenized sample, 10 µl of metmyoglobin, and 150 µl of chromogen were mixed together. Then 40 microliters of hydrogen peroxide were added to it. It was incubated for 5 minutes at room temperature. Then, its absorbance was read at the wavelength of 750 nm using an ELISA reader [23].

2.8 Statistical Analyses

The data are presented as mean ± standard error of the mean (SEM). SPSS software (v. 22.0) was used. Based on our data, one-way analysis of variance (ANOVA) was used to analyze the statistical significance between different groups. A p-value of 5% ($P < 0.05$) or lower was considered statistically significant.

3. Results

3.1 The Effects of BDL and OP on Liver Biomarkers

The levels of liver function indices, including bilirubin, ALP, ALT, and AST, significantly increased due to BDL surgery after four weeks compared to the sham group ($p < 0.001$), and treatment with OP in BDL + OP group significantly decreased liver biomarkers compared to BDL group ($p < 0.001$) (Table 1).

Table 1 The effects of BDL and OP treatment on hepatic indices in male mice.

Groups	Total bilirubin (mg/dL)	ALT (U/L)	ALP (U/L)	AST (U/L)
Sham	1.19 ± 0.07	95.5 ± 7.87	287 ± 16.08	112.66 ± 7.47
BDL	7.63 ± 0.33*	567.12 ± 23.77*	1078 ± 38.71*	748 ± 24.74*
BDL + OP	4.38 ± 0.18$^{\$}$	372 ± 23.80$^{\$}$	689.33 ± 27.24$^{\$}$	442 ± 21.46$^{\$}$

Total bilirubin levels, ALT, ALP, and AST significantly increased in BDL mice. OP treatment significantly reduced total bilirubin, ALT, ALP, and AST levels in mice. * $p < 0.001$, compared with the sham; $ $p < 0.001$, compared with the BDL. BDL: Bile duct ligation OP: Oleuropein ALT: Alanine aminotransferase ALP: Alkaline phosphatase AST: Aspartate transaminase.

3.2 The Effect of BDL and OP on Locomotor Activity

Mice in the BDL group showed a decrease in the total distance ($p < 0.001$), velocity ($p < 0.001$), and time spent in the central area ($p < 0.001$) compared to the sham group. OP increased the total distance ($p < 0.05$), velocity ($p < 0.05$), and time spent in the central area (p < 0.001) compared to the BDL group (Figures 1A, 1B, and 1D). BDL increased the time spent in the peripheral area ($p < 0.001$), and OP significantly decreased this time in the treated group ($p < 0.05$) (Figure 1C).

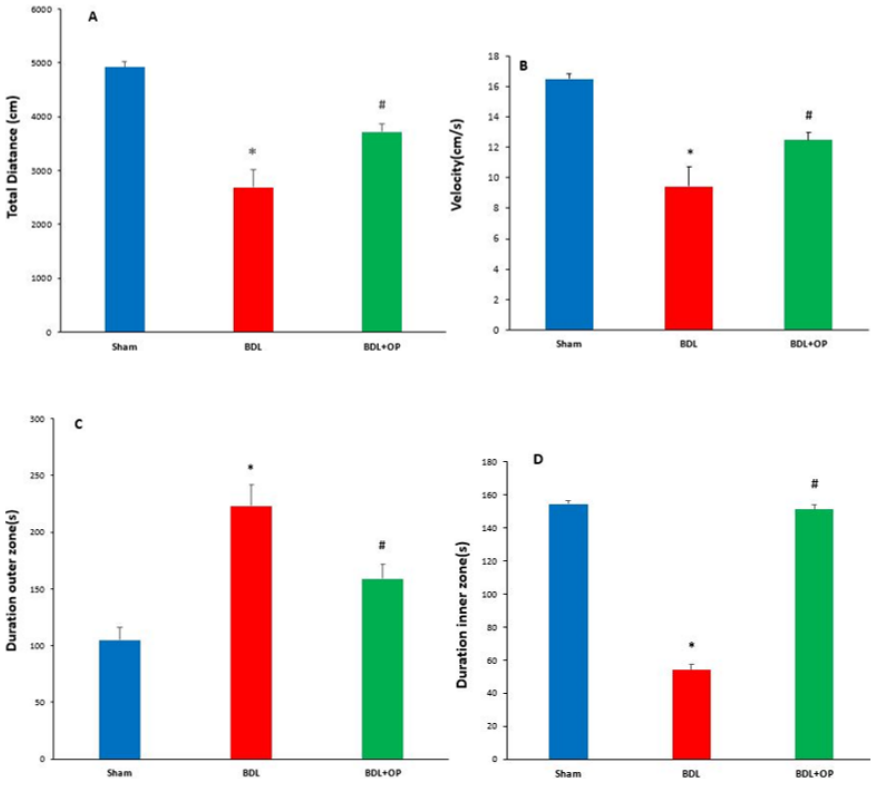

Figure 1 The effect of BDL on the locomotion behavior in open field tests of mice. The total distance, velocity, and time spent in the inner zone were decreased in the BDL group compared to the sham group, and OP significantly improved these parameters. There was a significant difference in time spent in the peripheral area for BDL mice as compared to the sham group ($p < 0.001$), and OP markedly diminished this parameter ($p < 0.05$). *$p < 0.001$ compared to Sham; #$p < 0.05$ compared to BDL + OP; ANOVA followed by Tukey's test. BDL: Bile duct ligation OP: Oleuropein.

3.3 The Effects of OP on Brain Injury Due to BDL in Male Mice

In the sham group, the neurons showed normal morphology in the motor cortex. In contrast, in the BDL group, some exhibited degenerative changes in the motor cortex, and the use of OP reduced the number of degenerative neurons in the BDL + OP group in the motor cortex (Figure 2).

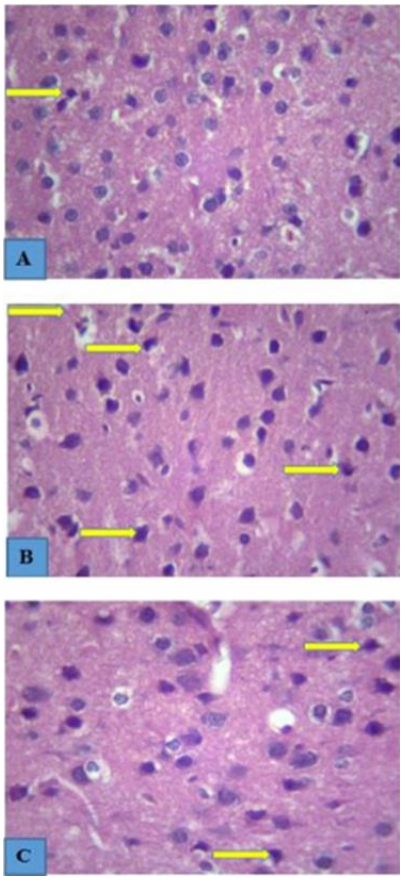

Figure 2 Photomicrographs showing cerebral cortex neurons of mice in different groups (A, B, C). The typical morphology of neurons, including a round nucleus with a light nucleus, prominent nucleolus, and light cytoplasm, is visible in the figure, and degenerated neurons show dark cytoplasm and shrinkage in the nucleus (yellow arrow). A: Sham, B: BDL, C: BDL + OP. Magnification: 400×.

3.4 The Effects of OP on MDA, SOD, and GPx on the Brain Tissue of Male Mice

Brain MDA in the BDL group was markedly higher than that of the Sham group ($p < 0.05$), and OP treatment reversed the MDA level back to the sham group ($p < 0.05$) (Table 2). BDL induction resulted in decreasing the antioxidant enzymes levels, SOD and GPx in the treated group compared to sham group ($p < 0.001$), and OP treatment was able to improve this reduction ($p < 0.001$) (Table 2). Brain TAC in the BDL group showed a significant decrease compared to the sham group ($p < 0.01$). Brain TAC in the BDL + OP group showed a significant increase compared to the BDL group ($p < 0.05$) (Table 2).

Table 2 Effects of BDL and the OP treatment on MDA and antioxidant enzymes of brain cortex in mice.

Groups	MDA (nmol/mg tissue)	TAC (nmol/$Fe2^{+}$/mg tissue)	SOD (U/mg protein)	GPx (U/mg protein)
Sham	0.07 ± 0.004	18.21 ± 0.70	0.046 ± 0.001	3.64 ± 0.10
BDL + Vehicle	0.16 ± 0.004***	14.23 ± 0.50**	0.031 ± 0.001***	2.57 ± 0.04***
BDL + OP	0.0125 ± 0.004###	16.84 ± 0.31#	0.038 ± 0.00##	2.97 ± 0.06#

BDL significantly increased the level of MDA in the brains of male mice. BDL reduced the TAC, GPx, and SOD levels in mice. Treatment with OP considerably diminished MDA in treated mice. The treatment could improve the TAC, GPx, and SOD levels in mice. * $P < 0.05$ vs. Sham; ** $P < 0.01$ vs. Sham. *** $P < 0.001$ vs. Sham. # $P < 0.05$ vs. BDL + Vehicle; ## $P < 0.01$ vs. BDL + Vehicle; ### $P < 0.001$ vs. BDL + Vehicle. BDL: Bile duct ligation; OP: Oleuropein; MDA: Malondialdehyde; GPx: Glutathione peroxidase; SOD: Superoxide dismutase 1.

4. Discussion

Current research data depicted that BDL in mice leads to an increased level of liver enzymes, a decreased level of brain antioxidant enzymes, behavioral impairments, and neuronal degeneration in the cerebral cortex. These can be improved by OP treatment.

Numerous researches have shown that BDL causes brain disorders [24, 25]. Although the mechanisms involved in the development of behavioral disorders are not fully known, oxidative stress, inflammation, and hyperammonemia are among the causes of these disorders [26]. In the brain of BDL animals, the level of free radicals increases [27] and can lead to behavioral disorders in the animals [25].

OP is a phenolic compound with antioxidant, antimicrobial, and anti-inflammatory activities [28] that has been shown to have neuroprotective effects [29, 30].

Previous reports have shown that using exogenous antioxidants can prevent behavioral disorders [29, 31]. According to these findings, in our study, BDL mice spent significantly less time at the center of the arena compared to the sham group, and the OP treatment reduced anxiety behaviors in the BDL group.

According to Tanaka [32], BDL increases bilirubin in rats. Our biochemical studies also showed that bilirubin levels and the functional parameters of mouse liver increased, and the use of OP reduced these markers.

In agreement with previous studies [33, 34], BDL led to oxidative stress in animals, increased level of MDA, and diminished antioxidant enzyme activities such as SOD and GPX. OP markedly increased antioxidant enzyme activities (GPx and SOD) and alleviated the detrimental effects of BDL-induced oxidative stress.

Neurodegeneration is one of the essential consequences of BDL [35], and preventing this harmful process is of great importance in protecting the brain. In earlier studies, BDL induction resulted in cortical neuron degeneration in male mice. OP treatment could protect the neurons from injuries due to its potent antioxidant and anti-inflammatory effects. One of the advantages of OP as a neuroprotective factor is its capability to cross the blood-brain barrier (BBB) [17], which

can be crucial for the emergence of its beneficial properties. In addition, OP had no toxic effects on neurons [17].

To better understand the exact mechanisms, it is suggested to investigate inflammatory cytokines such as tumor necrosis factor (TNF-α) and interleukin-6 (IL-6) and apoptotic markers, including caspase-3, Bcl-2, and Bax.

5. Conclusion

To sum up, the results of this study showed that OP improved the locomotor activity in the mice BDL model by reducing oxidative stress and neurodegeneration in the motor cortex, which might be due to its ability to cross the BBB and its antioxidant and anti-inflammatory properties easily. Further studies should be performed to elucidate the precise mechanisms.

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Author Contributions

Faezeh Kouhakan, Sepideh Ganjalikhan, Alireza Sarhadizadeh, Khadijeh Esmaeilpour, Khatereh Akbari, and Majid Asadi-Shekaari participated in data collection and drafting of the manuscript, data analysis, and performance of the interpretation. Faezeh Kouhakan, Sepideh Ganjalikhan, Alireza Sarhadizadeh, Khadijeh Esmaeilpour, Khatereh Akbari, Leila Jafaripour and Majid Asadi-Shekaari provided conception and design of the study, writing an article and revision of the article. All authors reviewed the manuscript.

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Competing Interests

There are no conflicts of interest that could be perceived to have influenced this study.

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