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Pretreatment with Naringenin ameliorates bile duct ligation induced injuries in male rats

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ABSTRACT

Hepatic encephalopathy (HE) is known to be a complex neurological syndrome. Former studies have shown that Naringenin (NG) can be beneficial for neurological impairments. Examples of these impairments are memory loss and motor dysfunction. In the present study, we have investigated the effects of NG on neuronal injures in bile duct-ligated rats. The male rats were divided into three groups including sham, bile duct ligation (BDL), and BDL + NG in a random manner. Behavioral, biochemical and histological studies were carried out for the purpose of evaluating neural damages. The results indicate that, BDL led to balance impairment and rise in the hepatic enzymes. NG had a preserving role in the treated group. Furthermore, NG pretreatment showed a decline in the neuronal injury in BDL + NG compared to the BDL group. The results showed that NG can have beneficial effects in the rat model of HE. This might be due to the anti-oxidant, estrogenic and anti-inflammatory properties of NG. Further studies are required in order to elucidate the precise mechanism.

Keywords: *Bile duct ligation; Hepatic encephalopathy; Naringenin; Rat; Neuroprotection.*

1. INTRODUCTION

Hepatic encephalopathy (HE) is a neuropsychiatric syndrome as a secondary result of liver insufficiency that leads to cognitive, behavioral, and personality dysfunctions by several mechanisms [1, 2].

High brain ammonia level is known to be the main reason leading to HE with a significant neuro-inflammation effects by triggering inflammatory pathways in brain endothelial cells due to the ammonia influence on activation of microglia. Ammonia is able to pass through blood-brain-barrier (BBB) and astrocytes serve as a detoxification agent by converting ammonia to glutamine so astrocytes swelling is assumed to be another pathological reason leading to brain edema and subsequently HE [3, 4].

Moreover, blockage of bile flow from the liver to intestine results in metabolic changes especially in mitochondrial functions. It also reduces fatty acids oxidation, and enhances the hydrophobic bile acid levels. These unwanted events activate the inflammatory response causing hepatocytes death and as a consequence cirrhosis [4, 5].

A flavonoid named Naringenin (NG) is found in several herbal sources like *Drynaria fortunei*, *Citrus aurantium*, *Citrus medica* and also in citrus fruits [6, 7]. Previous surveys unveil the great medical properties of NG including antioxidant, anti-inflammatory, anti-apoptotic, anti-ulcer, anti-osteoporotic and anti-carcinogenic [7, 8].

NG has been proved to have influences on blocking inflammatory factors, e.g., interleukin-6 (IL-6), interleukin-8 (IL-8) and TNF-a in animal models [7, 9]. There is also evidence related to NG impacts on central nervous system (CNS) disease including Alzheimer's disease, Parkinson's disease and epilepsy. Also in Parkinson's disease animal model, glial cell line-derived neurotrophic factor expression was elevated and TNF-α expression was declined in microglia [7]. There is a paucity regarding protective effects of NG on BDL rats so this work was designed to investigate its protective effect against BDL-induced injuries.

2. MATERIALS AND METHODS

In the current study male adult Wistar rats were used. The animals were housed under standard conditions of a standard vivarium. The housing was set to a 12h on-off light-dark cycle; free access to food and water and room temperature maintained at (20 ± 3 °C). The rats were randomly put into three groups (n=10) (sham, BDL surgery, and BDL surgery + NG). NG was purchased from Sigma-Aldrich (N5893) and the drug was administered (50 mg/Kg) intraperitoneally (i.p.) for a one week period prior to BDL. Sham and BDL surgery groups received saline (i.p.) in the same volume and based on the time schedule considered for BDL surgery + NG group. Ethics Committee of the Kerman University of Medical Sciences (KMUS) (Ethics Code: IR.KMUS.REC.1396.587) was approved in the study.

2.1. Surgical procedure of BDL.

The animals were anesthetized with ketamine (90 mg/kg, i.p.) and xylazine (20 mg/kg, i.p.) and the bile duct was ligated with 4–0 silk suture at two points and the abdominal wall closed in two layers. The common bile duct was identified and manipulated

but not ligated in sham operated rats. The animals were kept for six weeks after the surgery and they had ad libitum access to food and water.

2.2. Biochemical assays.

The animals were killed and blood samples were obtained by carotid bleeding under deep anasthesia. Biochemical markers of liver were analyzed in the blood plasma. These markers were aspartate transaminase (AST), alkaline phosphatase (ALP), alanine aminotransferase (ALT), total bilirubin and hepatic albumin. The testing was done using a commercially available kit.

2.3. Rotarod test.

For assessment of coordinated motor function impairment, computer aided accelerated rotarod was used. The rotarod was made of a suspended rod, rotated from 10 rounds per minute (RPM) to 60 RPM. Rats were put on the rod and sequentially tested in three sessions. Each session consisted of 5 min with a rest period of 30 min between each session. The duration (s) that the rats stayed on a rotating rod (latency to fall) was recorded [10].

2.4. Wire hang test.

The Wire Hang test is used for evaluating motor function, coordination and deficit in rodent models of CNS disorders. The test started with the animal hanging onto the wire. Two forelimbs were used for hanging. The latency to when the animal falls was recorded during 3 trials for each rat that intertrial interval was 5 min [11].

2.5. Histological study.

The rats were sacrificed six weeks after BDL under deep general anesthesia, brains were removed, fixed in 10% buffered-formalin for 48 h and embedded in paraffin. In order to asses the morphology of cortical neurons, the hematoxylin and eosin (H&E) staining technique was used. Coronal sections (6 µm) were cut from the cerebral cortex using a rotary microtome (Leika RM 2145). The sections were deparaffinized and stained with H&E. Cerebral cortex pyramidal neurons were manually evaluated and estimated in three microscopic fields (400 ×) of the sections from the cerebral cortex. Results are expressed as the number of degenerated neurons.

2.6. Statistical analyses.

Results are presented as mean ± standard error of the mean (SEM). Statistical software SPSS (v. 22.0) was used. Based on our results, the one-way ANOVA was used to determine the statistical significance between different groups. $P < 0.05$ was considered to be statistically significant.

3. RESULTS

3.1. Effect of BDL and NG on grasping ability in wire hang test.

The average of three sequential trials indicated a decline in the time of falling in BDL group as compared to the sham group in the wire hang test ($p < 0.05$, Fig. 1). In rats pretreated with NG, the duration staying on the wire significantly increased compared to BDL rats ($p < 0.05$). NG treated group had no significant difference in comparison to sham group.

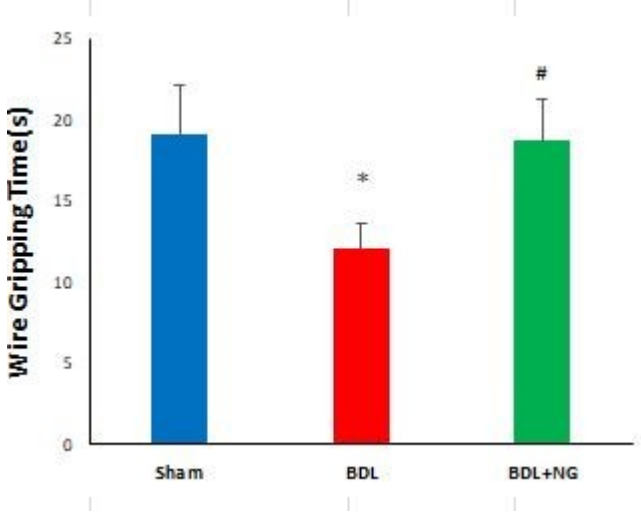

Figure 1. The effect of NG on muscle strength of rats. There were significant differences on latency to fall in muscle strength test. Rats of BDL model had a decreased time on rod which shows impairments of balance; administration of NG reversed this effect of BDL. * $p < 0.05$ compared to Sham; # $p < 0.05$ compared to BDL, ANOVA, followed by Tukey's test. BDL: bile duct ligation NG: Naringenin.

3.2. Effect of BDL and NG on motor coordination and balance skills in rotarod test.

There was statistically significant reduction in latency to fall in BDL group in three trails ($p < 0.001$, $p < 0.01$ and $p < 0.01$, respectively) compared to sham group. Rats of NG group stayed longer on the rotarod test compare to BDL rats in three trials ($p < 0.05$). Rotarod data showed that NG group had no significant difference as compared to the sham group (Fig. 2).

3.3. Biochemical assay.

According to our data, levels of biochemical indices including bilirubin, ALT, ALP, and AST were raised as a result of BDL surgery after six weeks and the NG administration counteracted these effects. On the other hand, the level of albumin markedly decreased in the BDL rats and NG can ameliorate this change (Table 1).

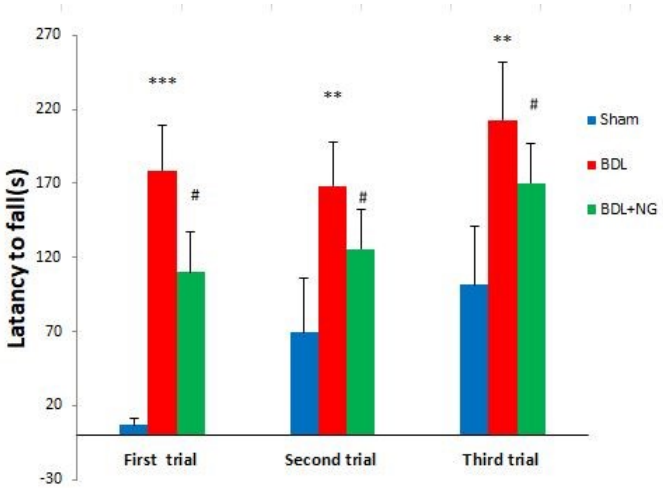

Figure 2. The effect of NG on balance functions of rats. There were significant differences on latency to fall in muscle strength test. Rats of BDL model had a decreased time on rod which shows impairments of balance; administration of NG reversed this effect of BDL. ** $p < 0.01$; *** $p < 0.001$ compared to Sham; # $p < 0.05$ compared to BDL, ANOVA, followed by Tukey's test. BDL: bile duct ligation NG: Naringenin.

3.4. Effect of BDL and NG on neuronal injury.

The number of degenerated neurons in the cortex of rats elevated significantly in the BDL group (Fig. 3) compared to the

sham group ($p < 0.001$). NG pretreatment significantly reduced the number of degenerated neurons in treated rats ($p < 0.01$).

3.5. Discussion.

The present study proved that BDL leads to motor impairments as well as increased hepatic enzymes levels and degenerated neurons in cerebral cortex of male rats. This can be reversed by NG pretreatment. Various studies have shown that BDL can induce motor and cognitive impairments [12-14]. The underlying mechanism of these impairments are not fully understood but neuro-inflammation, oxidative stress and excitotoxicity have been responsible candidates for these deficits [15].

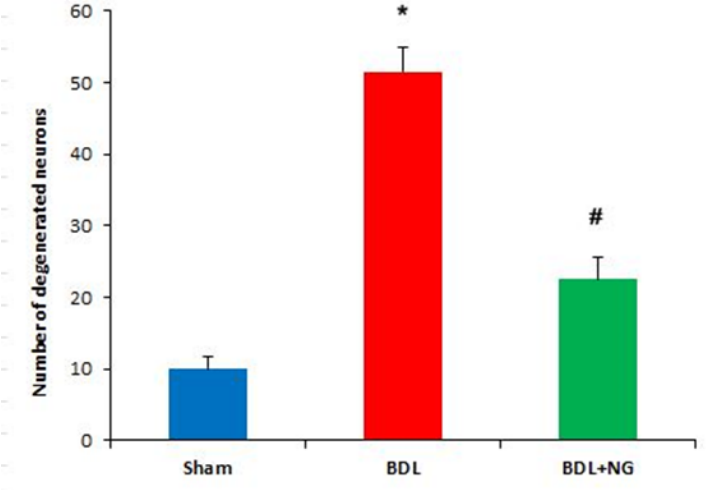

Figure 3. The effects of NG pretreatment on neuronal degeneration induced by BDL in rats. The figure reveals the quantitative analysis of cortical neurons in different groups. Data are the mean ± SEM. * p < 0.001 compared with the sham group; # p < 0.01 compared with BDL. BDL: bile duct ligation NG: Naringenin

Many studies have focused on the possible impact of oxidative stress in HE-induced neural disturbances [16, 17]. Finding the ways to decrease the oxidative stress in BDL-induced encephalopathy might help in diminishing the behavioral deficits and neurodegeneration. In addition, exogenous antioxidants that have the ability to inhibit reactive oxygen species (ROS) generation, prevent oxidative injury, and may lastly improve motor dysfunction.

In a previous study, it was shown that NG has neuroprotective activity against Huntington's disease symptoms such as weakness and rigidity of muscles motor and behavioral abnormalities. These observations confirm our findings that show NG improves and ameliorates BDL-induced impairment in motor function in rotarod and wire grip tests.

NG as a phytoestrogen is mainly found in citrus fruits). Antioxidant, anti-inflammatory and anti-apoptotic features of NG have been reported in different studies [6, 18]. On the other hand, there is evidence that NG has an estrogenic activity. This acitivity is done through interaction with estrogen receptors [19].

Increased oxidative stress and neuro-inflammation are among the highest complications of BDL that lead to neuronal injury [4, 20]. Previously, neuroprotective effects of NG have been investigated and in the current work NG could also protect cortical neurons against BDL-induced damages. An interesting point about NG is that it can easily traverse the blood-brain barrier (BBB) [21]. Based on our results, NG pretreatment declined neuronal degeneration. The result was consistent with its anti-apoptotic and antioxidant activities [22] and partly due to its estrogen-like activity. This observation is in agreement with our recent study [4].

In accordance with Huang's report, BDL significantly increased the levels of bilirubin, ALT, ALP, and AST that was related to liver injury in male rats [23].

NG pretreatment prevented the elevation in hepatic parameters compared to BDL group. Furthermore, the same beneficial effects of NG pretreatment was also observed on the increase of albumin levels compared to BDL group.

Table 1. The effects of BDL and NG pretreatment on hepatic indices in male rats.

Groups	Bilirubin total (mg/dl)	ALT(U/l)	ALP(U/l)	AST(U/l)	Albumin (g/dl)
Sham	0.68±0.07	95.2±14	640±25.29	132±12.04	3.02±0.18
BDL	6.39±0.87*	288.75±52*	1689±115.80*	530±38.15*	2.22±0.10*
BDL+NG	1.26±0.33$	127±26$	664.5±33.75$	241.25±74.85$	2.75±0.23

The level of total bilirubin, ALT, ALP, and AST significantly increased in BDL rats. The level of albumin was decreased in BDL rats. NG pretreatment significantly reduced the levels of total bilirubin, ALT, ALP, and AST in rats. * $p < 0.05$, compared with the sham; $ $p < 0.05$, compared with the BDL. BDL: bile duct ligation NG: Naringenin ALT: alanine aminotransferase ALP: alkaline phosphatase AST: aspartate transaminase

4. CONCLUSIONS

In conclusion, the ability of NG to show a neuroprotective effect in the rat BDL model might be due to its antioxidant, anti-inflammatory and estrogenic properties and its ability to easily cross the BBB. We suggest that further studies should be done to clarify the precise mechanisms.

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6. ACKNOWLEDGEMENTS

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