

THE POSSIBLE EFFECTS OF SILYMARIN ON CEREBRUM WITH EXPERIMENTAL HEPATIC ENCEPHALOPATHY IN RATS



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DOI: <https://doi.org/10.29121/granthaalayah.v8.i8.2020.946>

Article Type: Research Article

Article Citation: Ozgun Teksoy, Varol Sahinturk, Mustafa Cengiz, Behcet İnal, and Adnan Ayhancı. (2020). THE POSSIBLE EFFECTS OF SILYMARIN ON CEREBRUM WITH EXPERIMENTAL HEPATIC ENCEPHALOPATHY IN RATS. International Journal of Research - GRANTHAALAYAH, 8(8), 140-146. <https://doi.org/10.29121/granthaalayah.v8.i8.2020.946>

Received Date: 02 August 2020

Accepted Date: 26 August 2020

Keywords:

Hepatic Encephalopathy
Rat
DLPFC
Silymarin
Immunohistochemistry

ABSTRACT

Background: The relationship between liver diseases and neurological defects is well established. Hepatic encephalopathy (HE) has been seen both in people with acute liver failure (ALF) and chronic liver disease (CLF). HE is a complex neuropsychiatric syndrome that is seen in patients suffering from liver dysfunction. Silymarin (Sm) has antioxidant, anti-inflammatory, and anti-carcinogenic features. In this study, the possible protective effects of silymarin were investigated against dorsolateral prefrontal cortex (DLPFC) damage induced by thioacetamide (TAA).

Method: To achieve this, male Sprague Dawley rats (200-250 g) were randomly divided into four groups, with 7 animals comprising each group: the control group, 50 mg/kg TAA group, 50 mg/kg Sm + TAA group, and 100 mg / kg Sm + TAA group.

Results: Differences between the groups were determined by performing immunohistochemical analysis of the PFC. Bax, TNF- α , and TUNEL expression increased in the brain tissue of the experimental group where only TAA was administered.

Conclusions: It was observed that in high doses in particular (100 mg/kg Sm + TAA group), Sm was effective in preventing PFC damage caused by TAA. It was determined that 100 mg/kg Sm significantly reduces TAA-induced inflammation (TNF- α and H&E) and apoptosis (Bax, TUNEL) in brain tissue.

1. INTRODUCTION

Abnormal behavior and cognition impairment have been observed in the brains of patients suffering from acute and chronic liver failure, which is, in turn, followed by the detection of edema in the brain cells caused by increasing ammonia levels in the blood. This syndrome is called hepatic encephalopathy (HE) and is the result of several factors, defined as intrinsic (genetic) or extrinsic (viral, alcohol) [1]. According to the International Association of Hepatic Encephalopathy and Nitrogen Metabolism (ISHEN), thioacetamide (TAA), D-galactosamine, and carbon tetrachloride

have been commonly used to create an experimental animal model of HE [2]. TAA, used in the present study, metabolized in the liver [3]. The metabolizing process occurs in two stages: firstly, the TAA-S-oxide (TASO) appears followed by S, S-dioxide (reactive form, TASO₂). This metabolizing process is executed through hepatic cytochrome P450 enzymes and the FAD-containing monooxygenase (FMO) [4].

The cerebrum (telencephalon), responsible for controlling our emotions, hearing, vision, personality, and much more, is the largest part of the brain [5]. It is one of the areas most affected by HE precisely because it is such a big section of the brain [6], [7], [8], [9]. Our study is focused on the dorsolateral prefrontal cortex (DLPFC), the front most of the frontal lobe [10]. It is responsible for complex behavior such as planning, decision making, expression, etc [11], [12].

Several drugs are commonly used in HE treatment but, unfortunately, a host of side effects has also been reported alongside their use. For this reason, researchers are turning to herbal ingredients in their quest for a suitable treatment for HE. Silymarin (Sm), obtained from the seeds and fruits of *Silybum marianum*, is one of them. Sm is a mixture of isomeric flavonoids (silybin, isosilybin, and silychristin) [9]. It can be used in high doses without any resulting side effects in either humans or animals [13]. It has a hepatoprotective feature, while at the same time demonstrating free radical scavenging and cell membrane balancing activity [13], [14], [15], [16], [17]. Several studies have shown that Sm also has a neuroprotective effect [18]. This effect is largely related to the inhibition of oxidative stress molecules in the brain [19] but is also a result of the induction of pathways involved in neuroprotection, such as the inflammatory pathways [20], [21]. Moreover, a previous study demonstrated which Sm attenuates 6-OHDA-induced motor in-coordination in rats [22].

This study aims to show brain damage caused by TAA-induced liver damage as well as to throw some light on the protective effect of Sm on the brain in animal models with HE. To better understand this effect, the DLPFCs of rats were examined immunohistochemically via apoptotic markers.

2. MATERIAL-METHOD

2.1. CHEMICALS

TAA (Cat No: 62-55-5), DMSO (Cat No: 67-68-5), and Sm (Cat No:65666-07-1) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and immunohistochemistry (IHC) antibodies were obtained from Santa Cruz Biotech (Santa Cruz, USA).

2.2. ANIMALS

8 weeks-old male Sprague-Dawley rats (200-250g) were housed at 25°C and exposed to a 12 h light-dark cycle. Food and tap water were provided ad libitum. Animal experiments were performed according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (Reg. No. KU/IAEC/PhD/100 dated 26.07.2012). This study was carried out with the permission of the Eskişehir Osmangazi University Local Animal Ethics Committee (No: 513-2 / 2016).

2.3. THE EXPERIMENTAL DESIGN

The groups were determined in accordance with previous studies [23].

Untreated group: 0.5 mL of water with 0.2% DMSO was administered orally to each animal for the first 14 days, followed by an intraperitoneal injection of 0.5 mL serum physiologic for the remaining 14 days.

TAA group: 50 mg/kg TAA (1 mL/kg b.w.) was intraperitoneally injected to each animal for the second period of 14 days.

Low dose treatment group: 50 mg/kg Sm (1 mL/kg b.w.) was administered orally to each animal for the first 14 days, followed by 50 mg/kg TAA (1 mL/kg b.w.) intraperitoneally injected into each animal for the remaining second 14 days.

High dose treatment group: 100 mg/kg Sm (1 mL/kg b.w.) was administered orally to each animal for the first 14 days, followed by 50 mg/kg TAA (1 mL/kg b.w.) intraperitoneally injected to each animal for the remaining second 14 days.

5% dextrose containing 0.9% NaCl and potassium (20 mEq/L) was injected into all animals daily to prevent weight loss, hypoglycemia, and renal failure [24].

1 day after the last TAA injection, a laparotomy was drastically performed under sterile conditions and all rats were anesthetized with ketamine/xylazine (5/1 ratio); blood samples were then collected from the left ventricle of the heart and centrifuged at 3000 rpm for 10 mins after which serum was obtained.

2.4. IMMUNOSTAINING

Immunostaining was performed based on the standard procedure with some modifications. Zivic Rat Brain Slicers were used to cut the brain into slices. After fixing the cerebrum tissues (DLFPC) (56°C, 1 night), they were deparaffinized with xylene and then rehydrated in ethanol of various percentages. They were then immersed in fresh and ice-cold methanol containing 1% H₂O₂ for about 20 mins to eliminate the effect of endogenous peroxidase. Samples rinsed with PBS were incubated in blocking buffer (PBS containing 3% BSA, 0.1% Tween-20) at 25°C [25].

Samples were incubated with anti-Bax (Abcam, cat: ab53154) and anti-TNF- α (Abcam, cat: ab6671) antibodies (Millipore, cat: S7101) in a blocking solution for 1/2 day at 4°C and washed with PBS (25°C). This was then followed by incubation with a horseradish peroxidase (HRP) antibody (Abcam) (1:2500) in blocking solution for 120 minutes at 25°C. After washing with PBS, the samples were incubated with 0.2, 3, 3'-diaminobenzidine (DAB), and then later rinsed with distilled water. All samples were counterstained with hematoxylin. Immunoreactivity between groups was examined under a fluorescent microscope with a camera (Olympus-DP70 camera).

Apoptotic cells were detected according to the apoptotic detection kit guide, (Millipore, Cat No: S7101). After the sections had been sliced at 5 μ m thicknesses, they were treated with 20 μ g/ml proteinase K in 0.1 mol/l Tris-HCl buffer (pH 7.4) for 15 minutes. They were then incubated with 100 μ L equilibration buffer at 25°C for 15 min and later with rTdT incubation buffer (45 μ L equilibration buffer, 5 μ L nucleotide mix, 1 μ L rTdT enzyme) at 37°C-60 minutes in a humidified chamber. The reaction was completed by treating the samples with 2 x SSC for 15 minutes at 25°C. The samples were stained with 1 μ g/ml propidium iodide for analyzing in a fluorescence microscope (620nm). 500 healthy cells were examined in a fluorescence microscope and those that had a red appearance were considered TUNEL positive [26].

3. RESULTS AND DISCUSSIONS

This study has described how brain damage due to TAA-induced liver depredation was treated by 100mg/kg Sm. It was observed that apoptosis (Bax, TUNEL) and inflammation (TNF- α) reduced in the TAA group depending on the administration of Sm treatment. However, Bax, TUNEL, and TNF- α expression levels did not reduce in the 50 mg/kg Sm+TAA group (Figures 1-3).

The Bcl-2 family (Bcl-2, Bax, Bim, Bid, Bak, and Bcl-xL) plays an important role in initiating an intrinsic apoptotic pathway [26], [27], [28], [29], [30]. Bax, a pro-apoptotic member of the family, promotes cell death via permeabilization of the mitochondrial outer membrane. In contrast, Bcl-2 (an anti-apoptotic member) prevents apoptosis by blocking Bax activity [28], [29]. The balance between Bax and Bcl-2 can determine cellular fate. Mutagenic chemicals such as TAA trigger apoptosis, with some studies, successfully demonstrating increased expression of Bax and decreased Bcl-2 expression following TAA-induced hepatotoxicity [24]. Brain damage caused by TAA has also been demonstrated [30], [31], [32], [33], [34], [35]. Another study led by El-Ghazaly established that when TAA-induced Wistar HE rats are administered local or whole-body low dose γ radiation (0.5 Gy), increased caspase-3 expression level occurs in the brain immunochemically [32]. Khanna and Trigun (2016) found that 100mg/kg TAA in Wistar rats significantly increased the level of cerebrum Bax expression [33].

Tumour necrosis factor-alpha (TNF- α), a cytokine, plays a role in inflammation and is responsible for the process which results in cell death. As a result of excessive oxidative stress, neuronal apoptosis begins depending on mitochondrial dysfunction or TNF family receptors activation [34]. El-Marasy et al. (2018) showed that the TNF- α content in brain tissue was enhanced 1.46-fold compared to normal rats in TAA (100 mg/kg) induced-Wistar HE rats. They also noted that plasma TNF- α levels were significantly increased in Wistar rats given 150 mg/kg TAA when compared to the control group [36].

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) is a method used for apoptosis detection and it detects DNA fragmentation by labeling the 3'-hydroxyl terminal in dsDNA [37]. Bustamante and

colleagues found that the apoptosis rate in a rat MHE (minimal hepatic encephalopathy) model performed by surgery increased by 2.3 times compared to the control group [37].

Several studies have been conducted on the neuroprotective effect of Sm. Haddadi et al. treated neurotoxicity induced by 6-hydroxydopamine (6-OHDA) in Wistar rats with Sm daily, 100, 200, and 300 mg/kg injected into SNc (substantia nigra pars compacta) [26]. As a consequence, the Bcl-2 level increased while the Bax and TUNEL levels decreased. However, the caspase-3 level did not change significantly in any group. Our experimental results show that Silymarin may have neuroprotective effects on neurotoxicity caused by hepatic encephalopathy.

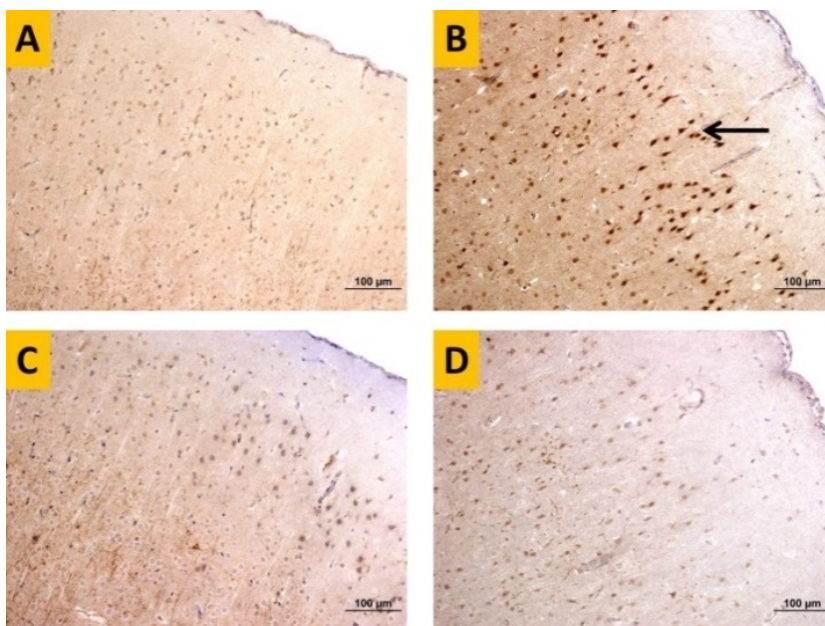


Figure 1: Bax immunoreactivity in neurons in brain cortex sections of rats belonging to experimental groups. Bax immunoreactivity is indicated by a neuron that reacts positively (arrow). A: Weak reaction in neurons in the control group. B: Strong positive reaction in the TAA group. C: Weak reaction in the TAA + low dose Sm group and D: Weak reaction in the TAA + high dose Sm group. All bars are 100 µm.

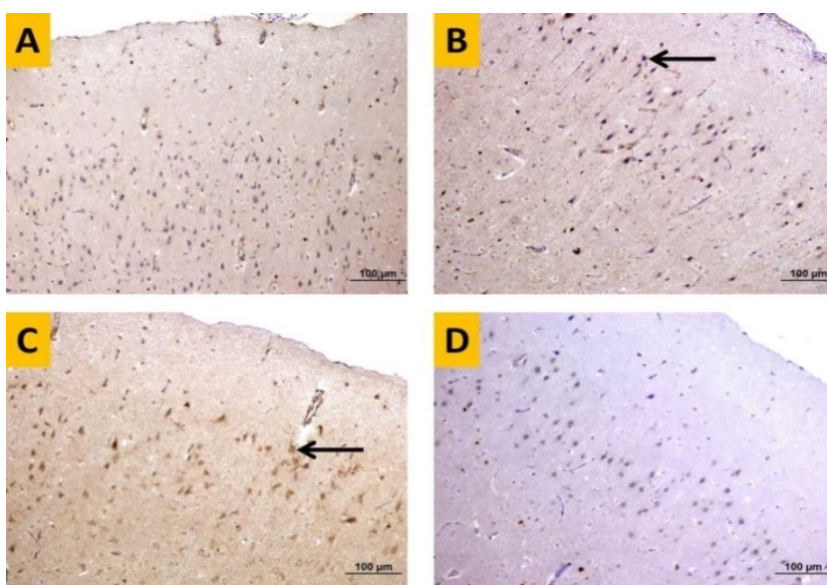


Figure 2: TUNEL immunoreactivity in neurons in the brain cortex sections of rats belonging to experimental groups. Some of the neurons that reacted positively are indicated by (arrows). A: Weak reaction in neurons in the control group. B: Strong positive reaction in the TAA treated group. C: Moderate reaction in the TAA + low dose Sm group and D: Weak reaction in the TAA + high dose Sm group. All bars are 100 µm.

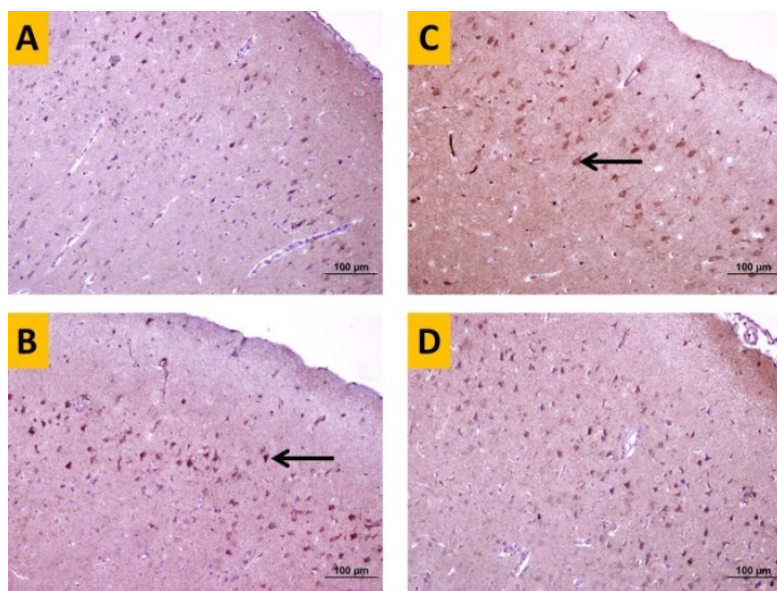


Figure 3: TNF- α immunoreactivity in neurons in brain cortex sections of rats belonging to experimental groups. Some of the neurons that reacted positively are indicated by (arrows). A: Weak reaction in neurons in the control group. B: Strong positive reaction in the TAA treated group. C: Moderate reaction in the TAA + low dose Sm group and D: Weak reaction in the TAA + high dose Sm group. All bars are 100 μ m.

4. CONCLUSION

Silymarin is an extremely good hepatoprotective agent. For this reason, it has the potential to be used as a protective agent in hepatic encephalopathy, which causes both liver and brain damage. Our experimental findings support this hypothesis. However, since the bioavailability of Sm is rather low, we propose two different strategies to increase its effectiveness. First, the active ingredients of Sm should be investigated more comprehensively, considering synergistic and antagonistic effects. Secondly, new materials containing Sm should be prepared to increase the bioavailability of Sm, such as sugar-coated tablets, its own micro emulsifying drug delivery system (SMEDDS), or beta-cyclodextrin inclusion.

SOURCES OF FUNDING

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

CONFLICT OF INTEREST

The author have declared that no competing interests exist.

ACKNOWLEDGMENT

This study (coded 2017-1621) from Özgün Teksoy's doctoral dissertation was supported by Eskişehir Osmangazi University.

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