

# A possible protective role of betain and omega-3 supplementation in traumatic brain injury



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## Investigation of possible protective role of betain and omega-3 supplementation in traumatic brain injury

**INTRODUCTION:** Due to irreversible damage following head trauma, many overlapping pathophysiological events occur including excitotoxicity, acidotoxicity, ionic imbalance, edema, oxidative stress inflammation and apoptosis.

**MATERIAL AND METHODS:** In this study, after the rats were separated in to groups these rats were fed throughout fourteen days with betaine, omega-3 or betaine+omega-3 combination in physiological limits prior to the trauma. After a closed head trauma, the damaged brain tissues were collected for biochemically and histologically analyses. This examination involved analyses of levels of caspase-3 and cytochrome C and neuron-specific enolase (NSE) levels in brain tissue.

**RESULTS:** These analyses showed that traumatic brain injury (TBI) caused an increase in the levels of caspase-3, cytochrome C and neuron-specific enolase (NED) in the brain tissues examined.

**DISCUSSION:** In this study, apoptotic and/or necrotic cell death via mitochondrial cytochrome C caspase pathway in traumatized cells and neuron-specific enolase (NED) increase indicative of neuronal damage confirmed the research hypothesis.

**CONCLUSION:** Level of the biomarkers induced by brain injury in the groups fed with betaine, omega-3 and betaine+omega-3 combination before the traumatic damage approximated to that of control group values, suggesting that these products may have a neuroprotective role.

**KEY WORDS:** Betain, Caspase-3, Cytochrome C and Neuron-specific enolase, Omega-3, Traumatic brain injury

## Introduction

Traumatic brain injury is a pathology that requires long-term treatment and care. It is also a common problem that concerns community health and one of the leading causes of death worldwide <sup>1,2</sup>. The incidence of head

trauma and associated mortality and morbidity risks are gradually increasing in today's daily accelerating social and technological life conditions. According to European Union statistics, the annual incidence of head trauma is 235/100.000 person and 2.7% of them result in mortality <sup>3</sup>. Evidence shows that average 1.7 million people in the US are exposed to head trauma each year, and about 5.3 million Americans continue to live with a TBI-related disability <sup>4</sup>. The main determinants of the outcome of traumatic injury (TBI) are primary factors including injury mechanism, severity of injury, lesion pathology, expanded involvement, involved brain region, and hemispheric dominance <sup>5</sup>. This damage is called primary damage and can result in severe disabilities, such

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as vegetative life and death, which is prevalent among more than 20% of the patient population <sup>6</sup>.

Traumatic brain injury occurs when the pathways of excitotoxicity, acidotoxicity, ionic imbalance, edema, oxidative stress, inflammation and apoptosis work together through complex interrelationships<sup>7</sup>. Primary damage usually causes irreversible damage to neurons, glial cells and blood vessels. The energy exchange initiated during the damage usually results in cerebral tissue deterioration and microvascular lesions. Along with other systemic factors, this damage opens the way to secondary damage by creating a hypoxic and ischemic condition <sup>8</sup>. These conditions lead to the transition from aerobic metabolism to anaerobic metabolism, resulting in lactate forming an acidosis state that activates pH-dependent calcium channels <sup>9</sup>. Excessive accumulation of glutamate also leads to reduction of ATP level and cause to ionic and proteolytic imbalance as like anaerobic metabolism. This causes sodium (Na<sup>+</sup>) and calcium (Ca<sup>+2</sup>) to enter into the cell via glutamate receptors and, consequently, necrosis and death of the cell <sup>8,10,11</sup>.

Apoptosis is, by definition, the cell death mechanism occurring through intrinsic and extrinsic mechanisms in the cytoplasm and can be genetically programmed in the regulation of tissue homeostasis triggered by free radicals, DNA damage, protease activation and ionic imbalance. Both pathways result in caspase-3 activation, which leads to the breakdown of cellular proteins necessary to maintain cell survival and integrity <sup>7</sup>. Especially caspase-3 is the essential protein that plays a central role throughout the apoptotic process. Glutamate also induces apoptosis by both stimulating the production of oxidative stress and mitochondrial reactive oxygen species (ROS) through pro-oxidant activity and via caspase-3 or mitochondrial cytochrome C release <sup>12,13</sup>.

Neuron Specific Enolase (NSE) is a basic glycolytic enzyme synthesized by neurons and neuroendocrine tissues. This is a dimeric enzyme composed of  $\alpha$  and  $\gamma$  subunits. NSE (gg-enolase) is an intracellular protein<sup>14</sup> found mostly in neuronal cytoplasm and in neuroendocrine cells in an insignificant concentration. The findings in some central nervous system diseases that cause neuronal damage suggested that NSE might be a specific indicator for neural damage <sup>15</sup>.

Nutritional-based therapies can represent a significant part of combination therapies as these agents have complemented the therapeutic effect on biological function and damage to the brain in several studies <sup>16</sup>. Previous studies showed that supplementation of essential nutrients for the metabolic cascade in the early stages of injury contributes to the functional recovery of patient <sup>4</sup>. Based on the theory developed in the light of this effect, this study was carried out with betaine and omega-3 in order to examine the effects of potential neuroprotective products consumed in the pre-trauma daily diet on the trauma-induced damage.

Also known as trimethylglycine, glycinebetaine, lysine or oxingerine, betaine is a quaternary ammonium com-

pound. betaine, which contains both amino acids with both positive and negative charges, is required for the conversion of homocysteine to methionine, stabilization of methionine levels, homocysteine detoxification and S-adenosylmethionine (SAM) production <sup>17</sup>. Having an osmolytic character, the main physiological role of betaine is to regulate transmethylation reactions. betaine is found in microorganisms, plants and animals and is an important component of many foods such as wheat, shellfish, spinach and sugar beets. betaine has two main physiological functions protecting cells under stress as organic osmolyte or acting in trans-methylation processes as a catabolic source of methyl groups in many biochemical events <sup>18</sup>. The highest betaine concentrations are found in the liver and kidney whereas it is found in low concentrations in the brain <sup>19</sup>.

Fish oil is rich in omega-3 and is known to be anti-inflammatory. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are predominantly omega-3 fatty acids <sup>20</sup>. It has a key role in mitochondrial function as well as in the structural component, modulation and viscosity of cell membranes <sup>21</sup>. Several studies showed that omega-3 exhibits anti-inflammatory and antiapoptotic properties although its neuroprotective effect is not fully explained. It is widely used in developed countries in the form of a fish oil capsule against hypertriglyceridemia, heart disease and macular degeneration<sup>22</sup>.

In the light of these data, the aim of this study was to identify possible neuroprotective activities of betaine and omega-3 in posttraumatic secondary trauma in rats fed with these products prior to the trauma.

## Material and method

This study was carried out with the rats obtained from the Medical and Surgical Experimental Research Center (TICAM) of Eskişehir Osmangazi University after approval of the Experimental Animals Ethics Committee of the Faculty of Medicine of the same university (446-2, 19.08.2015). A total of 40 Wistar albino adult male rats (weighing 260-280g) were divided into five groups (n = 8 for each).

### GROUPS AND DRUG ADMINISTRATION

There were five groups in the study: control group (n=8), TBI (Traumatic Brain Injury) group (n=8), TBI + betaine group (n=8), TBI + omega-3 group (n=8) and TBI + betaine + omega-3 Combination group (n=8). During the 14-day period before the TBI, the groups were respectively treated with 2 ml saline (0.9% NaCl); 2 ml saline (0.9% NaCl); betaine dissolved in saline at a dose of 800 mg/kg in 2 ml volume; 300 mg / kg omega-3; and betaine and omega-3 combination. The drugs were prepared freshly every day at the same

doses and applied by gavage. Rats were treated by the saline, betaine and omega-3 each day as described above and this process lasted throughout the fourteen days. The day after the last dose day (day fifteen) of the treatment all the rats were prepared for the head trauma.

#### HEAD TRAUMA MODEL AND COLLECTION OF SAMPLES

Marmarou's impact acceleration model was used to obtain a head trauma model<sup>23</sup>. Experimental animals were anesthetized by injecting intramuscularly with 70-mg/kg ketamine (Ketalar 50 mg/ml 10 ml vial, Pfizer Inc. Istanbul) and 7-mg/kg xylazine hydrochloride (Rompun 2% solution, 50 cc. Bayer-Türk Inc. Istanbul). The rats were placed in a prone position at a table. After the dorsal surfaces of their skulls were supported with 10 cm foam, a head impact was delivered to each rat via a free-falling 450 g cylindrical weight through a metal tube from a height of 100 cm onto the coronal and sagittal suture junctions in the cranium. Dropping of the cylindrical weight wasn't performed in the control group; however, other procedures were same for the control group rats.

Once the trauma model was administered, brain tissues were taken carefully from the trauma boundary into tissue bags for biochemical analysis without causing any additional damage or sacrificing the rats, and the tissues were then kept in a chest freezer (-80 °C temperature) until the examination time. Next, cardiac blood samples were taken from the animals for analyses to be carried out on the sera, and the sera were separated for use after being placed in biochemical tubes and centrifuged. In addition, for histopathological analysis, some tissues were fixed in formaldehyde until the moment of examination.

#### BIOCHEMICAL EXAMINATIONS

##### *Measurement of Brain Tissue caspase-3 Activity and cytochrome C Levels*

Measurements of brain tissue caspase-3 activity (CatNo 201-11-0281) and cytochrome C (Cat No 201-11-0628) levels were performed using the Sandwich ELISA kit from SunRed Corp (China, Shanghai). The tissues stored in the deep-freeze were homogenized with phosphate buffer and centrifuged at 3000 rpm for 20 minutes, the supernatant was tested for each brain tissue specimen according to the procedure specified by the kit. The results are given as ng/mg protein.

##### *Serum Neuron Specific Enolase (NSE) Analysis*

A serum NSE analysis was performed using the Sandwich ELISA kit (CatNo. 201-11-0542) supplied by

SunRedCorp (China, Shanghai). On the day when the cardiac blood samples were collected, the serum samples obtained from the blood samples which had been centrifuged at 3000 rpm for 20 minutes were removed from the deep-freeze and dissolved at +4 °C and then tested for each serum sample according to the procedure specified by the corresponding kit. The results are given in ng/ml.

##### *Determination of Tissue Protein Levels*

The measurement was performed using Bradford's method. This method involves spectrophotometrically quantifying the varying intensity of the colour blue at 595 nm that is produced by the Coomassie Brilliant Blue G-250 dye depending on the amount of protein in different concentrations<sup>24</sup>.

#### HISTOLOGICAL EXAMINATIONS

Hematoxylin-eosin dual staining was used in this study.

#### STATISTICAL ANALYSIS

SPSS 22.0 for Windows was used for statistical analysis. The results were expressed as mean ± standard deviation. Also, one-way ANOVA was used for statistical analysis of the groups, and Tukey test was used to determine the differences between the groups.

## Results

#### BIOCHEMICAL RESULTS

The graph in Fig. 1 shows the statistical analyses of the caspase-3 measurements performed on brain tissues of the five groups. caspase-3 activity of the TBI group significantly increased in comparison with the control group ( $p < 0.01$ ). In contrast, the level of caspase-3 activities in the other three treatment groups decreased in comparison with the TBI group ( $p < 0.01$ ). Among the groups, the closest caspase-3 activity to the control group was in the betaine+omega-3 combination group (Table I). Brain tissue cytochrome C levels increased in the TBI group but did not show a significant difference. However, the results were significantly lower in the other three groups compared to the TBI group. In the betaine group, on the other hand, this level showed a more significant difference than that in the control group ( $p < 0.05$ ) (Fig. 2, Table I).

Finally, as shown in Fig. 3, serum NSE activity of the TBI group was significantly higher than that of control group ( $p < 0.01$ ). In comparison with the TBI group, on

TABLE I - Brain tissue caspase-3 Activity and cytochrome C levels, serum NSE (Neuron Specific Enolase) Activity levels (s.d. standard deviation).

Groups	N	caspase-3 activity of brain tissues (ng/mg protein ± s.d.)	cytochrome C levels of brain tissues (ng/mg protein ± s.d.)	NSE activity of sera (ng/ml ± s.d.)
Control	8	15.52 ± 1.15	86 ± 1.88	16.78 ± 2.72
TBI	8	20.12 ± 2.10a	95.40 ± 11.15	23.37 ± 5.56a
TBI + betaine	8	16.19 ± 1.90b	71.42 ± 9.89b,d	19.74 ± 1.26
TBI + omega-3	8	16.10 ± 1.38b	79.40 ± 8.80b	18.27 ± 2.16b
TBI + Combined	8	15.35 ± 0.80b	81.84 ± 8.26c	17.99 ± 2.91b

a: Highly significant difference compared with the control group ( $p < 0.01$ ). b: Highly significant difference compared with the TBI group ( $p < 0.01$ ). c: Significant difference compared with the TBI group ( $p < 0.05$ ). d: Significant difference compared with the control group ( $P < 0.05$ ).

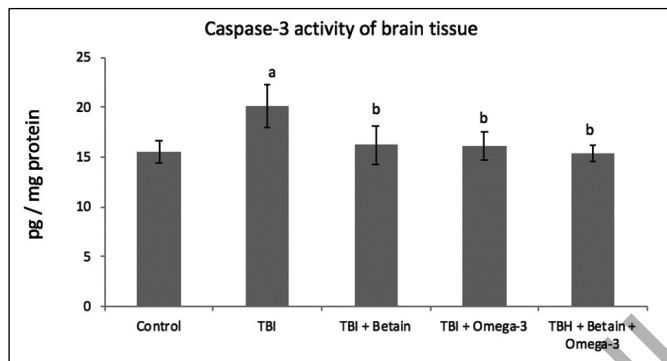


Fig. 1: a) Highly significant difference compared with the control group ( $p < 0.01$ ). b) Highly significant difference compared with the TBI group ( $p < 0.01$ ).

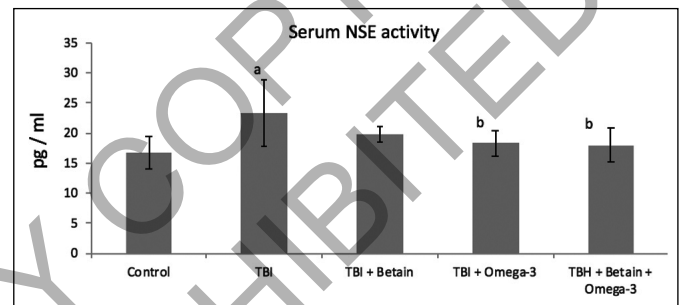


Fig. 3: a) Highly significant difference compared with the control group ( $p < 0.01$ ); b) Significant difference compared with the TBI group ( $p < 0.05$ ).

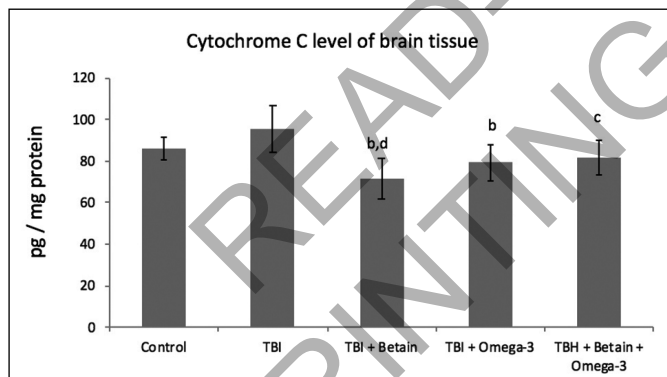


Fig. 2: b) Highly significant difference compared with the TBI group ( $p < 0.01$ ); c) Highly significant difference compared with the TBI group ( $p < 0.05$ ); d) Significant difference compared with the control group ( $p < 0.05$ ).

the other hand, the NSE activity of the betaine group was lower, but this decrease was not statistically significant. In contrast to these results, it is clear that the decreases in omega-3 and combined treatment groups were highly significant when compared to the TBI group, and that the results of the combined group were somewhat closer to the control group ( $p < 0.05$ ) (Fig. 3; Table I).

## HISTOLOGICAL RESULTS

As shown by the histological images of brain tissues in Fig. 4, normal cortical area neurons and glial cells are observed in the control group image. In the trauma group, on the other hand, numerous necrotic neurons and edema in the brain were noted in the cortical area. While edema decreased and less necrotic neurons were observed in the betaine group, edema continued to exist in the omega-3 group. In the group where drugs were used in combination to prevent TBI, edema was much more reduced and both necrotic and normal cell structures were observed (Fig. 4). These histological images showed that the best improvement was in the combined group, followed by the group given betaine as a therapeutic agent.

## Discussion

There are various studies on the primary damage following head trauma and neuronal degeneration resulting from its secondary damage that use nutrition-based therapies to support the healing process in the treatment of experimental brain injury in recent years<sup>16</sup>. The mechanism of the injury, the severity of the injury, the pathology of the lesion, the involved brain region

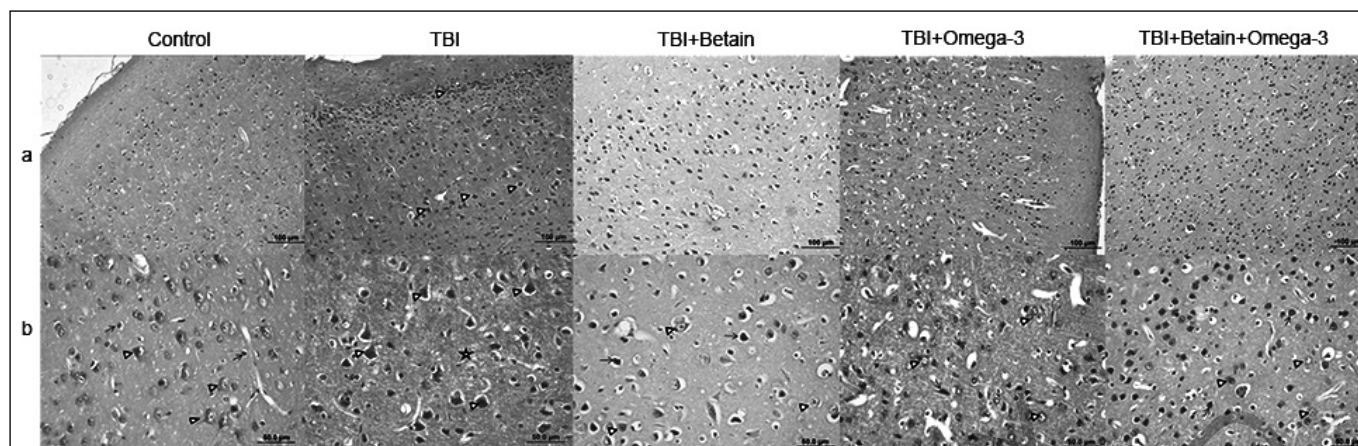


Fig. 4: The figure shows the histological image of brain cells stained with H&E cells. As can be seen in the figure, normal-looking neurons (▶) and glial cells (→) are observed in the control group. There are numerous necrotic neurons (▶) and edema (\*) in the brain in the cortical area in the TBI group. Necrotic neurons (→) are noted with normal-looking neurons (▶) in the cortical area against decreasing edema (→) in the group given TBI + betaine. Necrotic neurons (→) are observed along with normal-looking neurons (▶) in the cortical area against continuing edema (→) in the group given TBI + omega-3. Necrotic neurons (→) are seen along with normal-looking neurons (▶) in the cortical area against decreasing edema (→) in the group given TBI +betaine+omega-3. (H&E, a; scale bar: 100μm, b: scale bar: 50.0 μm).

and the hemispheric dominance are major determinants of the severity of damage and its sequelae. Many products emerging as a result of the complex relationships of the pathways of excitotoxicity, acidotoxicity, ionic imbalance, edema, oxidative stress, inflammation and apoptosis then determine the secondary damage<sup>7</sup>. These products in tissue and serum have been a popular research subject. In this study, we examined the effect of pre-trauma omega-3 and/or betaine dietary supplementation on neuronal damage following head trauma in rats by studying caspase-3 and cytochrome-C in brain tissue and NSE activity in serum.

Glutamate excitotoxicity triggers apoptosis by caspase-3 activation which is most important enzyme in apoptotic pathway, and cytochrome C release from mitochondria to cytosol<sup>13</sup>. Several studies showed that glutamate toxicity results in the loss of rapid ATP levels associated with mitochondrial dysfunction and consequent ROS production. Also, mitochondrial dysfunction activates caspase-3, which is a protease, and this acts as one of the key actors of the apoptotic cascade<sup>25</sup>. The cytochrome C oxidase is an essential electron transfer chain complex needing ATP and requires cytochrome C released after mitochondrial dysfunction for caspase-3 activation<sup>26</sup>. In a study, it was found to decrease the ATP level and cytochrome C oxidase activity in the glutamate injection group while it reversely affected the decrease in ATP level and cytochrome C oxidase activity in the KHG26693-treated group. This result proved it to be neuroprotective against oxidative stress caused by glutamate<sup>27</sup>. Activation of matrix metalloprotease (MMPs), which contribute to the deterioration of the blood brain barrier after TBI, leads to apoptosis and neuroinflammation via caspase-1/3 and IL-1 β release<sup>28</sup>.

caspase-3 inhibitors were shown to attenuate apoptosis induced by oxyHb in endothelial cells and to reverse chronic cerebral vasospasm in apoptotic subarachnoid animal models<sup>29,30</sup>.

Neuron-specific enolase (NSE) is a glycolytic cytoplasmic enzyme of neurons. It was previously used as a marker of stroke, traumatic brain injury, encephalitis, brain metastasis, subarachnoid haemorrhage (SAH) and neuronal damage in some neurodegenerative diseases. For this reason, it is important for clinicians<sup>31,32</sup>. High serum NSE levels in patients with SAH indicate an increased neuronal damage. Kacira et al. showed that CSF and serum NSE levels were significantly higher in patients with SAH than in control patients<sup>15</sup>.

betaine is critical for the structural and functional integrity of the cell membrane<sup>33</sup>. The main physiological role of betaine is being an osmolyte, which protects cells, proteins and enzymes from environmental stress. As another role, it takes part in methionine cycle as a methyl donor primarily in human liver and kidney. Inadequate uptake of methyl groups by nutrition leads to hypomethylation in many important pathways. Homocysteine levels increase and cause dyslipidemia. Eventually, changes in this liver metabolism may contribute to a variety of chronic diseases, including coronary disease, stroke, Alzheimer's disease, disorders of the nervous system such as dementia, disorders such as neural tube defects, and hepatic and vascular disease. Evidence suggests that betaine is a critical nutrient in the prevention of all these diseases<sup>18</sup>. In these diseases, betaine's cell protective properties may have a contributing role. betaine can be transported to the brain via the betaine-GABA transporter (BGT-1)<sup>34</sup>. betaine, which is also a molecular chaperone, protects the cells

against oxidative stresses and mitochondrial damage. betaine was also found to protect lysosomal membranes during a 30-day treatment period<sup>35</sup>. Kim et al. found that betaine was protective against necrotic damage in the cell<sup>36</sup>. Wu et al. reported that cholin and its metabolites, betaine and dimethylglycine, are important in pregnancy and infant neural development up to 18 months<sup>37</sup>. betaine regulates the immune response in osmotically stress-induced Kupffer liver macrophages by releasing TNF, forming phagocytosis and prostaglandin and suppressing cyclooxygenase 2 release<sup>38</sup>.

omega-3 acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are known to have anti-inflammatory effects. They are polyunsaturated oils found in plants and fish. They are rich in fish oil. Despite being controversial, their role in the prevention of cancer, heart disease and stroke is a popular research topic<sup>20,39</sup>. Although the mechanisms of how it occurs is yet to be understood, in Japan, Mishina et al. reported that daily diet intake of fish oil supplements might improve the prognosis of ischemic stroke patients<sup>40</sup>. In addition, Yoneda et al. reported that oral EPA could reduce symptomatic vasospasm and cerebral infarct frequency caused by cerebral infarction and aneurysmal SAH<sup>41,42</sup>. DHA plays an important role in the structure and function of brain cell membranes. In the brain, dramatic changes such as size, learning and memory changes of neurons were reported to occur when the amount of DHA decreases<sup>43</sup>.

El-Ansary et al. found that most of the measured parameters of brain poisoning in rats caused by the protective effects of omega-3 polyunsaturation were propionic acid, high levels of GABA (gammaaminobutyric acid), 5HT (serotonin), DA (dopamine), PE (phosphatidylethanolamine) PS (phosphatidylserine) and PC (phosphatidylcholine) and lower levels of IL-6, TNF- $\alpha$  and caspase-3<sup>44</sup>. Martin et al. showed that EPA (eicosapentaenoic acid) reverses the age-related increase in cytochrome C translocation and caspase-3 activity<sup>45</sup>. In their study of neurodegeneration associated with exposure to pre- and postnatal alcohol in the rat brain, Kusaol et al. showed that cytochrome C, caspase-3 and calpain levels decreased in the groups treated with betaine and consequently betaine and omega-3 had a therapeutic role in ethanol-induced neurodegeneration<sup>35</sup>.

In our study, caspase-3 and cytochrome C levels in brain tissue in trauma groups were significantly increased compared to the control group, indicating that apoptotic and/or necrotic cell death may be increased via traumatic mitochondrial cytochrome C caspase pathway. This result is compatible with the literature. Our histological findings also support the presence of a neuronal damage. The increase in serum NSE levels, which is an important biochemical biomarker of traumatic brain injury, indicates that brain damage occurred and that our trauma model was established sufficiently. In our study, neuronal damage caused by traumatic brain injury in the

betaine, omega-3 and combination groups was similar to that in the control group, and our morphological findings support this result.

## Conclusion

Our study confirms the neuroprotective effects of supplement nutrients such as betaine and omega-3. neuroapoptotic and/or necrotic processes after trauma can be prevented by supplement nutrients. Examination of post-traumatic oxidative stress and neuroinflammation in future studies will contribute to a better understanding of neuroprotective properties of betaine and omega-3.

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