

## Rat brain synaptosomes: In vitro neuroprotective effects of betaine against fluoride toxicity

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### Abstract

**Objective:** Fluoride increases the production of reactive oxygen and nitrogen derivatives, causing oxidative stress and cellular damage. Betaine, an antioxidant and an important methyl donor, has been reported to have potential protective effects on cardiovascular and neurodegenerative diseases in recent years. In this study, we aimed to investigate the neuroprotective effects of betaine treatment against cellular damage caused by fluoride exposure on rat synaptosomes.

**Material and Methods:** In the experimental period, 8 Wistar albino rats were decapitated and then their frontal cortices removed and divided into 4 equal cuts (total-32 cuts). Subsequently, an appropriate experimental procedure was applied to obtain the synaptosomal fractions. The determined doses of sodium fluoride (NaF) and betaine were administered in vitro at 37°C for 30 min. Synaptosomal glutathione (GSH), malondialdehyde (MDA) and nitricoxide (NO) levels, and also catalase (CAT) and Ca<sup>2+</sup>/Mg<sup>2+</sup> ATPase activities were measured spectrophotometrically.

**Results:** According to our results, NaF exposure caused a decrease in GSH levels, CAT and Ca<sup>2+</sup>/Mg<sup>2+</sup> ATPase activities, and also an increase in MDA and NO levels, significantly. MDA levels, NO levels and CAT activities were closed to the control group depends on the betain doses. The best recovery in terms of synaptosomal GSH levels and Ca<sup>2+</sup>/Mg<sup>2+</sup> ATPase activities was found at 0.5 mM betaine concentration (P <0.001).

**Conclusion:** Our results showed that betaine could be a potential neuroprotective therapeutic agent against synaptosomal fluoride toxicity.

**Keywords:** Fluoride, Betaine, Synaptosomes, Neurodegeneration, Oxidative stress

### Introduction

Fluoride (F), one of the most abundant elements on earth, can be found in rock and soil as well as in combination with other elements. Excessive F uptake causes fluorosis, which is an important health problem and characterized by defects in skeletal and tooth structure (1). The main cause of fluorosis is contaminated drinking water with organic and inorganic wastes. Since F in drinking water has an ionic structure, it is absorbed rapidly through the intestinal epithelium and interferes with metabolic processes by accumulating in the different organs of the biological systems (2). F intake at daily mean doses of 0.05 mg/L does not pose a risk to humans, while F exposure at doses ranging from 3 to 10 mg/L can cause significant health problems in various age groups (3).

Due to the electronegative character of F, which means that it is negatively charged and tends to form fluorine ions, it can pass through cell membranes via ion channels (4).

In vivo studies have found that F added to drinking water of rats causes toxic effects and accumulates in soft tissues such as lung, liver, heart, brain and kidney (5). F is a molecule with an anionic structure, which is easily permeable from membranes by binding to cations such as calcium and magnesium (6). F combined with cations has been shown to have direct effects on apoptotic processes characterized by impaired intracellular signaling mechanisms. It damages cell integrity by binding to Na<sup>+</sup>/K<sup>+</sup> ATPases and Ca<sup>2+</sup>/Mg<sup>2+</sup> ATPases found in the membranes, and also leads to enzyme degradations, reduction of intracellular calcium levels, deterioration of cell energy metabolism, depolarization of membranes and signal transduction (7). Calcium (Ca<sup>2+</sup>) is an element that plays a significant role in both metabolic processes and their interaction with the environment of the cell, and regulates various cellular responses.



Therefore, Ca<sup>2+</sup> plays a central role in maintaining viability, since it is an integrative component of many different signaling pathways in cells (8). F exposure disturbs the continuity of intracellular Ca<sup>2+</sup> homeostasis by causing inhibition of Ca<sup>2+</sup> pumps localized to cell membranes (9). Furthermore, F exposure has been shown to cause genotoxic effects with chromosome anomalies and DNA damage (10).

F can produce free oxygen and nitrogen species (ROS and RNS, respectively) by affecting the antioxidant metabolism (11). Furthermore, it has been shown that ROS and RNS interact with disulfide bonds in proteins, causing degradation of their synthesis and activities. In vitro and in vivo animal studies showed that F exposure causes oxidative stress by weakening the antioxidant defense system (12). F-induced ROS production reduces glutathione (GSH) levels as well as inhibition of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) (13). Increasing lipid peroxidation is also an important biomarker of oxidative stress. In vivo studies have been shown that F exposure enhances lipid peroxidation due to increased ROS production in rat brain tissues (14).

Betaine, which is known as trimethylglycine, is an important methyl donor derived from glycine amino acid. Betaine as an important osmolyte, can protect protein synthesis, enzyme activity and membrane integrity against the biotic and abiotic environmental stress conditions (15). Homocysteine, an important risk factor for cardiovascular diseases and neurodegenerative diseases, is converted to methionine by betaine. Moreover, betaine provides single carbon units for DNA synthesis and assists in the synthesis of choline, an important neurotransmitter (16). Metabolic anomalies linked to lack of betaine have been found to cause a variety of diseases such as cancer, cardiovascular disease and neurodevelopmental disorders (17). In animal model studies, increased lipid peroxidation, reduced GPx and GSH levels owing to oxidative stress have been shown to have neuroprotective effects by providing a significant improvement with betaine administration (18).

In this study, we aimed to investigate the neuroprotective effects of betaine against F toxicity, as studies related with fluorosis have become important in recent years. To verify our hypothesis, GSH contents, malondialdehyde (MDA) levels, CAT activities, nitric oxide (NO) levels and Ca<sup>2+</sup>/Mg<sup>2+</sup> ATPase activities were measured to test the neuroprotective effects of betaine following sodium fluoride (NaF) exposure of rat brain synaptosomes.

## Material and Methods

### Animals and Experimental Design

Eight healthy male Wistar albino rats weighing 300±50 g was supplied by Medical and Surgical Experimental Research Center, Eskisehir. Experimental procedures were carried out according to the decision of Animal Experiments Local Ethics Committee of Eskisehir Osmangazi University (Approval number: 651).

The rats were maintained under controlled conditions at 25°C ± 5°C and 50% ± 5% relative humidity with 12-hour periods (dark / light). Anesthesia was performed by intramuscular injection at 45±10 mg/kg ketamine + 10±5 mg/kg xylazine doses, and then the unconscious rats were decapitated. Rats' frontal cortex was removed and divided into 4 equal cuts (total 32 cuts) and the cuts were stored at -80°C until the day of the experiment.

In our previous work, we found that 80 mg/L NaF gave rise to toxicity on synaptosomes. In this study we also investigated the neuroprotective effects of betaine at concentrations of 0.25, 0.5 and 1 mM versus the toxicity caused by 80 mg/L NaF. The experimental group design was as described in Table 1.

**Table 1.** Design of experimental groups (n=6).

Group 1	Control
Group 2	80 mg/L NaF
Group 3	80 mg/L NaF + 0.25 mM Betaine
Group 4	80 mg/L NaF + 0.5 mM Betaine
Group 5	80 mg/L NaF + 1 mM Betaine

### Preparation of synaptosomal fractions

Rat brain synaptosomes were prepared according to Whittaker et al. (19). 32 brain cortical pieces obtained from healthy rats were distributed randomly into 4 experimental groups as it will be 6 pieces in each group (n=6). The cortical pieces were homogenized on ice in a solution containing 10 mM 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) and 30 µM sucrose. The homogenates were first centrifuged at 3000xg for 10 minutes at 4°C and then the supernatants were taken and centrifuged once more at 15000xg for 20 min at 4°C. The remaining pellets were re-suspended in saline and rat brain synaptosomal fractions were obtained. According to the determined experimental groups, synaptosomes were exposed to at 80 mg/L NaF and betaine at 0.25, 0.5 and 1 mM concentrations for 30 minutes at 37°C. Synaptosomal protein levels were measured according to the biuret method (20). This method is used to demonstrate the presence of peptide bonds in the samples. The reaction of copper (Cu<sup>2+</sup>) with the two peptide bonds is based on the principle of purple color formation, and the colored product was measured spectrophotometrically at 540 nm.

### Glutathione (GSH) contents

The Ellman reaction is based on the principle that the p-nitrophenol anion formed by reaction of thiol compounds with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) in an alkali environment is spectrophotometrically measured. GSH levels were measured spectrophotometrically at 412 nm according to Srivastava and Beutler method (21). Briefly, Reaction medium contains 0.1 ml sample, 2 ml 100 mM Tris HCl pH 8.4 and 0.1 ml Ellman's reagent (60 mg/100 ml, 0.1 M Tris-HCl buffer pH 7.0). The data for GSH levels were expressed as µmol/mg protein.

### Malondialdehyde (MDA) levels

The quantitative determination of lipid peroxidation is based on the color reaction between MDA and thiobarbituric acid (TBA). Synaptosomal MDA levels were measured at 532 nm according to the method reported by Ohkawa et al (22). In short, 0.6 ml rat synaptosomal fraction was added to sample 4 ml of sodium dodecyl sulphate (8%), and then 2 ml of acetic acid (%0.6, pH 6.5) and 2 ml of thiobarbituric acid solution (% 20, pH 4) was added to the reaction medium. The final concentration was adjusted to be 5 ml and heated in a water bath at 100°C for 60 minutes. After this process, it was centrifuged at 4000 rpm for 10 minutes and then spectrophotometric measurement was performed. The results were expressed as nmol/mg protein.

### Catalase (CAT) activities

Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), which is harmful to the cell, is converted to water and oxygenase by CAT. CAT activity measurement was based on the decrease in absorbance of H<sub>2</sub>O<sub>2</sub> at 240nm (23). In brief, the final concentration of the reaction medium adjusted to be 1 ml as follows: 60 mM sodium potassium phosphate (pH 6.5), 20 mM H<sub>2</sub>O<sub>2</sub> and 20 µL of sample. CAT was activated by the addition of H<sub>2</sub>O<sub>2</sub> to the reaction medium and the absorbance changes were spectrophotometrically monitored. The results were expressed as Unit/mg protein.

### Nitric oxide (NO) levels

The determination of NO levels is based on the measurement of nitrite and nitrate as a result of nitric oxide oxidation. The amounts of nitrite and nitrate in the samples are determined by two consecutive reactions. Initially, Nitrate is reduced to nitrite by means of enzymatic or non-enzymatic conversion. In the acidic reaction medium, the nitrite is diazotized with sulfanilamide and subsequently forms a purple azo compound with N-(1-naphthyl) ethylenediamine. The amount of nitrite was measured precisely according to the method also known as the Griess reaction (25). The results were expressed as µmol/mg protein.

### Ca<sup>2+</sup>/Mg<sup>2+</sup> ATPase activities

This method is based on the fragmentation of ATP at 340 nm in the presence of pyruvate kinase, phosphoenolpyruvate, lactate dehydrogenase and reduced nicotinamide adenine dinucleotide (NADH) in the reaction medium (25). To initiate the reaction, 0.5 M CaCl<sub>2</sub> was added to the medium containing 1 M ouabain. NADH oxidation was measured spectrophotometrically at 340 nm every 20 seconds for 5 minutes. The results were expressed as Unit/mg protein.

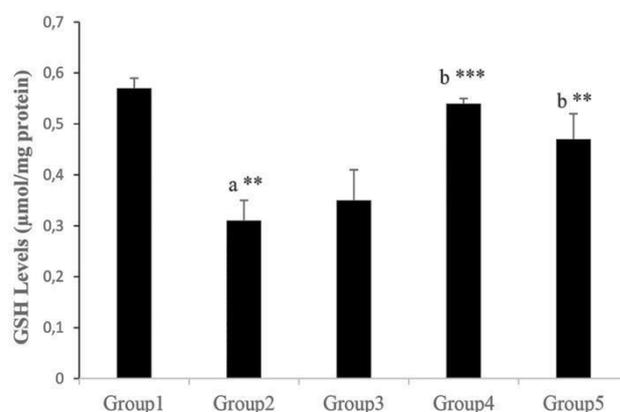
### Statistical analysis

All data were assessed for the normality by using the Kolmogorov-Smirnov and Shapiro-Wilk tests. One-way ANOVA was performed to determine the difference between the experimental groups and P <0.05 was considered significant. For multiple comparisons, the Tukey test was used when the variances were equal,

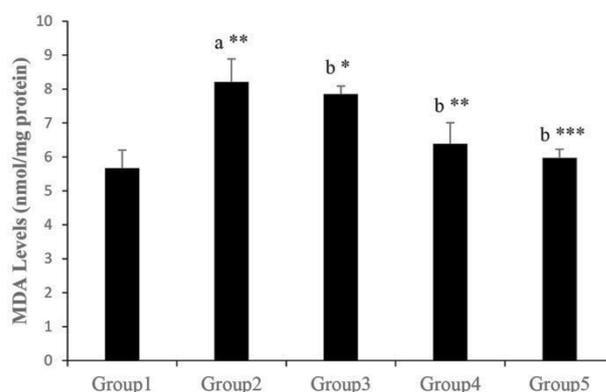
whereas the Tamhane's T2 test was used for analysis when the variances were not equal. Statistical analysis was performed by SPSS Version 21.

### Results

NaF exposure was found to cause a statistically significant decrease in GSH levels on synaptosomes compared to the control group (0.57±0.12) (Figure 1). All betaine doses provided protective effect against NaF toxicity and increased GSH levels. 0.5 mM betaine concentration among all doses resulted in the most improvement in GSH levels (0.54±0.11) against 80 mg/L NaF toxicity group (0.31±0.34) (P <0.001). GSH levels improved a statistically significant improvement when 0.5 and 1 mM betaine groups were compared with the 80 mg/L NaF group (P <0.001 and P <0.01, respectively). However, the 0.25 mM betaine concentration did not provide a statistically significant difference in GSH levels (0.35±0.16) compared with the 80 mg/L NaF group (P >0.05).



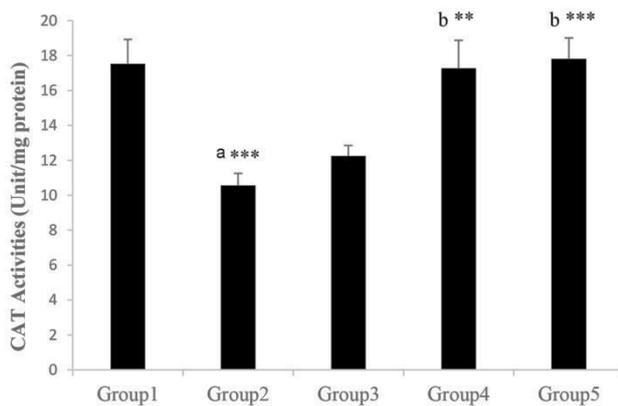
**Figure 1.** The protective effects of betaine on GSH levels against NaF-induced toxicity on rat brain synaptosomes. a: Comparison with the control group. b: Comparison with the 80 mg/L NaF group. \*P <0.05, \*\*P <0.01, \*\*\*P <0.001. All data are described as mean ± SEM.



**Figure 2.** The protective effects of betaine on MDA levels against NaF-induced toxicity on rat brain synaptosomes. a: Comparison with the control group. b: Comparison with the 80 mg/L NaF group. \*P <0.05, \*\*P <0.01, \*\*\*P <0.001. All data are described as mean ± SEM.

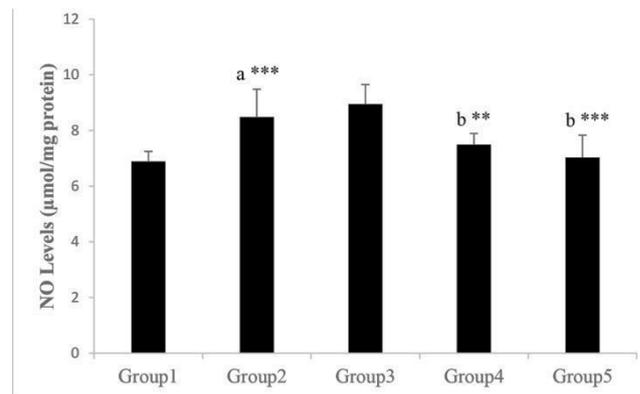
As shown in Figure 2, NaF exposure caused an increase in lipid peroxidation on synaptosomes, thus increasing MDA levels. MDA levels of 80 mg/L NaF group ( $8.21 \pm 2.68$ ) were significantly higher than control group ( $5.76 \pm 1.53$ ). All treatment doses of betaine has provided an amelioration by reducing effect at MDA levels and at 1 mM betaine concentration treatment group ( $5.97 \pm 1.26$ ) was almost obtained similar results to the control group ( $P < 0.001$ ). In addition, we can say that the increase in MDA levels against fluoride toxicity showed a dose-dependent decrease with betaine treatment.

NaF exposure showed a significant decrease in synaptosomal CAT activities compared to the control group ( $17.53 \pm 2.44$ ) ( $P < 0.001$ ). Reduced CAT activities were normalized by betaine treatment. The closest improvement to the control group was achieved at 1 mM betaine concentration treatment group ( $17.81 \pm 2.35$ ) ( $P < 0.001$ ). In addition, a statistically significant increase in CAT activities was found in 0.25 and 0.5 mM betaine concentrations treatment groups ( $12.25 \pm 1.61$  and  $17.28 \pm 1.89$ ;  $P < 0.05$  and  $P < 0.01$ , respectively). According to our results, decreased CAT activities owing to NaF toxicity showed a dose-dependent increase with betaine treatment (Figure 3).



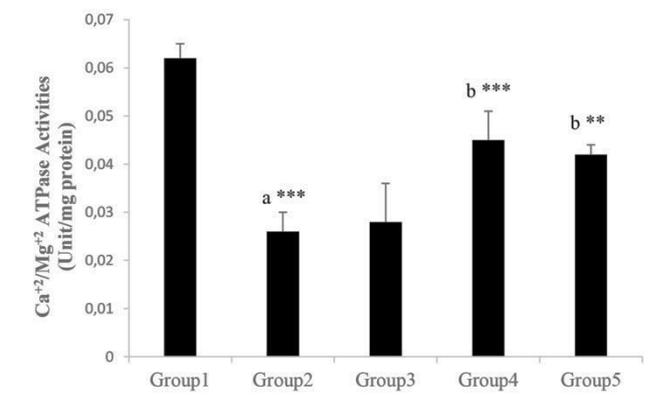
**Figure 3.** The protective effects of betaine on CAT activities against NaF-induced toxicity on rat brain synaptosomes. a: Comparison with the control group. b: Comparison with the 80 mg/L NaF group. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . All data are described as mean  $\pm$  SEM.

The 0.5 and 1 mM betaine groups resulted in a statistically significant reduction in NO levels ( $7.49 \pm 1.43$  and  $7.03 \pm 1.43$ , respectively;  $P < 0.05$ ) when compared with the 80 mg/L NaF group ( $8.48 \pm 2.77$ ). On the other hand, 0.25 mM betaine group ( $8.95 \pm 1.71$ ) did not cause a statistically significant decrease in NO levels ( $P > 0.05$ ). As shown in Figure 4, the highest decrease/improvement in NO levels after NaF exposure was obtained at 1 mM betaine concentration ( $P < 0.001$ ). 0.25 mM betaine concentration did not suppress the increase in NO levels caused by NaF. The 1 mM betaine concentration provided the closest ameliorating to the control group as compared to the 80 mg/L NaF group.



**Figure 4.** The protective effects of betaine on NO levels against NaF-induced toxicity on rat brain synaptosomes. a: Comparison with the control group. b: Comparison with the 80 mg/L NaF group. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . All data are described as mean  $\pm$  SEM.

NaF exposure caused a decrease in  $\text{Ca}^{2+}/\text{Mg}^{2+}$  ATPase activities compared to the control group ( $0.062 \pm 0.015$ ) (Figure 5).



**Figure 5.** The protective effects of betaine on  $\text{Ca}^{2+}/\text{Mg}^{2+}$  ATPase activities against NaF-induced toxicity on rat brain synaptosomes. a: Comparison with the control group. b: Comparison with the 80 mg/L NaF group. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . All data are described as mean  $\pm$  SEM.

The 0.5 and 1 mM betaine groups ( $0.045 \pm 0.007$  and  $0.052 \pm 0.013$ , respectively) showed a statistically significant improvement as compared to the 80 mg/L NaF group, but 0.25 mM betaine group ( $0.038 \pm 0.008$ ) did not provide a significant increase in  $\text{Ca}^{2+}/\text{Mg}^{2+}$  ATPase activity caused by the NaF. In this study, the best amelioration in  $\text{Ca}^{2+}/\text{Mg}^{2+}$  ATPase activities was found at 0.5 mM betaine treatment group.

## Discussion

In this study, neuroprotective effects of betaine against NaF toxicity on synaptosomes were investigated. The effects of NaF exposure on antioxidant/oxidant parameters and Ca<sup>2+</sup>/Mg<sup>2+</sup> ATPase activities were investigated and found to cause cellular damage by triggering oxidative stress. It has been determined that the antioxidant capacity also reduce due to increased reactive oxygen and nitrogen derivatives. Additionally, we determined that Ca<sup>2+</sup>/Mg<sup>2+</sup> ATPases, which play an important role in intracellular and extracellular signaling, were inhibited by NaF-induced oxidative stress. Given that the brain consumes oxygen much more than other organs and contains too much unsaturated fatty acids in its structure, it is extremely defenseless to oxidative stress. Therefore, we examined the neuroprotective effects of betaine, an important antioxidant and methyl donor, against NaF toxicity.

GSH, a component of the antioxidant mechanism, plays an essential role in protecting cellular integrity against peroxidative damage resulting from reactive oxygen species (26). Studies with Sprague-Dawley rats have been reported that betaine contributed to antioxidant mechanism by providing upregulation of GSH against increased oxidative stress in brain (27). In our result parallel with the literature, the protective effect of betaine contributes to the methylation pathways by increasing the formation of S-adenosylmethionine and provides the glycine requirement, which is the essential component for GSH synthesis (18).

The central nervous system has greatly polyunsaturated fatty acids, which are the main target of free oxygen radicals. In our previous study, we found that ethanol exposure increased MDA levels in synaptosomes by increasing oxidative stress and that betaine administered at 0.5% dose significantly reduced MDA levels (28). Our results of betaine treatment against NaF exposure are consistent with the literature.

H<sub>2</sub>O<sub>2</sub> derived from the superoxide anion is catalyzed to the water by CAT, so that the cell membranes are protected against oxidative damage (26). Since the F chemical structure is highly electronegative, in vitro and in vivo studies have been shown to cause oxidative stress-induced cellular damage by up-regulation of reactive oxygen species (12). It has been reported that F added to the drinking water of rats caused a decrease in GSH levels and CAT activities in brain tissue (13). Decreased CAT activity due to increased oxidative stress resulted in a significant improvement with betaine treatment (27). In other words, betaine helps protect cellular integrity by supporting antioxidant defense system.

NO is an important biological initiator of functional and metabolic processes for almost any organ. This gas modulates endothelial function, neurotransmission, immunity and cell death by activating intracellular cyclic guanosine monophosphate (cGMP) levels and guanylate cyclase, which increases cGMP-dependent protein kinase (29). Elevation of NO and cGMP has been associated with many pathophysiological processes. Consistent with the literature, we have found that NaF exposure significantly

increased NO levels compared to the control group (30). NO is formed by the activation of N-methyl-D aspartate (NMDA) receptors in neuronal cell membranes and causes biochemical effects by altering cGMP and intracellular calcium concentrations. It has been reported that NMDA receptors was stimulated by F (31), and based on this result, it is thought that NO synthesis may increase. Our data indicated that betaine treatment resulted in the inhibitory effect on NOS leading to a decrease in NO levels.

F binds to the proteins of ion channels in cell membranes and inhibits them, causing the deterioration of membrane potential. Calcium, which plays an important role in intracellular and extracellular signaling, triggers apoptosis either directly or indirectly by impairment of cellular calcium homeostasis with F exposure (32). Consistent with our previous study, Ca<sup>2+</sup>/Mg<sup>2+</sup> ATPase activities in erythrocyte membranes were reduced after ethanol exposure, and then betaine treatment was improved in Ca<sup>2+</sup>/Mg<sup>2+</sup> ATPase activities (33).

## Conclusion

F exposure has been shown to cause oxidative stress-induced neurodegeneration. In addition, experimental studies about fluorosis, which is the result of excessive F exposure, is increasing day by day. In this study, we found that betaine has neuroprotective effects against cellular damage caused by F. Betaine is taking significant steps towards becoming a new therapeutic agent, especially by giving positive results on neurodegenerative diseases. Nevertheless, there is a need to further study the action mechanisms of betaine in molecular and biochemical processes.

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**Ethical issues:** All Authors declare, Originality and ethical approval of research. Responsibilities of research, responsibilities against local ethics commission are under the Authors responsibilities. The study was conducted under defined rules by the Local Ethics Commission guidelines and audits.

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