The influence of melatonin supplementation against aluminum-induced toxicity in brains of male rats

Ömür KARABULUT BULAN 1, Bertan Boran BAYRAK 2*, Güner SARİKAYA-ÜNAL 1, Refiye YANARDAĞ 2

1 Department of Biology, Faculty of Science, İstanbul University-Cerrahpaşa, İstanbul, Turkey
2 Department of Chemistry, Faculty of Engineering, İstanbul University, Avvclar 34320 İstanbul, Turkey

* Corresponding Author. E-mail: bertanb@istanbul.edu.tr (B.B.B.); Tel. +90-212-473 70 70/17672.

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ABSTRACT: Aluminum (Al), an ubiquitous element in nature, enters the body primarily through gastrointestinal tract, respiratory system and skin. Being a powerful neurotoxin for human brain, Al was reported to be involved in the etiology of Alzheimer's disease due to its easy access and accumulation in the central nervous system. Melatonin (Mel) is a tryptophan-derived neurohormone in animals and plants, and produced in the pineal gland of all mammalian species. The present study examines the effects of Mel on Al-induced oxidative stress, inflammation, tissue factor production and brain damage in rat brain. Wistar albino rats were divided into four groups. Group I: control animals; Group II: rats injected with 10 mg/kg Mel; Group III: rats injected with 5 mg/kg Al₂(SO₄)₃ and Group IV: rats injected combination of Al and Mel (5 mg/kg Al₂(SO₄)₃ and 10 mg/kg Mel). Animals were injected three times a week for one month. At the end of the month, rats were sacrificed, their brains were removed. It was found that lipid peroxidation, protein carbonyl, advanced oxidation protein products, hydroxyproline levels, tissue factor, catalase, superoxide dismutase, glutathione reductase, myeloperoxidase, acetylcholine esterase, alkaline phosphatase, acid phosphatase and glucose-6-phosphate dehydrogenase activities were increased, while paraoxonase, arylesterase, sodium potassium ATPase activities and glutathione levels were decreased in the Al-treated group. Mel treatment reversed these changes by demonstrating significant antioxidant effects. Results indicated that Mel has potential therapeutic value against Al-induced oxidative stress in the rat brain tissue and these effects may be related to its antioxidant activities.

KEYWORDS: Aluminum; melatonin; brain; oxidative stress; tissue factor levels.

1. INTRODUCTION

Metals are essential for biological organisms. In brain, metal ion homeostasis is necessary for normal brain functions and its arrangement has been considered as one of the key factors in the progression of neurodegeneration [1]. Brain tissue is susceptible to oxidative stress, thus, indefensible to damages induced by various chemicals and metals.

Aluminum (Al) is a highly reactive element. Al can mainly be found in kitchenware such as foils, in medicine and pharmaceuticals such as antacids, in cosmetic materials such as deodorants, in foods and food additives such as tea, herbs, cheese, corn and spices [2]. Furthermore, this metal is widely used in homes, hospitals, schools, commercial and office buildings, packaging, construction, containers, electrical conductors and other equipment. Al and its salts are widely used for the treatment and purification of drinking water [3]. This element has been associated with various diseases [4] and implicated in acceleration of brain aging. Al has a fixed oxidation number and a strong pro-oxidant activity despite its non-redox status [5]. Researchers searched for a link between oxidative stress and Al accumulation in different tissues and organs such as bone, spleen, kidney and brain [6-8]. Brain is the most susceptible organ for Al absorption and toxicity [9]. Al passes through the blood-brain barrier and is stored in the brain’s cortex, cingulate bundles, corpus callosum, hippocampus and cerebellum [10,11] that subsequently affecting the physiology and function of all regions of the brain [12].


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275
Melatonin (Mel: N-acetyl-5-methoxytryptamine) is an indolamine, mainly synthesized by the pineal gland and many other organs during the night. Mel directly detoxifies various reactive oxygen (ROS) and nitrogen species (RNS) [13]. Thus, it plays an important role in scavenging free radicals and stimulates antioxidant system enzymes [14,15]. Several studies reported that Mel protects DNA, lipids, and proteins against the harmful effects of free radicals [16]. We aimed to investigate the effects of Mel in protection from Al-induced neural damage.

2. RESULTS

In Al-treated rats, reduced glutathione (GSH) levels were significantly less than the control group ($p < 0.05$). Administration of Mel to the Al in combination group resulted in an insignificant increase in GSH. Lipid peroxidation (LPO) levels in the brain tissues insignificantly increased in Al group. Protein carbonyl (PC), advanced oxidized protein products (AOPP) levels and tissue factor (TF) activity were remarkably raised in the Al group ($p < 0.05$; $p < 0.0001$, respectively). LPO, PC, AOPP levels and TF activity were reversed by the administration Mel ($p < 0.05$; $p < 0.0001$, respectively) (Table 1).

<table>
<thead>
<tr>
<th>Group</th>
<th>GSH (nmol GSH/mg prot)$^a$</th>
<th>LPO (nmol MDA/mg prot)$^a$</th>
<th>PC (nmol/mg prot)$^a$</th>
<th>AOPP (pmol/mg prot)$^a$</th>
<th>TF (sec)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28.91 ± 10.11</td>
<td>2.44 ± 0.97</td>
<td>3.16 ± 0.73</td>
<td>1.97 ± 0.59</td>
<td>100.75 ± 10.97</td>
</tr>
<tr>
<td>Control + Melatonin</td>
<td>26.17 ± 5.59</td>
<td>1.30 ± 0.43</td>
<td>2.46 ± 1.13</td>
<td>2.14 ± 0.70</td>
<td>119.17 ± 7.05</td>
</tr>
<tr>
<td>Aluminum</td>
<td>10.42 ± 4.71$^b$</td>
<td>3.52 ± 1.71</td>
<td>8.42 ± 4.56$^b$</td>
<td>11.59 ± 3.21$^b$</td>
<td>67.44 ± 7.97$^d$</td>
</tr>
<tr>
<td>Aluminum + Melatonin</td>
<td>13.88 ± 0.20</td>
<td>0.93 ± 0.61$^c$</td>
<td>3.98 ± 1.54$^c$</td>
<td>2.08 ± 0.62$^c$</td>
<td>126.00 ± 5.59$^e$</td>
</tr>
</tbody>
</table>

$^a$Mean ± SD.

$^b$p < 0.05 vs control group

$^c$p < 0.001 vs control group

$^d$p < 0.0001 vs control group

$^e$p < 0.0001 vs aluminum group

There was a significant increase in catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), and myeloperoxidase (MPO) activities of the brain in Al group ($p < 0.0001$; $p < 0.005$, respectively) (Table 2). Mel caused a remarkable decrease in brain’s CAT, SOD, GPx, GR and MPO activities in Al group ($p < 0.005$; $p < 0.0001$; $p < 0.05$, respectively) (Table 2).

<table>
<thead>
<tr>
<th>Group</th>
<th>CAT (U/mg prot)$^a$</th>
<th>SOD (U/g prot)$^a$</th>
<th>GPx (U/g prot)$^a$</th>
<th>GR (U/g prot)$^a$</th>
<th>MPO (mU/g tissue)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.94 ± 0.64</td>
<td>6.50 ± 1.54</td>
<td>41.93 ± 6.44</td>
<td>29.24 ± 5.50</td>
<td>5.59 ± 0.99</td>
</tr>
<tr>
<td>Control + Melatonin</td>
<td>4.67 ± 0.87</td>
<td>7.94 ± 2.80</td>
<td>61.30 ± 7.38</td>
<td>19.83 ± 12.57</td>
<td>5.49 ± 0.81</td>
</tr>
<tr>
<td>Aluminum</td>
<td>11.06 ± 2.14$^b$</td>
<td>17.86 ± 4.69$^b$</td>
<td>113.91 ± 24.11$^b$</td>
<td>37.57 ± 2.08$^e$</td>
<td>16.91 ± 3.58$^e$</td>
</tr>
<tr>
<td>Aluminum + Melatonin</td>
<td>6.31 ± 1.13$^c$</td>
<td>9.39 ± 1.83$^c$</td>
<td>61.30 ± 7.38$^d$</td>
<td>19.45 ± 6.14$^d$</td>
<td>5.28 ± 0.81$^c$</td>
</tr>
</tbody>
</table>

$^a$Mean ± SD.

$^b$p < 0.0001 vs control group

$^c$p < 0.005 vs aluminum group

$^d$p < 0.0001 vs aluminum group

$^e$p < 0.005 vs control group

$^f$p < 0.05 vs aluminum group
Xanthine oxidase (XO) and acetylcholine esterase (AChE) activities in the brain were significantly increased in the Al group \((p < 0.005, p < 0.001, \text{respectively})\). Mel supplementation to Al group reversed this effect in XO and AChE activities \((p < 0.005; p < 0.001, \text{respectively})\). On the other hand, paraoxonase (PON), aryl esterase (ARE) and sodium potassium ATPase \((\text{Na}^+/(\text{K}^+)-\text{ATPase})\) activities in Al group were found to be notably decreased in comparison to those in the control rats \((p < 0.005; p < 0.05; p < 0.001, \text{respectively})\). However, Mel administration to the Al group caused a significant increase in the brain’s PON, ARE and \(\text{Na}^+/(\text{K}^+)-\text{ATPase}\) activities \((p < 0.05; p < 0.0001, \text{respectively})\) (Table 3).

### Table 3. Brain tissue XO, PON, ARE, AChE and \(\text{Na}^+/(\text{K}^+)\)-ATPase activities for all groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>XO (U/g prot)</th>
<th>PON (U/g prot)</th>
<th>ARE (U/g prot)</th>
<th>AChE (U/mg prot)</th>
<th>Na(^+/(\text{K}^+))-ATPase (nmol P/mg prot/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.22 ± 0.28</td>
<td>11.02 ± 3.76</td>
<td>196.85 ± 29.90</td>
<td>0.83 ± 0.07</td>
<td>3.42 ± 1.32</td>
</tr>
<tr>
<td>Control + Melatonin</td>
<td>0.92 ± 0.43</td>
<td>6.98 ± 2.58</td>
<td>165.50 ± 47.12</td>
<td>0.83 ± 0.10</td>
<td>6.46 ± 2.87</td>
</tr>
<tr>
<td>Aluminum</td>
<td>3.18 ± 0.94(^a)</td>
<td>4.71 ± 0.95(^b)</td>
<td>123.20 ± 30.01(^c)</td>
<td>1.41 ± 0.23(^d)</td>
<td>1.21 ± 0.55(^e)</td>
</tr>
<tr>
<td>Aluminum + Melatonin</td>
<td>1.07 ± 0.19(^c)</td>
<td>6.44 ± 0.85(^d)</td>
<td>165.87 ± 7.18(^d)</td>
<td>0.88 ± 0.11(^e)</td>
<td>3.95 ± 0.85(^b)</td>
</tr>
</tbody>
</table>

\(^a\)Mean ± SD.  
\(^b\)\(p < 0.005\) vs control group  
\(^c\)\(p < 0.001\) vs control group  
\(^d\)\(p < 0.05\) vs control group  
\(^e\)\(p < 0.001\) vs control group  
\(^f\)\(p < 0.0001\) vs control group  
\(^g\)\(p < 0.0001\) vs Al group

There was a significant increase in alkaline phosphatase (ALP) and acid phosphatase (ACP) and glucose-6-phosphate dehydrogenase (G6PD) activities in the brain tissues in Al group \((p < 0.0001; p < 0.05, \text{respectively})\) (Table 4). However, Mel treatment reversed this effect, resulting in notable decreases in ALP, ACP and G6PD activities \((p < 0.0001; p < 0.05; p < 0.001, \text{respectively})\). Hydroxylproline (OH-proline) contents were significantly higher in Al group than those in the control group \((p < 0.0001)\). Nevertheless, Mel was capable of reversing this change in Al group \((p < 0.0001)\) (Table 4).

### Table 4. Brain tissue ALP, ACP and G6PD activities and OH-proline levels for all groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>ALP (U/g prot)</th>
<th>ACP (U/g prot)</th>
<th>G6PD (U/g prot)</th>
<th>OH-proline (µg/mg prot)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.06 ± 0.87</td>
<td>14.80 ± 2.75</td>
<td>20.58 ± 4.99</td>
<td>52.81 ± 11.96</td>
</tr>
<tr>
<td>Control + Melatonin</td>
<td>7.53 ± 1.12</td>
<td>15.02 ± 1.99</td>
<td>23.14 ± 6.41</td>
<td>41.05 ± 6.66</td>
</tr>
<tr>
<td>Aluminum</td>
<td>15.92 ± 1.18(^b)</td>
<td>23.29 ± 6.10(^d)</td>
<td>43.76 ± 10.04(^b)</td>
<td>82.46 ± 6.35(^b)</td>
</tr>
<tr>
<td>Aluminum + Melatonin</td>
<td>7.10 ± 1.53(^c)</td>
<td>17.88 ± 2.37(^c)</td>
<td>23.27 ± 8.13(^f)</td>
<td>57.30 ± 5.49(^c)</td>
</tr>
</tbody>
</table>

\(^a\)Mean ± SD.  
\(^b\)\(p < 0.0001\) vs control group  
\(^c\)\(p < 0.001\) vs control group  
\(^d\)\(p < 0.05\) vs control group  
\(^e\)\(p < 0.001\) vs control group  
\(^f\)\(p < 0.0001\) vs control group  
\(^g\)\(p < 0.0001\) vs Al group
3. DISCUSSION

Metals are needed for majority of enzymatic activities, mitochondrial function and myelination as well as neurotransmission. Toxic metals pose a global health risk because of their potential to contribute to a variety of diseases. Al is considered toxic to the central nervous, skeletal and hematopoietic systems. Al can cross the blood-brain barrier and cannot be eliminated from the brain. Brain, which is highly susceptible to oxidative stress, is vulnerable to damages induced by several chemical products, including metals [17]. High levels of ROS cause injuries by means of oxidative modifications of proteins, lipids and nucleic acid and neurons [18]. ROS was previously shown to cause apoptosis, inflammation and cell proliferation in various tissues.

Depletion of GSH, an important antioxidant in the brain, has been implicated in several neurodegenerative disorders [19]. In this study, GSH levels in rat brain were decreased under metal-induced stress. Decrease in GSH levels could be due to an increase in oxidative stress and exhaustion of GSH stores. However, Mel reversed GSH levels by exhibiting antioxidant effects in Al combination group.

Neuronal tissue MDA levels were assayed as an indicator of membrane oxidative damage. MDA levels increased in a way that suggests free radical attack on membrane lipids. Al caused notable oxidative damage by increasing the redox-active iron concentration in the brain [20]. In this study, the significant increase in the LPO levels in brain suggests that free radical-induced oxidative cell damage could mediate the toxicity of Al. Mel reduced the LPO levels in the brain. The oxidative damage in the brain was repaired in Al combination group following the administration of Mel.

Al administrations also caused a notable increase in PC and AOPP levels which are the markers of protein oxidation, and thus, an indicator of oxidative stress [21]. Oxidative modification of proteins leads to reduced specific protein function [22]. Oxidative modification of proteins may affect a variety of cellular functional proteins such as receptors, transport systems, enzymes as well as the signal transduction [23]. In the current study, PC and AOPP levels were increased in the Al group. These findings were consistent with results from other studies [24,25]. Application of Mel to the Al combination group reversed PC and AOPP levels. These results indicated that Mel could be effective in preventing oxidative protein damage.

Some organs, such as brain, lungs, heart, testes and skin and body fluids exhibit high TF activity [26,27]. As the clotting time is inversely proportional to the TF activity, the lengthening of clotting time is a manifestation of decreased TF activity [28]. Increased TF activity in many tissues is related to extensive cellular damage. In the current study, TF activity was significantly increased in rats administered Al. Increased TF activity showed that Al treatment could play a role in development of inflammation due to oxidative injury. Mel reversed this activity exhibiting notable antioxidant activity in the Al group. Decreased TF activity of the brain may have importance in terms of reducing the risk of thrombosis. It can be postulated that this reversal might be correlated to protection of membrane stability by Mel [29].

Increased antioxidant enzyme activity in the brain tissues of Al-treated rats appears to be a reaction to increased ROS generation [30]. These antioxidant enzymes may be alleviating the toxic effects of ROS. In our study, Al treatment caused a significant increase in the CAT, SOD, GPx and GR activities. Increased production of free radicals may subsequently increase the SOD activity resulting in production of higher amounts of H$_2$O$_2$. In organism, CAT activity increases in response to high H$_2$O$_2$ production. CAT detoxifies peroxides that are formed [31]. Similar results were reported by Gómez et al. [32]. Mel has been shown to stabilize mitochondrial functions and thus, will decrease the formation of ROS. Therefore, this study demonstrated the antioxidant properties of Mel in restoring the oxidative balance in the cell. GR catalyzes conversion of oxidized glutathione to its reduced form, by utilizing NADPH. In the present study, following the Al treatment, GR activity increased in the brain tissue. Mel returned GR to normal levels by demonstrating antioxidant effects in the Al group.

Neuroinflammation is an important contributor in the pathogenesis of neurodegenerative diseases [33]. MPO is an enzyme necessary for normal neutrophil function. This is released as a response to various stimulatory substances. Al exacerbates oxidative damage in brain that causes inflammation [34]. Increased MPO activity shows that Al-induced brain injury is a neutrophil-dependent inflammatory condition. Neutrophils play a role in oxidative damage via mechanisms that involve the activation of MPO enzyme systems. This enzyme activity increases due to oxidative stress. It has been shown that the MPO is enhanced in rats given Al by various researchers [35,36]. Our results demonstrated that in Al combination group toxicity, administration of Mel inhibited neutrophil infiltration and balanced oxidant-antioxidant levels in the brain tissue. It may be stated that Mel treatment could be used in individuals who are at risk of Al toxicity.

Increased XO activity may cause further tissue damage due to free radical generation. In this study, brain XO activity increased in the experimental group. Moumen et al. [37] showed that Al increased the XO...
activity in the brain tissue. Here, administration of Mel decreased XO activity, therefore, it can be suggested that Mel reduces oxidative stress. In a previous report regarding the ability of Mel to decrease XO activity in the brain, it was demonstrated that antioxidant properties of Mel are mainly related to the decrease of the pro-oxidant enzyme activities such as XO [38].

PON received significant attention due to its possible relationship with neurodegenerative disease [39,40]. These results proposed that enzyme was inactivated by increased oxidative stress and/or direct inhibition by Al. PON1 is an antioxidant and antiatherogenic enzyme [41]. PON1 protects the nervous system against toxicity. Hernández et al. [42] investigated whether environmental exposure to metals has any influence on PON1 activity or not. In the present study PON and ARE activities were reduced significantly in the brains of rats injected with Al. This might be due to the inactivation of the enzyme by Al. Administration of Mel could reverse PON and ARE activities in Al combination group.

AChE is an important regulatory enzyme. This enzyme is found in the brain, muscles, erythrocytes and cholinergic neurons [43] and this enzyme hydrolyzes the neurotransmitter acetylcholine. AChE activity was shown to increase in Al-treated rat brain [44-46]. This increase may be due to a direct neurotoxic effect exhibited via LPO of the plasma membrane, and such changes in plasma membranes may affect the integrity and functionality of the cholinergic system. Increases in AChE activity caused by Al treatment may lead to a reduction of cholinergic neurotransmission due to a decrease in acetylcholine levels in the synaptic cleft. In our study, AChE activity decreased significantly after treatment with Mel. This decrease might improve cholinergic neurotransmission by restoring acetylcholine levels.

Na⁺/K⁺-ATPase is very susceptible to structural changes in membrane [47]. Al administration increased LPO levels and, in return, LPO products inhibited integrated ion transport proteins. In several studies inhibitory effect of Al on Na⁺/K⁺-ATPase was observed in brain tissue [48,49]. A decrease in Na⁺/K⁺-ATPase activity can indicate membrane injury. In the present study, it was also observed that Al inhibited Na⁺/K⁺-ATPase. Failure of Na⁺/K⁺-ATPase has been implicated in the pathology of neurodegenerative diseases. Yu [50] showed the critical role of Na⁺/K⁺-ATPase in signal transduction and cell death pathways (apoptosis, necrosis and hybrid cell death). Here, Mel treatment significantly increased Na⁺/K⁺-ATPase activities in the brain. Mel might have prevented the inhibition of Na⁺/K⁺-ATPase activity by means of detoxification of ROS.

Increases or decreases in ALP activity are observed in a variety of pathologic processes [51]. ALP activity has been shown to alter inflammatory processes and tissue damage by Oktay et al. [52]. There was a significant increase in ALP activity in the Al group. Mel treatment decreased ALP activity in Al combination group. Increased ALP activity is an indication of inflammation and neutrophil activation caused by Al in the brain. Akinrinade et al. [53] and Sumathi et al. [49] also found increased ALP activity in rat brain after treatment with Al. Similarly, the present study showed that Al treatment led to significant elevation of ACP activity in brain. Increased ACP activity may result in phosphate accumulation within the lysosomes and an increase in catabolic rate. Mel treatment caused marked reduction in the ALP and ACP activities in Al combination group.

G6PD, an enzyme of the pentose phosphate shunt, produces the reducing NADPH for GSH generation [54]. This enzyme activity is increased in the brain of experimental animals treated with Al [55,56]. Increased G6PD activity indicates oxidation of glucose through the hexose monophosphate pathway, thereby facilitating increased production of NADPH for detoxification process. This, in return, leads to increased enzyme activities as a response to elevated brain peroxide metabolism. In the present study, Al significantly increased G6PD activity in the brain. Mel significantly decreased the G6PD activity in the Al combination group. The results suggested that Mel enhances pentose phosphate shunt pathway, generating a larger number of reduced NADP molecules for detoxification.

It was determined that the OH-proline levels in brain were increased in Alzheimer’s disease (AD) patients [57]. In the present study, brain OH-proline levels were increased in Al-treated group. OH-proline levels in the brain change as a consequence of the brain injury caused by Al. Here, Mel decreased the OH-proline levels in brain. It shows that the decrease of OH-proline levels prevented brain damage.

4. CONCLUSION

Al has adverse effects on human health. Its introduction could generate free radicals, resulting in elevation of neural LPO, PC, AOPP, TF and OH-proline levels and changes in enzymatic and nonenzymatic antioxidant levels. According to the results of our study, Mel may have significant protective effects against Al induced oxidative brain damage by inhibiting free radical production, supporting antioxidant redox system. Mel scavenges oxygen based reactants, stabilizes the mitochondrial membrane. Mel administration can be an effective treatment to alleviate the damage that Al produces.
In conclusion, Mel treatment modulates the antioxidant redox system and reduces brain oxidative stress induced by Al. For this reason, Mel may be a useful agent for the treatment neurodegenerative diseases such as AD.

5. MATERIALS AND METHODS

5.1. Animals

All experimental protocols were approved by the Animal Care and Use Committee of Istanbul University (Ethical Committee: 102/27.08.2009). Male Wistar albino rats, weighing 230-250 g were obtained from The Animal Laboratory of Institute of Experimental Medicine. The animals fed with a standard pellet and access to water was allowed ad libitum.

5.2. Experimental design

The animals were separated into four groups. Each group was consisted of 8 rats. Group I: control animals were injected intraperitoneally (i.p.) with physiological saline; group II: animals were injected subcutaneously (s.c.) with 10 mg/kg Mel; group III: animals were injected (i.p.) with 5 mg/kg Al$_2$(SO$_4$)$_3$; Group IV: animals were injected combination of Al and Mel. Animals were injected three times a week for 1 month. Mel was dissolved in a small amount of ethanol and diluted with physiological saline. In the last day of experimental period, rats were sacrificed by ketamine hydrochloride and the brain tissues were removed.

5.3. Biochemical assays

Brain tissue samples were homogenized in 0.9% NaCl solution. The clear supernatant was removed while the pellet was discarded and used for carrying out the total protein, GSH, LPO, PC, OH-proline and AOPP levels, TF activity, enzyme analysis.

Reduced GSH and malondialdehyde (MDA, LPO marker) levels in brain homogenates were determined according to Beutler [58] and Ledwożyw et al. [59], respectively. PC and AOPP levels were assayed according to Levine et al. [60] and Witko-Sarsat et al. [61] methods, respectively. The Quick’s method [28] was used for performing TF activity measurements in the brain tissue homogenates. Protein estimation was conducted by using Lowry method [62].

CAT activity was carried out in the brain tissue by the method described by Aebi [63]. SOD, GPx, GR, MPO, XO, PON, ARE, AChE, Na$^+$/K$^+$-ATPase, ALP and AC and G6PD activities and OH-proline levels were determined by spectrophotometric methods, respectively [64-75].

5.4. Statistical analysis

Biochemical results were evaluated using an unpaired t-test and ANOVA variance analysis using the NCSS statistical computer package. The values were expressed as mean ± SD. The value of $p < 0.05$ was regarded as significant.

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Conflict of interest statement: The authors declared no conflict of interest.

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