Neuroprotective effects of amantadine for experimental acute carbon monoxide poisoning

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Abstract. – OBJECTIVE: Amantadine is known to have a neuroprotective effect in many neurological diseases. This study aims at investigating the neuroprotective effect of amantadine in rats exposed to carbon monoxide (CO) poisoning. MATERIALS AND METHODS: Rats were maintained under standard experimental laboratory conditions and randomized into 4 different groups of 7 each namely control, amantadine only, CO exposure, and amantadine + CO exposure. For immunohistochemical analysis, tissues taken from the prefrontal and hippocampal regions were taken into formalin and kept for at least one day. Afterward, the tissue was followed and blocked for paraffin blocking. N-Methyl D-Aspartate (NMDA) levels in homogenates were studied by the Enzyme-Linked Immunosorbent Assay (ELISA) method. Superoxide dismutase (SOD) and catalase (CAT) activities in the supernatants were studied with commercial kits. Nitric oxide (NO) and Asymmetric Dimethyl Arginine (ADMA) levels were studied by the ELISA method. Enzyme activity values were calculated by dividing the protein values in the supernatants and normalizing them. RESULTS: CAT, SOD, NMDA, ADMA, and NO levels were statistically significantly different between the groups (p < 0.05). According to post-hoc pairwise comparison test results, the values of the control and amantadine groups for CAT, SOD, NMDA, ADMA, and NO parameters were significantly higher than that of CO group. Similarly, values in the control and amantadine groups were considerably higher than values for the amantadine + CO group. NMDA values were significantly lower in group amantadine + CO than in CO group (p: 0.049).

CONCLUSIONS: Apoptosis and endothelial damage after CO poisoning is a complex process, and amantadine administration has a limited contribution in preventing this process.

Key Words: Amantadine, Bcl-2, Carbon monoxide, Intoxication, N-Methyl D-Aspartate, Neuroprotective, Poisoning, Rat.

Introduction

Carbon monoxide (CO) is a colorless, odorless, tasteless, and non-irritant gas that can be easily absorbed from the lungs. It can be produced endogenously, and as a result of incomplete combustion of carbon-based fuels, such as wood, coal, gasoline, propane, and vinyl plastic, and may cause acute or chronic poisoning. The clinical presentation ranges from headache and dizziness to coma and death, and it has a mortality rate of 1-3%. CO diffuses rapidly through the alveolar membrane and preferentially binds to the iron ion in the heme with an affinity 230-300 times that of oxygen and forms carboxyhemoglobin (COHb). It causes a leftward shift in the position of the COHb dissociation curve, decreased oxygen-carrying capacity, and decreased oxygen release to peripheral tissue. CO toxicity is the result of a combination of tissue hypoxia-ischemia due to COHb formation and direct cellular damage by CO. In addition to
heme-containing proteins, CO also binds specifically to cytochrome-c oxidase and myoglobin. The affinity of CO for myoglobin is 60 times greater than that of oxygen and causes cardiac depression, arrhythmia, and potentially hypotension. Consequent to these processes, hypoxia elevates further. Organs with the highest oxygen demand, such as the brain and heart, become more susceptible to injury. The mechanism of brain damage caused by CO exposure is quite complex. Brain hypoxia induced by COHb is the main mechanism of various types of brain damage. Hypoxia produces various neurotoxic reactions, including increased glutamatergic transmission and activation of redox reactions. Acute CO poisoning can cause severe brain damage that can lead to high mortality and delayed neurological syndrome.

Maintaining normal physiology in tissues, damaged, dysfunctional or unnecessary cells are constantly cleared by regular cell death. If this usual cell death (apoptosis) is not triggered in normal circumstances, the results can be disastrous. One of the most important components of the orderly conduct of apoptosis is the B-cell lymphoma 2 (Bcl-2) protein family, which comprises both pro-apoptotic and anti-apoptotic members. The balance of the Bcl-2 protein group plays a key role in determining the balance between life and death. In CO poisoning, anti-apoptotic Bcl-2 proteins are negatively affected and apoptosis may occur.

Immediate-release amantadine (IR) was approved for use in the United States in 1966 as a protective agent against Asian Influenza. Amantadine is a non-competitive antagonist of the glutamatergic receptor type N-Methyl D-Aspartate (NMDA) receptor (Ki 5 10 1M). It may protect against secondary damage caused by the release of glutamate, an excitatory neurotransmitter. Amantadine facilitates presynaptic dopamine release and delays postsynaptic reuptake. This leads to increased neurotransmission in dopamine-dependent nigrostriatal, mesolimbic, and frontostriatal circuits, which is responsible for mediating arousal and cognitive functions and producing positive neurobehavioral effects. NMDA receptor antagonists have been previously studied in the prevention of secondary damage after CO poisoning. Publications recommending the use of amantadine due to CO damage have begun to emerge in the literature. Experimental studies have been carried out with different drugs in CO poisoning. However, studies related to the effect of amantadine, an NMDA antagonist, in CO poisoning are limited in the literature.

Cerebral hypoxia develops as a result of CO poisoning. Oxidative stress increases with the production of free oxygen radicals. Glutamate is the most important excitatory neurotransmitter in the brain and increased extracellular glutamate concentrations tend to cause neuronal degeneration. The NMDA receptor antagonist property of amantadine may prevent this neuronal degeneration. In this study, we hypothesize that amantadine can prevent neuronal damage in CO poisoning. The objective of this study is to investigate the effects of amantadine on superoxide dismutase (SOD), catalase (CAT), nitric oxide (NO), dimethyl Arginine (ADMA), and NMDA in rats with CO poisoning. As a secondary outcome, it is the detection of changes in the Bcl-2 immunopositive cell values of rats due to exposure to amantadine and CO.

**Materials and Methods**

The study was approved by Ankara Training and Research Hospital Animal Experiments Local Ethics Committee (ID: 0051, Date: 15.02.2019). Twenty-eight female Sprague Dawley rats with an average weight of 250 g obtained from the experimental animal’s laboratory of Ankara Hospital were used for the study. The CO gas used in the experimental protocol was prepared by Habaş Industrial and Medical Gases Production Industries Inc. (Izmit). CO gas was prepared at a concentration of 3000 ppm.

**Formation of Experimental Groups**

The rats were exposed to standard experimental animals’ laboratory conditions and a 12-hour fasting period. They were randomized by lot and grouped according to the following procedures:

*Group 1: Control group (n=7)*

This group was placed in an experimental cage and inhaled room air for 30 minutes. Following the expiration of this period, euthanasia was performed, and blood and tissue samples were taken.

*Group 2: Amantadine group (n=7)*

Intraperitoneal amantadine (45 mg/kg) was administered to this group of rats placed in an experimental cage and inhaled room air for 30 minutes. Euthanasia was later performed, and blood and tissue samples were taken.
Group 3: CO exposure group (n=7)
CO poisoning was created by breathing a mixture of CO gas at a concentration of 3000 ppm for 30 minutes at 3 L/min. Euthanasia was later performed and blood and tissue samples were taken.

Group 4: Amantadine group + CO exposure (n=7)
Following the administration of CO poisoning, this group received additional intraperitoneal amantadine (45 mg/kg). Thirty minutes later, euthanasia was performed, and blood and tissue samples were taken.

Immunohistochemical Analysis
Tissues taken from the prefrontal and hippocampal regions for immunohistochemical analysis were kept in formalin for at least 24 hours and blocked using paraffin. Sections of 5 µm thickness were taken from the blocks with a microtome and then placed on the slide. These sections were incubated overnight in an oven at 54°C and later freed from paraffin by passing them through xylol for 20 minutes twice. Afterward, the sections were rehydrated by passing through a series of decreasing concentrations of alcohols (100%, 90%, 80%, 70%) for 5 minutes each. The sections were washed twice with phosphate-buffered saline (PBS) and exposed to microwave irradiation in 250 mL of freshly prepared citric acid (pH 6.0) buffer to reveal antigenic epitopes, and later cooled at room temperature for 20 minutes. They were washed with PBS at room temperature three times for five minutes. Afterward, the sections were kept in a 3% H₂O₂ solution at room temperature for 20 minutes to saturate the endogenous peroxidase activity in the tissues. The sections were kept on hold with blocking solution for 7 minutes at room temperature to prevent (block) non-specific binding. The blocking solution was removed, and nuclear factor-kappa B (NF-kB) and caspase-3 primary antibodies were added to the samples and incubated overnight at 4°C in a humid environment. Appropriate isotype control antibodies or serum-containing them were used as a negative control, at the same concentration as the primary antibodies. The sections were then incubated with biotin-labeled secondary antibodies for 45 minutes at room temperature.

After the secondary antibody step, the streptavidin-peroxidase complex was added to the tissue samples and incubated at room temperature for 30 minutes. Following the washing steps with PBS, antigen-antibody complexes were made visible by dripping diaminobenzidine (DAB) solution. After washing it in water, it was stained with Mayer’s hematoxylin. The samples were then dehydrated by passing through increasing series of alcohols, cleared by passing through xylol, and covered with a closure solution. The resulting chromogenic reaction was then examined under a light microscope.

Collection and Preparation of Blood and Tissue Samples
Rats were sacrificed and blood samples were taken into a biochemistry tube by an intracardiac puncture. Biochemistry blood was centrifuged at 4,000 rpm for 10 minutes and their serums were separated and portioned. Brain tissues were washed with physiological saline after removing them, and right hemispheres were weighed, frozen in liquid nitrogen, and stored at -80°C. At runtime, all tissue samples were homogenized with a blade homogenizer in 1/6 phosphate buffer (pH; 7.4), and then, centrifuged at 20,000 x g to separate the supernatants.

Biochemical Parameters
Total protein levels in all homogenates and supernatants were studied spectrophotometrically using the Bradford method. NMDA levels in homogenates were studied by the ELISA method. SOD and CAT activities in supernatants were studied with commercial kits. NO, and ADMA levels were studied by the ELISA method. Enzyme activity values were calculated by dividing the protein values in the supernatants and normalizing them.

Preparation of Hippocampal Samples for Immunohistochemical Examination
After deep general anesthesia, the rat hippocampal tissue was rapidly collected and fixed with 4% paraformaldehyde. The hippocampal tissues were removed and post-fixed overnight at 4°C. The tissues were dehydrated through an ascending series of alcohol and embedded in paraffin through xylene. Sections from hippocampal tissues were prepared from paraffin blocks of 5-micrometer thickness. Hippocampal tissues were dewaxed, rehydrated, and rinsed in PBS pH 7.4 (3x5 min). For antigen retrieval, the slides were heated (7+5+5 min) in a microwave (750 Watt) by using a Citrate buffer (pH 6.0) solution. Endogenous peroxidase was blocked by soaking the sections in 3% v/v hydrogen peroxide/methanol for
20 min at room temperature followed by washing them with distilled water. They were thereafter rinsed in PBS pH 7.4 (3 × 3 min). Non-specific protein binding was minimized by covering the slides with a serum-free protein blocking reagent (Invitrogen, Carlsbad, CA, USA) for 10 min at room temperature. Sections were then incubated with the primary antibody (Mouse monoclonal anti-Bcl-2, Dako) overnight at 4°C. The slides were subsequently rinsed in PBS pH 7.4 (3 × 3 min) followed by incubation with biotinylated IgG (Invitrogen) for 20 min at room temperature. Visualization of the bound antibodies was carried out with DAB (Invitrogen) for 8-11 min at room temperature. All incubations were performed in a humidified chamber. Sections were counterstained in Harris hematoxylin, dehydrated, and mounted with DPX.

**Data Quantification**

Immunopositive cells were counted based on the method described in the previous report. For immunostaining, hippocampal sections were observed under a light microscope of 200 × magnification and used for quantification of the number of immunopositive cells of 1000 cells. The preparations were examined under the Nikon Eclipse Ni light microscope with Nikon DS-R12 digital camera using NIS-Elements 4.50 software.

**Statistical Analysis**

Statistical analysis was conducted with Statistical Package for the Social Sciences (Version 22.0, SPSS Inc., IBM, Armonk, NY, USA). Descriptive statistics were presented as mean ± standard deviation values. Distributions of the groups were evaluated with Shapiro-Wilk tests. The significance of the difference between more than two groups was evaluated using analysis of variance (ANOVA) test. Post-hoc tests with Bonferroni correction or Games-Howell were used to determine which groups differed with pairwise comparison. A value of $p < 0.05$ was considered statistically significant.

**Results**

According to the ANOVA test results; CAT, SOD, NMDA, ADMA, and NO values were statistically significantly different between the experimental groups ($p < 0.05$). According to post-hoc pairwise comparison test results, group control and group amantadine values in CAT, SOD, MDA, ADMA, and NO parameters were significantly higher than group CO values. Group control and group Amantadine values were significantly higher than group Amantadine + CO values. In addition, group Amantadine + CO values for NMDA parameters were significantly lower than group CO values ($p: 0.049$) (Table I) (Figure 1).

### Table I. Comparison of laboratory parameters according to rat groups with ANOVA.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Amantadine</th>
<th>CO</th>
<th>Amantadine + CO</th>
<th>$p$</th>
<th>Post-hoc $p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT</td>
<td>4.29±0.88</td>
<td>3.91±1.08</td>
<td>1.20±0.52</td>
<td>1.72±0.74</td>
<td>$&lt;0.001^*$</td>
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<td></td>
<td>1-4: $&lt;0.001^*$</td>
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<td>2-3: $&lt;0.001^*$</td>
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<td></td>
<td>2-4: $&lt;0.001^*$</td>
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<tr>
<td>SOD</td>
<td>1.14±0.17</td>
<td>1.26±0.31</td>
<td>0.38±0.14</td>
<td>0.44±0.21</td>
<td>$&lt;0.001^*$</td>
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<td>1-4: $&lt;0.001^*$</td>
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<td>2-3: $&lt;0.001^*$</td>
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<td>2-4: $&lt;0.001^*$</td>
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<tr>
<td>NMDA</td>
<td>0.98±0.35</td>
<td>1.31±0.24</td>
<td>3.87±0.80</td>
<td>3.09±0.36</td>
<td>$&lt;0.001^*$</td>
<td>1-3: $&lt;0.001^*$</td>
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<td>1-4: $&lt;0.001^*$</td>
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<td>2-3: $&lt;0.001^*$</td>
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<td>2-4: $&lt;0.001^*$</td>
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<tr>
<td>ADMA</td>
<td>66.3±19.8</td>
<td>62.4±12.8</td>
<td>452.3±101.3</td>
<td>428.9±115.0</td>
<td>$&lt;0.001^*$</td>
<td>1-3: 0.0011</td>
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<td>2-4: 0.0011</td>
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<td>NO</td>
<td>46.3±11.4</td>
<td>42.4±16.0</td>
<td>18.9±7.9</td>
<td>20.2±13.1</td>
<td>$0.001^*$</td>
<td>1-3: 0.0007</td>
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<td>2-4: 0.021</td>
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</table>

According to the ANOVA test results, Bcl-2 Immunopositive cells values were statistically different between the experimental groups ($p < 0.001$). According to post-hoc pairwise comparison test results, group control and group Amantadine Bcl-2 Immunopositive cells values were determined significantly higher than group CO ($p < 0.001$, $p: 0.006$) and group Amantadine + CO ($p < 0.001$, $p: 0.007$). There was no significant difference between the other groups ($p > 0.05$) (Table II) (Figure 2).

In the immunostaining performed with anti-Bcl-2 antibody in the hippocampus, widespread expression (arrows) is observed in the control group (Figure 3a). While the presence of immunopositive cells decreased in the CO group (Figure 3c), the presence of immunopositive cells in the Amantadine + CO group (Figure 3d) increased, albeit partially, compared to the CO group. It is seen that immune expression in the group given Amantadine (Figure 3b) was similar to the control group (bar scale 100 µm).

**Discussion**

In this study, in which the results of CO and amantadine application were evaluated, CAT and SOD, which play an important role in the elimination of free oxygen radicals levels decreased considerably, while the levels of NMDA with excitatory feature increased after CO exposure. In addition, a significant increase was observed in ADMA, which has a significant effect on endothelial function due to NO synthase inhibition levels after CO exposure. This situation is the opposite at the NO level. On the other hand, no significant effect was observed on other parameters except

<table>
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<tr>
<th>Table II. Comparison of Bcl-2 Immunopositive cells according to rat groups with ANOVA.</th>
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<tbody>
<tr>
<td><strong>Control</strong></td>
</tr>
<tr>
<td>(Group 1)</td>
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<tr>
<td>Bcl-2 Immunopositive cells</td>
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</table>

*Games-Howell. CO: Carbon monoxide
for the limited decrease in NMDA levels of aman-
tadine administration. This is also valid for Bcl-2 evaluated after immunohistochemical staining.

All organ systems related to CO poisoning are seriously affected by the process depending on the level of exposure. Although environmental exposure and many factors are effective in this, being affected at the cellular level is the key fac-
tor. It is known that CO poisoning causes both tissue hypoxia and direct cell changes, including inflammatory damage. Many pathways caused by hypoxia and subsequent reperfusion injury can complicate this situation even more. Antioxidants are defense systems in the body and are special-
ized to reduce or prevent damage caused by the formation of free oxygen radicals. SOD, a me-

Figure 2. Box plot graphs of Bcl-2. CO: Carbon monoxide.

Figure 3. Micrograph of immunohistochemical staining with anti-Bcl-2 antibody in the hippocampus of rats for all groups. a, Immunopositive cells (arrows) in the control group (Bar: 100 μm). b, Bcl-2 immunohistochemical group of Amantadine (Bar: 100 μm). c, Bcl-2 immunohistochemical group of CO (Bar: 100 μm). d, Bcl-2 immunohistochemical group of Amantadine + CO (Bar: 100 μm). CO: Carbon monoxide.
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Furthermore, it has been shown that the application of amantadine during CO poisoning does not contribute positively to these systems. Changes in ADMA and NO levels, which have an important effect on the regulation of vascular endothelial function, have a significant effect on neuronal cell damage after CO poisoning. The increase in ADMA is accepted as one of the coronary artery risk markers such as hypertension, hypercholesteremia, diabetes mellitus, and smoking. ADMA is an inhibitor of NO synthase. There is ample evidence that ADMA plays a fundamental role in maintaining vascular tone and structure. The increase of ADMA is accepted as one of the coronary artery risk markers, such as hypertension, hypercholesteremia, diabetes mellitus, and smoking. ADMA is also increased in traumatic brain injury and cerebrovascular diseases. However, we could not find any study evaluating ADMA levels and NO levels as a result of CO poisoning. Significant relationships were found between increased ADMA levels, increased oxidative stress, and endothelial dysfunction in various experimental animal models and diseases. Increased ADMA levels in endothelial cell cultures or patients with endothelial dysfunction are associated with increased production of reactive oxygen species. In a study conducted on children with traumatic brain injury, ADMA levels were observed to increase significantly in the cerebrospinal fluid. As a result, in addition to the release of free oxygen radicals, vascular endothelial damage, which is directly or indirectly related to this situation, further complicates CO poisoning. In this study, the increase in ADMA levels and the decrease in NO levels suggest that ADMA is an important parameter in the deterioration of endothelial function after CO exposure, and therefore, in the deterioration of neuron functions.

Although the mechanism of brain damage caused by CO exposure is quite complex, brain hypoxia induced by CO poisoning is the main mechanism of various types of brain damage. CO-induced toxicity leads to excitotoxicity, acidosis, nitrate stress, oxidative stress, inflammation, and apoptosis in nerve cells in the brain. The decrease of ATP activates intracellular proteases and lipases, resulting in cell death. In addition, the increase in glutamate results in the activation of NMDA receptors. This leads to CO-mediated cell dysfunction and apoptosis. The increase in NMDA receptors and the decrease in Bcl-2 immunopositive cells observed in the study suggest that NMDA receptors are an important factor in neuronal cell death after CO exposure. Although limited, the decrease in the number of NMDA receptors in rats given amantadine suggests that amantadine may be effective after CO exposure. However, studies with different time intervals of CO exposure and different amantadine doses are needed to clarify this issue. Another important issue is that the decrease in Bcl-2 proteins, which is an important parameter in the evaluation of apoptosis, correlates with the increase in NMDA receptors. This confirms the effectiveness of these two systems in apoptosis after CO exposure.

This study is without its limitations. Firstly, CO exposure was limited to a single period in the study, and this limits our assessment of how different exposure times will affect neuronal damage. Furthermore, keeping the dose of amantadine constant limited our evaluation of the effect of amantadine at different doses.

Conclusions

Considering that the mechanism of brain damage caused by CO exposure is a very complex process, cell damage mediated by free oxygen radicals and glutamate-mediated NMDA receptor activation are important factors in cell destruction. The decrease in Bcl-2 expression that develops after CO exposure plays an important role in cell destruction by disrupting the apoptosis balance, which ought to continue regularly in normal circumstance. Besides, ADMA-mediated reduction of NO, which has a key role in the preservation of endothelial function, suggests that in addition to direct CO hypoxia, circulatory hypoxia as a result of the deterioration of the vascular structure is fundamental to the development of this complex process. We draw inference that the administration of amantadine after CO poisoning has a limited effect in preventing this process.
Funding
None.

Conflict of Interests
Authors declare that they have no conflict of interest.

Ethical Approval
The study was approved by Ankara Training and Research Hospital Animal Experiments Local Ethics Committee (ID: 0051, Date: 15.02.2019).

Availability of Data and Material
The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors’ Contribution
E.N. Zengin: Conceptualization, Data curation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing. S. Kayır: Conceptualization, Data curation, Formal analysis, Methodology, Supervision, Visualization, Writing – original draft. G. Doğan: Conceptualization, Investigation, Resources, Visualization, Writing – original draft. M. Zengin: Methodology, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing. A. Akdağlı Ekici: Data curation, Formal analysis, Resources, Writing – original draft. M. Yalvaç: Data curation, Formal analysis, Investigation, Writing – original draft. O. Özcan: Conceptualization, Data curation, Formal analysis, Methodology, Supervision, Writing – original draft. O. Yağan: Conceptualization, Methodology, Supervision, Writing – review & editing. A. Alagöz: Methodology, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing.

ORCID IDs

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