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# Changes in biochemical and molecular genetic parameters in rats exposed to carbon dioxide and carbon monoxide

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**Abstract**

Objective criteria are essential in forensic toxicology for elucidating combustion-related deaths. Carbon monoxide (CO) and carbon dioxide ($CO_2$) impair oxygen delivery by competing with oxygen for hemoglobin binding and by disrupting cellular oxidative metabolism. This study aimed to evaluate biochemical changes in blood and brain tissues, neuroglobin gene expression in the brain, and histopathological alterations following CO and $CO_2$ exposure in rats, hypothesizing differential neuroglobin responses between the two gases. Twenty-four male Wistar albino rats (300 ± 50 g) were randomly divided into three groups and exposed to either carbon monoxide (0.05%), carbon dioxide (20%), or no gas (control) in a controlled glass isolation chamber. Blood and brain samples were collected for toxicological, genetic, and histopathological analyses. Blood gas analyses revealed intergroup differences in pH and bicarbonate levels, indicating altered oxidative balance. Antioxidant enzyme activity suggested increased oxidative stress in both exposure groups. Histopathological examination demonstrated more severe neuronal damage in the carbon monoxide-exposed group compared with the carbon dioxide and control groups. Neuroglobin expression levels showed intergroup variation but did not reach statistical significance. Both CO and $CO_2$ exposure induced oxidative stress-related changes, with carbon monoxide causing more pronounced neuronal injury. Although neuroglobin levels tended to increase following exposure, the differences were not statistically significant. Further experimental studies may enhance understanding of neuroglobin's role in forensic toxicology.

**Keywords:** Carbon monoxide poisoning, carbon dioxide poisoning, neuroglobin, genetic toxicology, forensic toxicology

## Introduction

Forensic Toxicology is an important branch of the practical applications of Forensic Medicine, as it provides measurable and objective results in elucidating forensic cases. The results of forensic toxicological analyses contribute to determining the origin of incidents and play a key role in distinguishing forensic cases. The advancement of analytical methods and the implementation of modern instrumentation in forensic toxicology not only help to solve problems encountered in daily practice but also contribute to scientific and technological progress.

The ability of toxicological analyses to produce objective results—often leaving no room for doubt—highlights their importance in forensic investigations. For instance, in forensic practice, livor mortis due to hypothermia may appear similar to that observed in carbon monoxide poisoning. Likewise, in cases where a burned body is found in an open area, the detection of carboxyhemoglobin (COHb) levels in the blood below lethal limits can complicate diagnosis and differential diagnosis [1-3].

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Exposure to toxic gases generally impairs tissue oxygenation, leading to a clinical condition known as asphyxia. The term asphyxia refers to a state of "airlessness" or "lack or absence of oxygen." Although the terms anoxia and hypoxia are sometimes used interchangeably, they differ in their underlying mechanisms and causes [4,5].

Among the toxic gases that induce asphyxia, carbon monoxide (CO) causes chemical asphyxia by preventing the transport and utilisation of oxygen in the blood and by the cells. Carbon monoxide poisoning represents the most common type of chemical asphyxiation caused by gaseous substances. CO is a colourless, odourless gas that is slightly lighter than air (molecular weight: 28.01 g/mol). It is an unsaturated carbon compound produced during the incomplete combustion of carbon-containing materials [6-9]. Carbon dioxide ($CO_2$), which holds a distinctive place among inert gases, constitutes approximately 0.4% of atmospheric air. $CO_2$, slightly heavier than air (molecular weight: 44.009 g/mol), is present at about 5% concentration in the alveolar air of healthy individuals. At atmospheric concentrations of around 3%, symptoms such as headache, drowsiness, dizziness, and weakness occur. The lowest concentration reported to cause death is approximately 25-30%, while sudden death may occur at concentrations of 60-80%. In fatal cases of carbon dioxide exposure, no specific macroscopic findings are expected at autopsy. Livor mortis typically appears dark purple, and marked congestion may be observed in internal organs.

Carbon monoxide, by contrast, induces asphyxia both by binding to hemoglobin—thus impairing oxygen transport to tissues—and through its metabolic effects, leading to histotoxic asphyxia at the cellular level. Consequently, there is no clear correlation between the degree of exposure, blood carboxyhemoglobin levels, and clinical manifestations in carbon monoxide poisoning. Therefore, new biomarkers, analytical techniques, and experimental data are needed to improve the understanding and differentiation of such toxic exposures [10,11].

The mechanism by which toxic gases induce asphyxia involves interference with oxygen transport and utilisation mediated by a family of oxygen-binding proteins known as globins. Globins are small respiratory proteins containing a heme group—a porphyrin ring with an iron atom—that allows the reversible binding of oxygen. In addition to their enzymatic functions, globin-structured proteins transport oxygen to the respiratory chain, thereby supporting aerobic metabolism. The best-known members of this family are myoglobin and hemoglobin. In the early 2000s, two additional globin types—neuroglobin and cytoglobin—were identified. In humans and other vertebrates, four types of functional globins (hemoglobin, myoglobin, neuroglobin, and cytoglobin) have been described, differing in their structural characteristics and tissue distribution. Based on this background, the present study was designed on the hypothesis that neuroglobin, a relatively newly identified molecule whose biological role continues to be investigated, may serve as a potential biomarker for differentiating cases of carbon monoxide poisoning [12-15]. In addition, one of the distinctive aspects of our study was to compare the oxidative and antioxidative status resulting from acute exposure to CO and $CO_2$, and to evaluate the biochemical outcomes of these effects. To the best of our knowledge, no previous experimental animal study has conducted such a comparative analysis. Therefore, we believe that the findings of this research will provide preliminary insights and guidance for future studies contributing to the scientific understanding of this topic. The aim of this study was to determine the changes in biochemical activity within the brain and blood tissues of rats exposed to carbon dioxide and carbon monoxide, and to evaluate the effects of such exposure on neuroglobin gene expression.

## Material and Methods

The experimental animal protocol was approved by the Ethics Committee for Animal Experiments of İnönü University Faculty of Medicine (No: 2014/A-90, Date: 12 November 2014). A total of 24 young male Wistar albino rats, weighing an average of 300 ± 50 g, were obtained from the İnönü University Experimental Animals Production and Research Center (İNÜ-DEHÜM). The rats were kept at INÜ-DEHÜM under controlled conditions for 15 days prior to the experiment, in rooms maintained at 21 ± 2°C, with a 12-hour light / 12-hour dark cycle and continuous ventilation by aspirators. During this acclimatization period, the animals were fed in accordance with standard laboratory procedures.

Before the experiment, the operation of the glass isolation system—designed to enable controlled exposure to carbon monoxide and carbon dioxide—was tested, and it was confirmed that the system functioned properly without technical issues. The carbon monoxide and carbon dioxide cylinders used in the experiment were supplied by HABAŞ A.Ş., Elazığ Regional Directorate, each accompanied by certified gas analysis documentation (Figure 1).

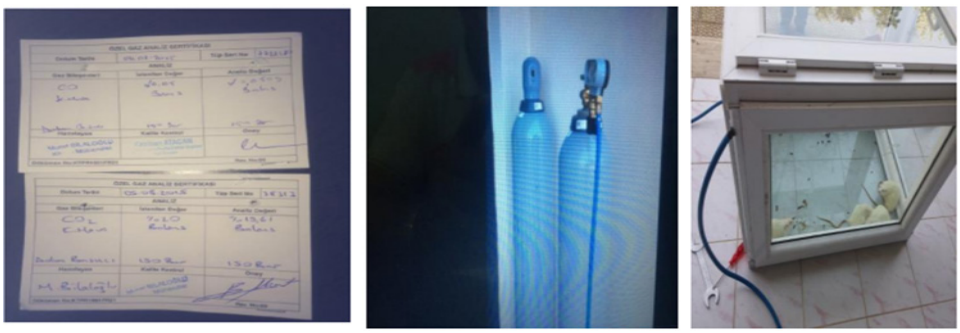

**Figure 1.** The isolated exposure system used in the experiment, the CO/$CO_2$ cylinders, and the certificates of the gases utilized

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The 24 rats used in this study were divided into three groups, each consisting of eight animals. In this experiment, carbon monoxide (0.05%) and carbon dioxide (20%) gases were used. The gas mixtures were buffered with dry air, and certification documents verifying the concentrations were available. The exposure duration was set at 15 minutes, and the gas flow rate was adjusted to 0.1 L/min (Table 1).

**Table 1.** Carbon dioxide and carbon monoxide doses, exposure duration, and experimental conditions applied to the study groups

| Group number | Number of rats | Gas used | Gas concentration | Exposure duration | Flow rate |
|---|---|---|---|---|---|
| **1** | 8 | Control | — | — | — |
| **2** | 8 | Carbon dioxide | 20% | 15 min | 0.1 L/min |
| **3** | 8 | Carbon monoxide | 0.05% | 15 min | 0.1 L/min |

### Experimental Procedure

#### Group 1 (Control)

Only standard monitoring procedures were performed for the control group. At the end of the experiment, analgesic and anaesthetic agents were administered. Whole blood was collected from the heart for the determination of antioxidant enzyme activities. Subsequently, decapitation was performed, and the brain tissue was excised. A portion of the collected blood was immediately sent to the Blood Gas Analysis Laboratory of İnönü University Turgut Özal Medical Centre for blood gas measurements. The remaining blood samples were stored at -86°C until the appropriate conditions for biochemical analysis were established. Portions of the excised brain tissue were allocated for genetic and biochemical analyses.

#### Group 2 (Carbon Dioxide Exposure)

The glass isolation chamber (dimensions: 90 × 60 × 70 cm) was inspected prior to the experiment. The experimental setup, including connection tubes, regulator manometers, and safety valves attached to the cylinders, was checked for functionality. Carbon dioxide gas was introduced into the system at a flow rate of 0.1 L/min, achieving an exposure concentration of 20% $CO_2$. Because $CO_2$ is heavier than air, it was introduced from the lower inlet tube of the chamber, while the upper outlet tube served as the exhaust port, and all other openings were sealed. This setup ensured uniform gas distribution throughout the chamber according to gas diffusion principles. Rats were exposed to the designated gas for 15 minutes, placed in the chamber in pairs.

#### Group 3 (Carbon Monoxide Exposure):

Similarly, the glass isolation chamber (dimensions: 90 × 60 × 70 cm) was checked before the experiment. The tubing system, regulator manometers, and safety settings were verified. Carbon monoxide gas was administered at a flow rate of 0.1 L/min, achieving an exposure concentration of 0.05% CO. Since CO is lighter than air, it was introduced from the upper inlet tube, with the lower outlet tube serving as the exhaust, while other openings were sealed. This configuration allowed homogeneous gas distribution throughout the exposure environment. Rats were exposed to the gas for 15 minutes, in pairs, inside the closed chamber system.

After completion of the experimental procedures described above, the rats were administered analgesic and anaesthetic agents and were subsequently decapitated in accordance with ethical and procedural standards. Blood and brain tissues were collected for genetic and biochemical analyses. The excised brain tissues were sectioned coronally into two parts: one half was allocated for the genetic phase of the study, while the remaining half, along with blood samples, was reserved for biochemical analyses. The collected specimens were properly transferred to the respective laboratories and stored at -86°C until analysis.

Whole blood for the determination of antioxidant enzyme activities was drawn directly from the heart. Following this procedure, decapitation was performed, and brain tissues were removed. A portion of the blood samples was immediately sent to the Blood Gas Analysis Laboratory of İnönü University Turgut Özal Medical Centre for blood gas measurements. The remaining samples were stored at -86°C until the appropriate analytical conditions were established. Portions of the brain tissue were also allocated for both genetic and biochemical assessments.

### Statistical Analysis

All biochemical data were analysed using SPSS for Windows, version 12.0. Following normality testing, one-way analysis of variance (ANOVA) was applied to compare differences between groups. Results are expressed as mean ± standard error, [16-21].

Statistical analyses of molecular genetic were performed using the MedCalc® software (version 11.4.2.0). The distribution of neuroglobin (Ngb) gene expression values in brain tissue was assessed using the Shapiro-Wilk test, which indicated that the data were not normally distributed ($p<0.05$). Therefore, the results were presented as median (minimum-maximum) values. Differences among the groups were evaluated using the Kruskal-Wallis test. $p<0.05$ was considered statistically significant.

## Results

### Biochemical and Toxicological Parameters

Whole blood was collected directly from the heart for the determination of antioxidant enzyme activities. A portion of the collected blood was immediately sent to the Blood Gas Analysis Laboratory of İnönü University Turgut Özal Medical Center for blood gas measurements, and the analyses were performed without delay (Table 2).

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**Table 2.** Blood gas analysis parameters and statistical comparisons among groups

| Blood gas analysis parameters | Control | $CO_2$ Group | CO Group |
|---|---|---|---|
| **$FO_2Hb$ (%)** | 9.60±5.00[a] | 8.38± 4.78[ab] | 7.38±5.82[ab] |
| **cK (mmol/L)** | 4.26±0.50[a] | 3.55 ±0.64[b] | 3.75± 0.42[ab] |
| **cNa (mmol/L)** | 137.50±2.61[a] | 138.5± 5.15[ab] | 139.62±3.15[ab] |
| **cCl (mmol/L)** | 114.12±7.23[a] | 112.75±5.92[ab] | 116.87±8.33[ab] |
| **$cHCO_3$ (mmol/L)** | 23.07± 2.01[a] | 21.37± 2.23[ab] | 19.87± 2.42[b] |
| **pH** | 7.30± 0.05[a] | 7.23± 0.05[b] | 7.24± 0.05[ab] |
| **$pCO_2$ (mmHg)** | 56.52±8.87[a] | 70.08±8.48[b] | 59.76±4.89[ab] |
| **$pO_2$ (mmHg)** | 13.93±3.91[a] | 11.66±5.56[ab] | 7.76± 4.19[b] |
| **Hb (g/dL)** | 12.60±4.42[a] | 16.26± 0.75[b] | 14.91±1.46[ab] |
| **Hct (%)** | 38.77±13.39[a] | 49.68±2.31[ab] | 41.76±11.60[ab] |

a: no statistically significant difference between groups, p>0.05; b: statistically significant difference between groups, p<0.05; ab: no significant difference but an increasing or decreasing trend is observed

The tissues removed from the deep freezer were weighed and placed into glass tubes. A 1.15% potassium chloride (KCl) solution was added at a 1:10 (g/mL) dilution ratio. While maintaining the samples at cold temperature, they were homogenized in a glass-Teflon homogenizer at 16,000 rpm for 3 minutes. In the prepared homogenates, tissue TBARS (MDA) and protein determinations were performed. The remaining homogenate was centrifuged at 3,500 rpm for 45 minutes at +4°C, and the resulting supernatant was collected. In these supernatants, GSH and protein levels as well as GSH-Px and CAT enzyme activities were measured. To the remaining portion of the supernatant, a chloroform/ethanol (3:5, v/v) mixture was added at a 1:1 (v/v) ratio, and the samples were mixed using a vortex. Subsequently, centrifugation was carried out again at 3.500 rpm for 45 minutes. In the upper chloroform/ethanol phase, SOD enzyme activity and protein concentrations were remeasured.

Tissue TBARS levels were determined spectrophotometrically according to the method described by Ohkawa et al. The principle of the assay is based on the measurement of the pink-colored complex formed by the reaction of malondialdehyde (MDA)—a secondary product of lipid peroxidation—with thiobarbituric acid (TBA) under aerobic conditions at pH 3.5. Tissue homogenates were incubated in a boiling water bath for one hour, and the absorbance of the resulting complex was measured spectrophotometrically at 532 nm (Table 3).

**Table 3.** Biochemical (oxidant/antioxidant) analysis findings

| Parameter | Control | $CO_2$ Group | CO Group |
|---|---|---|---|
| **TBARS (nmol/g tissue)** | 27.2 ± 2.36[a] | 26.7 ± 5.00[a] | 37.5 ± 2.82[b] |
| **GSH (nmol/ml)** | 95.8 ± 21.8[a] | 82.6 ± 16.4[ab] | 75.1 ± 13.1[b] |
| **CAT (kU/mg protein)** | 0.0114 ± 0.001[a] | 0.0047 ± 0.0007[b] | 0.0026 ± 0.0006[b] |
| **SOD (U/mg protein)** | 18.9 ± 3.86[a] | 15.6 ± 3.46[ab] | 14.5 ± 2.22[b] |
| **GPx (U/mg protein)** | 262.8 ± 88.5[a] | 243.4 ± 82.0[a] | 212.9 ± 29.2[a] |

TBARS, GSH, CAT, SOD, and GPx levels in the brain tissue of rats exposed to CO and $CO_2$ (n=8, mean ± SD). (a: no statistically significant difference between groups, p>0.05; b: statistically significant difference between groups, p<0.05; ab: no significant difference but an increasing or decreasing trend is observed.)

Superoxide dismutase (SOD) catalyzes the dismutation of toxic superoxide radicals ($O_2^-$), which are generated during oxidative energy production, into hydrogen peroxide ($H_2O_2$) and molecular oxygen. The determination of SOD enzyme activity in tissue samples was carried out according to the method described by Sun et al. Principle: The assay is based on the reduction of nitroblue tetrazolium (NBT) by superoxide radicals generated through the xanthine/xanthine oxidase system. The reduction of NBT by superoxide produces a colored formazan compound, which is measured spectrophotometrically at 560 nm, where the complex exhibits maximal absorbance. In the absence of SOD, superoxide radicals reduce NBT, yielding a dark blue-purple colour. When SOD is present, this reduction is inhibited, resulting in a lighter colour intensity proportional to the enzyme activity.

Glutathione peroxidase (GPx) is an enzyme that catalyses the reduction of hydrogen peroxide ($H_2O_2$) to water using reduced

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glutathione (GSH) as a substrate. GPx activity in tissue samples was determined according to the method described by Beutler. Principle: In the presence of $H_2O_2$, GPx catalyses the oxidation of GSH to oxidized glutathione (GSSG). The GSSG formed is then reduced back to GSH by glutathione reductase in the presence of NADPH. GPx activity is calculated spectrophotometrically at 340 nm, based on the decrease in optical density resulting from the oxidation of NADPH to $NADP^+$.

Catalase (CAT) catalyzes the decomposition of hydrogen peroxide ($H_2O_2$) into water and oxygen through its catalytic activity. CAT activity in tissue samples was determined according to the method described by Aebi. Principle: Hydrogen peroxide exhibits maximal absorbance at 240 nm. The degradation of $H_2O_2$ by catalase is monitored as a decrease in absorbance in the ultraviolet spectrum. The rate of this decrease is directly proportional to the enzyme activity.

Tissue GSH levels were measured using the dithiobis-nitrobenzoic acid (DTNB) method described by Ellman. Principle: 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) is reduced by sulfhydryl compounds to form a yellow-colored disulfide compound. The optical density of this compound is measured at 412 nm, and the GSH level is determined from the absorbance value.

Protein concentrations in the homogenate and supernatant fractions were determined using the method described by Lowry et al. Principle: In alkaline copper reagent, $Cu^{2+}$ ions form complexes with peptide bonds, with approximately one copper atom binding per seven or eight amino acid residues. When the Folin-Phenol reagent is added to this copper-treated mixture, a blue-purple colour develops, the intensity of which is measured at 700 nm.

### Histopathological Analyses and Findings

For histopathological examination, brain tissue samples were fixed in 10% formaldehyde solution. Routine tissue processing procedures were applied, and the specimens were embedded in paraffin blocks. Sections of 5 µm thickness were cut from the prepared blocks and stained using the hematoxylin-eosin (H&E) staining method. The stained sections were examined and photographed using a Leica DFC 280 light microscope equipped with the Leica Q Win Image Analysis System (Figure 2).

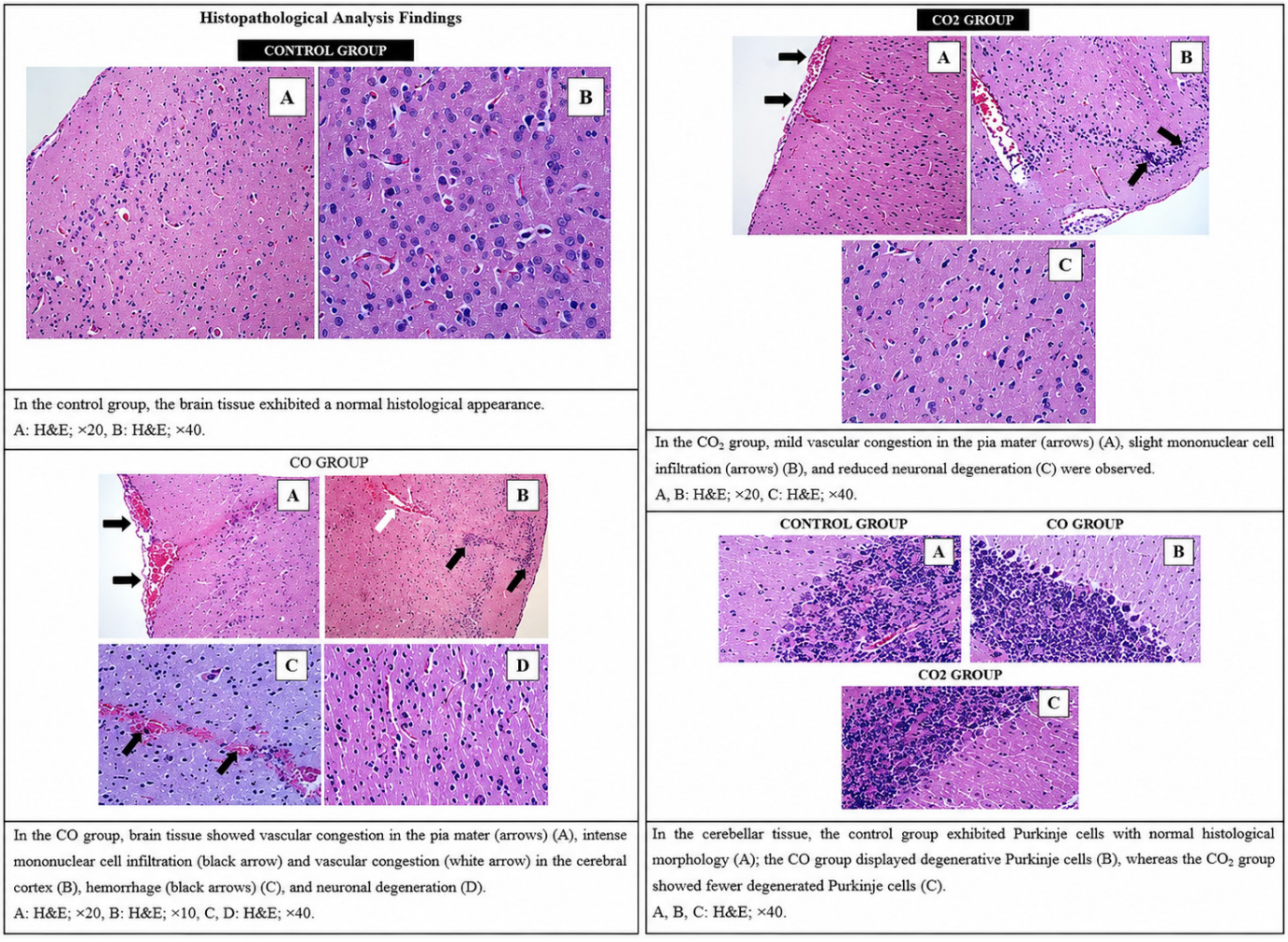


**Figure 2.** Histopathological analyses and findings

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**Molecular Genetic Analyses and Findings**

For the determination of neuroglobin (Ngb) mRNA levels in brain tissue, samples obtained from each group were cut into small pieces on ice under sterile conditions and placed into an RNA preservation solution. Total RNA was then isolated from these tissues using the High Pure RNA Tissue Kit (Roche, Lot No: 10156400). According to the manufacturer's protocol, approximately 25 mg of tissue was weighed and mixed with 350 µL of Lysis Buffer. The samples were homogenized on ice using a homogenizer at 13,500 rpm for approximately 1 minute. An equal volume of ethanol (½ of the lysate volume) was added to the homogenate, and the mixture was vortexed and centrifuged at 13.000 × g. After centrifugation, the collection tube beneath the column was discarded and replaced with a new one to continue the process. Subsequently, 100 µL of DNase solution was added to the column and incubated at room temperature for 15 minutes. Following incubation, 500 µL of Wash Buffer I was added and centrifuged at 8.000 × g for 1 minute at room temperature. Then, 500 µL of Wash Buffer II was added and centrifuged again under the same conditions. Finally, 300 µL of Wash Buffer II was added and centrifuged at 13.000 × g for 2 minutes at room temperature. The collection tube was discarded, and the column was transferred to a sterile Eppendorf tube. To elute the RNA, 100 µL of Elution Buffer was added to the column and centrifuged at 8,000 × g for 1 minute at room temperature. The eluate collected in the tube was obtained as total RNA.

Following RNA purification from brain tissue samples using the High Pure RNA Kit protocol, the integrity of total RNA was evaluated to determine whether any degradation had occurred. The RNA samples were subjected to electrophoresis on 1% agarose gel using 1× TBE buffer at 100 mV. RNA visualization was performed under ultraviolet light using the UVP ChemiDoc It² Imaging System. The presence of clear 28S and 18S ribosomal RNA bands confirmed that the isolated RNA was intact and free from degradation.

In addition, the purified RNA samples were analyzed spectrophotometrically to assess RNA concentration and purity. Measurements were performed using a Biotek spectrophotometer and Gen5 software at 260 nm and 280 nm in the UV spectrum. RNA concentrations were calculated in ng/µL, and samples with 260/280 absorbance ratios of approximately 2.0, indicative of pure RNA, were used for cDNA synthesis.

Complementary DNA (cDNA) synthesis was performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Lot No: 14585924) according to the manufacturer's instructions. Briefly, 220 ng of total RNA, 1 µL of Oligo(dT)$_{18}$ (50 pmol/µL), 1 µL of Random Hexamer Primer (600 pmol/µL), and nuclease-free water were added to a PCR tube to obtain a total volume of 13 µL. The mixture was incubated at 65°C for 10 minutes in a thermal cycler.

Subsequently, 4 µL of Transcriptor Reverse Transcriptase Reaction Buffer, 0.5 µL of Protector RNase Inhibitor, 2 µL of Deoxynucleotide Mix (10 mM of each dNTP), and 0.5 µL of Transcriptor Reverse Transcriptase enzyme were added, bringing the final reaction volume to 20 µL. The samples were mixed gently and subjected to the following incubation steps in a PCR machine: 25°C for 10 minutes, 55°C for 60 minutes, and 85°C for 5 minutes. The synthesised cDNA samples were stored at -20°C until further analysis.

Real-time PCR analysis was performed using the Roche LightCycler 96 system with the FastStart Essential DNA Probes Master Kit (Roche, Lot No: 10048800) and RealTime Ready Assays (β-Actin: Lot No: 90017829, Config No: 100081783; NGB: Lot No: 90017828, Config No: 100099177) containing hydrolysis probe primers (8 pmol/µL).

Each reaction was prepared in a final volume of 10 µL, consisting of 5 µL of master mix, 0.5 µL of RealTime Ready mix, 2 µL of PCR-grade water, and 2.5 µL of cDNA. All reactions were performed in triplicate.

The PCR conditions were carried out as recommended by the manufacturer: initial denaturation: 95°c for 10 minutes, denaturation: 95°c for 10 seconds, annealing: 60°c for 30 seconds, extension: 72°c for 1 second. These steps were repeated for 55 cycles. The primers and amplicon sizes used for the analysis of NGB gene expression are presented in Table 4 and Figure 3.

No statistically significant difference was found among the groups in terms of brain Ngb gene expression levels according to the Kruskal-Wallis test (p=0.143) (Table 5 and Figure 4).

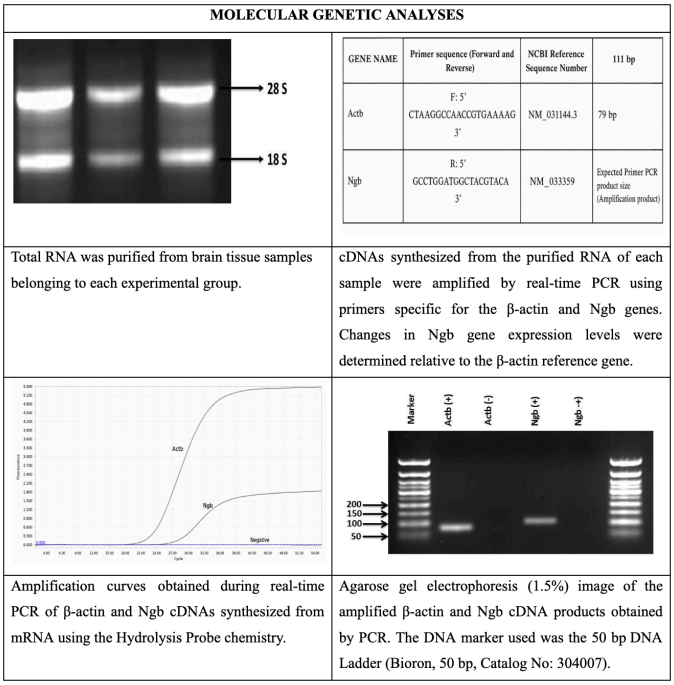


**Figure 3.** Molecular genetic analyses

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**Table 4.** Molecular genetic analyses and results

| Experimental groups | Sample no | Absorbence 260/280 | RNA amount ng/µL |
|---|---|---|---|
| **Control** | 1 | 2.0 | 190.4 |
| | 2 | 2.0 | 219.6 |
| | 3 | 2.0 | 146.0 |
| | 4 | 2.0 | 154.9 |
| | 5 | 2.0 | 221.7 |
| | 6 | 2.0 | 200.2 |
| | 7 | 2.0 | 188.9 |
| | 8 | 2.0 | 146.1 |
| **Carbon dioxide** | 9 | 2.0 | 219.1 |
| | 10 | 2.0 | 165.2 |
| | 11 | 2.0 | 204.6 |
| | 12 | 2.0 | 114.0 |
| | 13 | 2.0 | 200.6 |
| | 14 | 2.0 | 94.9 |
| | 15 | 2.0 | 125.7 |
| | 16 | 2.0 | 122.0 |
| **Carbon monoxide** | 17 | 2.0 | 136.7 |
| | 18 | 2.0 | 137.6 |
| | 19 | 2.1 | 183.5 |
| | 20 | 2.0 | 225.9 |
| | 21 | 2.1 | 170.4 |
| | 22 | 2.1 | 192.3 |
| | 23 | 2.1 | 88.9 |
| | 24 | 2.0 | 130.3 |
| Molecular genetic analyses and results | | | |

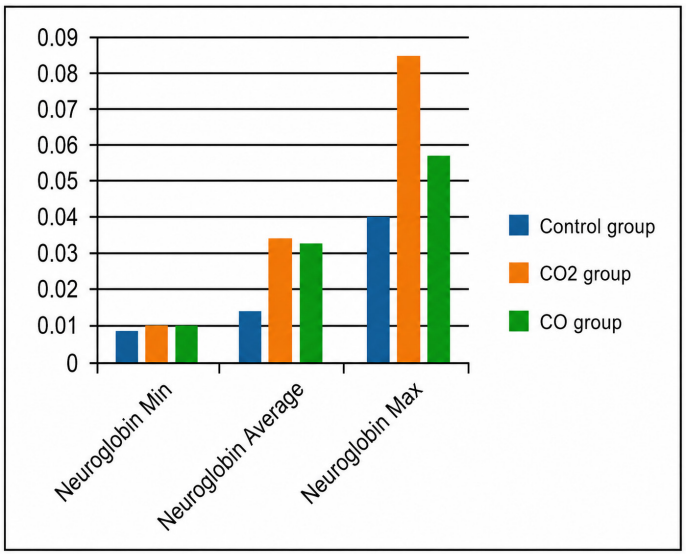


**Figure 4.** Brain Ngb gene expression results

**Table 5.** Brain Ngb gene expression values — minimum, maximum, and median levels of brain Ngb gene expression in study groups

| Groups (n=8) | Med (Min-Max) |
|---|---|
| **Control** | 0.014 (0.0089-0.039 |
| **CO2** | 0.035 (0.011-0.075) |
| **CO** | 0.034 (0.011-0.058) |

## Discussion

The findings from the blood gas and biochemical analyses revealed various alterations in metabolic processes associated with oxidative stress. The decreases observed in both pH and $HCO_3^-$ levels indicated an acidic environment in both exposure groups. Sodium ($Na^+$), chloride ($Cl^-$), and potassium ($K^+$) ions play crucial roles in maintaining acid-base balance. In the present study, significant alterations in these electrolytes were detected, with a notable decrease in potassium levels.

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These changes are known to be related to oxidative stress. Another remarkable observation in the blood gas results was the variation in COHb levels among rats exposed to the same concentration and duration of carbon monoxide, which is consistent with previous literature. The overall alterations observed were attributed to biochemical parameter changes induced by oxidative metabolism [22-24].

Oxidative stress represents an imbalance between lipid peroxidation markers such as TBARS and the antioxidant defence system, which includes enzymatic antioxidants (SOD, CAT, GPx) and non-enzymatic antioxidants (GSH). Free radicals and other reactive oxygen species (ROS) generated in tissues can normally be neutralised effectively by these antioxidant systems (SOD, CAT, GSH-Px, and GSH reductase). Disruptions in the oxidant-antioxidant balance result in increased ROS levels and oxidative damage. Oxidative stress may lead to irreversible cellular injury and inactivation of several enzymes. Free radicals, due to their high reactivity, interact with unsaturated fatty acids in cell membranes and initiate lipid peroxidation. The lipid peroxides formed are rapidly degraded, producing secondary products such as malondialdehyde (MDA). Because these intermediates are unstable and difficult to quantify, thiobarbituric acid reactive substances (TBARS), representing the MDA-TBA complex, were analysed as an indicator of lipid peroxidation in this study.

The biochemical results presented in Table 3 showed that the TBARS levels in the carbon monoxide group were significantly higher than those of the control and $CO_2$ groups, indicating enhanced oxidative damage. In contrast, antioxidant defence markers (GSH, SOD, and CAT) were significantly decreased in the CO-exposed rats. These findings suggest that exposure to carbon monoxide caused tissue injury in the brain through oxidative mechanisms. Although GPx levels did not show statistically significant differences among the groups, this may be due to a delayed enzymatic response, as GPx activity can increase during prolonged exposure. The observed tissue damage is likely due to the combined histotoxic and anaemic hypoxia caused by carbon monoxide, which binds to hemoglobin with an affinity 200-250 times greater than oxygen.

In the carbon dioxide-exposed group, TBARS levels did not differ significantly from the control group. While changes were observed in GSH, SOD, and GPx, these were not statistically significant. However, a significant decrease in CAT activity was detected. Because $CO_2$ has a lower affinity for hemoglobin compared to CO, the antioxidant parameters exhibited less variation. Catalase, a heme-containing enzyme found in peroxisomes and cytosol, catalyses the decomposition of hydrogen peroxide into water and molecular oxygen. The pronounced decrease in CAT activity may reflect a greater inhibitory effect of $CO_2$ on hemoglobin-bound catalase compared to other antioxidants [25-27].

Histopathological examination revealed normal histological architecture in the brain and cerebellar tissues of the control group. Neurons in the control group exhibited distinct euchromatic nuclei and nucleoli in hematoxylin-eosin (H&E)-stained sections. The cerebellar layers and Purkinje cells also appeared normal. In contrast, both CO and $CO_2$ groups exhibited histopathological alterations in the brain, including vascular congestion in the pia mater, mononuclear cell infiltration, hemorrhage, and neuronal degeneration characterised by cytoplasmic shrinkage, eosinophilia, and pyknotic nuclei. The severity of these lesions was greater in the CO group than in the $CO_2$ group. Furthermore, degeneration of Purkinje cells in the cerebellum was prominent in the CO group, while it was less marked in the $CO_2$ group. This difference likely reflects the more severe asphyxial injury in the CO group due to the combined histotoxic and anaemic hypoxia resulting from the strong hemoglobin affinity of carbon monoxide. As neurons and brain tissue have high oxygen requirements, they are particularly susceptible to hypoxic and toxic insults. Consequently, CO-induced chemical hypoxia caused more severe structural damage than $CO_2$ exposure [28-31].

Molecular genetic analyses of the brain tissue revealed wide variability in Ngb mRNA expression levels among individual rats within each group. Although mean Ngb expression levels appeared elevated in the exposure groups compared with controls, no statistically significant difference was found among the groups ($p=0.143$, Kruskal-Wallis test). Previous studies have shown that Ngb expression increases under certain conditions, exerting neuroprotective effects. In a 2004 study, Fordel et al. demonstrated that during hypoxic states with varying oxygen levels, the increase in neuroglobin expression was limited compared to changes in hypoxia-inducible factor 1α (HIF-1α), suggesting that Ngb synthesis may be regulated by additional mechanisms. The present study supports these findings: despite altered pH and oxidative stress conditions, the observed changes in Ngb mRNA levels were not statistically significant, indicating that the toxic gases tested did not exert a primary effect on Ngb gene expression.

Similarly, Picotti et al. (2009) examined the functional and structural stability of globin derivatives under varying pH conditions in vitro and found that neuroglobin exhibited greater stability than myoglobin at both pH 2 and pH 7. The results of the present study are consistent with these findings. In our experiment, although median Ngb gene expression levels increased in the CO and $CO_2$ groups ($CO_2$: 20%; CO: 0.05%; exposure duration: 15 min), the differences were not statistically significant ($p=0.143$). The mean carboxyhemoglobin (COHb) level measured in the CO group was $3.75 \pm 2.15\%$ (min=2.10; max=5.70). It is hypothesised that extending exposure duration or altering concentration may result in significant differences in Ngb mRNA expression in future studies. To our knowledge, this is the first in vivo comparative study to examine the effect of CO and $CO_2$ exposure on Ngb gene expression, and we believe that it will serve as a reference for future research in this field.

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## Conclusion

Research in genetic and biochemical toxicology typically aims to obtain measurable and reproducible data. Studies such as this, which integrate biochemical and molecular approaches, are essential for advancing forensic toxicology by bridging technology and multidisciplinary scientific inquiry. Reliable genetic and biochemical data may play a crucial role in elucidating causes of death and poisoning in forensic investigations. For instance, in a poisoning-related fatality, accurate genetic and biochemical toxicity analyses could provide decisive evidence for forensic interpretation. Therefore, we emphasise the importance of conducting multidisciplinary, technology-based studies in the field of forensic toxicology to enhance scientific understanding and practical application.

**Ethical Approval**
*Approved by the Ethics Committee for Animal Experiments of İnönü University Faculty of Medicine (No: 2014/A-90, Date: 12 November 2014).*

**Patient Consent**
*Written informed consent was waived due to the retrospective study.*

**Conflict of Interest**
*The authors declare no conflict of interest.*

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