Morpho-pathological and physiological changes of the brain and liver after ozone exposure

DENISA-IOANA CREŢU1), ALINA ŞOLOREA1), R. M. IGNAT1), ADRIANA FILIP2), CRISTINA BIDIAN2), AURICA CREŢU3)

1) Department of Histology
2) Department of Physiology
"Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca
3) Faculty of Physical Education and Sport, "Babeş-Bolyai" University, Cluj-Napoca

Abstract
This paper presents the consequences of long exposure to ozone in order to draw attention to this matter as far as the brain and liver are concerned. The material used was represented by two batches of 10 rats each that were daily exposed to ozone for 10 minutes at 0.5 ppm O₃. From the first group blood was collected after two weeks to determine the indicators of oxidative stress and samples of brain and liver were drawn for histological studies. Tissue changes were highlighted using Hematoxylin–Eosin and argentic impregnation. In addition, the brain and liver samples taken from study subjects were turned into homogeneous preparations in order to determine the intensity of oxidative stress occurred in these organs compared with the witness group. The second batch was exposed for a further two weeks, after which the same sampling techniques and determining methods as for the first group were applied. The results show a correlation between the values of malondialdehyde (MDA) and glutathione (GSH), obtained both in blood and in the homogeneous preparations, and the microscopic changes that implicate a pathological state. Therefore, cerebral edema was discovered in the brain hemispheres and the cerebellum indicating necrotic signs accompanied by a reduction in the molecular layer and Purkinje cells with pale core. The liver presented hepatocellular necrosis, extended from the port area to the centrolobular vein.

Keywords: ozone, oxidative stress, brain, liver, edema, necrosis.

Introduction
In relation to the elevated pollution levels on a global scale in the last couple of years, the noxious effects of ozone exposure – especially those concerning the respiratory system – represent a well-debated subject in scientific literature [1]. Ozone, also known as activated oxygen or trivalent oxygen, is an extremely reactive oxidizing agent because on one hand, it is unstable, and on the other hand, it can generate cytotoxic free radicals through the transfer of oxygen atoms to the body’s macromolecules. It is considered one of the six major air-polluting agents, with significant structural and functional impact on the respiratory, cardiovascular system, liver and brain, even in the smallest concentrations.

In nature, ozone can be found in wide concentrations at high altitudes, whereas the troposphere accumulates it in order to form the photochemical smog distinctive in intense chemical pollution combined with exposure to ultraviolet radiation. Ozone injures the epithelium of the respiratory tract, raising its permeability, induces inflammation, releases mediators and chemotactic factors, consecutive to edema, emphysema and pulmonary fibrosis. It causes vasoconstriction, increases sympathetic tonus, induces membrane lipid peroxidation and DNA lesions, reduces antioxidizing defense and generates inflammation mediators responsible for myocardial dysfunctions, arterial hypertension and arrhythmia. Its effects are systemic, the most common are pulmonary and cardiac, but hepatic and cerebral changes cannot be ignored, especially when exposed both to ozone and chemical pollutants.

The ozone’s toxic mechanisms imply electron transfer reactions, the generation of reactive oxygen species, oxidative processes that often take place together with other atmospheric pollutants: quinones, metallic compounds, aromatic compounds, ions, etc. The generated oxidative stress is accompanied by lipid peroxidation, cellular membrane lesions, mitochondrial dysfunction, protein and DNA-oxidation forming mutagen 8-hydroxyguanosine. The ozone’s toxicity leads to a reduction of antioxidizing reserves, decreases glutathione and creates a cellular redox state disturbance with important consequences concerning cellular functionality.

The present study sets out to evaluate the consequences of prolonged ozone exposure on vital organs such as the liver and brain, by determining oxidative stress parameters, as markers of oxidative aggression, and by analyzing histopathological modifications [2–5]. Knowing the ozone’s effects on the liver and brain is important because of the lack of data in scientific literature, and the perspective impact on the body’s homeostasis.
Material and Methods

Reagents

Ethanol and n-butanol were acquisitioned from Chimopar, Bucharest (Romania). KH₂PO₄, 2-thiobarbituric acid and 2,2-dithio-bisnitrobenzoic acid came from Sigma-Aldrich Chemicals GmbH (Germany) and EDTA-Na₂ from Merck KGaA, Damstadt (Germany).

Animal groups

The study was performed on three groups of Wistar rats, white, female, medium weight 200±10 g. The rats were acquisitioned from Biobase of “Iuliu Hațieganu” University of Medicine and Pharmacy, Cluj-Napoca. The animals were maintained 10 days prior to the experiment in the Physiology Department Biobase, on a 12 hours darkness/12 hours light regiment for acclimatization. The animals were received standardized diets and water ad libitum. The experiments the animals went through were in accordance with the Protocol for Animal Care, elaborated by the University’ Ethics Committee.

Group I and group II, each containing 10 animals, represented study groups, and group III, also consisting of 10 animals, represented control group.

Ozone exposure was conducted in the Laboratory for Experimental Research belonging to the Physiology Department, “Iuliu Hațieganu” University of Medicine and Pharmacy, Cluj-Napoca, with the help of the AIR O₃NE LABOR device, offered by S.C. TRIOX S.R.L., which are responsible for air ozoning, having the possibility to regulate the air’s O₃ concentration from 50 mg/m³ to 500 mg/m³. The device could function with a duration varying from one minute to nine hours. It aspirates a volume of air; ensures its physical processing, then releases it having the programmed O₃ concentration.

Group I was exposed to ozone (0.5 ppm concentration) 10 minutes/day for two weeks, and the second group also 10 minutes/day, but for four weeks. After the last ozone exposure, animals from each group were anaesthetized with a mix of Ketamine and Xylazine (90 mg kg⁻¹ Ketamine, 10 mg kg⁻¹ Xylazine), blood anaesthetized with a mix of Ketamine and Xylazine last ozone exposure, animals from each group were 10 minutes/day, but for four weeks. After the experiment in the Physiology Department Biobase, on a 12 hours darkness/12 hours light regiment for acclimatization. The animals were received standardized diets and water ad libitum. The experiments the animals went through were in accordance with the Protocol for Animal Care, elaborated by the University’ Ethics Committee.

Determination of MDA

Oxidative stress evaluation

The specimens were included in paraffin and stained with Hematoxylin–Eosin and Lithium Hematoxylin Carbonate (for the cerebral cortex and cerebellum), and Hematoxylin–Eosin and Sirius red (for the liver). Analysis of intracellular lipid deposits in hepatocytes was done on the same specimens, through frozen section and stained with Oil Red. After coloration, the specimens were analyzed using a Nikon YS2–H microscope with ×20 and × 40 lenses. The animals were closely monitored during the entire experiment; they were weighed daily, checked for motility, appetite and level of somnolence.

Determination of oxidative stress parameters was performed in the Laboratory of Oxidative Stress Study, within the Physiology Department, and the histopathological examinations in the Histology Department Laboratory, “Iuliu Hațieganu” University of Medicine and Pharmacy, Cluj-Napoca.

Statistical analysis

The data was analyzed using the program Analysis Toolpak. In order to calculate the variation for each rat group, the Fisher test was used, and in order to compare data obtained before and after ozone exposure of groups I and II, we used t-test: Two Sample Assuming Unequal Variances and t-test: Two Sample Assuming Equal Variances. p<0.05 was considered significant.

Results

Ozone exposure has important consequences in terms of oxidative stress markers as well as the normal structure
of cerebral, cerebellum and hepatic tissue. The obtained results were presented in Figures 1–13 and Table 1.

Table 1 – Oxidative stress parameter values in serum, cerebral tissue and hepatic homogenous

<table>
<thead>
<tr>
<th>Seric levels [µmol/L]</th>
<th>Group I</th>
<th>Group II</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum</td>
<td>Brain</td>
<td>Liver</td>
</tr>
<tr>
<td>MDA</td>
<td>2.67±0.48</td>
<td>0.15±0.11</td>
<td>0.21±0.03</td>
</tr>
<tr>
<td>–SH group</td>
<td>0.05±0.01</td>
<td>0.11±0.02</td>
<td>0.27±0.05</td>
</tr>
</tbody>
</table>

Ozone exposure for a period of 10 minutes/day for two weeks and four respectively, induces seric lipid peroxidation as well as macroscopic and microscopic alterations of the organs in study: liver, cerebral cortex and cerebellum. Macroscopically observing the groups that were exposed to ozone, we found they had developed hepatomegaly, with a dull gray-reddish color, on the surface and in section. Microscopically, histological studies, performed on liver harvested two weeks post ozone exposure, showed a mild stasis in portal area, accompanied by lymphocyte infiltration with periportal and intralobular migration tendencies (Figure 1). Sinusoid spaces appeared enlarged and the hepatocytes presented granular cytoplasmatic dystrophies (Figure 2).

Sections of liver harvested four weeks post-ozone exposure indicated an escalation of the degenerative alterations shown in group I. Nuclear modifications were revealed in hepatocyte cordons (small, hyperchromatic nucleus) (Figure 3), and the cytoplasm became foamy, transparent, indicating the occurrence of a vacuolar dystrophy (lack of color in Oil Red specimens indicated the presence of hydropic degeneration) (Figure 4). The inflammatory infiltration with round-nuclear cells was abundant, with clear signs of interface hepatitis (Figure 5). Also, we observed suggestive aspects for necrotic mediolobular hepatocyte transformation or apoptosis (Figure 6).

A marked peripheral fibrosis and an accentuation of perilobular septums were identified in a majority of Sirius red stained specimens (Figure 7).

Macroscopic evaluation of the brain did not reveal any clear modifications aside from attenuated cerebral fissures. However, the histological specimens of brain tissue harvested from two weeks ozone exposed rats showed an obvious cerebral edema, with great peri-cellular haloes, and in some cases, enlargement of the hematoencephalic barrier (Figure 8).

Study of sections obtained from four weeks ozone exposed rats showed similar alterations to those of group I, with a slight accentuation of the edema, stasis and an apparent cellularity growth of the molecular layer. Cerebellum specimens harvested from group I, stained with Hematoxylin–Eosin and Lithium Hemato-
xylin Carbonate indicated more obvious changes. The intensity of Purkinje cells color, both in the cytoplasm and nucleus, was reduced. We also observed a total absence of Purkinje cells in some zones of the cerebellum sections from four weeks ozone exposed rats, and the ones that were still present, had intense degenerative changes (Figure 9).

As for malondialdehyde, an indicator of lipid oxidation phenomenon, its evolution was similar in both groups, which indicated that the duration of pollutant exposure did not influence the intensity of oxidative alterations as much. Thus, the serum MDA levels at two and four weeks displayed a significant elevation compared to the control group ($p=0.02$) (Figure 10). Fisher test and Student $t$-test were used for group comparison. A dynamic determination of the –SH groups, at two and four weeks, faithfully reflected the alterations induced by pollutant exposure. The level of –SH groups decrease significantly statistically compared with the control group both at two weeks exposure ($p=0.0023$) and at four weeks ($p=0.0025$) (Figure 11).

Determination of the same parameters in the brain suggests an absence of significant modifications in the oxidizing/antioxidizing balance. Thus, the concentration of seric thiol groups did not differ statistically between the study groups and the control group ($p=0.3$ for group I, and $p=0.4$ for group II).

In the hepatic tissue MDA increased after two weeks of ozone exposure ($p=0.002$), but after four weeks MDA values significantly decreased ($p=0.0006$). The concentration of seric thiol groups decreased after 14 days, but at the end of the experiment the second group presented increased values ($p=0.0007$).

**Discussion**

"Bad ozone" formed in the lower atmospheric layers leads to photochemical pollution. When it surpasses certain limits, it is detrimental to life on Earth, because it reacts with vegetal and animal tissue and can also affect the human body irreversibly. The substances that underlie troposphere ozone formation are nitrogen oxides and volatile organic compounds. Being extremely reactive, they affect the gene expression implicated in stress and inflammatory response on the alveolar cell epithelium, they induce alterations of transcription factors, extracellular matrix, local antioxidizing defense, lipid metabolism enzymes, etc. [6]. Heart studies have shown that exposure to ozone aggravates preexistent conditions, and can lead to myocardial infarct [7, 8], and it can also escalate respiratory conditions [9, 10] concomitant with foreign bodies accumulation, septal stasis and pulmonary emphysema.

In this study, we observed the effects of repeated exposure to ozone on the liver and brain, by studying the oxidative stress markers and through histopathological exams. The presence of some seric modifications of oxidative stress markers, without variations in the cerebral tissue homogenous, suggests the existence of a local antioxidizing defense mechanism. It is possible for the hematoencephalic barrier to delay damage to the brain tissue by the free radicals excessively generated by ozone. Histological alterations in the cerebellum are, however, surprising, for specimens after four weeks exposure, due to the existence of zones absent in
cellularity. These changes are even more surprising considering the insignificant change in oxidative stress parameters compared to the control group. Scientific literature describes morphologic alteration of cerebellum Purkinje cells after ozone exposure [11], these alterations being interpreted because of degenerative changes with cellular lesions, induced by free radicals. In our study, the cerebral edema we recognized does not correlate with the accentuation of lipid peroxidation phenomenon and consecutive cellular membrane lesions. As the scientific literature suggests, a possible explanation of the cerebral edema would be the disruption of the Na+/K+ pump, and ionic imbalance [12].

Ozone exposure affected rat movements, which presented with uncontrollable motions 30 to 50 minutes. It seems that the presence of lipid peroxides, particularly MDA in brain, affects the black matter and basal ganglia, whose injury explains the uncontrollable motions in the animals [13–16].

The rats were carefully supervised through the whole experiment. In time, a loss of appetite was acknowledged, also weight loss (Figures 12 and 13) and somnolence due to elevated levels of serotonin [17] and acetylcholine [18].

Experiments performed on rats exposed for four weeks to 0.2 ppm; 0.5 ppm showed deteriorations in long term memory, decreased motility, and the adaptative activity build-up of CuZnSOD in brain and lungs. In doses of 1 ppm ozone, behavioral changes were more obvious in correlation with activity reduction in cerebral CuZnSOD [19].

The rats that were exposed for four hours at 1–1.5 ppm, had lost the primary and secondary dendrites of the granular cells in the olfactory bulb, whilst the control group presented no such alterations. Aside from these changes, we observed neuronal cytoplasmic vacuoles, inflammation of Golgi apparatus and mitochondria, as well as dilatations of rugose endoplasmic reticulum (RER). These findings show that oxidative stress alters the granular layer of the olfactory bulb, which we can correlate with functional changes [20].

The modifications we found in the liver concur with those of a reactive hepatopathy, with a rapid fibrotic transformation [21].

Conclusions

After two and four weeks of ozone exposure, we discovered the following:

• Morphological modifications in brain and liver.
• Seric oxidizing/antioxidizing imbalance, with an escalation of MDA levels, and reduction of antioxidizing reserve.
• Exposure to ozone in brain and liver determined an early appearance (two weeks) of some pathologic structural modifications. They exacerbated progressively within four weeks, but at different amplitudes.
• Brain analysis determined an enlargement of the hematoencephalic barrier and zones absent in Purkinje cells.

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References


Corresponding author
Denisa-Ioana Cretu, MD, PhD. Department of Histology, “Iuliu Hatieganu” University of Medicine and Pharmacy, 6 Pasteur Street, 400349 Cluj-Napoca, Romania; Phone +40264–595 433, e-mail: denysa_crt@yahoo.com

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