Exposure to Ozone Increases Executing Caspases 3 and 8 and Downregulates Bcl-2 in the Hippocampus, Frontal Cortex, and Cerebellum of Rats

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Research Article

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EXPOSURE TO OZONE INCREASES EXECUTING CASPASES 3 AND 8 AND DOWNREGULATES BCL-2 IN THE HIPPOCAMPUS, FRONTAL CORTEX, AND CEREBELLUM OF RATS

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ABSTRACT

Ozone (O₃) stands as one of the most prevalent atmospheric pollutants, arising from a photochemical reaction between volatile organic compounds (VOC), nitrogen oxides (NOx), and sunlight. According to the World Health Organization (WHO), exposure to O₃ levels above 100 g/m³ for 8 hours or longer proves harmful. O₃ triggers oxidative stress, resulting in lipid oxidation, inflammation, alterations in metabolic and cellular signaling, and potentially initiating cell death in vulnerable brain regions. Both, inflammation, and oxidative stress are recognized for their capacity to induce cell death, primarily through the apoptosis pathway. Apoptosis is programmed cell death characterized by caspase activation, DNA fragmentation, and the formation of apoptotic bodies. Various proteins intervene in this process along two pathways: intrinsic and extrinsic. This study aims to identify the expression of pro-apoptotic proteins and Bcl-2 in the frontal cortex, cerebellum, and hippocampus of rats exposed to O₃ acutely. Methodology: Two groups of twenty Wistar rodents (250-300 g) were established. The control group (n=10) was exposed to unrestricted polluted air for 12 hours, while the experimental group (n=10) was exposed to 1 ppm of O₃. After exposure, the animals were sacrificed for immunofluorescence and Western blot analysis. Using a t-test, the arbitrary units of pro-apoptotic proteins and Bcl-2 were compared between the two groups. Results and Conclusions: The investigation unveiled notable discrepancies in caspase-3 and 8, Bcl-2, and TUNEL. These findings lead us to conclude that acute O₃ exposure induces apoptosis in the hippocampus, cerebellum, and frontal cortex primarily via the extrinsic pathway.

KEYWORDS

apoptosis, ozone, toxicity, intrinsic pathway, extrinsic pathway, oxidative stress
INTRODUCTION

Air pollution is a global public health concern that primarily impacts urban areas. Its composition consists of PM2.5, gases, chemical compounds, and metals (Block, Michelle L. 2009). Outdoor air pollution is the ninth leading cause of mortality, contributing to 3.2 million deaths globally and inducing acute and chronic respiratory and cardiovascular diseases (Ozlem Kar Kurt, Jingjing Zhang 2016). O₃ and PM2.5 at ground level or in the troposphere are significant health hazards (González-Guevara et al. 2014; Frank J Kelly 2015; Zhang et al. 2019). Ozone is formed through a photochemical reaction involving volatile organic compounds (VOCs), nitrogen oxides (NOX), and sunlight. Since the turn of the century, the WHO (World Health Organization) has deemed ozone exposure levels >100 g/m3 or 60 ppb in an 8-hour daily average to be hazardous (World Health Organization 2022). Ozone is one of the principal tropospheric pollutants and one of the least controlled, so daily exposure exceeds prescribed limits in large cities such as Mexico City, New York, Delhi, and Beijing (Zhang et al. 2019; Peralta et al. 2021).

The pulmonary inflammatory response and oxidative injury are caused by increased soluble mediators, chemotactic factors, reactive oxygen species (ROS), and reactive nitrogen species (RNS) (Rubio and Paz 2003; Frank J Kelly 2015). Oxidizers such as O₃ can overwhelm antioxidant systems, resulting in lipoperoxidation, DNA damage, protein deamination, enzyme activity alterations, and peptide bond cleavage, all of which result in cell damage and necrosis or apoptosis (Rivas-Arancibia et al. 2003; Pereyra-Muñoz et al. 2006). Figure 1 demonstrates that after production, interleukins, prostaglandins, ROS, and RNS can readily travel through the vasculature to distal organs (Martínez-Lazcano et al. 2013). These effects also affect the Central Nervous System (CNS), resulting in deficits in learning, motor, and memory, neurodegeneration, and chronic brain plasticity and repair loss in vulnerable regions such as the olfactory bulb, striatum, cerebellum, frontal cortex, and hippocampus (Rubio and Paz 2003; Rivas-Arancibia et al. 2009; Farfán-García et al. 2014). However, specific biological damage pathways remain unidentified (Avila-Costa et al. 1999; Li et al. 2014).

![Passage of ozone oxidation products to the brain via systemic circulation. Ozone exposure triggers the activation and release of cellular oxidation-related products and the activation of immune cells. Cells such as macrophages, neutrophils,](image)
and pro-inflammatory cytokines can travel to other tissues, such as the brain, via systemic circulation. Once in the brain, they will activate inflammatory and cell death processes as well as promote reactive gliosis. PAF: Platelet activation factor. TNF: Tumor necrosis factor. INFγ: Interferon gamma; PGE2: Prostaglandin 2; IL-6: Interleukin 6; IL-8: Interleukin 8; ROS: Reactive oxygen species; RNS: Reactive nitrogen species

The effects of O₃, such as inflammation and oxidative stress, can result in neuronal apoptosis. Apoptosis is a homeostatic, energy-dependent cell death (Elmore 2007). This mechanism is unique to the activation of caspase, the fragmentation of oligonucleosomal DNA, and apoptosis (Zhao et al. 2001). Any initiator caspase activates caspase-3 and caspase-6, the main apoptosis-causing executor caspases. As we shall see, these caspases constitute the skeleton of the apoptotic pathway; consequently, their expression is essential for cell death. Nonetheless, multiple cell death pathways are possible without caspase activation (Broker et al. 2005; Zanna et al. 2005). The cell constantly monitors its external and internal environment via intrinsic and extrinsic pathways (Figure 2) and initiates apoptosis in response to any damage marker (Elmore 2007; Kaczanowski 2016).

**Fig. 2** Extrinsic and intrinsic apoptosis pathways. Extrinsic apoptosis is induced by death receptors ligands such as FAS or TNF. They bind to their receptor TRADD or FADD and then to the procaspase 8, forming the DISC activating caspase 8, which triggers the apoptosis activating the caspase 3, resulting in neuronal death. Intrinsic pathway triggers due to oxidative stress generated by excessive production of reactive oxygen species (ROS), the saturation of antioxidant systems, aerobic metabolism, and the activation of calmodulin pathway forming superoxide anion (•O₂⁻), hydrogen peroxide (H₂O₂), hydroxyl radical (•OH) and peroxynitrite (ONOO⁻), due to the entry of excessive Ca²⁺ which also stimulates the phosphorylation of kappa-N inhibitor (IκB) resulting in the upstream of Nf-κB which activates the cytokines transcription and other pro-inflammatory markers driving to chronic oxidative stress compromising the cell function, concluding in neuronal death.

The intrinsic pathway, also known as the mitochondrial pathway, destabilizes organelle dynamics. This is due to the release of cytochrome C into the cytoplasm by mitochondrial lesions (Susin et al. 1998)(Pawlowski and Kraft 2000; Cory and Adams 2002). This protein is present in mitochondria, which are essential to the electron transport chain of aerobic metabolism. The release of cytochrome C is regulated by a balance between pro-apoptotic (BAX and BAK) and anti-apoptotic (Bcl-2, Bcl-
xL, and Mcl-1) proteins (Pawlowski and Kraft 2000; Cory and Adams 2002) and molecular sensors including BIM, BID, BAM, Noxa, and Puma (BH3) (Avula 2011). In the absence of cell survival signals or lesions, like DNA damage that upregulates p53 (Vousden and Lu 2002), BH3 sensors activate and overexpress pro-apoptotic proteins. Thus, BAK/BAX channels and VDAC1 oligomerization generate the mitochondrial permeability transition pore (Kyle S. McCommis 2012), resulting in the release of cytochrome C into the cytoplasm and the activation of initiator caspase 9 (Ohtsuka et al. 2004; Michalak et al. 2008). When mitochondrial dynamics alter, anti-apoptotic proteins that prevent cytochrome C from leaving the organelle or inhibit procaspase-9 fail (Chau et al. 2000).

The extrinsic or death receptor-mediated pathway activates transmembrane receptors with cytoplasmic domains with pinpoint accuracy. Activation of apoptosis is most closely associated with the TNF-1 receptor and the Fas receptor or CD95, which are extensively investigated in immunology due to their ability to negatively select cells. These receptors' cytoplasmic domains change shape and engage intracellular proteins to form complexes (Guicciardi and Gores 2009) when activated by their ligands. Procaspase-8 interacts with this activated complex and transforms into caspase 10 in rodents and caspase 8 in humans (Widlak et al. 2005; Dickens et al. 2012).

These occurrences occur during the initial phases of apoptosis. Both pathways then converge into the signaling pathway for the execution phase. Initiated by initiator caspases 8, 9, and 10, the caspase cascade activates executioner caspases 3 and 6 regardless of the activated route (Lechardeur et al. 2000; Samejima and Earnshaw 2005). These caspases activate endonucleases, which disassemble the nucleus and eliminate DNA and RNA, and proteases, which degrade actin and tubulin in the cytoskeleton (Kothakota et al. 1997; Taylor et al. 2008). Finally, cytoplasmic vesicles disassemble the damaged cell into apoptotic bodies (Nagata 2018), which phagocytic cells consume.

This deleterious input induces apoptosis, a complex and tightly regulated cellular process that is dependent on the amount of pro- or anti-apoptotic proteins produced. During programmed cell death, the cell retracts, degrades the nuclear membrane and DNA, condenses chromatin, and attracts phagocyte lipids via signaling molecules (Lauber et al. 2003; Taylor et al. 2008). Multiple studies have demonstrated that mitochondrial dysfunction and endoplasmic reticulum stress are responsible for the mortality of hippocampus neurons after exposure to 0.25 ppm O₃ for 30 and 60 days (Rivas-Arancibia et al. 2009; Rodríguez-Martínez et al. 2016). However, it is unknown how acute inhalation of O₃ can induce CNS apoptosis. Consequently, this study investigates the role of caspase-3, caspase-8, Bax, Bel-2, cytochrome-c, and VDAC1 in the initial harm caused by acute ozone exposure in the frontal brain, cerebellum, and hippocampus of rats.

MATERIALS AND METHODS

Twenty Male Wistar rats weighing 250–300 g were our research subjects because females are more susceptible to O₃ physiological and airspace inflammation effects. All efforts were made to minimize the number of animals used and any potential pain or distress. Animals were handled and treated under institutional protocols that complied with national regulations (NOM-062-ZOO-1999) and international guiding principles (Council for International Organizations of Medical Sciences, CIOMS). Rats were individually housed and allowed to move freely in transparent cages with corn-cob bedding at 23±1°C under a 12-hour light-dark cycle (lights on at 07:00 h). Cages were cleaned once a week and kept dry. The rats were randomly divided into two groups (control and experimental) with an n = 10 per group. During the study, the subjects were allowed ad libitum access to food and water. The control group was transferred individually to hermetic chambers (30 x 25 x 30 cm) supplied with pollution-free air (1.7 l/min) under the same climatic condition and provided with free access to food and water for 12 hours. On the other hand, the experimental group was placed in individual chambers, provided with 1 ppm O₃ using a P15 TRIOZON generator (TRIOZON, Tlalnepantla, MX). O₃ concentration was measured and monitored constantly using a Serinus 10
(Ecotech, Melbourne, AU) ultraviolet light analyzer to maintain the 1ppm O3 concentration constant during the 12-hour exposure (an established parameter that triggers neuroinflammatory and neurotoxicity response). We exposed three rats per day because we only had three available chambers, taking us two weeks for the experimental phase.

Once we finished the previous step, rats were immediately sacrificed using two different techniques: ten rats (five control and five experimental) by decapitation for Western Blot and 10 rats (5 control and 5 experimental) by intracardiac perfusion for immunofluorescence. For the immunofluorescence procedures, five rats per group were anesthetized with one dose of sodium pentobarbital (50mg/kg; PiSA Agropecuaria S.A de C.V). Subsequently, the heart was exposed by making an incision at the xiphoid appendix level so that it could be perfused transcardially with heparin (5000 U/ml), followed by a Phosphate Buffer Saline (PBS) (AMRESCO, LLC) infusion of 300 ml through the right ventricle for 30 seconds and right atrial perforation. At the end of the PBS infusion, 4% paraformaldehyde (Sigma) fixation was performed using the same 300 ml volume at a temperature of 4°C. Afterward, craniotomy was performed to obtain the rats’ brain tissue from the skull, and was post-fixed for 24 hours in the same paraformaldehyde fixative at 4°C. After these procedures, tissue blocks were obtained by paraffin embedding for sagittal slicing in five µm slices using a microtome and the Paxinos and Watson rat brain atlas for guidance. Slides were baked overnight at 65 °C, followed by deparaffinization with xylene (30 baths in three stages, 5 minutes each) and rehydration through graded ethanol (First at 100% and then at 70%) to distilled water. Slides were washed in PBS and incubated for 48 hours at 4 °C.

Then, tissue was processed with their corresponding antibodies Bax (rabbit polyclonal, diluted 1:100, SC-493, Santa Cruz); cytochrome c (mouse monoclonal, diluted 1:100, SC-13156, Santa Cruz), Bcl-2 (mouse monoclonal, diluted 1:100, SC-7382, Santa Cruz), VDC-A (mouse monoclonal, diluted 1:100, SC-374343 Santa Cruz), caspase-3 (Alexa Fluor 1:100, SC-7272, Santa Cruz) and caspase-8 (mouse monoclonal, diluted 1:100, SC-56070, Santa Cruz) and incubated overnight at 4°C. After three washes of PBS for 5 minutes, the appropriate secondary antibody was applied, Rhodamine (Rhodamine Red, Anti-rabbit IgG, Jackson ImmunoResearch) for Bax, Rhodamine (Rhodamine Red, Anti-mouse IgG, Jackson ImmunoResearch) for caspase-3 and 8, cytochrome c and Bcl-2 thus giving the red fluorescence. FITC (FITC, Anti-mouse IgG, Jackson ImmunoResearch) for VDA-C showing the green fluorescence. Finally, DAPI with vectashield (ab104139) was added to all the slides. These sections were photographed and analyzed with Image-Pro Plus software (Media Cybernetics, Rockville, MD) adapted to an Olympus IX81-F3 (Olympus Corporation, Tokyo, Japan) microscope equipped with a Q-Imaging digital camera kit. The sections were observed with a 40X objective in a field of 520 mm2. The captured areas (Later 1.4 mm) were: Purkinje and granular neurons for cerebellum crus one, hippocampal neurons in Ammon's horn zone 3 or CA3; and neurons in the frontal cortex. All are captured in the most apical areas.

Once the captures were made, the protein densities were quantified using a Q-image. We performed a TUNEL assay using In Situ Cell Death Detection Kit, POD [11684795910 Roche], to detect and quantify apoptotic cell death, according to the manufacturer's instructions. The paraffin embedding slices were deparaffinized and rehydrated according to standard protocols. The brain tissue sections were incubated for 30 min at +21 to +37°C with a proteinase K working solution (10-20 μg/mL in 10 mM Tris/HCl, pH 7.4-8). We rinsed the slides twice with PBS, added TUNEL reaction mixture (50 μL) per sample, and then incubated for 60 min at +37°C in a dark and humidified chamber. After this procedure, the slides were rinsed 3 times with PBS solution, and we added 50 μL of Converter-POD, followed by incubation in a humidified chamber for 30 min at 37°C. Then, the slides were rinsed 3 times with PBS, and we added 50 μL of DAB substrate. The slides were incubated for 10 min at +15°C.
and rinsed 3 times with PBS. Finally, slides were analyzed in a drop of PBS under a fluorescence microscope using a wavelength range of 515-565 nm (green).

The Western blot technique was performed on five rats per group. Subjects were sacrificed by decapitation, extracting the frontal cortex, hippocampus, and cerebellum, placing them in an Eppendorf tube, and keeping them in a freezer at -87°C. Right after, tissues were homogenized with lysis buffer and protease inhibitor (Sigma); later, protein quantification by the Lowry method was performed using solution A (NaOH, 2% Na₂CO₃, 1% CuSO₄, 2% of sodium potassium tartrate, 10mg/20ml al 0.05% of albumin stock), solution B (Folin's reagent: H₂O, 1:1) and BSA (Bovine Serum Albumin) solution using a spectrometer. For every sample, a buffer cocktail and 2% 2-mercaptoethanol were added. Afterward, the tissue was heated in a water bath for 4 minutes; then, the samples were refrigerated for later use. The proteins were separated for SDS-polyacrylamide gel electrophoresis (10%) (Sigma Aldrich) and transferred to nitrocellulose membranes (Bio-Rad). Membranes were grouped based on their structure and were blocked with 5% skimmed milk in a TBS solution for 1 hour and incubated with the same antibodies used for immunofluorescence for anti-Bcl2, anti-caspase-3, anti-caspase-8, and anti-β-actin with a concentration of 1:1000 µL per membrane under a low shaking at 4°C. Membranes were washed for 4 cycles for 15 min each with a TBS-tween solution (250 ml of TBS and 125 µl of tween). After the washes, the corresponding secondary antibody with a concentration of 1:10000 µL per membrane was added and incubated for 2 hours. Then, the washing cycle was repeated and followed by 1 ml of the chemiluminescent solution (Sigma-Aldrich) to visualize and recognize the bands using a photographic plate (Kodak's BioMax MR Films). Finally, ImageJ software was used for quantifying optical density and compiling data using grayscales (8-bit image) and pixels as the measurement unit. We collected the error and standard deviation, gray mean, area, and density intensity for each protein. It is important to note that the immunofluorescence and Western Blot techniques were performed simultaneously. Therefore, the researchers involved in one process remained unaware of the results obtained in the other procedure until the end of the study.

STATISTICS

Pro-apoptotic proteins and Bcl-2 were expressed as mean ± standard error and analyzed using an independent samples t-test for both immunofluorescence and Western blot analysis. Levene's test was initially applied for both techniques to determine if variances were equal. As there was an equality of variances, we conducted an independent samples t-test in Excel.

RESULTS

The t-test conducted for immunofluorescence analyses revealed a significant increase in caspase-8 (Figure 3) and caspase-3 (Figure 4) activation in the O₃-exposed group compared to the control group in the frontal cortex, cerebellum, and hippocampus, respectively. Differences in caspase 3: cerebellum t (31) = 3.457, p = 0.0001, d = 0.6; frontal cortex t (28) = 3.230, p = 0.0015, d = 0.5; hippocampus t (15) = 2.822 p = 0.0064, d = 0.9. Differences in caspase 8: cerebellum t (30) = 5.605, p = 0.0000, d= 0.6; frontal cortex t (27) = 2.963, p = 0.0059, d = 0.5; hippocampus t (12) = 3.186, p = 0.003, d = 1.2. Additionally, significant changes in Bcl-2 expression (Figure 5) were observed in cerebellum t (29) = 3.456, p = 0.0008, d= 0.53; frontal cortex t (29) = 3.548, p = 0.0006, d = 0.7; hippocampus t (18) = 3.593 p = 0.001, d = 0.7. Furthermore, the TUNEL assay (Figure 6) displayed significant differences in immunopositivity among groups: cerebellum t (31) = 3.026, p = 0.0024, d = 0.8; frontal cortex t (27) = 6.042, p = 0.0000, d = 1.2; hippocampus t (13) = 2.695, p = 0.009, d = 1.3. However, intrinsic apoptotic proteins such as Bax, VDAC1, and Cytochrome-C had no significant differences or any effect size between both groups within the three analyzed structures. Furthermore, the most representative images are shown in this article, and the statistical significance of the results has been discussed previously.
Fig. 3 Caspase 8 expression in the frontal cortex. A) Immunofluorescence was taken in a field of 20µm with an approach of 40 x (p<0.05). In contrast with the control group, there was an overexpression of caspase 8 in the cells of frontal cortex rats exposed to acute O₃ (12 h). B) Western blot analysis of β–actin and caspase 8 (p<0.05). C) The graph bars show the mean number (±2 SE, 95% CI) of optical density arbitrary units (AU). kDa: kilodaltons
Fig. 4 Caspase 3 expression in the hippocampus. A) Immunofluorescence was taken in a field of 20µm with an approach of 40 x (p<0.05). Note that there were significant differences among groups in both techniques; thus, the O3 acute exposure increases the activity of caspase 3 in "Cornu Ammonis" CA1 of rats. B) Western blot analysis of β–actin and caspase 3 (p<0.05). C) The graph bars show the mean number (±2 SE, 95% CI) of optical density arbitrary units (AU). kDa: kilodaltons.

Fig. 5 Expression of Bcl-2 in the cerebellum. A) Immunofluorescence was taken in a field of 20µm with an approach of 40 x (p<0.05). There was a significant difference in the expression of Bcl-2 in the granular layer cells of the cerebellum among groups. B) Western blot analysis of β–actin and Bcl-2 (p<0.05). C) The graph bars show the mean number (±2 SE, 95% CI) of optical density arbitrary units (AU). kDa: kilodaltons.
Fig. 6 TUNEL assay of the cerebellum. Immunofluorescence was taken in a field of 20µm with an approach of 40 x (p<0.05). There were significant differences between groups showing cell positivity in the granular layer of the cerebellum in the group exposed to ozone. The graph bars indicate the mean number (SE±) of arbitrary units (UA).

The t-test in the Western Blot's protein bands analyses yielded similar results to the immunofluorescence results. Caspase 8 in cerebellum t (3) = 2.857, p = 0.03, caspase 3 in the hippocampus t (3) = 3.568, p= 0.001 and Bcl-2 in frontal cortex t (3), p=0.0009, exhibited a statistically significant higher concentration in the O₃-exposed group compared to the control group. However, the remaining proteins analyzed, such as Bax, Cytochrome-C, and VDAC1, showed no statistically significant differences in all structures.

**DISCUSSION**

According to the findings, short-term exposure to 1 ppm of ozone resulted in increased levels of both initiator caspase 8 and executor caspase 3 in structures such as the hippocampus, cerebellum, and frontal cortex of rats. By itself, caspase 3 does not differentiate whether the activated apoptotic
pathway is extrinsic or intrinsic since it can be started by caspase 8 or 9, respectively. With a discernible trend of caspase 8 overexpression in the experimental group, we can confirm that at least the extrinsic apoptotic pathway is being activated. This study indicates a prevalence of the extrinsic pathway in driving cell death, considering the absence of significant expression of pro-apoptotic proteins of the intrinsic pathway, such as BAX, VDAC1, and Cytochrome-C in these structures. Several factors may elucidate this molecular behavior; one of the reasons could be that the harmful central stimulus for ozone intoxication arises from the cell's external environment. It is worth recalling that intoxication by ozone exposure can initiate a whole cascade of pro-inflammatory cytokines within the lung region, along with immune cell activation. This unremitting pro-inflammatory cascade passes into the systemic circulation, having the capacity to reach other tissues, such as the brain. Once there, the blood-brain barrier suffers mainly endothelial damage and will then allow the passage of the pro-inflammatory products to the encephalic tissue. One of these products is TNF-α, a pivotal activator of cell death receptors, making it a potential key marker for damage in hypoxia-vulnerable structures such as the frontal cortex, hippocampus, and cerebellum. Another plausible reason for the limited involvement of the intrinsic pathway during acute O\textsubscript{3} exposure is the temporal aspect of its activity. The overheating of those systems cannot activate the intrinsic pathway because redox homeostasis is maintained, and antioxidant systems like Nrf2/ARE, SOD, and GST continue to function during acute O\textsubscript{3} exposure (Yan et al. 2016). Several reports (Re et al. 2014; Mumby et al. 2019) mention that the antioxidant activity of the Nrf2 pathway in response to low doses of O\textsubscript{3} is found after 3-6 weeks of exposure, beginning by increasing the activity of its repressor protein Keep 1 (cytosolic inhibitor). Cytochrome-C, an essential one-electron carrier, predicates its fate on its redox state—whether it exists in a reduced state, bearing an electron, or an oxidized state, devoid of an electron. Therefore, the apparent lack of activity Cytochrome-C within the experimental cohort can potentially be attributed to its association with the oxidative state it inhabits in which it is found since it must be oxidized to activate caspase 9 so intrinsic apoptosis can be initiated. However, Cytochrome-C could be reduced into the cytosol once released to maintain the cytosolic reserve against efflux. Investigations demonstrated that there are Cytochrome-C reducers such as ascorbate, superoxide, glutathione, NOS, neuroglobin, and cytochrome p450 reductase in an ozone exposure context the reduction reaction accelerates, lowering Cytochrome-C cytosolic concentration rapidly (Maureen O. Ripple, Michelle Abajian 2010). In other words, we might observe a neuroprotective behavior on behalf of the antioxidant enzymatic machinery that would prevent the propagation of apoptosis by the intrinsic pathway.

Turning to the BAX protein, previous research shows a discrepancy in space-time BAX and BAK activation because of its high relation to another member of the Bcl-2 family: Bcl-xL. This intricate dynamic eventuates in the relocation of BAX to the cytosol, effecting a regulatory process intended to stabilize the apoptotic machinery in an inert state (24,27). This could explain why it is not overexpressed in the experimental group. In contrast, the precise role of VDAC's role in apoptosis remains poorly understood, promoting debate in the scientific community if this protein opens or closes its channels at the apoptosis execution (45). There are 3 theories regarding VDAC's role; the first and the second lead to Cytochrome-c release, where VDAC induces the opening of mitochondrial external membrane channels and homo/hetero oligomerization promotes pore enlargement (46–48). The third theory, which could explain our findings, is the only one that defends that VDAC has a closure mechanism in apoptosis that leads to a high metabolite concentration that interferes with mitochondrial volume increase. This eventuality precipitates the disruption of the external membrane, culminating in the release of Cytochrome-c into the cytosol (Kyle S. McCommis 2012).

Although the intrinsic apoptotic pathway does not seem to be active in our results, the anti-apoptotic protein Bcl-2, which participates directly in this pathway by preventing the formation of the mitochondrial permeability transition pore and Cytochrome-C exit to the cytosol, is decreased in the experimental group in the three analyzed brain areas (Willis et al. 2003). The above leads us to believe that acute O\textsubscript{3} exposure has an added anti-protective effect, decreasing the chance of cell survival. This
result would reinforce the hypothesis that, although no pro-apoptotic activity of the intrinsic pathway was observed in rats exposed to ozone, this healing protective effect exerted by Bcl-2 would eventually lead to mitochondrial dysfunction with chronic ozone exposure.

Additionally, the TUNEL assay results were significantly positive for the O₃ group. This assay exposes one of the molecular characteristics of apoptosis: nuclear DNA fragmentation, facilitated by nucleases (Majtnerová and Roušar 2018). Our result confirms the caspases’ participation, as they are known to activate the nucleases in the execution phase (Kyrylkova et al. 2012). Finally, the results support all the clinical implications of O₃ exposure. Let us not overlook the fact that apoptosis assumes an essential role in the disease’s development within various neurological pathophysiological mechanisms, especially in neurodegenerative diseases. In these neurological diseases, pro-inflammatory markers such as TNF-α, IL-1β, IL-2, INFγ, NOS, and ROS are increased according to brain postmortem samples recollected (Gelders et al. 2018). Such markers are recognized as involved in cellular programmed death by the pro-oxidant effects O₃ exerts, provoking its exposure to be a significant risk factor for illness progression and development (Avila-Costa et al. 1999).

Several studies establish a link between ROS production due to O₃ and consequent disruptions in enzymatic function, lipoperoxidation, and protein oxidation, culminating in the loss of primary and secondary dendritic spines, provoking neurodegeneration (Croze and Zimmer 2018). Endoplasmic reticulum (ER) dysregulation and hippocampus stress are two cellular damage mechanisms in other acute and chronic neural diseases. Chronic O₃ exposure can imitate this mechanism by increasing intracellular calcium concentration and activation of the ATF6 pathway due to chronic oxidative stress, primarily due to the effect of increased ER calcium by TNF-α (Lauber et al. 2003). The ATF6 pathway regulates cell death, and when this system suffers a critical breakdown, Ca²⁺ entries into the ER and activates the apoptotic pathway by caspases induction (Galluzzi et al. 2014).

Likewise, it has been observed that the cerebellum is a structure that is quite affected because its neurotransmitter dynamics in newborn rats, particularly GABA and glutamate modulation on Purkinje cells, are altered by O₃ exposure (Gonzalez-Pina et al. 2008). These can lead to motor alterations such as ataxia and limited cortical brain recovery after damage. The behavioral area has also studied ozone effects in animals and humans; Wei-Cheng et al. (Wei-Cheng X., Xiao-Wen L., Qun-Bo W. 2013) found behavior alterations associated with neurotoxicity related to neuron apoptosis after administrating a high intrathecal dose of O₃. After 6 hours of ozone exposure in rats for 5 days, a significant increase in TNF-α and IL-6 in the frontal cortex was observed. Those cytokines induce an inflammatory response in the CNS with apoptosis activation (González-Guevara et al. 2014).

Furthermore, the domain of sleep has been subjected to investigation in the context of ozone intoxication rats. Studies have shown that exposures ranging from 0.5-1.0 ppm of O₃ can suppress rapid eye movement (REM) sleep and increase non-REM patterns, mainly due to decreased concentrations of acetylcholine in the hippocampus and increased concentrations of serotonin and dopamine in the brain stem (Huitrón-Reséndiz et al. 1994; Rubio and Paz 2003).

CONCLUSION

One of the main contemporary challenges stems from atmospheric changes resulting from excessive human activity, which significantly impacts public health. Tropospheric ozone is one of the primary pollutants in large cities, and its harmful pro-oxidant effects on pulmonary pathology have been widely studied; however, the mechanisms of damage in other tissues, such as the CNS, are still unknown.

The present study shows that acute exposure to ozone at levels of 1ppm can detonate the neuronal apoptotic machinery in rats’ frontal cortex, hippocampus, and cerebellum. This mechanism is due to the activation of the extrinsic pathway of apoptosis given by the initiator caspase 8 and the executor...
caspase 3. At the same time, an anti-protective effect is observed by the downregulation of Bcl-2 expression, an essential anti-apoptotic protein within the intrinsic apoptosis pathway. Although no participation of pro-apoptotic proteins of the intrinsic pathway such as BAX, VDAC, or Cytochrome-C was observed, we think that chronic exposure to oxidative stress by O$_3$ may be able to promote cell death by adding activation of this pathway, so long-term research needed to prove this hypothesis.

Furthermore, our study suggests that apoptosis activated by O$_3$ exposure can be considered a relevant cellular mechanism in which ozone intoxication can be involved in developing neurological disorders such as neurodegenerative or neurodevelopmental diseases.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Animals were handled and treated following institutional protocols from the committee of the National Institute of Neurology and Neurosurgery “Manuel Velasco Suárez” CICUAL, that complied with national regulations (NOM-062-ZOO-1999) and international guiding principles (Council for International Organizations of Medical Sciences, CIOMS).

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REFERENCES


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Data Availability

“The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.”