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Effect of oseltamivir and some vitamins on Dopamine, GSH and Lipid Peroxidation levels in young animal models exposed to O₃

Efecto de oseltamivir y algunas vitaminas en los niveles de dopamina, GSH y peroxidación lipídica en modelos de animales jóvenes expuestos al O₃

David Calderón Guzmán*, Jonas Attilus*, Hugo Juárez Olguín**, Ernestina Hernández García**, Norma Osnaya Brizuela*, Maribel Ortiz Herrera***, Gerardo Barragán Mejía*

ABSTRACT

Oseltamivir is used for the treatment of AH1N1 swine influenza in Mexico. The aim of the present study was to analyze the effect of oseltamivir on dopamine levels in patients exposed to vitamins A, D, and C as well as to ozone. For this, forty male Wistar rats (80 g) were divided into 6 groups. A combination of oseltamivir (20 vmg/kg) with A (39 vOUI.), C (30 mg) and D (60 UI.) vitamins with or without ozone 0.5 ppm were intraperitoneally administered for 5 days. The exposition to ozone was through a camera controlled. The animals were sacrificed and the brain dissected in cortex (I), hemispheres (II) and cerebellum/medulla oblongata (III) to measure dopamine, glutathione (GSH) and lipoperoxidation using fluorescence. Dopamine increased in I, II and III regions while GSH increased only in II and III regions and lipoperoxidation decreased in the same regions. This changes were significant ($p < 0.05$) in the groups that received oseltamivir and ozone. Administration of oseltamivir and ozone induces synergic effect on dopamine metabolism.

RESUMEN

El oseltamivir, para tratar influenza AH1N1 en México y su efecto sobre la dopamina en sujetos expuestos a ozono y vitaminas A, C y D, es desconocido, y por tal motivo fue el objetivo del presente trabajo. Se manipularon 40 ratas Wistar macho jóvenes (80 g) y se dividieron en seis grupos. El oseltamivir (20 mg/kg) y una mezcla de vitaminas A (390 UI), C (30 mg) y D (60 UI) se administraron con y sin exposición a ozono (0.5 ppm), i.p. durante cinco días. El ozono se aplicó con una cámara especial. Los animales fueron sacrificados, y el cerebro fue diseccionado en corteza (I), hemisferios (II) y cerebelo/tallo (III) para medir la dopamina, glutatión (GSH) y la lipoperoxidación con fluorescencia. En regiones I, II y III, la dopamina aumentó. En II y III GSH se incrementó y la lipoperoxidación disminuyó. Todos los cambios fueron significativos ($p < 0.05$) en los grupos tratados con oseltamivir y ozono. Se concluye que la administración de oseltamivir y ozono induce efecto sinérgico sobre el metabolismo dopaminérgico.

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Palabras clave:

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INTRODUCTION

Oseltamivir, a neuraminidase inhibitor, is effective for the treatment of both seasonal flu and H1N1 influenza A virus infections. Oseltamivir is generally well tolerated, and its most common adverse effects are nausea and vomiting. However, neuropsychiatric behaviors including jumping and falling from balconies by young patients being treated with oseltamivir have been reported (Yoshino, Nisijima, Shioda, Yui & Kato, 2008). Yoshino *et al.* (2008) suggested that the increase in dopamine during oseltamivir treatment may have caused abnormal behaviors in young patients. The pharmacological mechanism of the neuropsychiatric effects of oseltamivir in adults and in very young pediatric population remains unclear. Oseltamivir (Tamiflu) is now being stockpiled by Mexican government as a first line treatment for an anticipated outbreak of swine influenza caused by AH1N1. Which, came

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into effect in late March 2009 due to an outbreak of a respiratory illness that was later proved to be caused by H1N1 (S-OIV) virus – a novel swine-origin influenza A. 64% of chronic obstructive pulmonary disease (COPD) exacerbations are caused by respiratory infections including influenza (strains A and B). These infections affect the airway epithelium and provoke inflammatory and apoptosis events through mechanisms involving the generation of reactive oxygen species (ROS) (Mata, Morcillo, Gimeno & Cortijo, 2011) implicated in the deterioration of the patient's health during the course of the disease.

Environmental pollution or exposure to ozone generates reactive oxygen species and other oxidative stressors. Which, may initiate and augment inflammation, and may also result from inflammation (Dozor, 2010), and influence neurotransmitter systems (Biermann *et al.*, 2009). Many therapeutic strategies such as dietary changes and the use of antioxidant vitamins to decrease oxidative stress have been suggested (Chhabra, Yasir, Chaudhry & Shah, 2010), but above all, minimizing exposure of young children to environmental ozone remains paramount.

When patients are admitted in the Intensive Care Unit for complications of pneumonia resulting from seasonal flu, it is common to find the clinical conditions of vitamins in deficiency even when the minimum daily requirements had been met (Thompson *et al.*, 2003). Based on this, it was suggested that daily parenteral supplements of these elements should be higher than those recommended by the American Medical Association (AMA) (Henche-Morilla, Romero-Montero & Llorente-González, 1990). Likewise, there are some supplements with A, C and D vitamins like Aderogyl® for daily intake to offset this deficiency (Vademecum, 2009).

Glutathione (GSH) is the main redox equilibrium regulator which plays an important role in the protection of tissues suffering from damage by oxidative agents. It is also a ubiquitous reducing agent whose absence induces severe oxidative stress (OS) (Driver, Kodavanti & Mundy, 2000).

Recent studies indicated that the use of micronutrients induces defensive mechanisms to the brain by diminishing free radical-induced lipid peroxidation (Bediz, Baltaci, Mogulkoc & Öztekin, 2006). Free radicals (FR) are reactive oxygen or nitrogen species with impaired electrons, which may induce oxidative damage to biologically important molecules, though membrane lipids are the main target (Beckman, Beckman,

Chen, Marshall & Freeman 1990). The central nervous system (CNS) is particularly susceptible to this type of damage.

It is necessary to determine the effects of oseltamivir and vitamins under ozone conditions so as to establish methods for its safe administration. Consequently, the aim of this study is to determine the effect of these substances, under experimental ozone exposure, on dopamine levels and some biomarkers of oxidative stress in juvenile rat brain regions.

MATERIAL AND METHODS

Fourty male Wistar rats each with a weight of 80 g were recruited and divided into six experimental groups in absence and presence of ozone as follows: Group 1 ($n=6$), control, Oseltamivir (20 mg/kg); group 2 ($n=7$), Aderogyl® (150 ml/rat); group 3 ($n=7$); group 4 ($n=6$), control group ($n=7$), Ozone + Oseltamivir (20 mg/kg); group 5 ($n=7$), Ozone + Aderogyl® (150 ml/rat); group 6 ($n=7$). All treatments were given intraperitoneally every 24 h for 5 days. (Each 150 μ l Aderogyl solution contains vitamins A (390 UI.), C (30 mg) and D (60 UI)). The rats were sacrificed by decapitation 30 min after receiving the last dose of oseltamivir and Aderogyl®. The brains were extracted and preserved in NaCl 0.9% at 4 °C. Brain dissections were done in cortex, hemispheres, cerebellum/medulla oblongata and were homogenized in 10 volumes of TRIS-HCl 0.05 M, pH 7.4 for the assessment of lipid peroxidation (TBARS). An aliquot was combined with perchloric acid (HClO_4) 0.1 M, (50:50 v/v) to evaluate the levels of Glutathione (GSH) and dopamine. All experimental procedures were carried out with strict adherence to the rules for Laboratory Animals Use and Care Committee of international institutions.

Dopamine levels were measured from the supernatant of tissue homogenized in HClO_4 after centrifugation at 9000 rpm for 10 min in a microcentrifuge (Hettich-Zentrifugen, model Mikro 12-42, Germany), with a version of the technique reported by Calderon *et al.* (2008). An aliquot of the HClO_4 supernatant, and 1.9 ml of buffer (0.003 M octyl sulphate, 0.035 M KH_2PO_4 , 0.03 M citric acid, 0.001 M ascorbic acid), were placed in a test tube. The mixture was incubated for 5 min at room temperature in total darkness, and subsequently, the samples were read in a spectrofluorometer (Perkin Elmer LS 55, England) with 282 nm excitation and 315 nm emission lengths. The FL Win Lab version 4.00.02 software was used. Values were inferred in a previously standardized curve and reported as nM/g of wet tissue.

The levels of GSH were measured from a sample of the floating tissue homogenized in HClO_4 which was got after being centrifuged at 9000 rpm for 5 min (in a microcentrifuge Mikro 12-42, Germany), according to the technique reported by Hissin & Hilf (1976). 1.8 ml of Phosphate Buffer at pH 8.0 with EDTA at 0.2%, an aliquot of 20 μl of the floating tissue in HClO_4 , and 100 μl of ortho-phthalaldehyde (OPT) in concentration of 1mg/ml in methanol, were put in an assay tube and incubated for 15 min at ambient temperature in total darkness. At the end of incubation, the samples were read in a Perlin Elmer LS 55 spectrofluorometer with excitation longitude of 350 nm and emission of 420 nm. FL Win Lab version 4.00.02 software was used. The values were inferred in a previously standardized curve and were reported in nM/g of wet tissue.

Technique for the measurement of lipid peroxidation (TBARS) determination was carried out using the modified technique of Gutteridge & Halliwell (1990) as described below: From the homogenized brain in tris-HCl 0.05 M pH 7.4, 1 ml was taken and to it was added 2 ml of thiobarbaturic acid (TBA) containing 1.25 g of TBA, 40 g of trichloroacetic acid (TCA), and 6.25 ml of concentrated chlorhydric acid (HCL) diluted in 250 ml of deionized H_2O . They were heated to boiling point for 30 min (Thermomix 1420). The samples were later put in ice bath for 5min. and were centrifuged at 700 g for 15 min (Sorvall RC-5B Dupont). The absorbances of the floating tissues were read in triplicate at 532 nm in a spectrophotometer (Helios- α de UNICAM). The concentration of reactive substances to the thiobarbaturic acid (TBARS) was expressed in μM of Malondialdehyde/g of wet tissue.

For the analysis of results Kruskal-Wallis statistical test and analysis of variance (ANOVA) with their respective contrasts after being subjected to variances homogeneity test were used. The values of $p < 0.05$ were considered statistically significant (Castilla-Serna & Cravioto, 1991). To carry out the tests, JMP Statistical Discovery Software version 6.0.0 from SAS was used.

RESULTS

The levels of dopamine on brain regions (cortex, hemispheres and cerebellum/medulla oblongata) of group of young rats treated with oseltamivir and aderogyl® in the presence of air and ozone (figure 1) increase significantly ($p < 0.001$) in Kruskal-wallis test with respect to the control group in the presence or absence ozone. In the same regions, this biomarker decreased

significantly ($p < 0.02$) in the groups of rats that received oseltamivir alone with respect to the group that received oseltamivir plus aderogyl®.

Figure 2 shows the GSH levels in brain regions of young rats treated with oseltamivir and aderogyl® in the presence of air and ozone. GSH increased significantly under Kruskal-wallis test ($p < 0.001$), in hemispheres and cerebellum/medulla oblongata regions of the group that received oseltamivir in the presence of ozone as well as in those that received aderogyl® alone when both are compared with the control group.

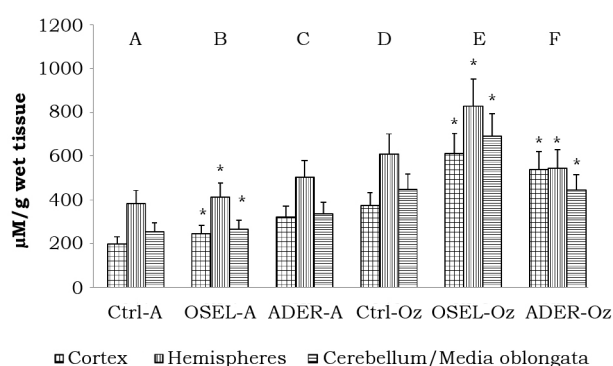


Figure 1. Dopamine levels brain regions of young rats treated with oseltamivir and aderogyl® in the presence of air (A) and ozone (Oz). Mean values \pm SD. Ctrl=Control, Osel=Oseltamivir, Ader=Aderogyl®. * $p < 0.05$ Kruskal-Wallis test. Cortex * $p < 0.0001$, F \neq C, D. E \neq B, D. B \neq C, Hemispheres * $p < 0.0001$, F \neq C. E \neq B, D, F. B \neq C, Cerebellum/Medulla oblongata * $p < 0.001$, F \neq C. E \neq B, D, F. B \neq C.

Source: Author's own elaboration.

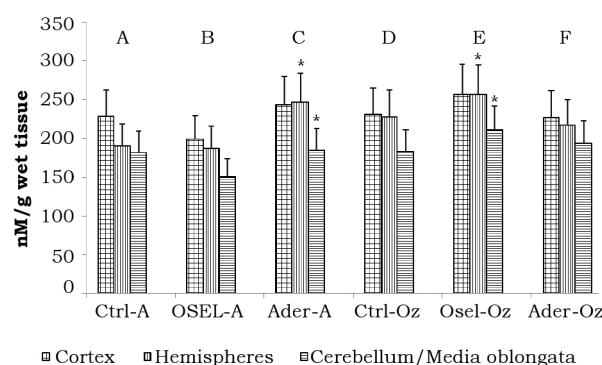


Figure 2. GSH levels in brain regions of young rats treated with oseltamivir and aderogyl® in the presence of air (A) and ozone (Oz). Mean values \pm SD. Ctrl=Control. Osel=Oseltamivir, Ader=Aderogyl®. * $p < 0.05$ Kruskal-Wallis test. Hemispheres * $p < 0.0001$, E \neq B, D and F. C \neq B, F, Cerebellum/Medulla, Oblongata * $p < 0.003$, E \neq B, D. C \neq B.

Source: Author's own elaboration.

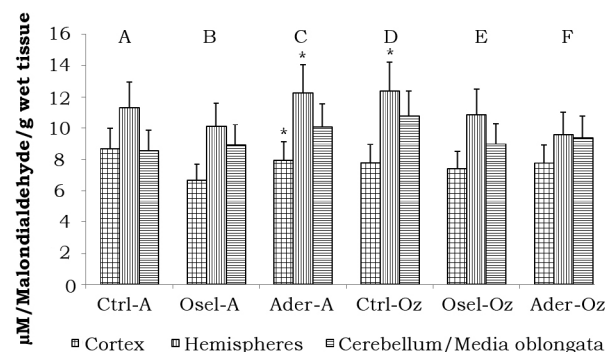


Figure 3. TBARS levels in brain regions of young rats treated with oseltamivir and aderogyl® in the presence of air (A) and ozone (Oz).

Mean values \pm SD. Ctrl = Control, Osel = Oseltamivir, Ader = Aderogyl®. * $p < 0.05$ Kruskal-Wallis test. Cortex * $p < 0.005$, C \neq B, Hemispheres * $p < 0.0001$, D \neq E, F, C \neq B.

Source: Author's own elaboration.

The concentration of lipid peroxidation on brain regions of young rats treated with oseltamivir and aderogyl® in the presence of air and ozone (figure 3) showed a significant decrease ($p < 0.001$), in cortex and hemispheres regions on the application of Kruskal-Wallis test in the group of rats that received oseltamivir in presence of air or ozone with respect to the control group. However, the group that received aderogyl® alone showed a significant increase ($p < 0.005$) in this biomarker.

DISCUSSION AND CONCLUSIONS

Neurological complications can occur following respiratory tract infection by the novel influenza A (H1N1) virus and the central nervous system side-effects observed with oseltamivir phosphate and active metabolite could be due to inflammation in the brain (Oshima, Nemoto, Kuramochi, Saitoh & Kobayashi, 2009). Ozone elicits a broad spectrum of airway antioxidant responses, with initial losses of vitamin C followed by a phase of augmentation of low-molecular-weight antioxidant concentrations at the air-lung interface (Behndig *et al.*, 2009).

The concentration of dopamine increased with oseltamivir treatment combined with ozone in all brain regions and this may probably due to the effects of this drug on neurons that contain dopamine hydroxylase and phenyletanolamine N-methyl transferase enzymes that change dopamine to noradrenaline and adrenaline, respectively (The Biochemical Pathways Charts [BPC], 1992). The dopamine levels decreased with the vitamin treatments in the group that received ozone exposition. These results suggest that there is a protective effect against the presence of external radicals and those

coming from endogenous metabolism as reported by Brook *et al.* (2009), and Valacchi, Pecorelli, Mencarelli, Maioli & Davis (2009). Indeed, dietary supplementation with antioxidant vitamins A, C and D, affords variable degree of protection against this enhancement.

The presence of ozone combined with oseltamivir induced increase of dopamine. Both substances could offer an additive effect. This increase is in accordance with the reports of Yoshino *et al.* (2008) who proposed that the increase in dopamine during oseltamivir treatment may have caused abnormal behaviors in young patients. Therefore, in cases where oseltamivir is prescribed to children, close observation is required.

We know that oseltamivir produces side effects on the central nervous system, especially when combined with other agents as ethanol (Izumi, Tokuda, O'Dell, Zorumski & Narahashi, 2007). It is necessary to take into account that there are genetic differences between Japanese and Caucasian patients. This gives rise to different levels of oseltamivir and/or oseltamivir carboxylate in the CNS and also accounts for the differences in their metabolism or pharmacological activity in the CNS as well as in the low capacity to convert oseltamivir to oseltamivir carboxylate in rat and human brains (Toovey *et al.*, 2008). Based on the results of the present study, we suggest that the combination of oseltamivir with ozone induces changes in the dopaminergic receptors and at the same time, provokes pro-oxidant effect in the brain.

The concentration of GSH in the brain was increase in ozone experimental groups that received oseltamivir, probably due to the accumulation of this drug in the brain. This is in accordance with the findings in the study of Morimoto *et al.* (2008) who suggested that the interindividual variation of P-glycoprotein activity may be an important factor determining susceptibility to the CNS side effect of this drug, and therefore might play a role in increasing the accumulation of oseltamivir in the brain, as a consequence of blood brain barrier immaturity in young animals (Johanson, 1980).

Some studies found that the presence of oseltamivir in brain reduced the generation of endogenous nitric oxide (NO) (Kacergius, Ambrozaitis, Deng & Gravenstein, 2006) suggesting that this increase could have uplifted the protective effect as was suggested by the findings of Ju, Chen, Liu, & Yang (2005), who proposed that S-nitrosoglutathione (GSNO), a potent endogenous antioxidant derived from the interaction between nitric oxide and glutathione, caused dose-dependent protective effects against amiloid beta (Abeta)/ceramide neurotoxicity via inhibition of caspase activation and production

of reactive oxygen species (ROS). This GSNO-mediated neuroprotection appeared to involve activation of cGMP-dependent protein kinase (PKG), phosphatidylinositol 3-kinase (PI3K), and extracellular signal-regulated kinase (ERK).

The levels of lipid peroxidation decreased in hemispheres and cerebellum/medulla oblongata of animals with ozone exposure by the administration of oseltamivir or vitamins. This biomarker showed opposite results with GSH levels. Decrease of lipoperoxidation was more evident in ozone exposed groups treated with oseltamivir and vitamins due to additive effects. These results suggest that the downward tendency of lipoperoxidation is due to the antioxidant effect induced by the carboxylate of oseltamivir, the active metabolite of the drug (Fuks, Ihama & Miyazaki, 2008), whose chemical characteristic is the attraction of electrons to the same molecule.

The results of the present study using a condition of ozone exposure suggest that oseltamivir and vitamins could be combined in the treatment of influenza A (H1N1) virus infection, because of induction of antioxidant effect in the brain. This is to say that if oseltamivir is administered to urban children with environmental ozone exposure, as first choice drug for the treatment of influenza, and these children consume vitamins in their daily diet, a beneficial effect on the brain will be induced by the combination of these substances as suggested by the results of this study.

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