EFFECT OF AN ANTIVIRAL AND VITAMINS A, C, D ON DOPAMINE AND SOME OXIDATIVE STRESS MARKERS IN RAT BRAIN EXPOSED TO OZONE

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Abstract - We measured the effect of an antiviral drug and vitamins on dopamine, 5-HIAA and some oxidative biomarkers in the brain of rats exposed to ozone. Groups of Wistar rats received intraperitoneally for 5 days: G1 (control) - 0.9% NaCl; G2 - oseltamivir (an antiviral); G3 - multivitamins (vitamins A, C and D). A similar assay was realized in rats exposed to ozone. The brain was excised and dissected into the cortex, hemispheres, cerebellum and medulla/oblongata to measure the levels dopamine, GSH and lipid peroxidation. Levels of 5-HIAA increased in the cerebellum/medulla oblongata of rats that received vitamins and ozone. Dopamine increased in all regions of the groups that received oseltamivir in the presence of ozone. GSH increased in the hemispheres and cerebellum/medulla oblongata only in groups that received oseltamivir and ozone. We found that vitamins and oseltamivir altered serotonin metabolism in the brains of young rats. Oxidative stress may be involved in these effects.

Key words: Dopamine, lipid peroxidation, oseltamivir, ozone, vitamins

INTRODUCTION

Oseltamivir, a neuraminidase inhibitor, is an effective antiviral, amply used for the treatment of both seasonal flu and H5N1 influenza A virus infections. The most common adverse effects are nausea and vomiting, however, neuropsychiatric behaviors such as jumping and falling from balconies have been reported in young patients (Yoshino et al., 2008), suggesting that the increase in dopamine in oseltamivir treatment may lead to abnormal behavior in young patients. The pharmacological mechanism of the neuropsychiatric effects of oseltamivir remains unclear not only in adults but also in the very young pediatric population. Today, oseltamivir is being stockpiled by Mexican governments as a first line of treatment for an anticipated outbreak of swine influenza caused by AH1N1, which came into effect in late March 2009 due to an outbreak of a respiratory illness that was later proved to be caused by the H1N1 (S-OIV) virus – a novel swine-origin influenza A. 64% of chronic obstructive pulmonary disease (COPD) exacerbations are attributed to respiratory infections, including influenza (strains A and B). These infections affect the airway epithelium, provoking inflammatory and apoptotic events through mechanisms involving the generation of reactive oxygen species (ROS) involved in the deterioration of a patient’s health during the course of the disease (Mata et al., 2011).

Environmental pollution or exposure to ozone generates reactive oxygen species and other oxida-
tive stressors, which may initiate and augment inflammation (Dozor, 2010), thereby altering the neurotransmitter systems (Biermann et al., 2009). Many therapeutic strategies to decrease oxidative stress have been suggested. Such strategies include dietary changes, use of antioxidant vitamins and minimizing the exposure of young children to environmental ozone (Chabra et al., 2010).

It is common to find the clinical parameters of vitamin deficiency, even when the minimum daily requirements are met, in patients admitted to the Intensive Care Unit due to complications of pneumonia as a consequence of seasonal flu (Thompson et al., 2003). For this, it was suggested that daily parenteral supplements of these elements should be higher than those recommended by the American Medical Association (AMA) (Hence-Morilla et al., 1990). Likewise, there are some supplements with A, C and D vitamins such as Aderogyl® for daily intake that are applied to offset this deficiency (PLM, 1999). GSH is the main redox equilibrium regulator and protector of the tissues suffering from damage by oxidative agents. It is also a ubiquitous reducing agent and the absence can occur in severe oxidative stress (OS) (Driver et al., 2000).

Recent studies have indicated that the use of micronutrients induces defensive mechanisms in the brain by diminishing free radical-induced lipid peroxidation (Bediz et al., 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 et al., 1990), and the central nervous system (CNS) is particularly susceptible to this type of damage.

Membrane lipids are known to strongly interact with the lipid bilayer structural proteins (Swapna et al., 2005), such as Na⁺-K⁺ ATPase, which is responsible for ion interchange across the membrane (Neault et al., 2001). The inhibition of Na⁺-K⁺ ATPase promotes the excitatory amino acid release in CNS (Hernandez, 1982), which induces antioxidant activity on damaged tissues (Muñoz et al., 2006). Therefore, it is necessary to determine the effects of oseltamivir and vitamins in order to establish methods for safe administration using experimental ozone exposure. The aim of this study rose from the above necessity and focused on determining the effects of these substances on dopamine levels and some biomarkers of oxidative stress in the brain regions of juvenile rats.

MATERIALS AND METHODS

Forty male Wistar rats, each weighing about 80 g, were divided into six experimental groups, three were maintained in the absence, and three in the presence of ozone, and were given oseltamivir and Aderogyl® as follows: Group 1 (n=6) control; group 2 (n=7) oseltamivir (20 mg/kg); group 3 (n=7) Aderogyl® (150 ml/rat). All treatments were given intraperitoneally every 24 h for 5 days. Each 150 μl of aderogyl solution contained a mixture of vitamins A (390UI), C (30mg) and D (60UI). The rats were killed by decapitation 30 min after receiving the last dose of oseltamivir and aderogyl® and the brains were extracted and placed in 0.9% NaCl at 4°C. Brain dissection was carried out in the cortex, hemispheres, cerebellum/medulla oblongata; samples were homogenized in 10 volumes of TRIS-HCl 0.05M, pH 7.4 for the assessment of lipid peroxidation (TBARS) and Na⁺, K⁺ ATPase. An aliquot of this was taken and combined with perchloric acid (HClO₄) 0.1 M, (50:50 v/v) to measure the levels of glutathione (GSH), dopamine and 5-HIAA. All experimental procedures were carried out according to the rules of the Laboratory Animals Use and Care Committee of international institutions.

Measurement of dopamine (DA)

The DA levels were measured in the supernatant of tissue homogenized in HClO₄ after centrifugation at 9000 rpm for 10 min in a microcentrifuge (Hettich Zentrifugen, model Mikro 12-42, Germany), based on the technique reported by Calderon et al. (2008). An aliquot of the HClO₄ supernatant and 1.9 ml of buffer (0.003 M octyl-sulphate, 0.035 M KH₂PO₄, 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) at 282 nm excitation and 315 nm emission. 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.

**Measurement of 5-hydroxyindol acetic acid (5-HIAA)**

The levels of 5-HIAA were measured in the supernatant of tissue homogenized in HClO₄ after centrifugation for 10 min in a microcentrifuge (Mikro 12-42, Germany), with a modified version of the technique reported by Beck et al. (1977). An aliquot of the HClO₄ supernatant and 1.9 ml of acetate buffer 0.01M pH 5.5 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 296 nm excitation and 333 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 nMoles/g of wet tissue.

**Measurement of glutathione (GSH)**

The levels of GSH were measured from a sample of the floating tissue homogenized in HClO₄ obtained after centrifugation for 5 min in a microcentrifuge (Mikro 12-42, Germany), according to the technique reported by Hissin and Hilf (1976). An aliquot of 20µl of the floating tissue in HClO₄ with 1.8ml of phosphate buffer at pH 8.0 with EDTA at 0.2%, and 100µl of ortho-phthalaldeide (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 the incubation, the samples were read in a Perkin Elmer LS 55 spectrofluorometer (excitation 350nm; emission of 420 nm). FL Win Lab version 4.00.02 software was used. The values were obtained from a previously standardized standard curve and were reported in nM/g of wet tissue.

**Measurement of lipid peroxidation (TBARS)**

TBARS determination was carried out using the modified technique of Gutteridge and Halliwell (1990), as described below. From the homogenized brain in TRIS HCl 0.05 M pH 7.4, 1 ml was taken and mixed with 2 ml of thiobarbituric acid (TBA) containing 1.25 g of TBA, 40 g of trichloroacetic acid and 6.25 ml of concentrated HCl diluted in 250 ml deionized H₂O. The mixture was heated to boiling (Thermomix 1420). The samples were placed in an ice bath for 5 min and centrifuged at 700 g for 15 min (Sorvall RC-5B Dupont). The absorbance of the floating tissues was read in triplicate at 532 nm in a spectrophotometer (Helios de UNICAM). The concentration of reactive substances to the thiobarbituric acid (TBARS) was expressed in µM of malondialdehyde/g of wet tissue.

**Measurement of total ATPase**

The technique was carried out by using approximately 1 mg of the brain homogenate in 0.05M TRIS HCl at pH 7.4. This was incubated for 15 min in a medium that contained 3 mM MgCl₂, 7 mM KCl, 100 mM NaCl, with or without ouabain 0.06 mM; 4 mM of TRIS-ATP was added to the homogenate after 15 min of incubation; the sample was incubated for 30 min at 37ºC with agitation in a Dubnoff Lab-conco water bath. The reaction was stopped by the addition of 100 µl of 10% trichloroacetic acid. The samples were centrifuged at 3500 rpm for 5 min at 4 ºC (Calderon-Guzman et al., 2005), and an aliquot of the floating tissue was used to measure inorganic phosphate (Pi) using the method proposed by Fiske and Subarrow (1925). The absorbance of the floating tissue was measured at 660 nm using Helios of UNICAM spectrophotometer. Total ATPase absorbance was then measured in the absence of ouabain, and their activity was expressed in µM Pi/g of wet tissue/min.

**Analysis of results**

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

RESULTS

The levels of dopamine in brain regions of young rats treated with oseltamivir and aderogyl® in the presence of air and ozone is shown in Fig. 1. The concent-

Fig. 1. Dopamine levels in brain regions of young rats treated with oseltamivir and aderogyl® in the presence of air (A) and ozone (O). Mean values ± SD. Ctrl = Control, Osel = Oseltamivir, Ader = Aderogyl®. $^*p<0.05$ Kruskal-Wallis test.

Fig. 2. 5-HIAA levels in brain regions of young rats treated with oseltamivir and aderogyl® in the presence of air (A) and ozone (O). Mean values ± SD. Ctrl = Control, Osel = Oseltamivir, Ader = Aderogyl®. $^*p<0.05$ Kruskal-Wallis test.
Concentration of DA in the cortex, hemispheres and cerebellum/medulla oblongata increased (p<0.001) after the Kruskal-Wallis test for the groups of rats that received oseltamivir or aderogyl® plus ozone, compared to the control groups. In the same regions, this biomarker decreased significantly (p<0.02) in the groups of rats that received oseltamivir when compared with the group that received aderogyl®. The concentration of 5-HIAA increased (p<0.05) in cerebellum/medulla oblongata of the group that received vitamins and ozone (Fig. 2).

The levels of GSH in the hemispheres and cerebellum/medulla oblongata increased in young rats...
treated with aderogyl® and oseltamivir in the presence of ozone and in those treated with aderogyl® in the presence of air under the Kruskal-Wallis test (p<0.001) when compared with the control group (Fig. 3).

The concentration of lipid peroxidation in the young rats treated with oseltamivir and aderogyl® in the presence of air and ozone decreased (p<0.001) in the cortex and hemisphere regions on application of the Kruskal-Wallis test compared to the control group (Fig. 4). In addition, in the same regions this biomarker increased (p<0.005) in the group treated with aderogyl®.

The activity of total ATPase increased in the hemisphere regions of the groups that received oseltamivir or aderogyl® combined with ozone, while in the cerebellum/medulla oblongata regions this activity declined in the group that received oseltamivir in the presence of ozone; the Kruskal-Wallis test showed differences (p=0.007) with respect to the control group (Fig. 5).

**DISCUSSION AND CONCLUSIONS**

It has been reported that neurological complications can occur following respiratory tract infection by the novel influenza A (H1N1) virus, and that the central nervous system side effects observed with oseltamivir phosphate and its active metabolite are probably due to their inflammatory effect (Oshima et al., 2009). Ozone elicits a broad spectrum of airway antioxidant responses, with an initial loss of vitamin C, followed by a phase of augmentation of low-molecular-weight antioxidant concentrations at the air-lung interface (Behndig et al., 2009).

In our study, the concentration of dopamine increased with oseltamivir treatment in presence of ozone in all brain regions. Probably this effect occurred on neurons that contain dopamine hydroxylase and phenylethanolamine N-methyltransferase, in whose metabolic pathways dopamine is changed to noradrenaline and adrenaline, respectively (BPC, 1992). The levels of dopamine decreased in rats with vitamin treatments in the group exposed...
to ozone. These results are in accordance with the reports of Brook et al. (2009) and Valacchi et al. (2009) who suggested that there is a protective effect against the presence of endogenous metabolic and external radicals. Indeed, dietary supplementation with antioxidant vitamins A, C, and D provides a variable degree of protection against this enhancement.

The presence of ozone combined with oseltamivir induced an increase of dopamine. This means that both substances have an additive effect. Its increase agrees with the reports of Yoshino et al. (2008), who found the same increase in their study and proposed that this increase in dopamine during oseltamivir treatment may have caused abnormal behaviors in young patients. Consequently, the prescription of oseltamivir in the treatment of viral ailments in children requires a strict measure of observation and vigilance.

The levels of 5-HIAA decreased with the administration of vitamins in the absence of ozone and increased when exposed to this substance. These findings are in accordance with previous studies made in our lab (Barragan-Mejia et al., 2002), and with the study of Gonzalez and Paz (1997), where the occurrence of an increase of 5-HIAA in the midbrain during ozone exposure was reported to affect the metabolism of major neurotransmitter systems after 1 h of exposure. This suggests that ozone alters the metabolism of serotonin.

Oseltamivir produces side effects on the central nervous system, especially when it is combined with other agents such as ethanol (Izumi et al., 2007). Besides, it is necessary to consider that there are genetic differences between Japanese and Caucasian patients that result in different levels of oseltamivir and/or oseltamivir carboxylate in the CNS. These genetic differences influence the metabolism and pharmacological activity of the drug in the CNS. Perhaps similar things occurred in the present study because the capacity for converting oseltamivir to oseltamivir carboxylate in rat and human brains was low (Toovey et al., 2008).

Based on the results of the present study, it can be suggested that the combination of oseltamivir with ozone induces changes in dopaminergic receptors and at the same time, provokes a pro-oxidant effect in the brain.

The concentration of GSH in the brain increased in the experimental groups that received oseltamivir in the presence of ozone. This effect may be due to the accumulation of this drug in brain as suggested by Morimoto et al. (2008). These authors proposed that the interindividual variation of P-glycoprotein activity might be an important factor, determining susceptibility to the CNS side effect of this drug, and therefore possibly playing a role in increasing the accumulation of oseltamivir in the brain because of the immature blood brain barrier in young animals (Johanson, 1980).

Some studies suggest that the presence of oseltamivir in the brain reduced the generation of endogenous nitric oxide (NO) (Kacergius et al., 2006), meaning that this increase could hike up a neuroprotective effect as was suggested by the findings of Ju et al. (2005). The later proposal that S-nitrosothiol (GSNO), a potent endogenous antioxidant derived from the interaction between nitric oxide and glutathione, caused dose-dependent protective effects against amyloid beta (Abeta)/ceramide neurotoxicity for inhibition of caspase activation and production of reactive oxygen species (ROS). It seemed that the activation of cGMP-dependent protein kinase (PKG), phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated kinase (ERK) are involved in this GSNO-mediated neuroprotection.

The levels of lipid peroxidation decreased with the administration of oseltamivir or vitamins on the hemispheres and cerebellum/medulla oblongata of animals with ozone exposure. This is contrary to the result obtained with GSH levels in the same condition. The decrease of lipoperoxidation was more evident in ozone-exposed groups treated with oseltamivir and vitamins, due to the 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 (Fuке et al., 2008), whose chemical characteristic is the attraction of electrons to the same molecule.

On the other hand, the total ATPase activity increased after administration of oseltamivir in the hemisphere regions; the basal state was recovered in the presence of vitamins. This suggests that the substances administered exert an effect on the presence of intracellular ions, particularly calcium and sodium ions (Brase, 1990), thereby modifying the membrane potential, probably through the dopamine transporter, a membrane protein specifically expressed by dopaminergic neurons, which is regulated by Zn\(^{2+}\) that directly interacts with the protein (Пilf et al., 2009).

The results of the present study under conditions of ozone exposure suggest that oseltamivir and vitamins could be combined in the treatment of influenza A (H1N1) virus infection because they induce an antioxidant effect in the brain. This means that if oseltamivir is administered to urban children with environmental ozone exposure as the first choice of 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.

REFERENCES


Diccionario de Especialidades Farmaceuticas (PLM) (1999): Refermed, Edición 55, Mexico DF.


