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EVALUATION OF THE ANTIOXIDANT EFFECT OF OLANZAPINE IN COMBINATION WITH N-ACETYLCYSTEINE IN A MOUSE MODEL OF MK-801-INDUCED SCHIZOPHRENIA

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Summary

Introduction: Schizophrenia is a chronic condition affecting 1% of the population. One of the main theories about its etiology points out that hypofunction in N-methyl-D-aspartate (NMDA) glutamate receptors induces a loss of balance between the production of oxidative species generated in cellular metabolism and the antioxidant defense systems, which generates a state of oxidative stress. N-acetylcysteine (NAC) has been proposed as an adjuvant agent to potentiate the efficacy of atypical antipsychotics such as olanzapine (OLZ), improving the oxidative processes of the disease. **Methods**: Thirty mice divided into five experimental groups were administered MK-801 (an NMDA antagonist) as a model of schizophrenia. The involvement of oxidative stress was evaluated by measuring lipid peroxidation and reduced glutathione concentration at the level of the frontal cortex. Results: MK-801 administration produced increased lipid peroxidation and decreased reduced glutathione concentration at the level of the frontal cortex. Both OLZ and NAC treatments, administered alone or in combination, decreased lipid peroxidation and increased reduced glutathione in the frontal cortex. **Discussion**: These data suggest that OLZ and NAC treatment, given alone or in combination, regulate oxidative damage inherent to the disease and could be an option for patients with chronic psychosis or with poor response to current treatment regimens. However, further studies are required to demonstrate their efficacy and safety.

Keywords: *schizophrenia model, MK-801, olanzapine, N-acetylcysteine, lipoperoxidation, reduced glutathione*.

Background

Schizophrenia is a chronic condition that affects 1% of the population worldwide, that is, up to 1 in 300 people suffer from it.[1] Some reports indicate that the incidence rate of this disease has remained stable over time among different populations, which may suggest that it affects individuals in a similar way.[2] However, during the last decade it has been observed that there is variability in the incidence and prevalence of this condition.[3] In this regard, factors that may increase the risk of developing schizophrenia have been identified, such as genetics and a history of perinatal complications that alter proper neurological development during the gestational period.[4]

Because symptomatology usually begins in early adulthood, schizophrenia represents a significant economic and social burden.[4] This condition is considered one of the most serious psychiatric disorders, primarily because of the high mortality rates of those who suffer from it; it is estimated that patients with schizophrenia have a life expectancy up to 20 years shorter than the general population.[5,6,7] As a clinical syndrome, schizophrenia is characterized by a wide range of signs and symptoms that present differently in each patient.

Positive symptoms are the presence of hallucinations, usually auditory or visual, and delusional ideas, as well as language and behavioral disturbances. These symptoms are usually the first to be identified and the reason for seeking medical attention.[4] There are other symptoms, known as negative symptoms, which manifest as loss of motivation, loss of interest and social withdrawal, and are often associated with poor functioning and decreased quality of life for the patient. In general, these symptoms have a poor response to current treatment regimens and contribute to an increased

risk of chronic degenerative diseases, such as cardiovascular disease and diabetes mellitus.[5,6,7] Finally, cognitive symptoms constitute alterations in attention, working memory and executive functions.[4]

Regarding the etiology of this condition, it is suggested that there is a hypofunction in the N-methyl-D-aspartate (NMDA) glutamate receptors, a theory supported mainly by evidence in animal models. According to this theory, upon acute and chronic administration of an NMDA receptor antagonist such as MK-801 (dizocilpine), behavioral characteristics that reproduce the signs of this condition are observed.[8,9] Within the pathophysiology of schizophrenia, strongly involved brain areas have been localized, such as the prefrontal cortex (PFCx), responsible for complex cognitive processes through the interconnection of sensory and motor cortical areas and subcortical structures.[10,11,12] The PFCx is mainly composed of glutamatergic pyramidal neurons (75 to 80% of the total neuronal cells) and GABAergic interneurons.[7] On the other hand, oxidative stress has been reported to play a significant role in the pathophysiology of various psychiatric diseases, such as schizophrenia and bipolar affective disorder.[13,14] This process causes functional alterations that may contribute to the development of a greater propensity to relapse, a reduced response to treatment and an increase in functional and cognitive decline.[15] Oxidative stress is observed when there is an imbalance between the production of free radicals (FR) and the capacity of antioxidant defenses to counteract them. Excess FR produces alterations in the molecular structure of cellular components, causing damage to proteins, lipids and DNA.[16,17,18] FR damage to polyunsaturated fatty acids in cell membranes is called lipid peroxidation (LP). Antioxidant defenses and molecules such as reduced glutathione (GSH) - a non-protein tripeptide composed of the amino acids glutamate, cysteine and glycine[21, 16, 17] - contribute to the redox balance of the cell.[19,20]

Antipsychotic drugs are the central treatment for schizophrenia; their antipsychotic mechanism of action is due to the regulation of dopamine D2 receptors at the striatal level.[4,22,23] It is important to note that, despite the availability of various drugs, it has been reported that 20-30% of patients show a lack of response to treatment,[24,25] so the development of safe and effective alternatives remains a challenge.

Olanzapine (OLZ) is a second-generation atypical antipsychotic drug that has a better tolerability profile compared to typical antipsychotics such as haloperidol. OLZ causes less extrapyramidal effects and less sedation effect and hyperprolactinemia, not only at moderate antipsychotic doses but also at higher doses. In addition, superior improvement of negative symptoms has been reported with its use compared to typical antipsychotics.[26,27] Initially, this drug was approved for the treatment of patients with schizophrenia and, subsequently, for patients with bipolar affective disorder and treatment-resistant depression.[28] However, its use as a first-line agent is limited in patients with comorbid conditions that increase metabolic risk, because it is not only consistently associated with weight gain, but also carries a high cardio-metabolic risk by significantly increasing fasting triglyceride levels and insulin resistance, leading to increased blood glucose levels.[29,26] According to studies performed in animal models with MK-801, atypical antipsychotics have been shown to be superior to typical antipsychotics in reversing cognitive deficits induced by NMDA receptor blockade, as they generate improvement in spatial learning, reversal learning and recognition memory.[30,31,32] Specifically, OLZ was observed to partially reverse these cognitive deficits, as well as the MK-801-induced decrease in the expression of phosphorylated GluN1 and GluN2B subunits of the NMDA receptor. [33,34,35]

On the other hand, in an in vitro model performed with rodent hypothalamic cells, the use of OLZ was associated with increased oxidative stress. Because of this, a combined therapy with an antioxidant could represent a good treatment option.[36]

N-acetylcysteine (NAC) is a clinically approved mucolytic that, being a precursor of glutathione, can cross the blood-brain barrier, and acts as an antioxidant molecule by decreasing FR levels and restoring GSH depletion.[17,37,38,39] It has been shown to play an important role in various psychiatric disorders, as it is involved in the modulation of processes such as neuro-inflammation, oxidative stress and the regulation of glutamate and dopamine neurotransmitter systems.[17,14,15,40,41] Due to this evidence and its good safety profile, NAC has been considered as an adjuvant agent that potentiates the efficacy of atypical antipsychotics such as OLZ, and regulates the oxidative and cell death processes of both the disease itself and those related to the prolonged use of high doses of OLZ. Consequently, combination treatment constitutes a viable option for patients with schizophrenia or associated psychoses, particularly those with a chronic course and poor response to current treatment regimens.[17,42,43] Based on this information, the aim of the present study was to evaluate the antioxidant effect of therapy alone or in combination (OLZ + NAC) in a mouse model of MK-801-induced schizophrenia.

Material and methods

Animals

Thirty CD1 strain albino mice, adult males weighing 25 to 30 g, donated by the Universidad Autónoma Metropolitana Unidad Xochimilco biotherium, were maintained under standard biothermal conditions, with 12:12 light-dark cycles, a temperature of 22°C, and free access to water and food. All animal handling procedures were performed in accordance with the regulatory guidelines for the care and use of laboratory animals according to NOM-062-ZOO-1999. Likewise, the protocol was registered at the Instituto Nacional de Neurología y Neurocirugía Manuel Velasco Suarez under number 38/20.

Experimental design and pharmacological treatment

The animals were divided into 5 experimental groups with the following characteristics:

- Healthy: intact animals,
- MK-801/Veh: they were treated with 0.9% saline (i. p.) every 12 hours for 14 days prior to the administration of MK-801 (dizocilpine) (0.25 mg/kg, i. p.), as well as an NMDA receptor antagonist to induce the schizophrenia model following the methodology of Aquino-Miranda et al.,[8]
- MK-801/OLZ: animals treated with olanzapine (4 mg/kg, i. p.) every 12 hours at doses of 0.25 mg/kg (i. p.), as reported by Reddy, et al.,[35] for 14 days prior to MK-801 administration, to induce adverse effects due to OLZ effect, as reported by Ng et al.,[44]
- MK-801/NAC: mice treated with N-acetylcysteine (80 mg/kg, i. p.) every 24 hours for 14 days prior to administration of MK-801 (0.25 mg/kg, i. p.),
- MK-801/OLAZ+NAC: animals that received treatment with the combination of olanzapine (4 mg/kg, i. p.) every 12 hours and NAC (80 mg/kg, i. p.) every 24 hours, for 14 days prior to the administration of MK-801 (0.25 mg/kg, i. p.).[44]

Determination of serum glucose levels

Since it has been reported that OLZ treatment can develop insulin resistance,[26] on day fourteen serum glucose levels were assessed in all animals at the conclusion of treatment administration, three hours after MK-801 administration. A Roche Accu-chek glucometer was used.

Lipid Peroxidation assay

Mouse frontal cortex was obtained to measure fluorescent end products of LP using the technique described by Triggs and Willmore[45] and modified by Diaz-Ruiz et al.[46] The frontal cortex was homogenized in 3 mL of saline (0.9% NaCl), subsequently, it was separated into 1 mL aliquots, to which 4 mL of a chloroform-methanol mixture (2:1, v/v) was added. After stirring, the mixture was kept on ice for 30 min to allow phase separation. Finally, the fluorescence of the chloroformic phase was measured in a Perkin-Elmer LS50B luminescence spectrophotometer at 370 nm excitation and 430 nm emission. The sensitivity of the spectrophotometer was adjusted to 150 fluorescence units with a standard quinine solution (0.1 g / mL). The results were expressed as international fluorescence units per gram of fresh tissue.

Determination of the concentration of reduced glutathione.

Three hours after MK-801 administration, the animals were sacrificed by decapitation (after anesthesia with sodium pentobarbital) and frontal cortex dissection was performed. GSH standard was prepared in 0.1 M sodium phosphate and 5 mM EDTA buffer (pH 8) and kept on ice until use. The o-phthalaldehyde (OPA) solution was prepared in reagent grade absolute methanol just prior to use. Samples were homogenized in 3.75 mL of EDTA-phosphate buffer (pH 8.0) plus 1 mL of HPO3 (25 %). The homogenates were centrifuged at 3,000 x g for 15 min, the supernatants were separated into 500 μL aliquots and 4.5 mL of phosphate buffer plus 100 μL of o-phthalaldehyde were added. Samples were incubated at room temperature for 15 minutes and measured in a Perkin-Elmer LS50B fluorescence spectrophotometer at 350 nm excitation and 420 nm emission.[47]

Statistical analysis

The mean values ± standard error were obtained. The sample size estimate for this randomized, controlled experimental study was obtained by the following formula:

$$\left[\frac{(Z_{\alpha} - Z_{\beta})\,\sigma}{\mu_{\tau} - \mu_{2}}\right]^{2}$$

In this formula, n is the number of subjects for each treatment group, $\mu\tau - m2$ is the detectable difference between the means of the two groups, σ is the common standard deviation of each group, and $Z\alpha$ and $Z\beta$ are the values including alpha in the two tails and beta in the lower tail of the standard normal distribution, as described by Greenberg et al. in 1998. $\sigma 2$, $\mu\tau$ and $\mu 2$ were estimated with data from a pilot trial.

In all cases, an exploratory analysis of the data was performed to determine whether there was a normal distribution, using the Kolmogorov-Smirnov test, and homogeneity of variances, using Levene's test. Once this was determined, parametric

or nonparametric statistical tests were used according to the assumptions of each analysis. In this sense, a one-way ANOVA test was performed followed by Dunnett's post hoc test; statistical significance was established with $p<0.05$, using SPSS 20.0 software.

Results

Serum glucose levels

The basal serum glucose value was 128.50 ± 6.29 (mg/dL) and 131.20 ± 12.62 in the case of the MK-801 group without treatment. With respect to the groups with MK-801 and OLZ, NAC and OLZ+NAC treatments, the values were 153.60 ± 17.39, 128.60 ±13.48 and 146.60 ±24.47, respectively. An increasing trend was observed only in the group of mice that received olanzapine treatment alone, as well as a decreasing trend in the mice that received the OLZ+NAC combination, with no statistically significant differences.

Levels of lipid peroxidation

Figure 1 shows the results of the analysis of LP levels assessed in the frontal cortex 14 days after administration of the treatments and 3 h after injection of MK-801. The basal level of LP end products in healthy animals was 441.52 ± 66.53, and 630.50 ± 58.68 in MK-801 vehicle-treated mice, an increase of 42% being observed, this difference being statistically significant. In all groups treated with OLZ, NAC and its combination (OLZ+NAC) and MK-801 a statistically significant ($p<0.05$) decrease in LP of 35.22, 29.55 and 51.43%, respectively, compared to the control group (MK-801/Veh) is shown. The values included are the mean ± standard error and are expressed in international fluorescence units per gram of fresh tissue.

Reduced glutathione levels

GSH levels were assessed in the frontal cortex three hours after MK-801 administration. The results are shown in Figure 2. The basal GSH level in healthy animals was 0.99 ± 0.12 (mm/mg), whereas in MK-801 vehicle-treated mice it was 0.50 ± 0.07; a decrease of 49.5% was observed, this difference being statistically significant. In all groups treated with OLZ, NAC and its combination (OLZ+NAC) and MK-801 we observed statistically significant ($p<0.05$) increases in GSH levels of 66, 72 and 46%, respectively, compared to the control group (MK-801/Veh).

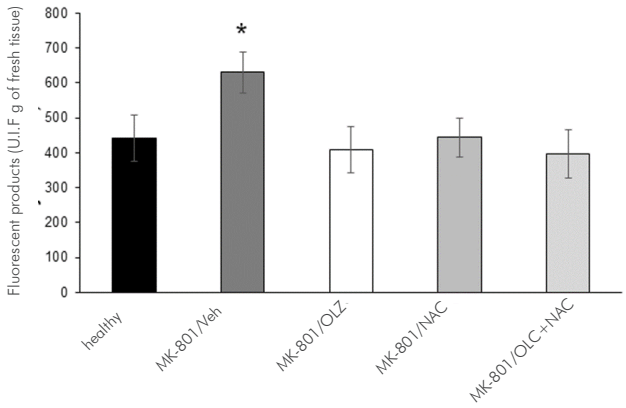

Figure 1. Graph showing the results of lipid peroxidation levels evaluated in the frontal cortex. Values are the mean ± E.E. of 5 to 6 animals per group and are expressed in international fluorescence units (I.F.U.) per gram of fresh tissue. Healthy: intact animals; MK-801/Veh: animals with MK-801 and vehicle; MK-801/OLZ: mice with MK-801 and OLZ at doses of 4 mg/kg (i.p.) administered every 12 hours for 14 days; MK-801/NAC: mice with MK-801 and NAC at doses of 80 mg/kg (i.p.) administered every 24 hours for 14 days; MK-801+ OLZ/NAC: animals with MK-801 and with both treatments at the doses mentioned above. Results of one-way ANOVA followed by Dunnett's test * $p<0.05$.

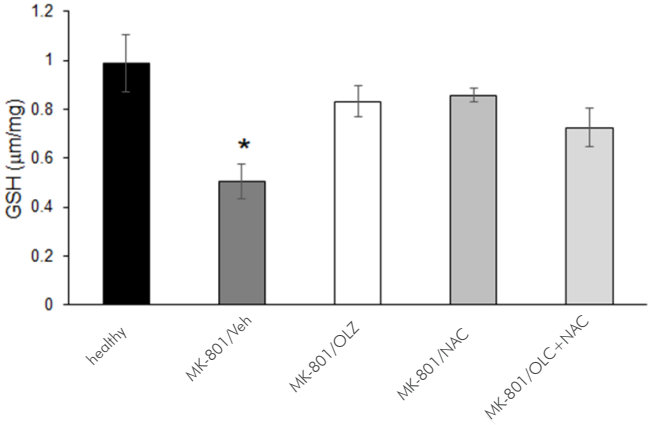

Figure 2. Graph showing the results of reduced glutathione (GSH) levels evaluated in the frontal cortex. Values are the mean ± S.E. of 5 to 6 animals per group and are expressed in micro moles per milligram. Healthy: intact animals; MK-801/Veh: animals with MK-801 and vehicle; MK-801/OLZ: mice with MK-801 and olanzapine at doses of 4 mg/kg (i.p.) administered every 12 hours for 14 days; MK-801/NAC: mice with MK-801 and NAC at doses of 80 mg/kg (i.p.) administered every 24 hours for 14 days; MK-801/OLA+NAC: animals with MK-801 and both treatments at the doses mentioned above. Results of one-way ANOVA followed by Dunnett's test * $p<0.05$.

Discussion

Hypofunction of NMDA glutamate receptors is one of the main theories on the etiopathogenesis of schizophrenia. Hypofunction in these receptors is associated with alterations in memory, cognition and neuronal plasticity, and with alterations in glutamate release in specific neuronal structures, such as the hippocampus and frontal cortex.[48] Based on the above, this study used the model of acute administration of MK-801 in mice according to the methodology reported by Aquino-Miranda et al,8 as this is a standardized and reproducible model that causes glutamatergic hypofunction, oxidative stress and cell death, as observed in schizophrenia.[48]

We report an increase in lipoperoxidation in the MK-801 group that was treated with vehicle, demonstrating that MK-801 increases oxidative stress and damage to cell membranes. These findings are in agreement with those reported in the study by Ozyurt et al.,[49] in which intraperitoneal administration of MK-801 in rats increased oxidative stress in the frontal cortex, with increased lipoperoxidation observed.

Likewise, our findings demonstrated that NAC treatment decreases lipid peroxidation after MK-801 administration. This effect is supported by the results of the study by Turkmen et al,[50] in which they tested the antioxidant effect of NAC after MK-801 administration in mouse testis, observing that NAC treatment increased the activity of antioxidant defenses and decreased the oxidative stress induced by MK-801. On the other hand, it should be considered that NAC administered in high doses can be pro-oxidant, so the doses chosen for our model were established according to the assays performed by Smaga et al, [51] in whose study NAC was administered in doses of 50 and 100 mg/kg via intra peritoneal route in Wistar rats, both acutely and chronically, for 10 days. The results showed that both doses have an antioxidant effect, since they increased the efficacy of the antioxidant defense mechanisms in the prefrontal cortex without altering the endogenous antioxidant status of the brain of the animals that received the chronic or acute doses of NAC and those that were not subjected to oxidative stress. Our findings are in agreement with these results, since the 80 mg/kg dose was observed to decrease lipid peroxidation compared to the untreated control group (Mk-801/Veh).

It is important to note that most of the studies performed with NAC have been in animals. However, it has recently been tested in more than 20 clinical trials as an adjuvant treatment in different neuropsychiatric conditions. This is the case of the study conducted by Nucifora et al.[52] in patients with schizophrenia and bipolar affective disorder, in which a notable decrease in serum GSH levels was reported in patients compared to controls, and an association was observed between the decrease in these levels and the magnitude of psychotic symptoms. On the other hand, decreases of up to 27% ($p<0.05$) of total GSH in the cerebrospinal fluid of patients with schizophrenia without pharmacological treatment compared to controls have been reported.[53] Along the same lines is a randomized double-blind, placebo-controlled clinical trial in patients with schizophrenia treated with conventional antipsychotics, who received 1200 mg of NAC as adjuvant treatment. The study reported that treated patients showed significant improvement on the PANSS scale for both positive and negative symptoms, as well as in cognitive performance in areas such as attention, working and short-term memory, executive functioning and processing speed.[54]

These findings are in agreement with the results of our study, since the administration of MK-801 in mice that did not receive treatment with antipsychotic, antioxidant, or the combination, generated a statistically significant decrease in reduced glutathione in the frontal cortex, suggesting an increase in oxidative stress and possible cellular damage. Therefore, treatment with OLZ and NAC, administered alone or in combination, reduces lipid peroxidation levels and increases GSH levels, possibly regulating the oxidative damage inherent to this disease. This indicates that the administration of NAC could be considered an adjuvant treatment, mainly in patients with a chronic course. Likewise, the results of this research protocol support the existing theory related to the role of oxidative stress and neuronal apoptosis in psychiatric diseases such as schizophrenia. Our results would also reinforce the possible modification of current treatment regimens, especially in patients with a chronic course of the disease and with poor response to such regimens.

Conclusions

Given the findings of the present study, we can conclude that MK-801 increases lipid peroxidation levels and decreases GSH levels in the frontal cortex. Likewise, treatments with OLZ at a dose of 4 mg/kg and with NAC at a dose of 80 mg/kg, administered alone or in combination, reverse these effects, i.e., they are effective in decreasing oxidative stress in the mouse frontal cortex.

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Conflicts of interest

The authors of this work declare that there are no conflicts of interest.

Credit

All authors have contributed to the study design, ethics committee approval and drafting, critical revision, and final version of the manuscript. RLA: drafting of the manuscript, standardization of the mouse model of schizophrenia; IPN: review of the manuscript, advice for standardization of biochemical methods; CR: study conception and design, statistical analysis; AMB, EM and NM: treatment administration, sample collection, standardization of biochemical methods; ADR: drafting of the manuscript, advice for statistical analysis, study conception and design.

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