The Antipsychotic Ziprasidone has Cito-Genotoxic and Pro-Inflammatory Effects Influenced by Val16ala-Sod2 Gene Polymorphism, ASEAN Journal of Psychiatry, Vol. 22 (S1), October-November 2021: 1-9

Original Article

THE ANTIPSYCHOTIC ZIPRASIDONE HAS CITO-GENOTOXIC AND PRO-INFLAMMATORY EFFECTS INFLUENCED BY VAL16ALA-SOD2 GENE POLYMORPHISM

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Abstract

Objectives: In immune cells, especially macrophages some oxidant molecules present a key role on inflammatory response trigger by pathogens and non-pathogens substances. For this reason, basal genetic superoxide-hydrogen peroxide (S-HP) imbalance as caused by Val16Ala-SOD2 single nucleotide polymorphism (SNP) could has some influence on side effects induced by pharmacological drugs. This could be de case of Ziprasidone (ZIP), a second-generation antipsychotic (SGA) used to treat some psychiatric and neurodegenerative diseases that seems to act on oxidative-inflammatory metabolism. To test this hypothesis, an in vitro study using human peripheral blood mononuclear cells (PBMCs) carrying different Val16Ala-SOD2 genotypes was performed. In standardized 72h cell cultures, the effect of ZIP exposure at plasmatic therapeutic concentration in oxidative (including level of DNA oxidation quantified by 8-deoxiguanosine) and inflammatory markers were analysed. Results showed that AA-PBMCs that have basal higher HP levels presented cito-genotoxic effect when ZIP-exposed, whereas VV-PBMCs presented higher levels of proinflammatory cytokines. The whole of results indicated some pharmacogenomic action of Val16Ala-SOD2 SNP despite in vitro methodological constrains. ASEAN Journal of Psychiatry, Vol. 22 (1): January – February 2021: XX XX.

Keywords: Pharmacogenomics, Oxidative stress, Inflammation, Gene polymorphis, Antipsychotics

Introduction

Psychosis symptom have important association with schizophrenia and, bipolar disorders also affecting patients with other neurodegenerative diseases, such as Parkinson [1], dementias been associated with stress response blood biomarkers and inflammation as well as on indicators of redox dysregulation, and DNA damage [2-4]. The pharmacological control of psychosis symptoms especially is conducted mainly from use of second-generation antipsychotics (SGAs). However, SGAs treatment has been also consistently associated metabolic side effects, such as obesity and, diabetes type 2 associated with peripheral oxi-inflammatory states [5-8]. However, these metabolic side effects seem to be attenuated by ziprasidone use (ZIP), a drug novel antipsychotic approved for clinical use [9].

ZIP is a bezidoxazole-derivative with effective action on positive and negative symptoms in the schizophrenia that is a selective monoaminergic antagonism by dopamine D2 and 5-HT2A receptors, also presenting for 5-HT1A, 2C,1D receptors and by 1- and 2- adrenergic and H1-histaminergic receptors inhibiting synaptic reuptake of serotonin and norepinephrine [9]. In pharmacokinetics terms, the absolute ZIP bioavailability at 20-mg oral dose is 60% under feeding condition. Plasmatic ZIP therapeutic range from 50 to ng/mL been highly metabolised in humans, with less than 5% of the administered dose being excreted in an unchanged form [10].

Despite, ZIP to present attenuated risk of development of metabolic diseases, other side effects associated with oxi-inflammatory states have been described in the literature including: allergic events associated hypersensitive reactions, chest pain and diffuse urticaria [11-13], respiratory failure [14], Kounis syndrome

that is an allergic reaction preceding and leading to an acute coronary syndrome [15], hypertensive reactions [16]. An in vitro study also described that ZIP could activate a RAW-macrophage cells line by induction of higher levels of cellular proliferation and proinflammatory cytokines such as IL-1, IL-6, TNFα, INFγ [17]. Although there are studies suggesting that this antipsychotic causes relevant cytogenetic changes, there is a need to clarify how much basal oxidative states of peripheral cells could affect both the cyto-genotoxic effects and the pro-inflammatory effects of this antipsychotic [18-20].

The relevance of this complementary study is associated with studies that suggest that baseline oxidative status could directly influence the risk of development of some non-transmissible chronic diseases, as well as drug and food response. This is the case of a single nucleotide polymorphism (SNP) located in the superoxide dismutase manganese dependent gene (Val16Ala-SOD2, rs4880) that its two homozygous genotypes (AA and VV) has been associated with chronic pro-inflammatory diseases [21,22] and that in vitro and in vivo studies indicated potential pharmacogenetic and nutrigenetic effects [23-25]. In this context, the present investigation performed in vitro analysis of potential ZIP effect on oxidative metabolism and inflammatory response of human PBMCs carrying different Val16Ala-SOD2 genotypes.

Materials and Methods

Chemical Reagents and Equipment's

The drugs and reagents used in the experiments, including Dulbecco Modified Eagle Medium (DMEM, 1640), RPMI 1640 culture mediums, caffeine, ziprasidone, catechin and theophylline and other chemical reagents, were

purchased from Sigma-Aldrich (San louis, MO, USa). Materials used in all cell cultures experiments were purchased from Vitrocell-Embriolife (Campinas, São

Paulo, Brazil) and Gibco-Life Technologies (Carlsbad, CA, USA) including fetal bovine serum, heat-inactivated horse serum, penicillin and streptomycin. Molecular biology reagents were obtained from Qiagen (Hilden, North Rhine-Westphalia, Germany), Invitrogen (Carlsbad, CA, USA), and Bio-Rad Laboratories (Hercules, CA, USA). Vacutainer® was purchased from BD Diagnostics (Plymouth, UK) and cytokines kits for Elisa immunoassays were purchased from Biomyx Technology (San Diego, Ca, USa). Part of this in vitro study used commercial murine RAW 264.7 macrophages cells that were obtained from American Type Culture Collection (ATTC, Manassas, USA) by the Cell Bank of Rio de Janeiro, which thawed and provided aliquots of these cells for the study. This non-profit non-governmental organization also issued a certificate that the line was not contaminated. Analyses involving the

measurement of absorbance or fluorescence were performed by use of SpectraMax i3x Multi-Mode Microplate Reader equipment (Molecular Devices, Sunnyvale, California, USA).

Val16AlaSOD2 SNP PBMCs and ZIP - General Protocol

Potential in vitro pharmacogenetic effects of Val16Ala-SOD2 SNP on PBMCs inflammatory response triggered by ZIP exposure was evaluated using blood samples of healthy subjects previously genotyped by this polymorphism. Barbisan et al [26] genotyped 120 healthy undergraduate students that accepted to donate blood samples as asked by researchers. All subjects signed consent term related to project previously approved by an Ethics

Committee at the Universidade Federal de Santa Maria, Brazil (process number: 0332.0243.000-11). To ensure that all triplicate tests were performed, volunteers underwent more than one blood collection on different days.

The PBMCs from blood samples were obtained and prepared similar to the protocol described by Barbisan et al. Briefly, blood sample (20 mL/subject) were collected by venepuncture from 18 volunteers randomly selected from the original bank (20.49 years old) years old) carrying different Val16Ala-SOD2 genotypes (6-VV, 6AV and 6-AA). Therefore, the volunteers were not necessarily the same as those who took part in previous in vitro studies that investigated some effect of Val16Ala-SOD2 SNP.

However, it is important to point out that in the moment of study these volunteers were not intaking

any medication for chronic disease treatment or vitamin supplements. Moreover, no-smokers, and did not present previous cardiovascular medical histories or hypertensive disorders, did not take psychiatric drugs, and had no metabolic disease or other morbidity that could affect the results. The Val16Ala-SOD2 genotype was determined by the polymerase chain reaction using a direct total blood cell sample and Tetra- Primer ARMS-PCR assay, as described by Barbisan et al.

Blood samples collected were transferred to tubes containing Ficoll Histopaque (2:1) and centrifuged for 20 min at 900g in order to collect the PBMCs that were harvested in the interface. The PBMCs were washed with phosphate buffer by centrifugation and pelleted cells were transferred to culture

medium containing 1 mL RPMI 1640 supplemented with 10% foetal calf serum (FCS) and 1% penicillin/ streptomycin at a cell density of1 × 106 cells/mL. Further, PBMC cultures were incubated at 37ºC and 5% CO2 for 24 h before performing any experiment. PBMCs carrying different Val16Ala-SOD2 were exposed to ZIP at 75 ng/mL that is the estimated plasmatic therapeutic concentration of this drug and that previously Duarte et al. described pro-inflammatory effects on RAW macrophages cell. All analyses were performed in 72 h cell cultures.

Assessment of cellular proliferation

One of the characteristics of the activation of mononuclear cells is the increase in the rate of cellular proliferation in relation to a group of nonactivated cells. For this reason, in this protocol the proliferative rate in 72 h cell cultures was performed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction spectrophotometric assay, as described previously by Barbisan et al. Briefly, MTT was dissolved in 5 mg/mL phosphate-buffered saline (PBS) and was added to a 96-well plate containing the sample treatments. Further, the plate was incubated for 1 h at 37□C. Culture supernatant was removed, and the cells were resuspended in 200 µL of dimethyl sulfoxide (DMSO). Reaction was spectrophotometrically read at 560 nm absorbance.

Oxidative stress markers assessments

The follow pro-oxidants molecules were quantified here: nitric oxide (NO), that is considered a key molecule in the early inflammatory response, that was spectrophotometric quantified by a colorimetric assay used to detect organic nitrate [27]. The Griess assay detects nitrite formed by the spontaneous oxidation of NO under physiological conditions involving azo coupling between diazonium species, which are derived from sulfanilamide and NO2, and naphthylethylenediamine [28]. A colorimetric produce measure at 540 nm is obtained from this reaction that is proportional of NO level present in the sample. Barbisan et al. described detailed the NO quantification performed here.

As Val16Ala-SOD2 SNP is directly associated with imbalance of oxidative molecules (superoxide anion and hydrogen peroxide), in the first protocol were also quantified and compared the levels of follow variables associated with oxidative stress: lipid peroxidation (LPx) and protein carbonylation (PCarb). LPx was spectrophotometrically estimated through the formation of thiobarbituric acid reactive substances (TBARS) as previously described by Jentzsch et al. and PCarb quantified according to Levine et al, using 532 nm and 370 nm wavelengths, respectively.

Antioxidant and Immunological assays

The levels of 8-deoxyguanosine that is a marker of DNA oxidation (oxDNA), cytokines, antioxidant enzymes levels SOD, catalase (CAT), glutathione peroxidase (GPX) and IL-1, IL-6, TNFα and IL-10 were quantified in 72 h cell cultures in both protocols conducted with PBMCs and RAW macrophages. These measures were performed by immunoassay tests using Quantikine Human Immunoassays kits as manufactures instructions. Briefly, all reagents and working standards were prepared and the excess microplate strips were removed, before adding 50 µL of the assay diluent RD1W to each well. Next these procedures, 100 µL of standard control for our sample was added per well, after which the well was covered with an adhesive strip and incubated for 1.5 h room temperature. Each well was subsequently aspirated and washed twice, for total of three washes. The antiserum of each molecule analysed here was

added to each well and covered with a new adhesive strip before being incubated for 30 in at room temperature. The aspiration/wash step was repeated, and the conjugate of each antioxidant enzyme (100 µL) was added to each well and incubated for 30 min at room temperature. The aspiration/wash step was repeated before adding 100 µL of substrate solution to each well, followed by incubation at room temperature for additional 20 min. Finally, 50 µL of stop solution was added to each well and the optical density was determined within 30 min using a microplate reader set to 450 nm [29].

Statistical Analysis

Data treatment obtention were performed according to in vitro good practices presumptions described by Griesinger et al., 2016. As currently is used, all assays were conducted in independent triplicate, and for this analysis data obtained were normalized by the mean of cell growth and transformed as % of control. Statistical analyses were performed using Graph Pad Prism Software (6.0 version). Data were presented as % mean ± standard deviation (SD) of negative or positive control group. Treatments were repeated, at least 5-times in each 96-well plate. The upper and lower values of 2-SD range found in these repetitions were considered outliers

and excluded of the analysis, because generally these outliers generate

relative SD > 10% indicating presence of some experimental imprecision. software. Comparison among treatments were performed by one- or two-way analysis of variance followed by Tukey's post hoc test. All tests comparisons with < 0.05 were considered statistically significant. In results showed in Figures different letters identified statistical differences ($p \leq 0.05$) among treatments.

Results

Analysis of PBMCs carrying different Val16Ala-SOD2 genotypes showed similar values of NO levels whereas, just V-cells (AV and VV) showed significant increase in the cellular proliferation. At contrary, AA-cells present lower values of cellular proliferation that AA-control group (Figure 1).

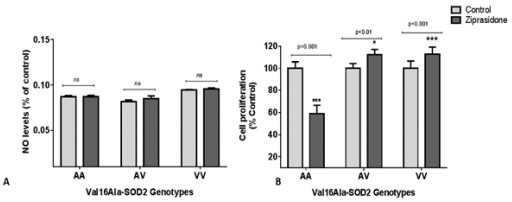

Figure 1: Comparison of (A) nitric oxide (NO) levels and (B) cellular proliferation rate in peripheral blood mononuclear cells (PBMCs) carrying different Val16Ala-SOD2 SNP genotypes (AA, AV and VV) ziprasidone (ZIP) exposed in 72h cell cultures by two-way analysis of variance followed by Tukey post hoc test. Data are presented by comparison between control and treated cells of each genotypes and statistically significant differences were identified by $p < 0.05$ = *; $p < 0.01$ = ** and $p < 0.001$ = ***.

The Figure 2 presents comparison between cytokines levels of 72 h ZIP-exposed and non-exposed PBMCs carrying different Val16Ala-SOD2 genotypes. The ZIP-exposure triggered higher levels of pro-inflammatory cytokines in PBMCs cells independent of SOD2 genotypes. However, this effect was slightly low in AA- than VV-cells. At contrary, a lowering effect of ZIP on IL-10 anti-inflammatory cytokine was observed when PBMCs. Again, this result was similar in all PBMCs cultures independent of Val16Ala-SOD2 genotype (Figure 2).

The effect of ZIP on oxidative metabolism variables considering PBMCs carrying different Val16Ala-

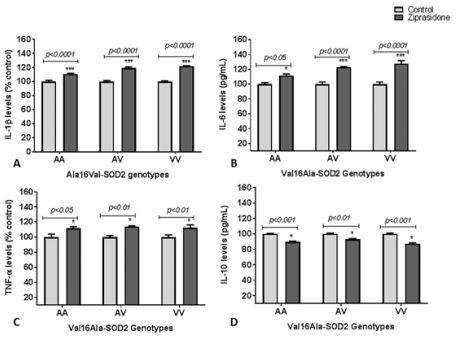

Figure 2: Comparison of different cytokines: (A) Interleukin 1 beta (IL-1β);(B) IL-6; (C) tumor necrosis factor alpha (TNF-α) and (D) IL-10 in peripheral blood mononuclear cells (PBMCs) carrying different Val16Ala-SOD2 SNP genotypes (AA, AV and VV) ziprasidone (ZIP) exposed in 72h cell cultures by two-way analysis of variance followed by Tukey post hoc test. Data are presented by comparison between control and treated cells of each genotypes and statistically significant differences were identified by $p < 0.05$ = *; $p < 0.01$ = ** and $p < 0.001$ = ***.

SOD2 genotypes are presented in Figure 3. ZIP induced significant higher levels of oxidative stress markers. Analysis indicated potential genotoxic action since levels of ox DNA were higher in all PBMCs ZIP-exposed. However, higher levels of this marker were observed in AA-PBMCs ZIP exposed (Figure 3A, 31 ± 3 % of control group) than AV-PBMCs (Figure 3B, 120 ± 4 % of control group) and VV-PBMCs (Figure 3C, 114 ± 3 of control) ($p < 0.001$). Despite ZIP trigger increase in the LPx levels, comparison among genotypes showed that this effect was lower in the AV-PBMCs (114 ± 3 of control group) than homozygous PBMCs (AA= 122 ± 2 and VV= 121 ± 2 of control) ($p < 0.001$). Protein carbonylation were elevated when cell cultures were ZIP-exposed, however, the increase in the levels of this oxidative markers were similar among SOD2 genotypes. The ZIP effect on antioxidant enzymes showed increase in the levels of SOD in a similar way among genotypes. At contrary, AA-PBMCs showed higher CAT levels in cultures ZIP-treated (124 ± 3 % of control) than V-allele cells (AV =107 ± 2, VV=110 ± 3 % of control) ($p < 0.001$). On ZIP-exposure, GPX-levels were also higher in AA-PBMCs (124 ± 3 % of control) than V-allele cells (AV =115 ± 2, VV=114 ± 3 % of control) ($p < 0.001$).

Discussion

In the present investigation was described potential pro-genotoxic and pro-inflammatory action of ZIP, an antipsychotic with attenuated metabolic alterations side effects. Moreover, these effects seem to be intensified by basal superoxide-hydrogen peroxide imbalance triggered by Val16Ala-SOD2 gene. In this case, whereas AA-PBMCs present higher levels of some oxidative markers, including ox DNA, VV-PBMCs present higher levels of some inflammatory molecules than other genotypes.

Before these results to be discussed more deeply it is important to point out that, despite methodological limitations associated with in vitro studies, in vivo analysis of gene and diet influence on antipsychotic drugs represent a challenge since there are a great number of variables that can to influence the results and the data interpretation. For this reason, in vitro protocols could serve as an exploratory approach to infer genetic and environmental variables influence on inflammatory response triggered by antipsychotic drugs.

The protocol performed here that evaluated Val16Ala-SOD2 SNP influence on ZIP oxidative and inflammatory response was based in previous evidences suggesting occurrence of a basal superoxide-hydrogen peroxide imbalance determined by both homozygous genotypes, AA and VV. In the Val16Ala-SOD2 SNP, a change of valine (GTT) to alanine (GCT) on 16 codon causes a protein structural modification affecting the SOD2 non-active protein transport into mitochondria [30]. Therefore, this structural modification affects the SOD2 efficiency, since A-allele produces an alpha helix protein that ingress easily in to mitochondria became an active SOD2 enzyme, whereas V-allele produces a beta-sheet protein that is partially arrested in the mitochondrial membrane. In these terms, previous investigation estimated that AA produce approximately 40% more active SOD2 enzyme than VV genotype [31].

Although AA has higher SOD2 enzyme this phenotype is not beneficial considering that AA-SOD2 dismutates higher concentrations of superoxide anion in hydrogen peroxide but did not occur a concomitant elevation in the GPX levels that, subsequently catalyze hydrogen peroxide in water. The excess of basal hydrogen peroxide could be a key explanation for the association between AA genotype with risk of several cancer types. A recent meta-analysis that included

33,098 cases and 37,831 controls from 88 studies estimated association between SOD2 SNP with overall cancer risk [32]. Previous studies also described that AA genotype could be more sensitivity to environmental prooxidant agents such as UV, methylmercury, moderate static magnetic fields and others.

In this work, a drastic reduction in the number of cells 72 h cultures was initially observed. This result could indicate a strong immunosuppressive effect of ZIP on AA cells or a markedly cytotoxic effect. In this cell's higher levels of oxDNA that is a marker of DNA damage was observed than other V-allele cells. Other prooxidant molecules also presented high levels in AA-PBMCS as well as antioxidant enzymes.

Other hand, high levels of proinflammatory cytokines occurred in AA-cells indicating a proinflammatory state. Therefore, the whole of results suggest that ZIP could present a cito-genotoxic effect on AA-PBMCs.

In the present investigation, VV-PBMCs presented higher production of inflammatory markers than A-allele cells when ZIP-exposed. The VV-genotype of Val16Ala-SOD2 SNP is related to low SOD2 efficiency that maintain elevate basal levels of superoxide anion in the cells. Despite superoxide to be less membrane soluble than hydrogen peroxide, this molecule has highly affinity for NO that is, virtually produced by all cells of the body. The reaction between superoxide anion and NO generates potent oxidative products, in especial peroxide nitrite that causes extensive peroxidation on lipid molecules present in the cellular membrane and other cellular compounds [33].

In fact, macrophages are very sensitive cells been are able to sense and clear structures that contain exogenous or endogenous pathogen-associated molecular pattern (PAMPs) and also danger-associated molecular patterns (DAMPs). These properties are closely linked with production and regulation of potentially harmful oxidant molecules, as well as cytokines. For this reason, macrophages are important producer's superoxide/hydrogen peroxide and nitric oxide. However, this production is highly controlled according the macrophages status and microenvironmental condition. Therefore, elevated basal levels of superoxide could represent a greater sensitivity of macrophages to trigger and maintain a given inflammatory response. This process could explain the potential cause of the association between VV genotype and risk of several types of metabolic diseases related to chronic low-grade inflammation [34,35]. Among these we can cite hypercholesterolemia, obesity, cardiovascular diseases including angina pectoris and stroke and with diabetes type 2 complications risk including retinopathy, nephropathy lipid profiles alteration.

Complementary in vitro and in vivo investigations showed that VV-genotype is associated with chronic inflammatory patterns including high levels of proinflammatory cytokines, such as IL-1β, IL-6 and TNFα, and lower levels of Il-10, an anti-inflammatory cytokine. Furthermore, investigations also has been described that VV-genotype could to has some pharmacogenomic influence on proinflammatory or anti-inflammatory response triggered by pharmacological drugs or bioactive molecules present in some foods. For example, a study performed by Duarte et al. showed that hypercholesterolemic patients who present a low-efficiency SOD2 enzyme (VV-genotype) exhibited an attenuated response to rosuvastatin compared with the A-allele patients. The effect of rosuvastatin on inflammatory and fibrinolytic biomarkers was also less intense in the VV patients. Barbisan et al. also described that Li anti-inflammatory in vitro effects could be directly influenced by Val16Ala-SOD2 SNP that was more attenuated in VV-PBMCs than A-allele cells [36-40].

Therefore, whole of results described here corroborate the potential proinflammatory ZIP effect on humans PBMC cells similar a previous study performed by Duarte et al in RAW macrophage cells. Moreover, this effect has some differences according to unbalanced homozygous genotypes: AA or VV. Whereas basal higher hydrogen peroxide levels seems to potentialize ZIP genotoxic and oxidative stress action, in cells with basal higher superoxide anion occur a potentialization of inflammatory response by increase in the PBMCs proliferation and in the levels of proinflammatory cytokines.

The results described here suggest that both, homozygous Val16Ala-SOD2 genotypes could be associated with negative effects triggered by ZIP-intake. It is relevant to comment that, adverse drug reactions in patients causes more than 2 million hospitalizations including 100,000 deaths per year in the United States. These reactions are associated with multiple factors including genetic variables. For this reason, the pharmacogenomics concept was developed and consolidated in order to increase the efficacy and safety of pharmacological drugs. Generally, negative effects have been associated with detoxification cytochrome P450 genes [41-44]. However, the results described here also suggest a potential role of oxidative imbalance triggered by genetic factors on modulation of negative side effects of drugs, such as ZIP.

Despite in vitro methodological constrains results suggest that Val16Ala-SOD2 could present some pharmacogenomic action on ZIP side effects related to immune and oxidative metabolism. However, if this effect could be found in relation to other antipsychotics drugs is an open question that needs to be elucidated by future investigations. The results described here also indicate that it may be that environmental factors, such as the consumption of foods rich in bioactive molecules, antioxidants and anti-inflammatories could attenuate the side effects in the immune system related ZIP-exposure [45-48].

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Received: 14 October *2021* *Accepted:* 24 November *2021*