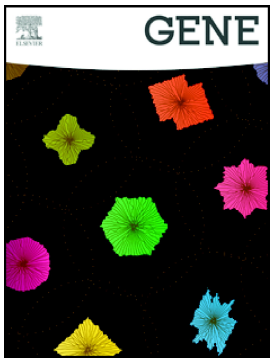

Genetic or pharmacological superoxide-hydrogen peroxide imbalances modulate the in vitro effects of lithium on glycogen synthase kinase-3β

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Genetic or Pharmacological Superoxide-Hydrogen Peroxide Imbalances Modulate the *in vitro* Effects of Lithium on Glycogen Synthase Kinase-3β

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Abstract

Introduction: Lithium (Li), a mood stabilizer used to treat bipolar disorder (BP) symptoms has important anti-inflammatory effects by downregulation of glycogen synthase kinase-3 beta (GSK-3β). However, sometime Li effect is not efficient in some patients suggesting genetic interference. Previous investigations described association between a genetic superoxide-hydrogen (S-HP) imbalance caused by a superoxide dismutase manganese dependent gene polymorphism (Val16Ala-SOD2 SNP, rs4880) and differential anti-inflammatory response of some drugs and bioactive molecules. Therefore, we postulated here that S-HP imbalance could present some effect on GSK-3β modulation by Li. **Methods**: to test this hypothesis, a genetic and a pharmacological S-HP imbalance protocols were performed. In the two protocols, immune cells were activated by phythohemaglutin (PHA). The first one, used peripheral blood mononuclear cells (PBMCs) cultures carrying different Val16Ala-SOD2 genotypes, and the second used a commercial macrophage cell line RAW 264.7. Macrophages were exposed to paraquat to induce high S levels (VV-like cells) or porphyrin, that is a SOD2-like molecule that increase dismutation of S into HP (AA-like cells). In both

protocols the Li effects on GSK-3β gene and protein modulation as evaluated in 24 h cultures. The inflammatory activation was also analyzed by cellular proliferation in 72 h cell cultures. **Results:** as expected PHA exposure triggered a strong upregulation of GSK-3β gene expression ($p \leq 0.001$), and Li exposure showed GSK-3β gene downregulation from 0.7 mEq/L concentrations. However, Li modulatory effects on GSk-3β gene and protein expression was directly influenced by basal S-HP balance. Presence of high S-basal levels (VV genotype and VV-like cells) induced attenuated Li anti-inflammatory effects in comparison with balanced and AA and AA-like cells ($p< 0.001$). Despite methodological limitations related to *in vitro* assays, the whole of results suggested that Li anti-inflammatory effects is influenced by S-HP basal state and is plausible that its influence could contributes to resistance of some patients to Li treatment or to increase of intensity of some side effects Li-associated.

Keywords: PBMC; genotype; inflammation; SOD2, bipolar disorder, oxidative metabolism

Introduction

Bipolar disorder is a psychiatric disorder associated with a chronic inflammatory pattern, oxidative stress, and mitochondrial dysfunction (Czarny et al., 2017). Glycogen synthase kinase 3 beta (GSK-3β) is a key molecule that is associated with these changes that are see in bipolar disorder patients. GSK-3β is a serine/threonine kinase, which is expressed in all mammalian tissues and was initially identified as a phosphorylating and inactivating agent for glycogen synthase. Presently, evidence has shown that GSK-3β has pleiotropic effects and plays a central role in cellular signaling and the regulation of cell proliferation. This enzyme is essential in the central nervous system, regulating neurotransmission, synaptic plasticity, apoptosis, and neurogenesis (Muneer, 2016; Cole, 2013; Muneer, 2017). Dysregulation of GSK-3β triggers its hyper activation and has been associated with the pathophysiology of several diseases, including bipolar disorder (Cole, 2013; Muneer, 2017). Moreover, the elevated levels of GSK-3β observed in patients with bipolar disorder have been related to chronic inflammatory processes that stimulate the production of several pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and inhibit the production of

interleukin 10 (IL-10), an anti-inflammatory cytokine (Luca et al., 2016; Muneer et al., 2016).

The symptoms of bipolar disorder can be controlled by lithium (Li) where some of its pharmacological effects appear to involve inhibition of the enzymatic activity of GSK-3β (Muneer et al. 2016). Several studies have suggested that inhibition of GSK-3β could, at least in part, explain the beneficial effects of Li on bipolar disorder, and its superiority in terms of clinical efficacy when compared with other pharmacological drugs (Luca et al., 2016). However, only about 30% of patients with bipolar disorder are responsive to Li, suggesting that its action has different levels of magnitude. Differences in Li efficacy are probably influenced by cellular micro-environmental factors, such as basal oxidative metabolism which is genetically determined, and, therefore, differs between individuals (Sutton et al., 2003).

Genetic studies have described an important association between the superoxide-hydrogen peroxide (S-HP) imbalance on inflammatory and oxidative response. This is due to a single nucleotide polymorphism (SNP) located in codon 16 of the superoxide dismutase manganese-dependent enzyme (MnSOD, SOD2), which resides in the mitochondrial targeting sequence (MTS) that is responsible for the import of inactive-SOD2 into the mitochondrion. At this codon, alanine is substituted for valine, causing a protein conformational change. The V-allele produces an SOD2 containing a beta sheet; therefore, the enzyme is not able to enter into the mitochondrion easily, and, as a result, is partially trapped in the membrane of this organelle. This phenotype decreases the availability SOD2 enzyme, with the VV-genotype producing higher superoxide levels than the A-allele genotypes (AA or AV). In contrast, the A-allele produces an SOD2 protein that has an alpha-helix, with the result that the AA-

genotype produces higher hydrogen peroxide levels than the V-allele genotypes (VV or AV) (Sutton et al., 2003; Bresciani et al., 2015).

Prior investigations have suggested that both of these SOD2 homozygous genotypes are associated with the risk of chronic diseases (Bresciani et al., 2015). Moreover, *in vitro* studies have suggested that Val16Ala-SOD2 SNP is associated with differential inflammatory and oxidative responses to pharmacological drugs, as well as the bioactive molecules found in some foods (Montano et al., 2009; Barbisan et al., 2014; Capeleto et al., 2015; Schott et al., 2017). A clinical assay has also described the pharmacogenetic effect of the Val16Ala-SNP on resistance to rosuvastatin treatment in hypercholesterolemic patients (Duarte et al., 2016).

Recent *in vitro* studies have also suggested that the S-HP imbalance associated with Val16Ala-SOD2 could affect the expression of some immunosenescence markers. Whereas AA-cells exhibit an intense short inflammatory response, VV-cells show a higher chronic, inflammatory response (Barbisan et al., 2017). The potential effects of S-HP imbalance on cellular function have also been evaluated using a pharmacological model that used paraquat to generate higher superoxide levels, and porphyrin, which acts as an SOD2-like enzyme, to generate increased ROS levels (Azzolin et al., 2016; Berto et al., 2015).

Presently, the effect of S-HP levels on the inhibition of GSK-3β by Li are not known; therefore, here, we conducted an *in vitro* study using both genetic and pharmacological approaches. Both of these approaches evaluated the effect of an S-HP imbalance on the Li induced inhibition of GSK-3β expression both at the mRNA and the protein levels. In the first protocol, peripheral blood mononuclear cells (PBMCs) with different Val16Ala-SOD2 SNP genotypes were exposed to Li, and in the second

protocol, a pharmacological S-HP imbalance was induced in the RAW-264.7 macrophage cells, followed by exposure to Li.

Materials and methods

Chemical reagents and equipment

All chemicals and solvents used in this study were from Sigma Aldrich (Saint Louis, MO, USA), MnTBAP (Porphyrin) was from Merck Millipore (Billerica, MA, USA). Materials used in cell culture were purchased from Vitrocell-Embriolife (Campinas, São Paulo, Brazil) and Gibco-Life Technologies (Carlsbad, CA, USA). Molecular biology reagents were obtained from Qiagen (Hilden, North Rhine-Westphalia, Germany), Invitrogen (Carlsbad, CA, USA), and Bio-Rad Laboratories (Hercules, CA, USA). Assay kit to estimate total GSK-3β levels in cell lysates was obtained from Thermo Fischer Scientific (Waltham, Massachusetts, United States). The protocols involving spectrophotometric analysis were performed using a 96-well microplate reader (SpectraMax M2/M2e Multimode Plate Reader; Molecular Devices- Sunnyvale, CA, USA).

Ethical issues, genotyping, and blood sample collection

This study is part of a research project previously approved by the Ethics Committee at the Universidade Federal de Santa Maria, Brazil, and all volunteers signed consent terms and agreed to donate blood samples to perform *in vitro* assays (process number: 0332.0243.000-11).

To obtain PBMCs, the blood samples were first collected by venipuncture from 18 healthy adult subjects (18 ± 22 years old), with 6 donors per genotype (6- VV; 6-AV; 6-AA), all of whom were students of the Federal University of Santa Maria, with a very similar lifestyle, and ate meals at the university restaurant. All subjects lived in the same region of Brazil (Rio Grande do Sul), were non-smokers, non -obese, were not taking any chronic medications or vitamin supplements, had no 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., 2014.

PBMCs *in vitro* cell culture

Blood samples (20 mL) were collected by venipuncture using EDTA vacutainers and then transferred to tubes containing Ficoll Histopaque (2:1). The tubes were centrifuged for 20 min at 900g and the PBMCs at the interface were harvested. The PBMCs were washed with phosphate buffer by centrifugation (10 minutes at 900 g), and the supernatant was discarded. The pelleted cells were transferred to culture medium containing 1 mL RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 1% penicillin/ streptomycin at a cell density of1 × 10^6 cells/mL. The PBMC cultures were incubated at 37ºC and 5% CO_2 for 24 h before performing experiments.

General *in vitro* design

Two protocols were performed to evaluate the influence of S-HP imbalance on the Li inhibition of GSK-3β. The first protocol involved the genetic S-HP imbalance

present in PBMCs isolated from subjects with the different Val16Ala-SOD2 genotypes. The second protocol involved a pharmacological imbalance created in the RAW 264.7 macrophage cell line by exposure to paraquat, a generator of the superoxide anion, and porphyrin, which acts as an SOD2-like molecule, to increase ROS levels. This latter pharmacological model has been previously described by Azzolin et al. (2016). Figure 1 shows an overview of the general experimental design for the two approaches. Since Li is an anti-inflammatory molecule, both PBMCs and RAW cells were activated using 125 μg/mL phytohemagglutinin (PHA), as previously described by Barbisan et al. (2017) where they analyzed the effect of the Val16Ala-SOD2 genotype on the chronic inflammatory response of PBMCs. PHA was chosen as the PBMC mitogenic activator since it is a lectin that is found in several types of foods habitually consumed by humans, including peas, peanuts, soybeans, dry beans, broad beans, dry peas, lentils, as well as others, and can often trigger a non-microbiological inflammatory response (Bouchenak et al., 2013). Pharmacological S-HP imbalance in the macrophage cells was confirmed by a quantification of superoxide and reactive oxygen species (ROS) levels in 24-h cell cultures. Cells that were exposed to paraquat, and so had higher superoxide levels, were referred to as VV-like cells, whereas cells exposed to porphyrin, which had higher ROS levels, were referred to as AA-like cells. In the two models, both GSK-3β gene and protein expression were determined in 24 h cell cultures. Differential inflammatory modulation by Li and the S-HP imbalance association was confirmed by analysis of the proliferative rate in 72-h cell cultures.

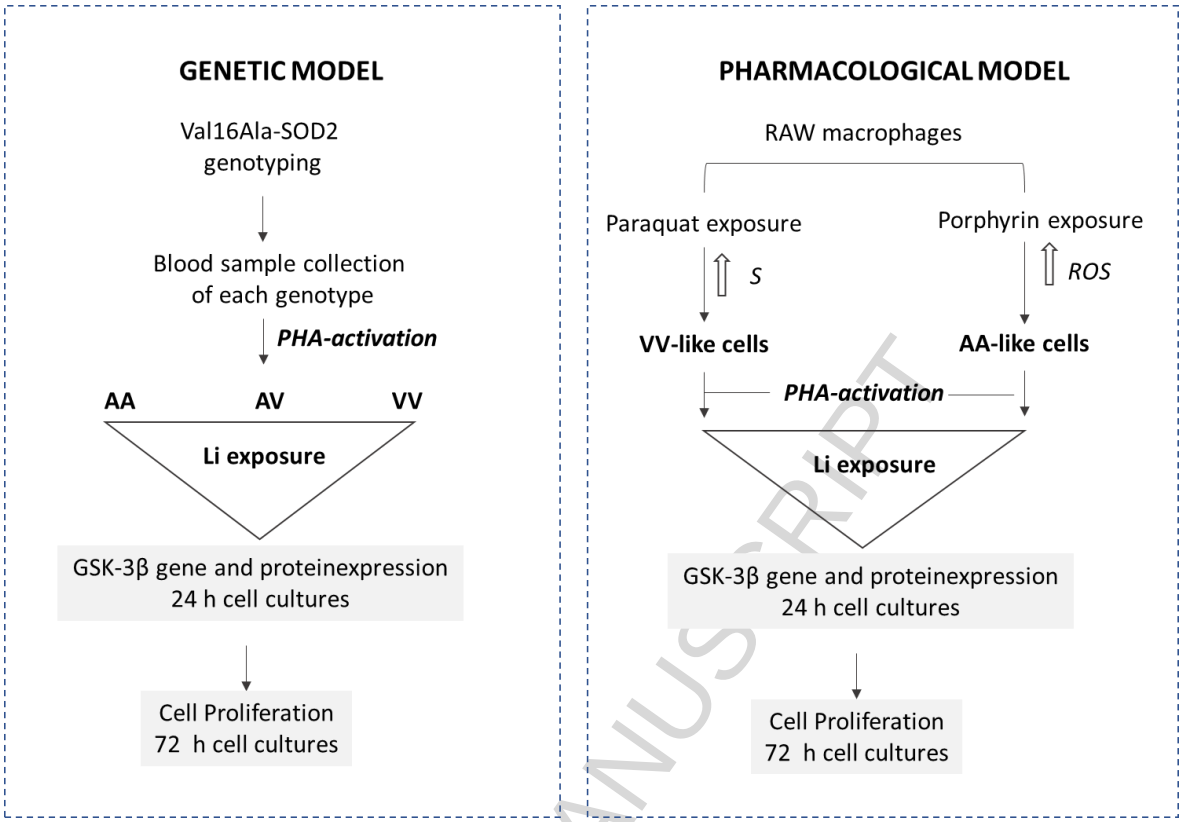

Figure 1 General experimental design used to evaluate the impact of S-HP imbalance on the Li inhibition of GSK-3β. The first protocol used Val16Ala-SOD2 SNP PBMCs cells that had a genetic S-HP imbalance due to changes in the efficiency of SOD2 enzyme transport into the mitochondrion. VV-PBMCs had higher basal S levels whereas AA-PBMCs had higher basal ROS levels. The second protocol used RAW 264.7 macrophage cells that were exposed to paraquat in order to generate higher S levels (inducing a VV-like oxidative state), or that were exposed to porphyrin in order to generate higher ROS levels (inducing an AA-like oxidative state).

S-HP Genetic Imbalance Protocol

To confirm that an S-HP imbalance affected GSK-3β gene and protein expression, as well as the inhibitory effect of Li, a pharmacological protocol was used similar to that described by Azzolin et al. (2016). However, although Azzolin et al. used colorectal cancer cells, here we elected to use a mouse macrophage cell line (ATCC® TIB-71™ — Rio de Janeiro Cell Bank, RJCB 0212, Brazil). We opted to use a non-human cell line in order to avoid the potential effect of the Val16Ala-SOD2 SNP. Macrophages were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1%

antibiotics (penicillin/streptomycin), and 1% antifungal agent (Amphotericin B). The cells were maintained at 37°C in an atmosphere of 5% CO_2. Following this, cell suspensions were placed in the wells of a 96-well microplate (1×10^5 cells/well). As described above (see Figure 1), the cells were treated with either paraquat or porphyrin, in order to generate an S-HP imbalance. To confirm successful establishment of a pharmacological imbalance, the S and ROS levels were quantified in 24-h cell cultures.

Superoxide levels were quantified using a colorimetric assay that produces a formazan salt through the reaction between nitroblue tetrazolium (NBT) chloride, following a protocol previously published by Morabito et al. (2010). Briefly, the cells were seeded in a 96-well plate, diluted in 1× PBS, treated with 10 μL of NBT solution (10 mg/mL), homogenized, incubated at 37°C for 3 h, and centrifuged. Following this, 75 μL of supernatant was removed, and the same volume of DMSO was added to each well. After incubation for 20 min at 37°C, 75 μL of the cell suspension was transferred to another 96-well plate, and the absorbance measured at 540 nm.

The production of reactive species, principally H_2O_2, was determined using the 2′–7′-dichlorofluorescein diacetate (DCFDA) assay. In this assay, the DCFDA is hydrolyzed by intracellular esterases to DCFH, which is trapped within the cell. This non-fluorescent molecule is then oxidized to fluorescent dichlorofluorescein (DCF) by cellular oxidants. The fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 525 nm, and the data were expressed as picomoles/mL of 2′,7′-dichlorofluorescein (DCF) production from 2′,7′-dichlorofluorescin (Barbisan et al., 2014).

Lithium treatments

All experiments were performed using pure lithium, obtained from Sigma-Aldrich (St. Louis, MO, USA). The lithium was diluted directly in culture medium, since this salt is water soluble. Concentrations of lithium, recognized as being effective in the treatment of psychiatric symptoms (Schatzberg and Nemeroff, 2009), were used to determine the concentrations used in the cell culture experiments. The dose response curve was constructed using two low and two high concentrations of Li that were related to the following therapeutic doses: 0.17, 0.35, 0.7, 1.4 and 2.8 mEq.

Quantitative real time PCR (qRT-PCR) to assess the gene expression of GSK-3β

Total RNA was extracted using Trizol, following the manufacturer's instructions (Ludwing-Biotec, Rio Grande do Sul, Brazil). The concentration of the extracted RNA was measured using a Thermo Scientific NanoDrop™ 1000 Spectrophotometer 532 nm. The RNA (1 μg/mL) was treated with 0.2 μL DNAase (Invitrogen Life Technologies, Carlsbad, CA, USA) at 37°C for 5 min, followed by heating at 65°C for 10 min. The cDNA was then generated by reverse transcription using 1 μL of Iscript cDNA and 4 μL of Iscript Mix (Bio-Rad Laboratories, California, United States). The reaction consisted of the following steps: heating at 25°C for 5 min, at 42°C for 30 min, and at 85°C for 5 min, followed by incubation at 5°C for 60 min. Real time PCR was performed as previously described by Barbisan et al. (2014) using a QuantiFast SYBR® Green PCR Kit and a Rotor Gene®(Qiagen, Hilden, Germany) . The specific sequences of the human GSK-3β primers were: Forward: 5′-CTCCCCCGTTGACATAGTCG-3′ and Reverse 5′-GCAGGAACCTGGAGTTTTCA-3′. The specific sequences of the mouse GSK-3β primers were: Forward: 5′-CTCTGGCCACCATCCTTATC-3′ and Reverse: 5′-

CACGGTCTCCAGCATTAGTATC-3′. Beta-actin (β-actin) was the housekeeping gene used to normalize the gene expression of all samples tested.

Quantitation of GSK-3β protein levels by immunoassay

The levels of the GSK-3β protein were quantified in cell culture supernatant using an ELISA immunoassay following the manufacturer's instructions (Thermo Fischer Scientific). Briefly, this kit uses a monoclonal antibody to GSK-3β, that is used to pre-coated the wells of a microplate in order to bind the GSK-3β in the sample. After one hour of incubation, the sample was removed, and the wells washed, after which a rabbit polyclonal antibody to GSK-3β, was added to bind to the GSK-3β captured on the plate. The polyclonal antibody detects a non-phosphorylated region on GSK-3β, and therefore detects GSK-3β regardless of its phosphorylation state. After a one hour incubation, the excess antibody was washed out, and a secondary antibody conjugate was added, and the samples were incubated for one hour. The excess conjugated antibody was then washed out, and the substrate added. After a further one hour incubation, a stop solution was added and the color generated was measured at 450nm using a plate reader.

Cell proliferation assay

The inflammatory status of the cells was confirmed by measuring the proliferative rate of the cell cultures at 72 h. In general, in activated cells the proliferation rate is higher than in non-activated cells. This last experiment was conducted to validate the anti-inflammatory effects of Li, and the potential impact of S-HP its ability to inhibit GSK-3β. Cell proliferation was determined using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] spectrophotometric assay, as described by dos

Berto et al. (2015). For this assay, the cells were incubated for 1 hour with MTT reagent. After the formazan salt that was formed was dissolved in dimethylsulfoxide (DMSO), its absorbance was measured at 560 nm in a 96-well microplate.

Statistical analyses

Statistical analyses were performed using the program GraphPad Prism 5 with a one-way analysis of variance followed by Dunnet's *post hoc* test, or a two-way analysis of variance followed by a Bonferroni *post hoc* test when the interaction between Val16Ala-SOD2 and Li or between S-HP pharmacological imbalance was analyzed. P values ≤ 0.05 were considered to be statistically significant.

Results

As expected, exposure of AV-PBMCs to different concentrations ofLi resulted in the down-regulation of GSK-3β gene expression. This effect was observed in cells exposed to > 0.7 mEq/L Li (Figure 2). From these data, we selected 0.7 mEq/L Li as the concentration that was used to evaluate the potential effect of Val16Ala-SOD2 SNP on its ability to inhibit GSK-3β.

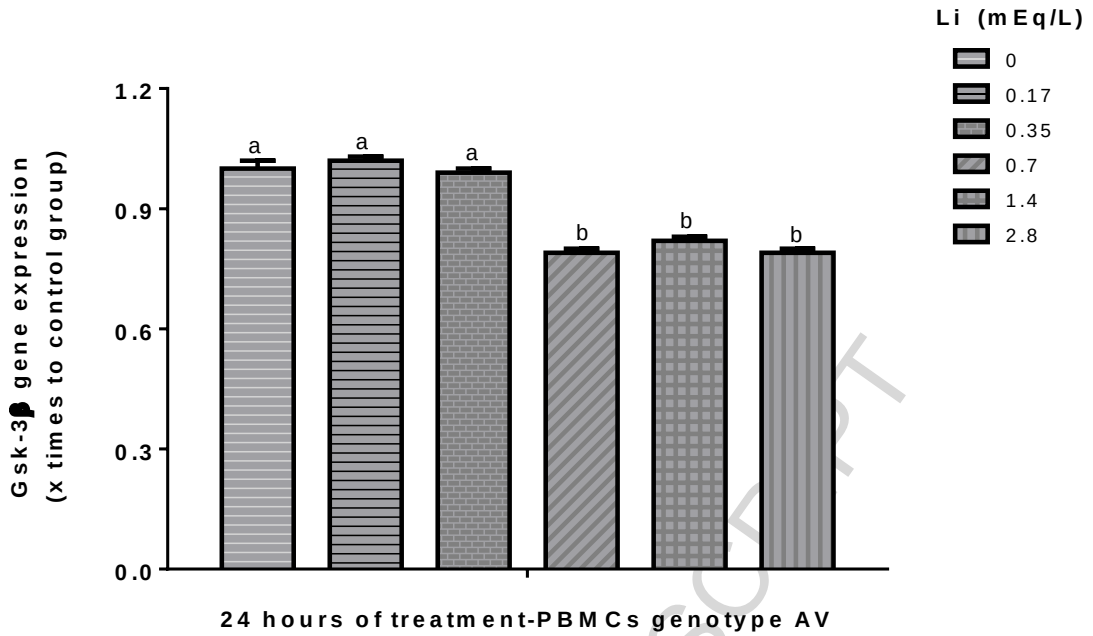

Figure 2 Effect of different concentrations of Li on GSK-3β gene expression levels in PBMCs. Gene expression levels were normalized using β-actin as the housekeeping gene. A value lower than 1 indicates gene downregulation, whereas a value higher than 1 indicates gene up-regulation, relative to the control group. Measurement of GSK-3β levels was performed in 24 h cell cultures and compared using a two-way analysis of variance followed by a Bonferroni *post hoc* test. Statistical differences among the different GSK-3β treatment groups are identified by the different letters (a, b). $p \leq 0.05$ was considered as being statistically significant.

Before the analysis of the effect of an S-HP imbalance caused by the Val16Ala-SOD2 SNP on the ability of Li to inhibit GSK-3β, the levels of GSK-3β expression were compared among the different genotypes. As a result, the VV-cells had higher levels of GSK-3β than either the AA cells or the AV-cells (Figure 3A).

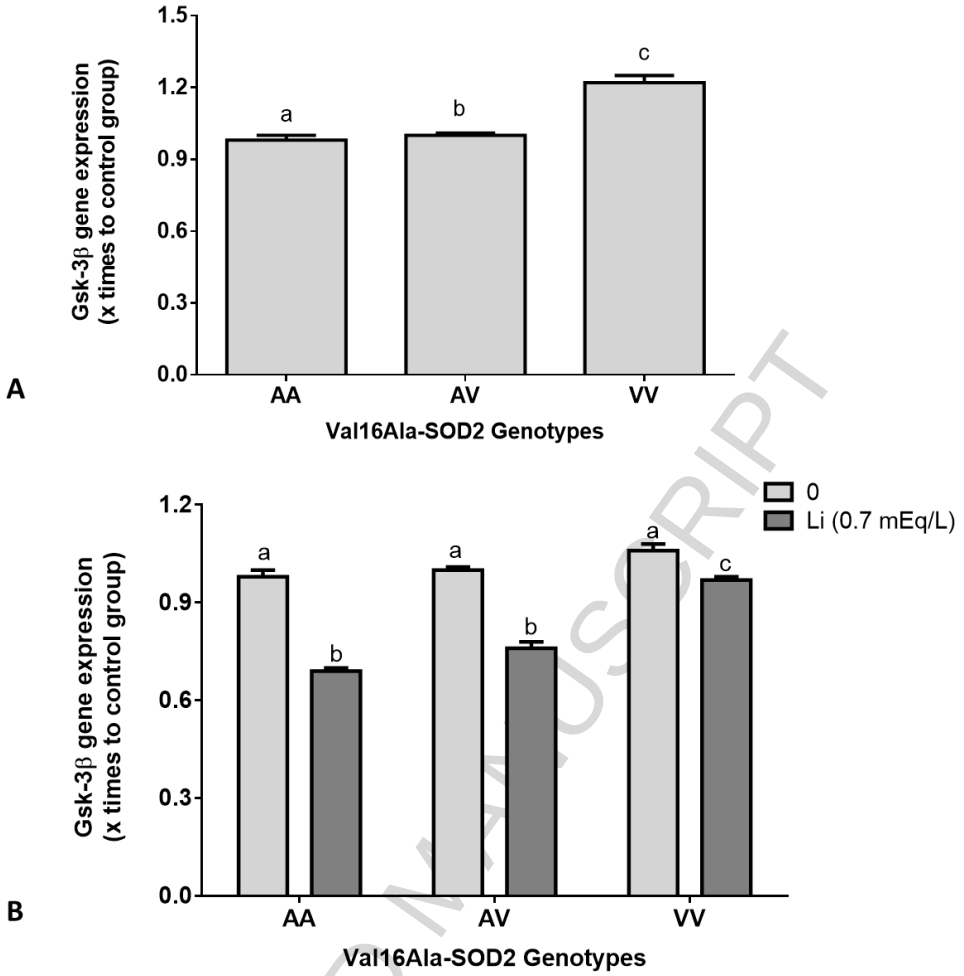

Figure 3 Level of GSK-3β gene expression in activated PBMCs from subjects with different Val16Ala-SOD2 genotypes (AA, AV, and VV) (B) Effect of Li on GSK-3β gene expression in PBMCs from subjects with different Val16Ala-SOD2 genotypes. Gene expression was normalized using β-actin at the housekeeping gene. A value lower than 1 indicates gene downregulation, whereas a value higher than 1 indicates gene up-regulation, relative to the control group. Statistical differences were determined by analysis of variance and are identified by the different letters (a, b). $p \leq 0.05$ was considered as being statistically significant.

The extent of the downregulation of GSK-3β gene expression was found to depend on the Val16Ala-SOD2 genotype. The Li effects on GSK-3β gene expression were greater in AA and AV cells than in VV cells (Figure 3B).

The impact of the different Val16Ala-SOD2 genotypes on GSK-3β protein levels in activated PBMCs, exposed to Li or not, was also assessed. The data showed that there was a more pronounced decrease in GSK-3β levels in A-allele PBMCs (AA

or AV) than in VV-PBMCs, since the latter showed no significant difference in GSK-3β protein levels between following Li treatment. (Figure 4).

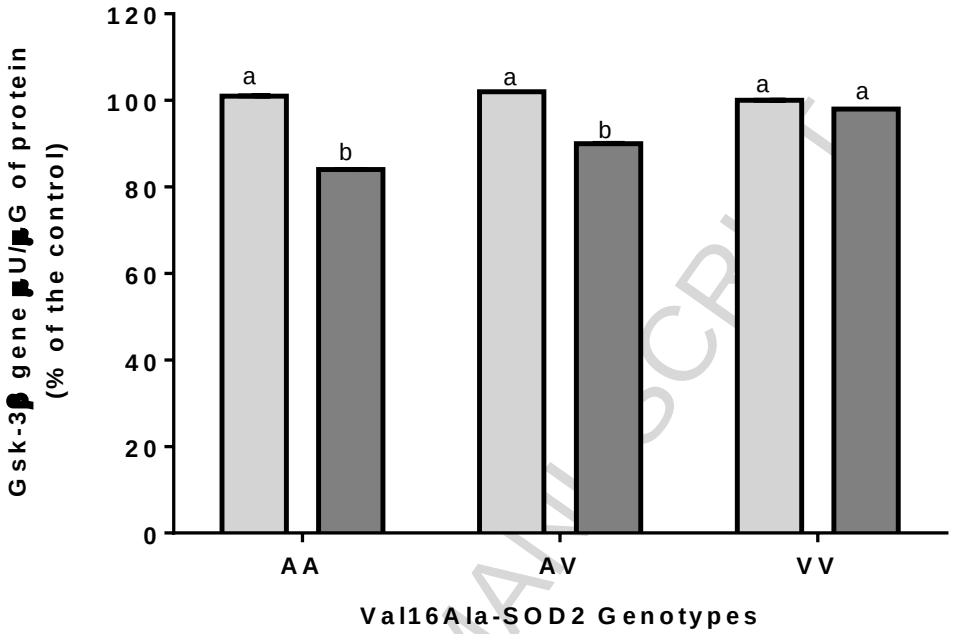

Figure 4 Effect of Li on GSK-3β protein expression in PBMCs from subjects with different Val16Ala-SOD2 genotypes. Data are expressed as percentage of the control genotype. Protein levels were measured after 24 hours in cell culture. The data were compared by a two-way analysis of variance, followed by Bonferroni *post hoc* test. Significant differences are identified by the different letters (a, b). $p \leq 0.05$ was considered as being statistically significant.

To perform the second *in vitro* protocol, an S-HP imbalance was established in macrophage cells, using paraquat treatment to generate VV-like cells and porphyrin treatment to generate AA-like cells. Both S levels (Figure 5A) and ROS levels (Figure 5B) were increased in a concentration-dependent manner, following treatment with paraquat or porphyrin, respectively. Based on these data we elected to use 0.1μM paraquat to generate VV-like cells and 1μM porphyrin to generate AA-like cells.

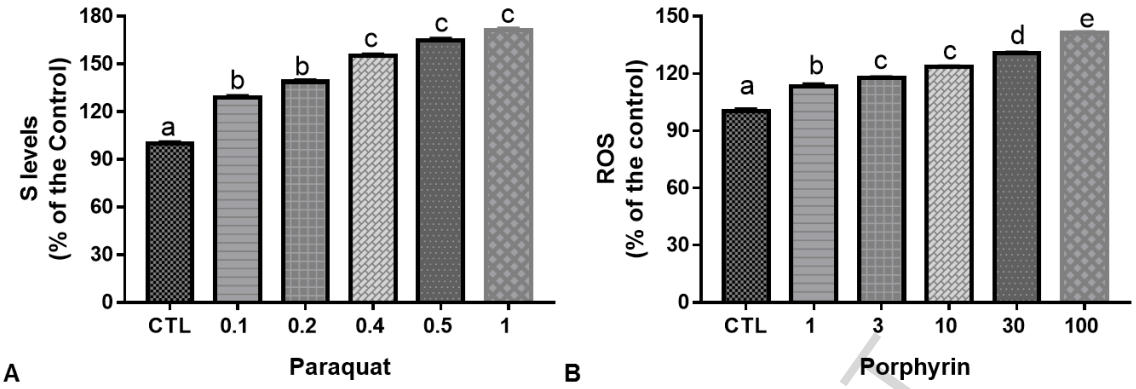

Figure 5 Effect of different paraquat (A) and porphyrin (B) concentrations on activated mouse RAW 264.7 macrophages after 24 h of exposure. The data are presented as % of the untreated control group. A statistical comparison was performed by analysis of variance two way followed by Dunnet's test. Statistical differences $p \leq 0.01$. Different letters are used to identify treatments with significant differences at $p < 0.01$.

The effect of a pharmacological S-HP imbalance the ability of Li to inhibit GSK-3β gene and protein expression was also determined and the results are shown in Figure 6. Compared to untreated activated RAW 264.7 macrophages, Li treatment of RAW264.7 macrophages without an S-HP imbalance (AV-like genotype), or with an S-HP imbalance (AA-like or VV-like genotype), resulted in a significant downregulation of GSK-3β gene expression (Figure 6A). Compared to the balanced cells (AV-like), this effect was more pronounced in AA-like cells and less pronounced in VV-like cells. Similar results were observed when the GSK-3β protein levels were also evaluated (Figure 6B).

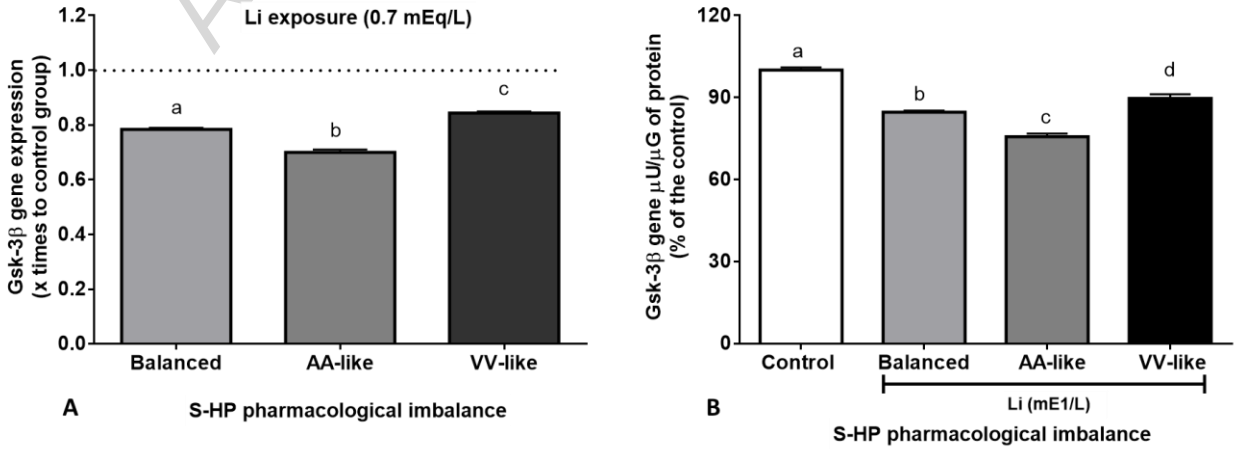

Figure 6 Effect of 24 h Li treatment on GSK-3β gene (A) and protein (B) expression in RAW 264.7 macrophage cells. The balanced macrophages were left untreated (to give an AV-like genotype) whereas an S-HP imbalance was created by treating the macrophages with either 1 µM porphyrin (to create AA-like cells) or with 0.1 µM paraquat (to create VV-like cells). Statistical significance was evaluated using an analysis of variance two way. The different letters indicate significant differences ($p \leq 0.05$).

Finally, cell proliferation was evaluated in 72 hour cell cultures in order to confirm that the culture conditions triggered an inflammatory response in the RAW 264.7 macrophages (Figure 7). The data confirmed that PHA treatment did indeed induce a proliferative state, and that Li treatment triggered an anti-inflammatory response in these macrophage cells. However, the S-HP imbalance influenced the intensity of these responses. AA-like cells had a greater response to PHA exposure than balanced cells, whereas VV-like cells showed an intermediate response. However, in the presence of Li, AA-like cells showed similar behavior to balanced cells, with a decrease in cell proliferation. In contrast, the VV-like cells were somewhat resistant to the anti-inflammatory effect of Li.

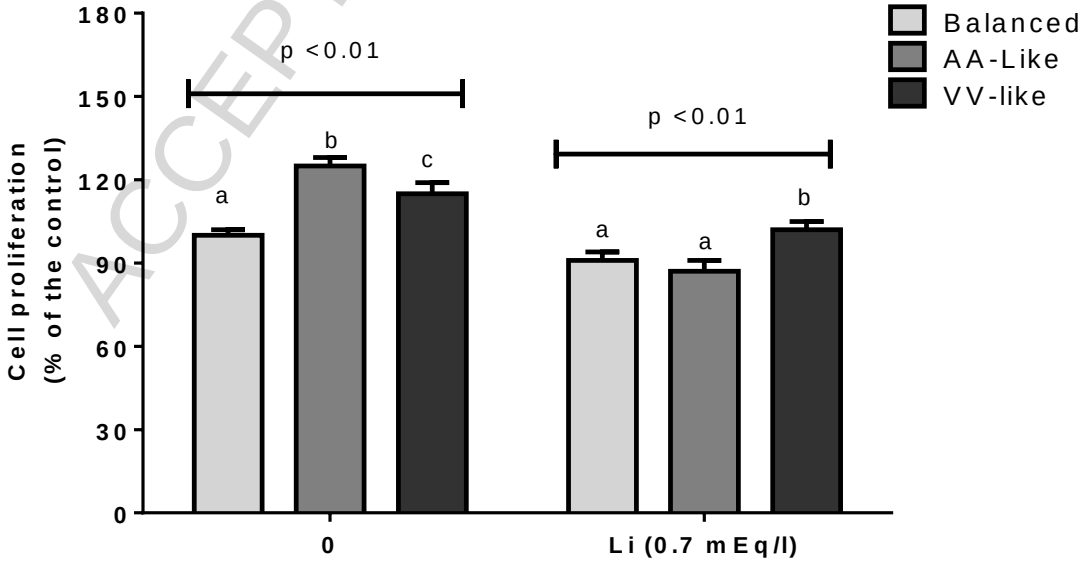

Figure 7: Effect on the inflammatory response to PHA in 72 h cell cultures of RAW 264.7 macrophages, and the anti-inflammatory effect of Li. Balanced RAW 264.7 macrophages or macrophages with an S-HP imbalance obtained by porphyrin treatment

(AA-like cells) or paraquat treatment (VV-like cells) were stimulated with PHA in the presence or absence of Li. Cell proliferation was used as a readout of the inflammatory response. Treatments were compared using a two-way analysis of variance followed by a *post hoc* Bonferroni test. The different letters (a, b, c) indicate statistical differences for each treatment at $p < 0.05$.

Discussion

In the present study, we investigated the potential influence of an S-HP imbalance on the Li anti-inflammatory response by analyzing GSK-3β gene and protein expression levels. We found that an S-HP imbalance, either genetically or pharmacologically triggered, had an impact on the anti-inflammatory effects of Li, being attenuated in VV- and VV-like cells that had higher basal S levels than AA or AA-like cells. We consider these results relevant since Li is a first-choice pharmacological treatment in bipolar disorder. In fact, Li has been used to treat bipolar disorder for a long time (since about 1960) and remains as the primary mood stabilizing medication (Crossley and Bauer, 2007; Nassar and Azab, 2014).

Prior evidence has also shown that Li has important anti-inflammatory effect; currently it is accepted that this effect occurs as a result of the ability of Li to inhibit the GSK-3β enzyme (Nassar and Asab, 2014). Despite this important Li effect, a considerable number of patients do not respond or tolerate Li treatment (Glitin, 2016); the reason for this is unknown, and in addition there are several side effects that have been reported that limit the use of Li in the treatment of bipolar disorder (Glitin, 2016). The differential effect of Li in some psychiatric patients suggests the occurrence of some pharmacogenetic influence. However, until now the main candidate gene have included genes involved in the therapeutic action of Li, and in general the results have not been reproducible (McCarthy et al., 2010; Can et al., 2014; Hou et al., 2016).

Since Li acts via the inhibition of GSK-3 β and an oxidative imbalance affects the inflammatory response, we hypothesized that genetic polymorphisms in the

oxidative pathway could be associated with a differential Li anti-inflammatory response. Prior studies have suggested that a SNP located in the SOD2 gene could directly affect the inflammatory state of cells (Montano et al., 2012). Complementary studies have also strongly suggested that the S-HP basal state can interfere with the cellular response to drugs, as well as the bioactive molecules present in some foods (Montano et al., 2012; Barbisan et al., 2014; Capeleto et al., 2015; Duarte et al., 2016).

The relevance of an S-HP imbalance on the modulation of inflammation has been thoroughly examined in a previous *in vitro* study that exposed PBMCs, derived from subjects with different Val16Ala-SOD2 genotypes (VV, AV, AA), to persistent PHA activation over 15 cell culture passages. This study clarified the relevance of an S-HP imbalance on the inflammatory response, showing that high basal HP-levels found in PBMCs from subjects with the AA- genotype had a more intense and short inflammatory response, whereas the high basal S-levels found in PBMCs from subjects with the VV-genotype sustained their inflammatory state for longer. In fact, the VV-inflammatory pattern seems to be similar to chronic inflammatory states that are associated with some non-transmissible diseases, such as bipolar disorder (Barbisan et al., 2017).

Based on the fact that humans have a large number of SNPs genes that could have some influence on cellular S-HP imbalance, a pharmacological S-HP model was designed using murine macrophage cells. This *in vitro* S-HP pharmacological model was previously used by Azzolin et al. (2016) and Berto et al. (2015) in colorectal and prostate cancer cells, respectively. These authors investigated the effect of an S-HP imbalance on the cancer proliferative state and the potential influence of the S-HP imbalance on resistance to chemotherapy. Both studies suggested that there was an important influence of the S-HP imbalance on cancer cell biology. However, whereas in

colorectal cancer cells, the high HP levels associated with an AA-like phenotype, induced high surveillance and proliferative state, whereas in prostate cancer cells a contrary effect of the S-HP imbalance was observed. Despite these different effects of the S-HP imbalance in different cancer cells, taken together these reinforce the relevance of this basal state in cellular biology, and possibly in the inflammatory response.

The results described here also suggest that the Val16Ala-SOD2 SNP could influence the effect of Li on GSK-3β, since, following Li treatment, AA-cells showed a strong down-regulation of both the GSK-3β gene and protein compared with AV-cells or VV-cells. The premise that supports this investigation came from prior studies suggesting an important effect of the Val16Ala-SOD2 genotype in inflammation. This effect was initially reported by Montano et al, (2012) in an *in vitro* study that showed higher levels of pro-inflammatory cytokines were present in PBMCs derived from subjects with the VV genotype, compared to the AA genotype. A complementary study confirming the effect of the Val16Ala-SOD2 genotype in the inflammatory response, was reported by Duarte et al, (2010) who studied inflammatory markers in hypercholesterolemic patients having the different SOD2 genotypes.

In Duarte et al., (2016) study, the VV-genotype subjects also had higher levels of pro-inflammatory cytokines, as well as higher levels of oxidative markers compared to the AA-genotype subjects. Subsequently, a clinical assay was conducted to assess if Val16Ala-SOD2 SNP could affect the pharmacological response of hypercholesterolemic patients to rosuvastatin treatment. The data also showed that VV-genotype subjects had higher levels of pro-inflammatory cytokines and were more resistant to decreased lipid levels on rosuvastatin treatment compared with A-allele subjects (Duarte et al., 2016). Considering the pharmacogenetic effects of Val16Ala-

SOD2 found for rosuvastatin treatment (Duarte et al., 2016), it might be possible to find similar results with respect to Li treatment. However, compared to hypercholesterolemic patients who practice a controlled lifestyle and pharmacological use of other drugs, patients with bipolar disorder are more difficult to include in pharmacogenetic studies. For this reason, we opted to conduct the present study using only *in vitro* protocols.

A causal mechanism involving a potential S-HP imbalance on GSK-3 levels could be considered as being two potentially correlated processes. First, macrophages principally produce S/HP and NO, when they are activated. An elevation in basal S levels related to the VV-genotype could predispose these macrophages to a "low-grade inflammatory state" or "a chronic inflammatory state in the presence of molecules or pathogens inductors" (Weigert et al., 2018; Barbisan et al., 2017). Second, inflammatory processes are triggered through GSK-3, which induces high levels of NF-κB, a key molecule that triggers an increase in the levels of pro-inflammatory cytokines, such as IL-1, IL-6, TNF-α, etc. Both endogenous and exogenous molecules, such as Li, are able to inhibit GSK-3. Inhibition of GSK-3 causes an elevation in the levels of the Creb transcription factor, which triggers the production of anti-inflammatory cytokines. Moreover, a decrease in the levels of GSK-3 has been associated with an increase in the levels of antioxidant enzymes, including SOD2 (Valerio et al., 2011). Since subjects with the VV-genotype have higher basal S levels than subjects with the A-allele genotypes, it is possible that the anti-inflammatory process, which involves an increase in the levels of antioxidant enzymes, could be impaired, contributing to the maintenance of a chronic inflammatory state, and that there are potentially higher GSK-3 levels in subjects with the VV genotype than the other genotypes described in the present study.

Currently, GSK-3β is recognized as a being a major regulator of the balance between pro-inflammatory and anti-inflammatory mediators in immune cells, including

microglia (Beurel and Jope, 2009). The protein is ubiquitously expressed, and is unusually active in resting, non-stimulated cells. In mammals, at least three proteins (α, β1, and β2), are generated from two different genes; GSK-3α and GSK-3β, are widely expressed at both the RNA and protein levels. Despite this, the control of GSK-3 activity occurs by complex mechanisms that depend on specific signaling pathways (Medina et al., 2011), some inhibitors like Li are also able to induce a direct downregulation of GSK-3β gene expression (Mendes et al., 2009). With respect to this, studies examining the pharmacological modulation of GSK-3β gene expression and protein levels are therefore relevant. Moreover, Li exerts its anti-inflammatory effects by suppression of pro-inflammatory molecules such as interleukin 1 beta (IL-1β), tumor necrosis factor-α (TNF-α), and the enhancement of anti-inflammatory cytokines such as IL-10 (Nassar and Azab, 2014). Some previous studies have also described an effect of Li on nitric oxide (NO) production as a consequence of GSK-3β enzyme inhibition (Wang et al., 2013; Azab et al., 2017).

However, although the relevance of S production during the inflammatory process is broadly accepted, prior studies involving the GSK-3β enzyme and S and HP modulation are still incipient. In the inflammatory process, for example in the presence of an antigen or in response to injury, high S levels are produced by the NAD(P)H oxidases found in all cell types that participate in the inflammatory response (leukocytes, endothelial cell, and other vascular cells, as well as others). Concomitantly, NO levels are also elevated in activated immune-cells, especially macrophages. Both, superoxide and NO exert their effects on the inflammatory response through multiple mechanisms, also inducing a high level of pro-inflammatory cytokines and macrophage proliferation (Guzik et al., 2003).

Considering the weight of evidence previously published in the literature around the Val16Ala-SOD2 SNP, it is plausible to accept that AA-genotype cells have an intense response when exposed to Li, triggering a more effective downregulation of GSK-3β at both the protein and gene levels. The reason for this appears to be that they have higher HP levels and lower S levels than VV-genotype cells. Moreover, the anti-inflammatory effect of Li on AA-PBMCs was confirmed by the decrease in cell proliferation rate in 72- h cultures. Very similar data for the effects of lithium on cell proliferation were found for macrophages treated with either paraquat or porphyrin. Cells treated with lithium and porphyrin (AA-genotype mimic) showed lower proliferation (72 hours) than cells treated with lithium and paraquat (VV-genotype mimic).

In contrast, VV-cells had higher S levels, and this reduces the inhibitory effect of Li on GSK-3β gene expression and protein level. In fact, some authors have observed that SOD2 overexpression triggers T-cell activation (Kamiński et al. (2013), and Barbisan et al (2017) have shown that AA-PBMCs responded more rapidly when exposed to phytohemagglutinin (PHA) than VV-PBMCs (Bresciani et al., 2015).

Figure 8 shows a schematic that summarizes the data found here. The figure shows the potential Li and S-HP interactions in the inflammatory response of PBMCs, as well as in macrophages with a pharmacologically induced imbalance in S-HP. In a cellular environment with low S levels and high HP levels, the Li effects on GSK-3β gene expression, protein levels, and the inflammatory pattern (evaluated by the cell proliferation rate) are fast and more intense. In a cellular environment with high S levels, the Li effects are slower, and less intense, and consequently the Li effect on the general inflammatory pattern (evaluated by the cell proliferation rate) is low.

Despite the methodological constraints associated with *in vitro* studies, we consider these results to be relevant. It is clear that GSK-3β gene and protein levels are related to the S-HP balance in immune cells, and moreover that there is a potential pharmacogenetic effect of Val16Ala-SOD2 on Li responsiveness in patients with bipolar disorders. However, this second issue needs to be clarified further in human studies that evaluate the relevance of this polymorphism in the treatment of bipolar disorder with Li.

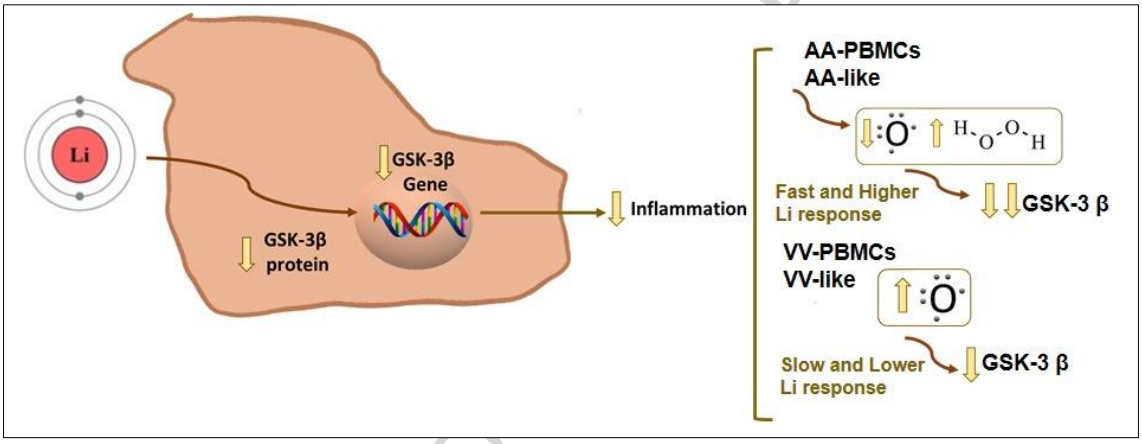

Figure 8 General scheme showing the inhibitory effects of lithium (LI) on GSK-3β gene expression and protein level associated with its anti-inflammatory action and the potential interaction with Val16Ala-SOD2 SNP homozygous genotypes (AA and VV) that cause a superoxide-hydrogen peroxide imbalance in immune cells resulting in a differential anti-inflammatory response to Li.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committees and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Author contributions

Conceptualization: FB; GW; IBMC

Formal analysis: FB; IBMC

Funding acquisition: EER; MMMFD; IBMC

Investigation: FB; VFA; CFT; GCM; MMMFD

Methodology: FB; VFA; CFT; GCM; PASPL; IBMC

Resources: FB; MHM; VB;IBMC

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Abbreviations list

GSK-3β: Glycogen synthase kinase 3 beta

TNF-α: Tumor necrosis factor alpha

IL-10: Interleukin 10

Li: Lithium

S: Superoxide

HP: Hydrogen peroxide

SNP: Single nucleotide polymorphism

MnSOD/SOD2:Superoxide dismutase manganese-dependent enzyme

MTS: Mitochondrial target sequence

Val: Valine

Ala: Alanine

PBMCs: Peripheral blood mononuclear cells

MnTBAP :Porphyrin

PHA: phytohaemagglutinin

ROS: Reactive oxygen species

FBS: Fetal bovine serum

DCF: Dichlorofluorescein

DCFDA: 2′–7′-dichlorofluorescein diacetate

mEq: Milliequivalents

L: Liter

IL-1β: Interleukin 1 beta

NO: Nitric oxide

Highlights

-The anti-inflammatory effects of lithium are influenced by superoxide-hydrogen (S-HP) imbalance.

- Elevated basal levels of superoxide decreased the anti-inflammatory effects of Lithium.

- Unbalance may be related to resistance of some patients to lithium treatment.