GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

In vitro effects of Ala16Val manganese superoxide dismutase gene polymorphism on human white blood cells exposed to methylmercury

T.D. Algarve[1], F. Barbisan[2], E.E. Ribeiro[3], M.M.M.F. Duarte[4], M.F. Mânica-Cattani[1], C.P. Mostardeiro[2], A.F. Lenz[1] and I.B.M. da Cruz[1,2,4]

[1]Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, Universidade Federal de Santa Maria, Santa Maria, RS, Brasil
[2]Programa de Pós-Graduação em Farmacologia, Universidade Federal de Santa Maria, Universidade Federal de Santa Maria, Santa Maria, RS, Brasil
[3]Universidade Aberta da Terceira Idade, Universidade do Estado do Amazonas, Manaus, AM, Brasil
[4]Laboratório de Biogenômica, Departamento de Morfologia, Centro de Ciências da Saúde, Universidade Federal de Santa Maria, Universidade Federal de Santa Maria, Santa Maria, RS, Brasil

Corresponding author: T.D. Algarve
E-mail: thais.algarve@gmail.com

Genet. Mol. Res. 12 (4): 5134-5144 (2013)
Received October 30, 2012
Accepted April 26, 2013
Published October 29, 2013
DOI http://dx.doi.org/10.4238/2013.October.29.7

ABSTRACT. Environmental contamination by methylmercury (MeHg) is an enormous public health problem in world regions such as Amazonia. MeHg toxic effects seem to be influenced by environmental and genetic factors. However, few studies have evaluated the genetic influences of MeHg toxicity in humans. Therefore, the aim of this study was to evaluate the genetic influence of Ala16Val manganese superoxide dismutase gene polymorphism (Ala16Val-MnSOD) on the cytotoxic effects of *in vitro* human leukocytes exposed to MeHg. Subjects were selected from 100 individuals aged 26.4 ± 7.3 years genotyped to Ala16Val-MnSOD

polymorphism (AA = 6, VV = 6, and AV = 12) to perform *in vitro* testing using white blood cells (WBCs). Reactive oxygen species production was measured using 2',7'-dichlorofluorescein diacetate fluorimetric assay, and cell viability was measured using MTT assay on WBC samples from the same subjects that were both exposed and not exposed to MeHg (2.5 µM for 6 h). The results showed that AA- and VV-WBCs exposed to MeHg did not display increased reactive oxygen species levels compared to those in cells that were not exposed. However, AV-leukocytes exposed to MeHg displayed increased ROS levels. Cellular viability comparison among genotypes exposed to MeHg showed that the viability of AA-WBCs was lower than that of VV-WBC, with mean values of 3.46 ± 0.13 and 3.08 ± 0.77 (standard error), respectively (P = 0.033), whereas heterozygous cells (AV) displayed intermediate values. This difference was likely due to the higher basal H_2O_2 production of AA-WBCs compared to that of other genotypes. These results suggest that the Ala16Val-MnSOD polymorphism has toxicogenetic effects in human cells exposed to MeHg.

Key words: Ala16Val manganese superoxide dismutase polymorphism; Methyl mercury; Cell viability; Toxicogenetic

INTRODUCTION

Methylmercury (MeHg) is a well-known potential threat to human health owing to its capacity to cause systemic toxicity that damages the body through neurotoxic effects; motor function impairment (Clarkson and Magos, 2006); immune, kidney, and cardiovascular system dysfunction (Moszczyński et al., 1998; Rutowski et al., 1998; Virtanen et al., 2007); and genotoxicity (Grotto et al., 2009).

The process responsible for triggering MeHg toxicity is incompletely understood and likely involves metabolic alterations such as increased oxidative stress (Su et al., 2008) and inflammatory response (Havarinasab and Hultman, 2005). Previous investigations have described the relationship of MeHg to changes in the activities of antioxidant enzymes such as superoxide dismutase (SOD) (Ariza et al., 1998) and catalase (CAT) (Hussain et al., 1999). It also induces the production of reactive oxygen species (ROS), especially hydrogen peroxide (H_2O_2) and superoxide anion radical ($O_2^{\bullet-}$) (Lund et al., 1991; do Nascimento et al., 2008), which promotes oxidative stress and lipid peroxidation. Populations exposed to high Hg levels such as those that occur in Amazonian communities have displayed oxidative stress biomarkers measured in whole blood, plasma, and hair, as well as significant inverse relationships between exposure and antioxidant enzymes such as glutathione peroxidase (GPx) and CAT have clearly demonstrated an association between Hg exposure and oxidative stress (Grotto et al., 2010). However, the dynamic of the Hg toxicological process in human cells and tissues involving oxidative metabolism is difficult to determine owing to the influence of several environmental and gene factors.

Conversely, gene polymorphism studies related to oxidative stress triggered by Hg intoxication metabolism have focused mainly on glutathione (GSH)-related enzymes (Engström

et al., 2011). However, to define genetically susceptible risk groups completely, research is also needed on the genes/proteins involved in the toxicodynamics - i.e, the mechanisms causing adverse effects in the organism (Gundacker et al., 2010). Therefore, gene polymorphism studies related to other antioxidant enzymes may improve understanding of the differences in Hg susceptibility among individuals.

Of particular interest is the manganese SOD enzyme (MnSOD), a primary antioxidant enzyme in the mitochondria that converts ROS into oxygen and H_2O_2 (Ferreira and Matsubara, 1997). ROS in mitochondria are produced via an oxidative phosphorylation pathway involved in energy production in the mitochondrial electron transport chain. Complexes I and III are the primary sources of $O_2^{\bullet-}$ production in mitochondria. Superoxide is released into the matrix from complex I, whereas it is released into both the matrix and the inter-membrane space by complex III (Murphy, 2009). In addition to being a major site of ROS production, mitochondria are a target for ROS and reactive nitrogen species and are compromised by severe or prolonged oxidative stress (Zorov et al., 2006). These conditions instigate a vicious cycle that amplifies mitochondrial ROS, whereby mitochondrial ROS/reactive nitrogen species cause oxidative damage to mitochondrial DNA leading to further mitochondrial dysfunction and oxidant generation (Fukai and Ushio-Fukai, 2011).

The human MnSOD enzyme gene displays a diallelic polymorphism in the mitochondrial targeting sequence (MTS) in which alanine 16 (GCT) is replaced with a valine (GTT) - the Ala16Val polymorphism (Ala16Val MnSOD). This polymorphism affects the import of MnSOD into the mitochondria by altering the conformation of its leader signal (Zelko et al., 2002). A study performed by Sutton et al. (2005), has suggested the Ala-MnSOD precursor generates 30-40% more MnSOD homotetramer than that generated by the Val-MnSOD precursor. Therefore, the Ala-MnSOD mitochondrial targeting sequence allows efficient MnSOD import into the mitochondrial matrix, whereas the Val-variant causes partial arrest of the precursor within the inner membrane and decreased formation of the active SOD tetramer in the mitochondrial matrix. The Ala16Val polymorphism results in 3 genotypes: AA, VV, and AV (Sutton et al., 2005).

Several studies, including our own investigations and those of other researchers, have suggested that the Ala16Val MnSOD polymorphism is associated with various oxidative stress-dependent pathologies. Interestingly, in some cases, the disease risk is associated with the A allele or AA genotype - for example, in prostate cancer (Taufer et al., 2005), breast cancer (Bica et al., 2009), and other cancers. Disease risk is linked to the V allele or VV genotype in diabetes microvascular complications (Tian et al., 2011), obesity (Montano et al., 2009), hypercholesterolemia (Duarte et al., 2010), and metastatic potential of breast cancer (Bica et al., 2010).

Recently, we began testing whether this polymorphism influences responses to toxic agents in the human body. The first *in vitro* protocol that evaluated whether cell carriers of Ala16Val MnSOD polymorphism display differential responses when exposed to ultraviolet radiation (UV) showed that human VV-lymphocyte cell cultures had lower viability, lower mitotic index, and higher lipoperoxidation levels than those of AA genotypes in both control and UV-exposed groups (dos Santos Montagner et al., 2010). Furthermore, this polymorphism has *in vitro* influence on antioxidant drug response (Costa et al., 2012). Therefore, we postulated that Ala16Val MnSOD polymorphism influences the viability of white blood cells (WBCs) exposed to MeHg *in vitro*.

MATERIAL AND METHODS

Reagents

All of the chemicals used for the biochemical and molecular analyses in this study were purchased from Sigma (St. Louis, MO, USA), Invitrogen (Carlsbad, CA, USA), Mo Bio (Carlsbad, CA, USA), BD Diagnostics (Plymouth, DN, UK) and Cultilab Co. (Campinas, SP, BR).

Subject selection

To test MeHg cytotoxicity in human WBCs in carriers of different Ala16Val genotypes, we analyzed ROS production and cell viability. Blood was obtained from healthy adult subjects enrolled at Universidade Federal de Santa Maria in a Brazilian region with no history of MeHg exposure (Rio Grande do Sul). Initially, we selected 100 young, healthy people (age, 26.4 ± 7.3 years old) and considered the following factors for inclusion: non-smoking, not obese, no chronic use of medication or vitamin supplements, no previous cardiovascular medical history or hypertensive disorder, and no metabolic diseases or other morbidity that could affect the results. The research study described herein was approved by the ethics committee of the Universidade Federal de Santa Maria (No. 23081.015838/2011-10). All blood cell donors signed a consent form.

Ala16Val MnSOD genotyping

Blood samples were collected from the study group via Vacutainer® tubes with EDTA. Genomic DNA was isolated using a DNA Mini Purification Kit. Genotypes of Ala16Val MnSOD gene polymorphism was assessed via polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) as described by Costa et al. (2012).

In vitro treatments

A subgroup of the initial study group was asked to donate blood again for *in vitro* tests because a previous study performed by Montano et al. (2009), has shown an association between the Ala16Val polymorphism, dietary behavior, and health conditions. The 8 mL blood samples were collected via venipuncture using tubes with heparin, which were centrifuged within 1 h of collection for 15 min at 3000 rpm. Then the WBC were collected and resuspended in PBS buffer with 2% glucose at a final concentration of 1 x 10^6 cells/mL. Two treatments were performed for each sample: negative control and concentrated exposure to 2.5 µM MeHg (Shenker et al., 2002). The samples were maintained in sterile conditions at 37°C for 6 h.

Cell viability assays

Cell viability assays were performed using a 96-well plate. Cytotoxicity was evaluated using a 3-(4,5dimethylthiazol-2-yl)-2,5-diphenyltetrazolic bromide reduction assay described by Mosmann (1983), the absorbance of each well was measured at 570 nm (SpectraMax M2/M2e Multi-mode Plate Reader, Molecular Devices Corporation, Sunnyvale, CA, USA). All tests were performed in triplicate for each sample.

Reactive oxidative species (ROS) WBC production

Intracellular ROS production was detected in WBC samples using the 2',7'-dichlorofluorescein diacetate (DCFDA). The fluorescence was measured at an excitation of 485 nm and an emission of 520 nm (SpectraMax M2/M2e Multi-mode Plate Reader, Molecular Devices Corporation, Sunnyvale, CA, USA). All tests were performed in triplicate for each of the samples tested (LeBel et al., 1992; Halliwell and Whiteman, 2004).

Statistical analysis

All analyses were carried out using SPSS, version 18.0 (SPSS Inc., Chicago, IL, USA). Cytotoxicity in WBC samples from different Ala16Val MnSOD donors treated with and without MeHg was compared using 1-way analysis of variance followed by post hoc Tukey's test. All P values were 2-tailed. The alpha value was set to <0.05 to determine statistical relevance.

RESULTS

The MnSOD genotype frequencies were as follows: AA = 31% (31), VV = 23%, and AV = 46%. The calculation of possible deviation from Hardy-Weinberg equilibrium, which was used to assess the c2 goodness of fit, showed that the samples were in genetic equilibrium. From this sample, we selected 24 volunteers (AA = 06, VV = 06, and AV = 12) with similar lifestyle behaviors to perform the WBC *in vitro* exposure to avoid possible environmental interferences.

Initially, we evaluated the potential toxicity of MeHg treatment considering the whole of the WBC samples. We observed that after *in vitro* treatment, the viability of WBCs exposed to MeHg decreased significantly to 89.1 ± 15.4% (P = 0.022). The viability was also compared among WBC samples with different Ala16Val MnSOD genotypes without MeHg treatment. After 6 h, the WBC viability under *in vitro* conditions showed differences among Ala16Val MnSOD genotypes. The absorbance in the AA [3.46 ± 0.13 standard error (SE)] and AV (3.23 ± 0.8 SE) groups was significantly higher than that in the VV group (3.08 ± 0.77 SE P = 0.033), indicating that VV cells presented lower viability than that of AA and AV cells independent of MeHg treatment. ROS production was also higher in VV than in AA and AV control groups (VV = 477.7± 137.45; AA = 343.9 ± 184.3; AV = 297.0 ± 155.7 P = 0.0001).

MeHg WBC ROS and cytotoxicity were influenced by the Ala16Val MnSOD genotypes. As shown in Figure 1, in the AA-WBC samples, the ROS production was similar between the control and MeHg treatments (P = 0.532). Similar results were also observed in VV-WBC samples (P = 0.516). However, in the heterozygous (AV-WBC), the ROS production increased 27% when exposed to MeHg (P = 0.037).

Despite the AA-WBC present similar ROS production between the control and MeHg treatments, cell viability decreased significantly in the presence of MeHg (P = 0.020). As seen in Figure 2, these results were not observed in the VV-WBC and AV-WBC groups. Cell viability was similar between the control and MeHg treatments (P = 0.145 and P = 0.759, respectively).

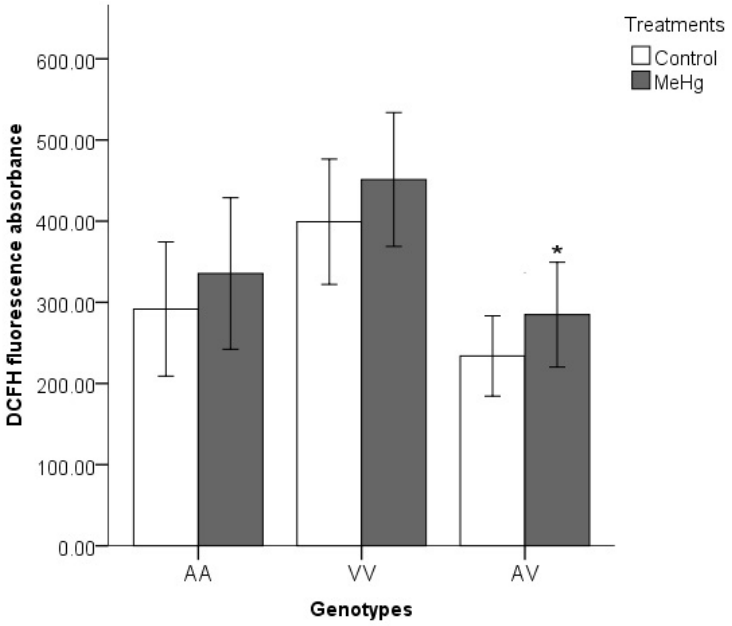

Figure 1. Reactive oxygen production (ROS) comparison among white blood cell carriers (WBC) and different Ala16Val MnSOD genotypes. The asterisk indicates a significant increase from the untreated control ($P < 0.05$).

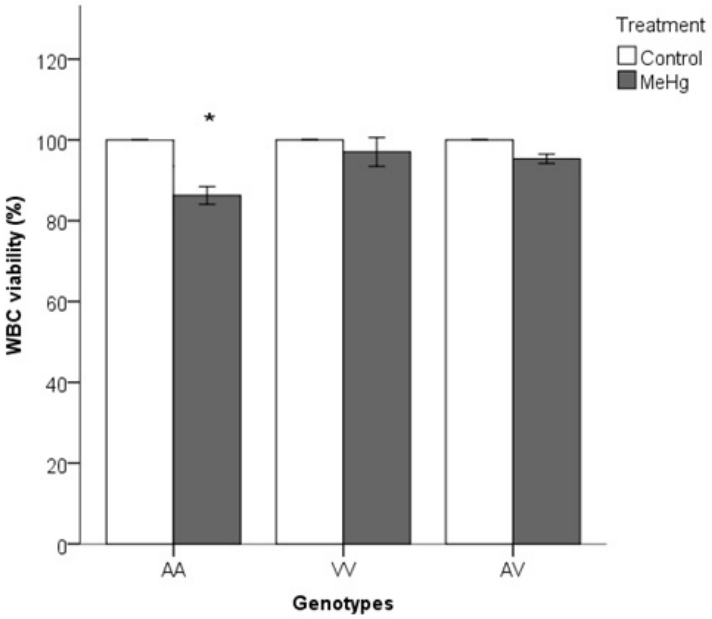

Figure 2. Viability (% in relation to control group considered as 100% viability) between WBC with different Ala16Val MnSOD genotypes exposed to MeHg compound. The asterisk indicates significant decrease from the untreated control ($P < 0.05$).

DISCUSSION

In vitro MeHg exposure in WBCs obtained from human subjects increase ROS and decreased cell viability. The results corroborate investigations performed in animals and *in vitro* protocols that have shown an increase in cellular MeHg-induced oxidative stress and cytotoxicity effects. *In vivo* and *in vitro* studies have demonstrated that MeHg-exposed systems in various species display an increase in reactive species such as $O_2^{\bullet-}$, H_2O_2, and nitric oxide (Farina et al., 2011). Excessive free radical formation has been implicated as a causative factor in neurotoxic damage associated with a variety of metals, including MeHg. MeHg-induced systems increase ROS formation in astrocytes as well as in the presence of superoxide. Hydroxyl scavenger antioxidant molecules attenuate MeHg-induced ROS formation (Shanker et al., 2005). Bussolaro et al. (2010) have investigated the hepatocytes of the tropical fish *Hypostomus commersoni* and observed that MeHg deregulates the redox intracellular milieu leading to cell death and oxidative stress.

The severity of toxic effects depends on the dose, duration, and timing of exposure. Humans, however, show a greatly varying susceptibility toward toxic substances even under comparable exposure situations. Several biological and non-biological factors (e.g.; age, gender, diet) can modulate individual susceptibility to toxic compounds. Gene variation plays a certain role because it constitutes various detoxifier phenotypes ranging from individuals with regular enzyme and detoxification functions to poor metabolizers with low or no enzyme activity to high metabolizers (Gundacker et al., 2010).

In humans, cytosolic GSTs catalyze intracellular conjugation reactions between GSH and various electrophilic substrates, among them Hg. These GSH conjugates can be easily effluxed from cells. Concomitant GSH depletion must be compensated by *de novo* synthesis (Dickinson and Forman, 2002). Thus, the interplay of all GSH system components is required for GSH affine mercury to be rapidly bound and removed from the cell. Evidence suggests that gene variants can influence mercury toxicokinetics. This genetic background can therefore potentially explain some of the individual vulnerability toward mercury toxicity (Gundacker et al., 2010).

Mercury is capable of altering the expression and activity of many genes/proteins, but functional proof of that influence is lacking. Many studies of Hg toxicokinetic and detoxification pathways have analyzed the impact of gene knockout/knockdown or a gene sequence variation (mostly single-nucleotide polymorphisms) (Gundacker et al., 2010), although none of these polymorphisms is as directly related to Hg toxicity as much as SODs are by ROS toxicity pathways. The major daily source of ROS in the cell is the mitochondria, in which MnSOD is located to protect against $O_2^{\bullet-}$ and the Ala16Val polymorphism executes its role.

Our results were directly influenced by the Ala16Val MnSOD genotypes of WBC samples. VV-WBCs exposed to MeHg presented no increase in ROS production, nor did they display cell viability similar to that in the control group, suggesting a protective role against mercury intoxication. However, AA-WBCs displayed similar ROS production with and without MeHg exposition, whereas cells exposed to MeHg displayed decreased cell viability compared to that of the control group. The heterozygous genotype (AV) was the intermediate phenotype of AA- and VV-WBCs because it displayed an increase in ROS production but no decreased cell viability. These results may be related to H_2O_2 production, which is lower in the VV genotype compared with that in the AA genotype.

The MnSOD enzyme belongs to a set of antioxidant defense mechanisms that prevent high levels of free radicals, limiting deleterious effects to macromolecules in the cell. Because su-

peroxide radicals are produced by nicotinamide adenine dinucleotide phosphate oxidase, xanthine oxidase, nitric oxide synthase, lipoxygenase, and mitochondrial enzymes, they must be highly controlled by the cell. A superoxide radical is converted by SOD to H_2O_2, which in turn is reduced to water by CAT, GPx, and peroxiredoxins. Thus, MnSOD (as well as others SODs) is considered a first line of defense against the toxicity of superoxide anion radicals (Fukai and Ushio-Fukai, 2011).

This enzyme is synthesized in the cytoplasm and directed to the mitochondria by a peptide signal. Therein, it is involved in dismutating superoxide radicals generated mainly by the electron transport chain from cellular respiration. The Ala16Val MnSOD polymorphism in the peptide signal region has 2 homozygous genotypes with unbalanced MnSOD activity because the AA genotype present higher efficiency of enzyme transport into the mitochondria than VV genotype. In biochemical terms, the AA genotype converts a higher quantity of superoxide radicals in H_2O_2. However, unlike SOD1, MnSOD does not show allosteric inhibition by H_2O_2 production, and the excess H_2O_2 produced by AA can undergo spontaneous conversion or react with transition metals, producing a hydroxyl radical ($^{\bullet}OH$) (Beyer and Fridovich, 1987). Because the hydroxyl radical presents high affinity to the DNA molecule causing mutations, the Ala allele or genotype AA has been associated with an increased risk for breast cancer (Taufer et al., 2005; Bica et al., 2009).

Conversely, the VV genotype does not display efficient superoxide radical dismutation in H_2O_2. The superoxide radical is extremely reactive, mainly with nitric oxide, leading to the production of the strong oxidant peroxynitrite, which has a higher affinity for the lipids of the membrane that causes lipoperoxidation. The VV genotype has been associated with carotid atherosclerosis (Kakko et al., 2003) and higher oxidized low-density lipoprotein levels in Brazilian subjects with synergic effects in type II diabetes (Gottlieb et al., 2005), obesity (Montano et al., 2009), and hypercholesterolemia (Duarte et al., 2010).

The results described herein and those of our previous studies showed that *in vitro* lymphocyte exposure to UV radiation produced lower toxicity in AA cells compared with that in VV cells (dos Santos Montagner et al., 2010). Therefore, we postulated that an MnSOD gene presents a dual protective characteristic dependent on toxic agents (Figure 3). This dual characteristic is similar to that observed in the association between the Ala16Val MnSOD gene polymorphism and susceptibility to non-transmissible diseases (Bica et al., 2010).

Studies supporting this hypothesis are still incipient. However, Lund et al. (1991) have observed that Hg compounds such as mercuric ion present mitochondrial GSH as a principal intracellular target and increase H_2O_2 approximately 4-fold in rat kidney mitochondria. The authors suggest that H_2O_2 formation by mercuric ion may lead to oxidative tissue damage, such as lipid peroxidation, which is observed in Hg-induced nephrotoxicity.

Another question that needs to be answered is related to WBCs as cell potentially affected by Hg exposure. Studies using animal models have demonstrated that Hg affects immune function in a complex manner. This influence depends on both the species of Hg used and the genetic background under which exposure takes place. In genetically susceptible mouse strains, inorganic and organic Hg species, including MeHg, induce autoimmunity, resulting in a lupus-like condition (Havarinasab and Hultman, 2005). Investigations suggest that humans may also be susceptible to the immunotoxic effects of MeHg exposure, which has been associated with an increased risk of lupus and greater severity of scleroderma (Cooper et al., 2004). Among the toxic effects of MeHg on polymorphonuclear leukocytes was stimulation of ROS formation, including superoxide radicals and H_2O_2 (Jansson and Harms-Ringdahl, 1993). Therefore, WBCs can be a good model for *in vitro* evaluation of the potential influence

of human genetic polymorphisms in responses to Hg contamination. Based on these results, complementary investigations may improve understanding of the biochemical influence of MnSOD modulation in cellular MeHg toxicity.

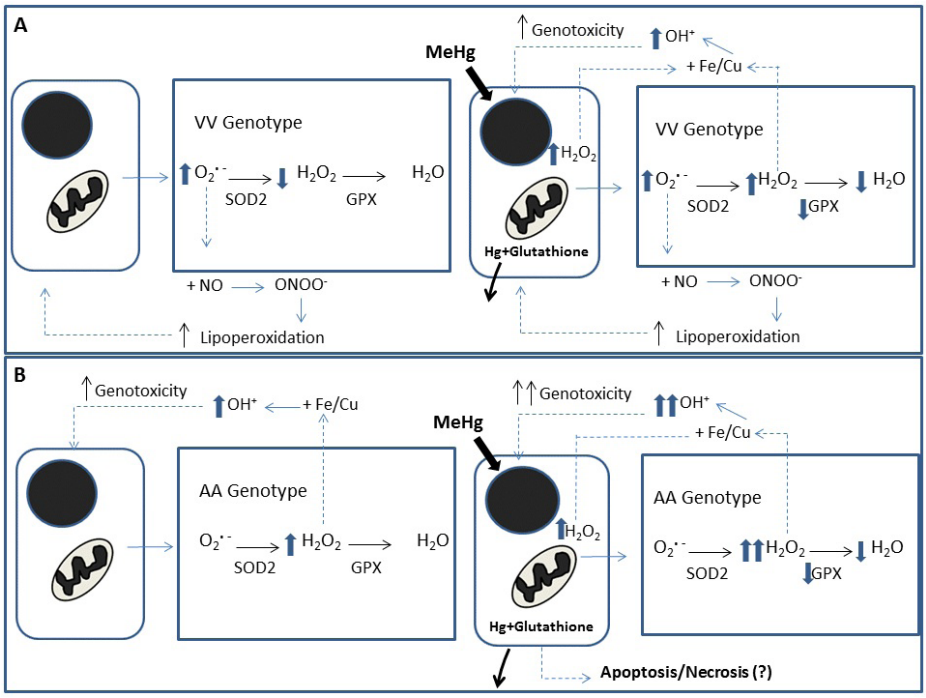

Figure 3. Synthesis of the potential protective role of human VV genotypes of Ala16Val MnSOD gene polymorphism on toxic effect caused by MeHg acute exposition. **A.** VV genotype metabolizes smaller rates of $O_2^{\bullet -}$ than AV and AA genotypes, which can react to NO producing $ONOO^-$, resulting in lipoperoxidation. When Hg enters into the cell, MeHg rapidly binds to reduced glutathione (GSH) besides binding other thiol groups (-SH) present in proteins. Not only does GSH deflect mercury from binding to target proteins inside the cell, but it also serves as a means of removing mercury from the cells. As GSH is consumed through mercury binding, lower amounts of this molecule are available to prevent H_2O_2 toxic effects, which in turn (by Fenton's reaction or spontaneously) produce $^{\bullet}OH$ that leads to DNA damage. **B.** AA genotype presents the highest $O_2^{\bullet -}$ dismutation rates between genotypes resulting in excessive production of H_2O_2 that needs to be processed by glutathione (mitochondria and cytoplasm) and catalase (cytoplasm). However, H_2O_2 production rates exceeding enzymatic metabolization capacity in physiological conditions, result in DNA damage. This genotype, in the presence of Hg, is exposed to a higher amount of H_2O_2 than they are used to, leading to an increase in DNA damage, reaching the cell apoptosis/necrosis.

The study described herein presents some methodological concerns. The choice to perform a short test was based on the idea that the protocol included a large number of subjects to evaluate toxic effects in *in vitro* human blood samples exposed to MeHg. However, the protocol used herein did not permit the analysis of various oxidative and antioxidant biomarkers in the samples exposed or not exposed to MeHg. However, these limitations can be solved through complementary cell culture studies that search causal mechanisms to discover whether gene polymorphism has toxic response influence as observed in the present study.

Because human studies involving controlled toxicological exposure are ethically inappropriate, studies that integrate genetic polymorphism and *in vitro* protocols as performed here can, despite their methodological limitations, be useful to our understanding of the toxicogenetic effects of compounds such as Hg.

ACKNOWLEDGMENTS

Research supported by grants and fellowships of the Brazilian research support agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (#300969/2009-0), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (#1006741), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior.

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