Toxicology in Vitro 24 (2010) 1410–1416

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Toxicological effects of ultraviolet radiation on lymphocyte cells with different manganese superoxide dismutase Ala16Val polymorphism genotypes

Greice Franciele Feyh dos Santos Montagner [a], Michele Sagrillo [b], Michel Mansur Machado [a], Renata Chequeller Almeida [c], Clarice Pinheiro Mostardeiro [c], Marta Maria Medeiros Frescura Duarte [d], Ivana Beatrice Mânica da Cruz [a,c,e,*]

[a] Programa de Pós-Graduação em Bioquímica Toxicológica, Universidade Federal de Santa Maria, Av. Roraima 1000, Prédio 18, 97105900 Santa Maria, RS, Brazil
[b] Centro Universitário Franciscano – UNIFRA, Rua dos Andradas, 1614, 97010-032 Santa Maria, RS, Brazil
[c] Programa de Pós-Graduação em Farmacologia, Universidade Federal de Santa Maria, Av. Roraima 1000, Prédio 21, 97105900 Santa Maria, RS, Brazil
[d] Universidade Luterana do Brasil – ULBRA, BR 287, Km 252, Trevo Maneco Pedroso, Boca do Monte, Cx. Postal 21834, 97020-001 Santa Maria, RS, Brazil
[e] Universidade Federal de Santa Maria, Av. Roraima 1000, Prédio 19, Office 3126, 97105900, Santa Maria, RS, Brazil

ARTICLE INFO

Article history:
Received 2 March 2010
Accepted 19 April 2010
Available online 24 April 2010

Keywords:
ALa16Val MnSOD polymorphism
Oxidative stress
DNA damage
Ultraviolet radiation
Lymphocyte culture
Genotoxicity

ABSTRACT

The aim of this study was to investigate whether there is a differential response of lymphocytes from healthy MnSOD genotype subjects to oxidative stress. We used UV radiation as a toxic agent due to its genotoxic effects associated with chromosome aberrations caused by breaks in the DNA strands. Cellular growth rate, cell viability, mitotic index, chromosomal instability and biomarkers of oxidative metabolism were analysed in lymphocyte cells from healthy adults with different Ala16Val MnSOD polymorphisms that produce tree genotypes: AA, VV and AV. We found a differential response to UV exposure in cultures of lymphocyte cells from Ala16Val genotype donors. In general, AA cell cultures presented higher viability and mitotic index and lower TBARS levels than VV and AV cells for both the control and UV exposure groups. However, when we compared the DNA damage among the three genotypes, AA lymphocyte cells presented the highest damage from UV exposure. These data suggest that the Ala16-Val polymorphism affects the response of cellular oxidative metabolism in different ways.

1. Introduction

Endogenous anti-oxidant enzymes are vital to the regulation of oxidative stress within cells. One of the primary cellular anti-oxidants, superoxide dismutase (SOD), catalyses the conversion of superoxide (O_2^-) to hydrogen peroxide (H_2O_2), which can then be removed by catalase (CAT), glutathione peroxidase or peroxiredoxins (Emerit, 1994).

There are three distinct types of SOD activity found in human cells: two Cu/Zn-SOD enzymes and a mitochondrial matrix homotetrameric MnSOD (Martin et al., 2009). Several lines of evidence suggest that MnSOD is crucial for life of organisms (Li et al., 1995). MnSOD is directly involved in mitochondrial superoxide catalysis, and investigations have suggested that MnSOD acts as a tumour suppressor gene (Oberley et al., 1984). Data suggest that superoxide dismutase derivatives can significantly inhibit both the production of ROS and metastatic tumour growth (Hyoudou et al., 2008). However, *in vitro* studies have shown that a number of cancer cell lines contain elevated levels of SOD and decreased levels of CAT, and that this change in steady-state levels of H_2O_2 correlates with increased metastasis, proliferation and resistance to apoptosis (Chuang-Man et al., 2001; Skrzydlewska et al., 2003).

Epidemiologic evidence has also linked a single nucleotide polymorphism in MnSOD (SOD2 gene, rs4880) that increases the enzyme's activity to the risk of developing several cancers. This polymorphism occurs due to a single nucleotide substitution at nucleotide 47, changing the encoded amino acid from Ala (GCT) to Val (GTT); it is known as the Ala16Val polymorphism in the mitochondrial targeting sequence (MTS) (Shimoda-Matsubayasgi et al., 1996; Millikan et al., 2004). The Ala16Val polymorphism results in three genotypes: AA, VV and AV.

Investigations using an *in vitro* Ala MnSOD precursor generated 30–40% more of the active, matrix-processed MnSOD homotetramer than the Val MnSOD precursor. The Ala-MTS allowed efficient MnSOD import into the mitochondrial matrix, whereas the Val variant caused partial arrest of the precursor within the mitochondrial

* Corresponding author at: Programa de Pós-Graduação em Bioquímica Toxicológica, Universidade Federal de Santa Maria, Av. Roraima 1000, Prédio 18, 97105-900 Santa Maria, RS, Brazil. Tel.: +55 55 32208736; fax: +55 55 32208239.
E-mail addresses: greiceijui@ibest.com.br (G.F.F. dos Santos Montagner), sagrillomr@yahoo.com.br (M. Sagrillo), michelmachado@globo.com (M.M. Machado), chequeller@hotmail.com (R.C. Almeida), claricem@unijui.edu.br (C.P. Mostardeiro), duartmm@hotmail.com (M.M.M.F. Duarte), ibmcruz@hotmail.com (I.B.M. da Cruz).

0887-2333/$ - see front matter Crown Copyright © 2010 Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.tiv.2010.04.010

inner membrane and decreased formation of the active MnSOD homotetramer in the mitochondrial matrix (Sutton et al., 2003).

Several studies, including investigations performed by our team, have suggested that the Ala allele is associated with an increased risk of breast (Ambrosone et al., 1999; Egan et al., 2003; Eras-Erdogan et al., 2009; Bica et al., 2009) and prostate cancers (Taufer et al., 2005; Woodson et al., 2003; Bica et al., 2009). Moreover, the Val allele has been associated with an increased risk of lung (Wang et al., 2001) and bladder cancers (Hung et al., 2004), as well as with cardiomyopathy (Hiroi et al. 1999), higher oxidised LDL levels (Gottlieb et al., 2005) and obesity (Montano et al., 2009). In a recent study performed by us on the lymph node status of women with breast cancer, we showed that even though the AA genotype is well established as being associated with an increased risk of breast cancer, the VV genotype was actually associated with a higher metastatic potential (Bica et al., 2010).

These studies suggest a dual nature of the anti-oxidant enzyme system in the progression of chronic degenerative diseases that needs to be explored with complementary investigations. In this context, the aim of this study was to investigate whether there is a differential response of lymphocytes from healthy MnSOD2 genotype subjects to oxidative stress. Cellular growth rate, cell viability, mitotic index, chromosomal instability and biomarkers of oxidative metabolism were analysed in lymphocyte cells from healthy adults with different Ala16Val MnSOD gene polymorphisms.

2. Material and methods

2.1. Design

From a previous genetic population analysed for several epidemiological aspects associated with Ala16Val gene polymorphism frequency (Taufer et al., 2005; Montano et al., 2009; Bica et al., 2009), we selected healthy subjects that did not smoke, drink, or use chronic medication and had different genotypes (AA, VV and AV) without any diseases or morbidity conditions that could influence the lymphocyte *in vitro* analysis. The mean age was 36 ± 4.6 years old. Since, a previous study performed by Montano et al. (2009) showed no influence of the Ala16Val polymorphism in dietary behaviour, we chose volunteers with similar lifestyle variables related to diet and physical activity to avoid possible environmental interferences. Additionally, we asked volunteers to avoid consuming any anti-oxidant-containing food 24 h before blood collection. The list of main foods that were not consumed by the volunteers included salads, fruits and juices (natural or manufactured). As studies involving polymorphisms and *in vitro* assays are still incipient, it is difficult to assess a statistical power test, and the choice of the number of enrolled subjects was justified based on practical and experimental reasons.

For this reason, we believe that the sample size is sufficient for statistical comparisons. We used 4–6 subjects for each Ala16Val genotype.

2.2. Blood sample collection

Peripheral blood samples were collected after 12-h overnight fasting by venipuncture using top Vacutainer® (BD Diagnostics, Plymouth, UK) tubes with heparin. One aliquot of each sample was used for cell culture, and the other aliquots were for the biochemical and DNA damage analysis. Blood specimens (5 ml) were routinely centrifuged within 1 h of collection for 15 min at 2500*g*, and aliquots of plasma and erythrocyte samples were stored at −20 °C until biochemical analysis of the cell cultures. A blood sample (10 ml) was used for cytogenetic and biochemical analysis of the lymphocyte cultures.

2.3. Cell culture and UV radiation

Lymphocytes obtained through gradient centrifugation were immediately transferred to culture media containing 1 ml RPMI 1640 (GIBCO) with 10% foetal calf serum (FCS) and 1% penicillin/streptomycin. Four culture tubes per subject were prepared, and two samples tubes were exposed directly to UV radiation, as previously described by Eldridge et al. (1992), using a 254 nm peak wavelength (germicidal lamp). The incident dose rate was 28.7 J/m^2/s of UV light for 15 min (UV treatment).

We used UV exposure because this agent mainly induces pyrimidine dimers and relatively very few strand breaks, although some transient ones are generated during excision repair of the dimers. The UV exposure time was chosen from a previous test that evaluated oxidative biochemical effects at 0, 20, 60 and 120 min of UV exposure in lymphocyte cell cultures. At 15 min of UV exposure, we observed higher lipid peroxidation and decreased catalase and superoxide dismutase levels without cellular death compared to levels prior to UV exposures. The cells were maintained in suspension culture at 37 °C in a humidified 5% CO_2 atmosphere in RPMI 1640 growth medium (RPMI 0 medium) for 72 h. Cells not exposed to UV were used as controls.

2.4. Mitotic index and chromosomal instability

After UV exposure, one replicate of each treatment was used to investigate the mitotic index and chromosomal instability using G-band cytogenetic analysis (Yunis, 1996). At least 50 mitoses were analysed in each sample.

2.5. Single cell gel electrophoresis (comet assay)

The alkaline comet assay was performed as described by Singh et al. (1995) in accordance with the general guidelines for use of the comet assay (Tice et al., 2000; Hartmann et al., 2003; Nadin et al., 2001). One hundred cells (50 cells from each of the two replicate slides) were selected and analysed. Cells were visually scored according to tail length and received scores from 0 (no migration) to 4 (maximal migration). Therefore, the damage index for cells ranged from 0 (all cells with no migration) to 400 (all cells with maximal migration). The slides were analysed under blind conditions by at least two different individuals.

2.6. Cell viability

The cytotoxic effects of UV radiation on 72-h lymphocyte cell cultures from different Ala16Val MnSOD genotypes were determined by the loss of membrane integrity using the Trypan blue dye exclusion method (Burow et al., 1998). At least 300 cells were counted for each survival determination. Cell viability was expressed as a percentage of the control value.

2.7. Biochemical analysis

Biochemical analysis associated with oxidative metabolism was performed using blood samples collected from the study subjects. We analysed the biochemical basal values and 72-h lymphocyte culture values. Total polyphenols were spectrophotometrically determined in plasma by reading the absorbance at 750 nm (Folin–Ciocalteau method) and using gallic acid as a standard, as described by Chandra and de Mejia (2004). Total phenol concentrations of plasma samples were determined after a procedure of acid extraction/hydrolysis and protein precipitation with 0.75 mol L^{-1} metaphosphoric acid (MPA). For hydrolyzing the conjugated forms of polyphenols, hydrochloride acid was added to the sample, followed by sodium hydroxide in methanol. This step breaks the links

Table 1
Characteristics baselines and biomarkers of oxidative metabolism in blood samples after 12 h overnight fasting of healthy volunteers with different SOD2 genotypes.

Variables	AA (mean ± SD)	VV (mean ± SD)	AV (mean ± SD)	*p*
Age (mean ± SD)	22.65 ± 3.67[a]	22.04 ± 3.08[a]	22.78 ± 3.99[a]	0.673
BMI (kg/m^2)	21.76 ± 2.35	22.08 ± 2.39	22.87 ± 2.44	0.546
TBARS (nmol/mL erythrocytes)	8.05 ± 3.46[a]	23.82 ± 11.03[a]	14.74 ± 12.87[a]	0.173
Thiol groups (mmol/mL erythrocytes)	226.82 ± 70.42[a]	212.93 ± 80.19[a]	268.87 ± 91.68[a]	0.710
SOD (uSOD)	53.22 ± 4.57[a]	40.49 ± 6.41[b]	56.73 ± 5.60[ab]	0.036
Polyphenols (mg/mL plasma)	4.44 ± 0.33[a]	5.87 ± 0.75[b]	4.70 ± 0.83[ab]	0.049
Catalase (μmol of H_2O_2/mL eritrocytes/min)	31.28 ± 8.74[a]	29.02 ± 3.67[a]	33.26 ± 7.86[a]	0.753
Protein carbonyl (nmol/mg protein)	0.09 ± 0.11[a]	0.08 ± 0.02[a]	0.07 ± 0.01[a]	0.135
Acid ascorbic (nmol/mL plasma)	18.24 ± 9.96[a]	19.80 ± 11.78[a]	20.82 ± 5.51[a]	0.923

SD = standard deviation. Different letters in each treatment represent significant differences among genotypes using analysis of variance followed by Tukey test. *p* values < 0.05 were considered significant.

of polyphenols with lipids and provides a first extraction of polyphenols. MPA was used in this procedure for removing plasma proteins. The final extraction of polyphenols was performed by adding a 1:1 (v/v) solution of acetone:water. The results were expressed as the gallic acid equivalent (GAE) in mg L^{-1}. Thiol groups were determined as described by Ellman (1959). Lipid peroxidation was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS) (Ohkawa et al., 1979). Total blood SOD (E.C.1.15.1.1) activity was measured spectrophotometrically according to Boveris and Cadenas (1997). One unit of activity is defined as the amount of enzyme required to inhibit the rate of epinephrine autoxidation by 50%. Catalase activity (EC 1.11.1.6.) was determined according to Aebi (1984). One unit of catalase activity was defined as the activity required to degrade 1 μmol of hydrogen peroxide in 60 s. The total ascorbic acid concentration was measured according to a method adapted from Jacques-Silva et al. (2001). Protein carbonyls were measured according to the method previously described (Morabito et al., 2004). Results were expressed as nanomoles of carbonyl groups per mg protein.

2.8. Statistical analysis

All analyses were carried out using the statistical package for social studies SPSS version 12.0 (SPSS Inc., Chicago, IL). Two statistical analyses were performed. The first one compared the mean values among different genotypes without or with UV exposure using an analysis of variance followed by a *post hoc* Tukey test. The second analysis compared the effect of UV exposure in each genotype's lymphocyte culture using the Student's *t* test. We chose these statistical tests because a previous statistical analysis performed using the Kolmogorov–Smirnov test showed normal distribution of the variables investigated here. All *p* values were two-tailed. The alpha value considered to be statistically significant was $p = 0.05$.

3. Results

Baseline characteristics of healthy volunteers with different Ala16Val gene polymorphism genotypes are presented in Table 1. Biomarkers of oxidative metabolism in blood samples from different genotypes were similar with the exception of SOD and polyphenol levels after 12-h overnight fasting. AA carriers presented higher SOD levels than VV carriers. However, plasmatic total polyphenols were higher in VV carriers than AA carriers. The heterozygous genotype (AV) presented intermediary MnSOD and total polyphenol levels.

3.1. Mitotic index and chromosomal instability

As expected, the mean number of metaphases was, in general, higher in the control (106.2 ± 43.1) than in UV-exposed (60.8 ± 38.2) lymphocyte cultures ($p = 0.01$) independent of Ala16-Val polymorphism.

Additionally, we found significant differences in the mitotic index between the Ala16Val genotypes (Fig. 1). In the control group, cell cultures from AA lymphocytes presented a higher metaphase count (203.8 ± 7.5) than the AV (91.0 ± 1.4) and VV (27.5 ± 2.9) lymphocyte cultures ($p = 0.001$). The UV-treated group showed maintenance of these patterns among genotypes (AA > AV > VV metaphase counts).

The metaphase count was significantly decreased in AA lymphocyte cultures, from 203.8 ± 7.5 in the control group to 101.3 ± 2.5 in the UV group ($p = 0.001$). In AV cultures, the metaphase count was decreased from 91.0 ± 1.4 to 69.40 ± 2.6, and in VV cultures, it was decreased from 287.5 ± 2.9 to 9.5 ± 1.2 ($p = 0.0001$).

However, we did not observe chromosomal instability or apoptosis in lymphocyte cultures from healthy subjects with different Ala16Val genotypes after UV treatment.

3.2. Cell viability

In the present study, we did not observe differences in cell viability between the control (84.52 ± 5.53%) and UV groups (82.78 ± 7.88%) ($p = 0.556$); this result was independent of genotypes. When we analysed the lymphocyte cell cultures grouped by genotypes, we observed that the AA lymphocyte cell cultures presented higher cell viability than VV and AV cultures. However,

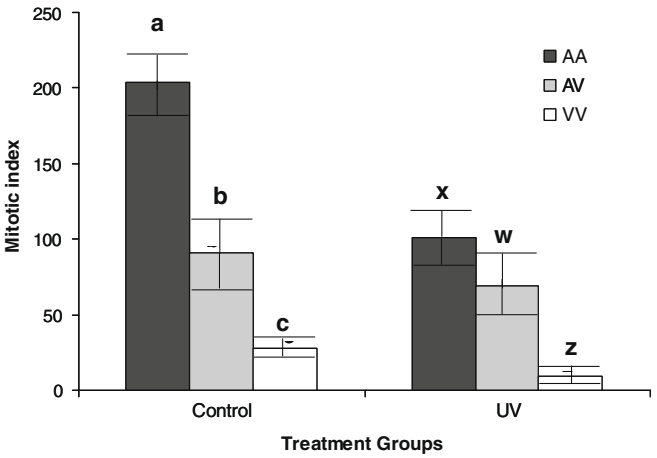

Fig. 1. Mitotic index comparison among A16V-SOD2 gene polymorphism genotypes (AA, AV and VV) in lymphocytes cell cultures in without (control) and with ultraviolet radiation (UV) exposition. Different letters in each treatment represent significant differences among genotypes using analysis of variance followed by Tukey test, $p < 0.05$ (a,b,c to control group and x,w,z to UV exposition group).

the higher AA viability when compared to other genotypes was not maintained in the lymphocyte cultures exposed to UV radiation (Table 2).

3.3. Oxidative stress biomarkers

When we compared the oxidative biomarkers among the three genotype cultures, we did not observe significant differences. As the study involves a genetic trait (Ala16Val polymorphism), and allelic variation can result in a co-dominant, recessive or dominant phenotype, we performed an additional analysis comparing VV + AV cells to AA cells.

Table 2
Viability lymphocyte cell culture (%) considering UV radiation and A16V-SOD2 genotypes.

Genotypes	Control		UV radiation	
	Mean ± SD	*p*	Mean ± SD	*p*
AA	89.22 ± 2.69[a]	0.049	86.79 ± 4.84[x]	0.493
AV	83.23 ± 5.09[b]		81.10 ± 5.27[x]	
VV	79.98 ± 5.01[b]		79.68 ± 3.47[x]	
Total	84.52 ± 5.53		82.78 ± 4.88	

SD = standard deviation. Different letters in each treatment represent significant differences among genotypes using analysis of variance followed by Tukey test, $p < 0.0$ (a,b,c to control group and x,w,z to UV exposition group).

We observed significant differences in TBARS levels between AA and VV + AV cultures exposed to UV radiation. UV exposure of AA lymphocyte cells caused a decrease in TBARS levels when compared to the same cells in the control group. The TBARS concentration was 9.89 ± 5.23 nmol of MDA/106 cells in the AA control lymphocyte culture and 3.69 ± 0.89 nmol of MDA/106 cells in the UV-exposed cell culture ($p = 0.05$). In contrast, cells from VV and AV subjects presented a significant increase in TBARS levels when exposed to UV radiation. The TBARS concentration was 5.44 ± 2.98 nmol of MDA/106 cells in the VV + AV control lymphocyte cultures and 11.66 ± 6.43 nmol of MDA/106 cells in the UV-exposed cell cultures ($p = 0.039$). Therefore, the results showed a recessive effect of the A allele, because two A alleles were needed to yield lower TBARS concentrations.

The other oxidative biomarkers investigated presented similar concentrations after 72 h of cell growth independent of UV exposure or Ala16Val genotype/allele.

DNA damage induced by UV exposure was evaluated using the comet assay under alkaline conditions. Table 3 shows the comet class and damage index, and Fig. 2 shows the mean values of the DNA tail moment in the lymphocyte cell cultures from different Ala16Val genotypes with or without UV exposure. The damage index was higher in the UV-exposed AA culture cells than in the control group of the same genotype ($p = 0.05$). The VV and AV cell cultures presented a similar damage index between UV-exposed and control groups. In the case of AA lymphocyte cells, DNA

Table 3
DNA migration in the comet assay for the assessment of genotoxicity of UV exposition in lymphocyte culture cell from donors with different A16V-SOD2 genotypes.

Genotypes	Subjects	Comet class frequency (/100 nucleus analyzed)					Index damage
		0	1	2	3	4	
AA							
1	Control	62	28	4	4	2	0.38
	UV	50	26	8	4	12	0.50
2	Control	52	28	10	4	6	0.48
	UV	48	28	6	12	6	0.52
3	Control	84	10	2	0	4	0.16
	UV	58	26	6	6	4	0.42
4	Control	58	24	2	8	8	0.42
	UV	54	28	6	6	6	0.46
Mean + SD	Control	62 ± 6[a]	25 ± 6[a]	4 ± 2[a]	5 ± 3[a]	4 ± 2[a]	0.37 ± 0.07[a]
	UV	53 ± 4[b]	28 ± 2[a]	7 ± 1[b]	4 ± 2[b]	8 ± 3[b]	0.47 ± 0.04[b]
VV							
1	Control	56	30	4	2	8	0.44
	UV	58	26	10	2	4	0.42
2	Control	74	14	4	2	6	0.26
	UV	62	26	6	0	6	0.38
3	Control	56	22	10	4	8	0.44
	UV	50	30	6	4	10	0.50
Mean + SD	Control	62 ± 9[a]	22 ± 8[a]	6 ± 3[a]	3 ± 1[a]	7 ± 1[a]	0.38 ± 0.10[a]
	UV	56 ± 6[a]	27 ± 2[a]	7 ± 2[a]	2 ± 1[a]	7 ± 3[a]	0.43 ± 0.06[a]
AV							
1	Control	66	20	8	2	4	0.34
	UV	80	14	2	0	4	0.20
2	Control	60	18	2	14	6	0.40
	UV	62	16	0	8	14	0.38
3	Control	82	10	4	2	2	0.18
	UV	54	36	2	6	2	0.46
4	Control	76	20	4	0	0	0.24
	UV	70	30	0	0	0	0.30
Mean + SD	Control	66 ± 7[a]	20 ± 2[a]	4 ± 2[a]	6 ± 3[a]	4 ± 2[a]	0.34 ± 0.07[a]
	UV	67 ± 9[a]	23 ± 9[a]	2 ± 1[a]	5 ± 3[a]	5 ± 3[a]	0.33 ± 0.11[a]

SD = standard deviation; Index damage: $\sum$(1,2,3,4 comet class)/100. Different letters mean statistical differences between control and UV treatments for each genotype. 0 = nucleus without DNA damage.

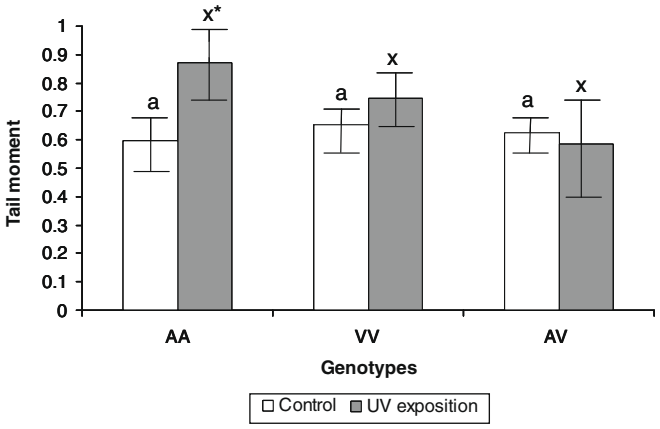

Fig. 2. DNA damage to UV exposition in lymphocyte cell culture from subjects with different A16V-SOD2 genotypes. DNA damage was measured by comet assay. The values represent the mean of four culture cells to AA, VV and AV genotypes ± SE with and without UV exposition. Different letters in each treatment represent significant differences among genotypes using analysis of variance followed by Tukey test, $p < 0.01$ (a,b,c to control group and x,w,z to UV exposition group). An asterisk (*) denotes $p > 0.01$ statistical differences between Tail moment of AA lymphocytes cultures cells without and with UV exposition using Student t test.

damage was significantly increased when compared with the control group ($p = 0.014$) and when compared to lymphocytes from the AV and VV genotypes ($p = 0.042$).

4. Discussion

We report herein a differential response of lymphocyte cell cultures from donors of different Ala16Val genotypes to UV exposure. Before we discuss the main results obtained here, it is important to note that the basal condition of lymphocyte cells showed differences in SOD and polyphenol content that were Ala16Val genotype-dependent. In this case, AA and AV presented higher SOD levels than the VV genotype. These data are consistent with a previous publication by Martin et al. (2009) that evaluated functional effects in cryopreserved human hepatocytes and found a reduction in MnSOD catalytic activity.

However, we did not find any literature report regarding the association between blood polyphenol content and Ala16Val polymorphism. Plasma polyphenol is a general measure to evaluate the quantity of non-enzymatic anti-oxidant compounds. Since we chose cell donor volunteers with similar dietary patterns, the results showing higher polyphenol content in the VV and AV genotypes are intriguing. Therefore, future investigations need to be performed to elucidate the nature of the variation in polyphenol content associated with Ala16Val polymorphism.

Considering the lymphocyte cell cultures, in general, AA cell cultures presented higher viability and mitotic index and lower TBARS levels than VV and AV cells in both the control and UV-exposed groups. However, when we compared DNA damage among the three genotypes, AA lymphocyte cells presented a statistical significance to highest damage from UV exposure. However, the number sample analyzed here is small and just a few cells wrongly assigned to comet class could to change the result. For this reason, a complementary study need to be performed to confirm if the Ala16Val polymorphism affects cellular oxidative metabolism in ways that are independent of non-enzymatic anti-oxidants.

As previously noted, the basal values in our experiment showed similar levels of oxidative biomarkers with the exception of SOD levels, which were highest in AA subjects, and polyphenol total levels, which were highest in VV subjects. However, these differences disappeared after a 72-h culture period, indicating that the oxidative biomarkers measured were similar in all samples that were cultivated under similar conditions.

We believe that the most important result from the present study is the differential oxidative response associated with the Ala16Val polymorphisms despite the environmental similarities of the cell cultures. Apparently, the AA lymphocyte cell culture presented high DNA damage, and the VV and AV lymphocyte cell cultures presented high lipid peroxidation levels. As the AA cultures suffered the most DNA damage, these results corroborate with the previous suggestion that this genotype is more susceptible to oxidative agents than the VV or AV genotypes.

Our results are in accordance with the hypothesis of Ambrosone et al. (1999) that the MnSOD polymorphism affects the mitochondrial targeting of the enzyme, modifying the ability of mitochondria to defend against oxidative stress due to ROS. As the amphiphilic helical structure is essential for correct transport, it is predicted that the possible explanation for association between the A allele and neoplasias is the simultaneous accumulation of H_2O_2, at least in the absence of H_2O_2-scavenging enzymes such as glutathione peroxidase and catalase. In the presence of transition metals, H_2O_2 in turn gives rise to generation of potent free radicals and hence, mitochondrial damage.

In a previous study performed by our team, we found an association between AA and DNA damage in peripheral blood cells of the elderly, as well as an association of this genotype with breast and prostate cancer and the immunosenescence profile (Taufer et al., 2005). Additional investigations have shown an association between the AA genotype and male and female breast cancer (Bica et al., 2009).

Bica et al. (2009) suggested that there is a "paradox" in the MnSOD polymorphism, since this variation causes an imbalance in anti-oxidant modulation mainly in homozygous cases (AA and VV), either increasing superoxide levels in VV subjects or hydrogen peroxide levels in AA subjects. In both cases, environmental interactions could increase or decrease the risk of non-transmissible morbidities such as neoplasias and cardiovascular diseases.

It is interesting to point out that some studies have suggested that MnSOD suppresses cell growth in several tumour cells *in vivo* and *in vitro* (Soini et al., 2001; Oberley, 2005). Kim and colleagues have described the manganese superoxide dismutase enzyme as a potential mechanism behind the inhibition of tumour cell growth (Kim et al., 2001). The study showed an apparent decrease in MnSOD levels in numerous tumour cell lines when compared to their non-malignant counterparts. These data led to the proposal that MnSOD could exert a tumour-suppressive effect in oestrogen-dependent human breast cancer cells. Considering these results, Bica et al. (2010) studied the effect of the Ala16Val polymorphism on regional lymph node status in breast cancer from evaluating 188 controls and 93 cases of invasive breast cancer. The results showed that the VV genotype presented higher metastatic potential than the AA genotype.

The results described here corroborate the suggestion of Bica et al. (2010); we observed two oxidative stress situations that could explain the greater susceptibility of the AA genotype to cancer as well as the metabolic susceptibility of the V allele. In this last case, it is important to mention the oxidative effect of UV exposure shown by the higher lipid peroxidation levels observed in the VV and AV genotypes.

Investigations showed that UV radiation increased ROS, activating signalling pathways involved in regulating cell growth, differentiation and proliferation (Martin et al., 2009). UV exposure may affect diverse biological functions including DNA replication, repair, cell cycle control and chromatin remodelling. All of these facts may contribute to the increased risk of cancer, particularly skin cancer. UV exposure induces H_2O_2 production (Syed et al.,

2006), inducing lipid and protein oxidation at the plasma membrane, which may cause a loss of membrane fluidity, inactivation of enzymes, and alteration of ion permeability. These changes may cause cell rupture by an osmotic process (Bommareddy et al., 2005). Therefore, V allele carriers cannot be considered to have anti-oxidant protection when compared to AA genotype carriers.

Furthermore, a possible explanation for the higher lipid peroxidation levels associated with the V allele is related to increased levels of superoxide radical as a result of an inefficient MnSOD enzyme. Superoxide anion produced by NAD(P)H oxidases have some important metabolic functions. Superoxide is present in all cell types that participate in the inflammation process (leukocytes, endothelial, other vascular cells, etc.) and may lead to toxic effects when produced at high levels during oxidative burst. However, in a chronic state of unbalance, as occurs in VV carriers, the superoxide old present important oxidative reactions. For example, superoxide has a high affinity to nitric oxide (NO), a reactive oxygen species that is produced by virtually all cells and is associated with several biological effects including the regulation of immune responses. When the superoxide anion reacts to molecules of nitric oxide, peroxynitrite (OONO–) is produced. Peroxynitrite (PN), formed by the diffusion rate-limited combination of nitric oxide (·NO) and superoxide ($O_2^{\cdot-}$) free radicals (Alvarez et al., 2002), has been proposed to be a key contributor to oxidative damage, mainly because its highly reactive decomposition produces nitrogen dioxide ($\cdot NO_2$), hydroxyl radical (·OH) and carbonate radical ($CO_3^{\cdot-}$). These PN-derived radicals can oxidise proteins, nitrate tyrosine residues and induce cell membrane lipid peroxidation (Denicola and Radi, 2005). However, future studies need to be performed to determine if the possible causal mechanism associated with higher lipoperoxidation in VV genotypes involves peroxynitrite production.

We know that there is a potential for variation in results related to the Comet assay and other biochemical tests performed in this study. However, since protocols that integrate genotypic analysis and cell culture are still incipient, we opted to select subjects who had a health profile and lifestyle that were very similar. Additionally, we conducted all experiments at the same time in order to minimise possible microenvironmental variations. We believe that the results reported here could contribute to toxicological studies, because we suggest the differential response is associated with oxidative stress metabolism. In summary, although our study involved a relatively small number of subjects, our results suggest that Ala16Val genotypes may present differential oxidative stress responses that could to be related to the development of chronic diseases. Complementary investigations need to be performed to observe the response of Ala16Val genotypes to other oxidative stress agents, as well as to anti-oxidant compounds, to elucidate molecular and biochemical mechanisms associated with the nature of the polymorphism unbalance.

Acknowledgements

This research was supported by the Conselho Nacional de Pesquisa (CNPq) of Brazil and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, 166/08). The authors would like to express their gratitude to Maria Izabel de Ugalde Marques da Rocha, Maria Fernanda Manica-Cattani, Thais Doeler Algarve and Olmiro Cezimbra de Souza Filho.

References

Aebi, H., 1984. Catalase in vitro. Methods Enzymol. 105, 121–127.

Ambrosone, C.B., Freudenheim, J.L., Thompson, P.A., Bowman, E., Vena, J.E., Marshall, J.R., Graham, S., Laughlin, R., Nemoto, T., Shields, P., 1999. Manganese superoxide dismutase (MnSOD) genetic polymorphism, dietary antioxidants, and risk of breast cancer. Cancer Res. 59, 602–606.

Alvarez, M.N., Trujillo, M., Radi, R., 2002. Peroxynitrite formation from biochemical and cellular fluxes of nitric oxide and superoxide. Methods Enzymol. 359, 353–366.

Bica, C.G., da Silva, L.L.M., Toscani, N.V., da Cruz, I.B.M., Sá, G., Graudenz, M.S., Zettler, C.G., 2009. MnSOD gene polymorphism association with steroid-dependent cancer. Pathol. Oncol. Res. 15, 19–24.

Bica, C.G., Silva, L.L.M., Toscani, N.V., Zettler, C.G., Gottlieb, M.G.V., Alexandre, C.O.P., Graudenz, M.S., Cruz, I.B.M., 2010. Ala-16-Val manganese-dependent superoxide dismutase (MnSOD) gene polymorphism correlates to breast cancer regional lymph node status. Cancer Gen. Cytogen. 196, 153–158.

Bommareddy, A., Hora, J., Cornish, B., Dwivedi, C., 2005. Chemoprevention by alpha-santalol on UVB radiation-induced skin tumour development in mice. Anticancer Res. 27, 2185–2188.

Boveris, A., Cadenas, E., 1997. Cellular source and steady-state levels of reactive oxygen species. In: Clerch, L., Massaro, D. (Eds.), Oxygen, Gene Expression and Cellular Function, vol. 105. Marcel Decker, pp. 1–25.

Burow, M.E., Weldon, C.B., Tang, Y., Navar, G.L., Krajewski, S., Reed, J.C., Hammond, T.G., Clejan, S., Backman, B.S., 1998. Differences in susceptibility to tumour necrosis factor alpha-induced apoptosis among MCF-7 breast cancer cell variants. Cancer Res. 58, 4940–4946.

Chandra, S., de Mejia, E.G., 2004. Polyphenolic compounds, antioxidant capacity, and quinine reductase activity of an aqueous extract of *Ardisia compressa* in comparison to mate (*Ilex paraguariensis*) and green (*Camellia sinensis*) teas. J. Agric. Food Chem. 52, 3583–3589.

Denicola, A., Radi, R., 2005. Peroxynitrite and drug-dependent toxicity. Toxicology 208, 273–288.

Egan, K., Thompson, P., Titus-Ernstoff, L., Moore, J., Ambrosone, C., 2003. MnSOD polymorphism and breast cancer in a population-based case-control study. Cancer Lett. 199, 27–33.

Eldridge, S.R., Gould, M.N., Butterworth, B.E., 1992. Genotoxicity of environmental agents in human mammary epithelial cells. Cancer Res. 52, 5617–5621.

Ellman, G.L., 1959. Tissue sulphydryl groups. Arch. Biochem. Biophys. 589, 70–77.

Emerit, I., 1994. Reactive oxygen species, chromosome mutation and cancer. Free. Radic. Biol. Med. 16, 99–109.

Eras-Erdogan, N., Akbas, E., Senli, H., Kul, S., Çolak, T., 2009. Relationship between polymorphism in the manganese superoxide dismutase gene and breast cancer. Mutat. Res. 680, 7–11.

Gottlieb, M.G.V., Shwanke, C.H.A., Santos, A.F.R., Jobim, P.F., Müssel, D.P., da Cruz, I.B.M., 2005. Association among oxidised LDL levels, MnSOD, apolipoprotein E polymorphisms, and cardiovascular risk factors in a south Brazilian region population. Genet. Mol. Res. 4, 691–703.

Hartmann, A., Agurell, E., Beevers, C., Brendler-Schwaab, S., Burlinson, B., Clay, P., Collins, A., Smith, G., Speit, G., Thybaud, V., Tice, R.R., 2003. Recommendations for conducting the in vivo alkaline comet assay. Mutagenesis 18, 45–51.

Hiroi, S., Harada, H., Nishi, H., Satoh, M., Nagai, R., Kimura, A., 1999. Polymorphism in the MNSOD and HLA-DRB1 genes are associated with nonfamilial idiopatic dilated cardiomyophaty in Japanese'. Biochem. Biophys. Res. Commun. 261, 332–339.

Hung, R., Boffetta, P., Brennan, P., Malaveille, C., Gelatti, U., Placidi, E., Carta, A., Hautefeuille, A., Porru, S., 2004. Genetic polymorphisms of MPO, COMT, MnSOD, NQO1, interactions and bladder cancer risk. Carcinogenesis 25, 973–978.

Hyoudou, K., Nishikawa, M., Kobayashi, Y., Ikemura, M., Yamashita, F., Hashida, M., 2008. SOD derivatives prevent metastatic tumour growth aggravated by tumour removal'. Clin. Exp. Metastasis 25, 531–536.

Jacques-Silva, M.C., Nogueira, C.W., Broch, L.C., 2001. Diphenyl diselenide and ascorbic acid changes deposition of selenium and ascorbic acid in liver and brain of mice. Pharmacol. Toxicol. 88, 119–125.

Kim, K.H., Rodriguez, A.M., Carrico, P.M., 2001. Potential mechanisms for the inhibition of tumour cell growth by manganese superoxide dismutase. Antioxid. Redox Signal 3, 361–373.

Li, Y., Huang, T.T., Carlson, E.J., Melov, S., Ursell, P.C., Olson, L.J., Yoshimura, M.P., Berger, C., Chan, P.H., Wallace, D.C., Epstein, C.J., 1995. Nat. Genet. 11, 376–381.

Martin, R.C.G., Li, Y., Liu, Q., Jensen, N.S., Barker, D.F., Doll, M.A., Hein, D.W., 2009. Manganese superoxide dismutase V16A single-nucleotide polymorphism in the mitochondrial targeting sequence is associated with reduced enzymatic activity in cryopreserved human hepatocytes. DNA Cell Biol. 28, 3–7.

Millikan, R.C., Player, J., Cotret, A.R., Moorman, P., Pittman, G., Vannappagari, V., Tse, C.J., Keku, T., 2004. Manganese superoxide dismutase Ala-9Val polymorphism and risk of breast cancer in a population-based case-control study of African American and whites. Breast Cancer Res. 6, R264–R274.

Montano, M.A.E., Lera, J.P.B., Gottlieb, M.G.V., Schwanke, C.H.A., da Rocha, M.I.U.M., Manica-Cattani, M.F., dos Santos, G.F., da Cruz, I.B.M., 2009. Association between manganese superoxide dismutase (MnSOD) gene polymorphism and elderly obesity'. Mol. Cell. Biochem. 328, 33–40.

Morabito, F., Cristiani, M., Saija, A., Stelitano, C., Callea, V., Tomaino, A., Minciullo, P.L., Gangemi, S., 2004. Lipid peroxidation and protein oxidation in patients affected by Hodgkin's lymphoma'. Mediators Inflamm. 13, 381–383.

Nadin, S., Vargas-Roig, L., Ciocca, D., 2001. A silver staining method for single-cell gel assay. J. Histochem. Cytochem. 49, 1183–1186.

Oberley, L.W., 2005. Mechanism of the tumour suppressive effect of MnSOD overexpression. Biomed. Pharmacother. 59, 143–148.

Ohkawa, H., Ohishi, H., Yagi, K., 1979. Assay for lipid peroxide in animal tissues by thiobarbituric acid reaction'. Anal. Biochem. 95, 351–358.

Shimoda-Matsubayasgi, S., Matumine, H., Kobayashi, T., Nakagawa-Hattori, Y., Shimizu, Y., Mizuno, Y., 1996. Structural dimorphism in the mitochondrial

targeting sequence in the human manganese superoxide dismutase gene. Biochem. Biophys. Res. Commun. 226, 561–565.

Singh, N., McCoy, M., Tice, R., Schneider, E., 1995. A simple technique for quantification of low levels of DNA damage in individuals cells. Exp. Cell Res. 175, 184–191.

Soini, Y., Vakkala, M., Kahlos, K., 2001. MnSOD expression is less frequent in tumortumour cells of invasive breast carcinomas than in situ carcinomas or non-neoplastic breast epithelial cells. J. Pathol. 195, 156–162.

Sutton, A., Khoury, H., Prip-Buus, C., Cepanec, C., Pessayre, D., Degoul, F., 2003. The Ala16Valgenetic dimorphism modulates the import of human manganese superoxide dismutase into rat liver mitochondria'. Pharmacogenetics 13, 145–157.

Taufer, M., Peres, A., de Andrade, V.M., De Oliveira, G., Sá, G., do Canto, M.E.P., dos Santos, A.R., Bauer, M.E., da Cruz, I.B.M., 2005. Is the Val16Ala manganese superoxide dismutase polymorphism associated with the aging process? J. Gerontol. A: Biol. Sci. Med. Sci. 60, 432–438.

Tice, R.R., Agurell, D., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C., Sasaki, Y.F., 2000. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing'. Environ. Mol. Mutagen 35, 206–221.

Wang, L., Miller, D., Sai, Y., Liu, G., Wain, J., Lunch, T., Christiani, D., 2001. Manganese superoxide dismutase alanine-to-valine polymorphism at codon 16 and lung cancer risk'. J. Natl. Cancer Inst. 93, 4471–4473.

Woodson, K., Tangrea, J., Lehman, T., Madali, R., Taylor, K., Snyder, K., Taylor, P., Vitamo, J., Albanes, D., 2003. Manganese superoxide dismutase (MnSOD) polymorphism, alpha-tocopherol supplementation and prostate cancer risk in the alpha-tocopherol, beta-carotene cancer prevention study (Finland)'. Cancer Causes Control 14, 513–518.

Yunis, J.J., 1996. High resolution of human chromosomes. Science 1976, 1268–1270.