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Artículo/Article

THE *BER* PATHWAY GENES AND *PON1* POLYMORPHISM: INFLUENCE ON DNA DAMAGE IN AGRICULTURE-EXPOSED WORKERS

POLIMORFISMOS DE LA VÍA BER Y DEL GEN PON1: INFLUENCIA EN LOS DAÑOS CELULARES EN LOS AGRICULTORES EXPUESTOS

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ABSTRACT

Pesticide exposure represents a potential risk for human health. Farmers from Rio Grande do Sul (the southernmost state of Brazil) use different kinds of such substances and are therefore constantly exposed to those chemical compounds. The aim of this study was to evaluate the influence of polymorphisms in *PON1* and in two BER genes *(XRCC1* and *OGG1* polymorphisms) in cell damage, analyzing a group of pesticide-exposed farmers. Genotyping of the three polymorphisms was performed by PCR/RFLP. DNA damage was evaluated by Comet assay (ID) and Micronuclei test (MN) in 107 pesticide-exposed workers and in 73 non-exposed people. Statistically significant differences between cases and controls, considering data from ID, MN and genotyping analysis, were tested by the non-parametric Mann-Whitney *U* test. *PON* polymorphisms showed influence in cell damage in the pesticide-exposed group, considering the frequency of MN: homozygotes *Gln/Gln* presented a higher incidence of MN when compared to Arg/-($p<0.05$). Control individuals with *XRCC1 194 Arg/Trp* presented better results considering ID and MN ($p<0.05$). Heterozygote individuals for *OGG1 326 Ser/Cys* showed lower incidence of MN ($p<0.05$). The results from the *PON* and *XRCC1* genotypes combined showed an effective repair for individuals Gln*/Gln* + *Arg /Trp* ($p< 0.05$). The *PON* and *OGG1* genotypes combined showed an effective repair for individuals Gln/Arg + Cys/- ($p=0.01$). Our results demonstrated that the metabolizing gene *PON* and the *BER* genes may have an influence on exposure to pesticides. Combinations of metabolizing and repair genotypes modulate the final result of incorporation of DNA damage after exposure. These results demonstrate the importance of considering together metabolizing and repair pathways on the individual susceptibility to incorporate DNA damage after exposure to xenobiotics.

Keywords: Occupational exposure, Polymorphisms, NER, PON, MN and Comet Assay.

RESUMEN

La exposición a pesticidas puede representar un potencial riesgo para la salud humana. Así, el objetivo de nuestro trabajo fue evaluar la influencia de los polimorfismos del gen del metabolismo (*PON1*) y de los genes de la vía BER (*XRCC1* y *OGG1*) en los daños celulares ocasionados por la constante utilización de diferentes

pesticidas por los viticultores de la región nordeste del estado de Rio Grande do Sul. Por PCR/RFLP se obtuvo el genotipo de los tres genes. El daño en el ADN fue evaluado por el ensayo Cometa (ID) y por el test de micronúcleos (MN) en los 107 individuos expuestos y 73 no expuestos a pesticidas. Las diferencias en ID, MN y las diferencias entre el genotipos dentro de los grupos control y expuestos, presentaron una desviación de la normalidad, por lo que fueron testeados por el test no paramétrico *U* de Mann-Whitney. El polimorfismo *PON* mostró una influencia en el grupo expuesto, considerando la frecuencia de MN ($p<0,05$). Los homocigotos *Gln/Gln* presentaron mayores incidencias de MN. Individuos control con el genotipo *XRCC1 194 Arg/Trp* tuvieron mejores resultados considerando ID y MN ($p<0,05$). Individuos heterocigotos para *OGG1 326 Ser/Cys* mostraron menores incidencias de MN ($p<0,05$). Los resultados con los genotipos *PON* y *XRCC1* combinados presentaron una mayor eficacia en la reparación en los individuos que presentaban el genotipo Gln*/Gln* + *Arg /Trp* ($p< 0.05$). Los genotipos combinados *PON* y *OGG1* presentaron una mayor eficiencia de reparación para individuos Gln/Arg + Cys/- ($p=0,01$). Nuestros resultados demostraron que el gen del metabolismo *PON1* y los genes de la vía BER pueden influir en la repuesta a la exposición a pesticidas. La combinación de genotipos de genes de metabolismo y de reparación modula la incorporación de daño en el ADN después de la exposición. Estos resultados demuestran la importancia de considerar juntas las vías del metabolismo y BER en la susceptibilidad individual de incorporar daños al DNA después de la exposición a los plaguicidas.

Palabras claves: Exposición ocupacional, polimorfismos, BER, PON, MN y Ensayo cometa.

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INTRODUCTION

Exposure of individuals to a variety of environmental chemicals may result in serious toxicological consequences, such as cellular alterations which, if not repaired, can lead to the development of mutations, apoptosis, uncontrolled cellular proliferation and diseases (Keshava and Ong, 1999; Lucas *et al.*, 2001; Goode *et al.*, 2002). Workers of various occupational settings are exposed to hazardous substances. Some of these substances may increase the chance of recurrent diseases in different production sectors (Fritschi *et al.*, 2005; De Celis *et al.,* 2005).

Epidemiological studies have shown increased incidence of cancer and other diseases, such as Parkinson disease and reproductive outcomes in the vineyard. These workers are exposed to complex mixtures of pesticides. An increased frequency of chromosomal aberrations in pesticide-exposed populations also suggest a genotoxic effect caused by this group of xenobiotics (Gauthier *et al.*, 2001; Ji *et al.*, 2001; Shaham *et al.,* 2001;Zeljezic and Garaj-Vrhovac, 2001; Zheng *et al.*, 2001; Grover *et al.,* 2003).

Genes and proteins involved in metabolization/detoxification of xenobiotics, as well as those involved in DNA repair, are usually used as potential markers of susceptibility for the development of several diseases in which the aetiology is related to exposure to environmental hazards. Polymorphisms in such genes have been linked with an increased risk of cancer in several case-control studies. (IARC, 1999; Wilkinson and Clapper, 1997).

The metabolism of organophosphorous substances has become important since their widespread use as pesticide began over 40 years ago. Hydrolysis of paraoxon is catalyzed by serum paraoxonase (PON)/arylesterase, an enzyme that is associated with the lipoprotein fraction of serum. There is a 10-40 fold difference in serum PON activity between individuals, which is genetically determined by polymorphisms. Humans may differ in susceptibility to parathion poisoning de-

pending on PON allelic status. The allele *Gln 192* codifies a low activity enzyme in contrast to the Arg *192* allele, that codifies a high activity enzyme (Humbert *et al.,* 1993). It has been suggested that individuals with low enzyme levels may be more susceptible to the toxic effects of organophosphorous substances.

At least five DNA repair pathways operate on specific types of DNA damage: Base Excision Repair (BER), Nucleotide Excision Repair (NER), Homologous Recombination, Non Homologous End-joining Repair and the Mismatch Repair pathways. Several genes involved in these repair pathways present polymorphisms which can be related to individual susceptibility to incorporate DNA damage (Saffi and Henriques, 2003).

The *XRCC1* and the *OGG1* genes, involved in Base Excision Repair (BER), have a key role in the efficient repair of DNA damage caused by ionizing radiation and oxidative stress. Mutations in *XRCC1* result in an increased sensibility and an increased number of chromosomal aberrations (Thompson and West, 2000) The 194Arg/Trp and 399Arg/Gln polymorphisms in *XRCC1* were associated with an increased risk of developing an adverse response to RT in breast cancer patients (Moulan *et al.*, 2003). OGG1 326 Ser/Cys polymorphism may alter the impact of some environmental factors on stomach cancer development (Takezaki *et al.*, 2002).

Pesticide exposure represents a potential risk for human health. Farmers from Rio Grande do Sul (the southernmost state from Brazil) use different kinds of such substances and are therefore constantly exposed to those chemical compounds. The aim of this study was to evaluate the influence of polymorphisms in *PON1* and in two BER genes *(XRCC1* and *OGG1* polymorphisms) in cell damage, analyzing a group of pesticide-exposed farmers.

MATERIALS & METHODS

Sample

The study involved a total of 173 individuals (men) from Caxias do Sul (in the north east region of Rio Grande do Sul). Among those, 108 individuals were agricultural workers exposed to pesticides (cases) and 65 were non-exposed individuals (controls). All the individuals who participated in the study were asked to answer a Portuguese version of a questionnaire from the International Commission for Protection against Environmental Mutagens and Carcinogens (Carrano and Natarajam, 1988) and to participate in a face-to-face questionnaire, which included standard demographic data (age, gender, etc.) as well as questions related to medical issues (exposure to X-rays, vaccinations, medicines, etc.), life style (smoking, coffee and alcohol drinking, diet, etc.) and their occupation (number of working hours per day, time exposed to organic solvents, use of protective measures, etc.).

The control individuals were fireman or had bureaucratic jobs in the same region from which the exposed individuals came from. None of them had been exposed to pesticides or any other potential genotoxic agents for the past few months, and they had no previous occupational exposure to genotoxicants.

Methods

Comet Assay

The alkaline Comet assay was performed as described by Singh *et al.* (1988) with the modifications suggested by Tice *et al.* (2000). Blood cells (5 μl) were embedded in 95 μl of 0.75% low-melting point agarose and when the agarose had solidified the slides

were placed in lysis buffer (2.5M NaCl, 100mM EDTA and 10mM Tris; pH 10.0-10.5) containing freshly added 1% (v/v) Triton X-100 and 10% (v/v) dimethyl sulfoxide (DMSO) for a minimum of 1 hour and a maximum of two weeks. After treatment with lysis buffer, the slides were incubated in freshly-made alkaline buffer (300 mM NaOH and 1 mM EDTA; pH >13) for 20 min and the DNA was electrophoresed for 20 min at 25 volts (0.90 V/cm) and 300 mA after which the buffer was neutralized with 0.4 M Tris (pH 7.5) and the DNA stained with ethidium bromide (2 μg/ml). The electrophoresis procedure and efficiency for each electrophoresis run was checked using negative and positive internal controls consisting of whole human blood collected in the laboratory, the negative control being unmodified blood and the positive control 50 μl of blood mixed with 13 μl (8×10^{-5} M) of methyl methanesulfonate (CAS 66-27-3 - Sigma, St. Louis, MO, USA) and incubated for 2 hours at 37°C. Each electrophoresis run was considered valid only if the negative and positive controls yielded the expected results.

Images of 100 randomly selected cells (50 cells from each of two replicate slides) were analyzed for each person. Comet image lengths (IL) (i.e. the nuclear region + tail) were measured in arbitrary units using a calibrated eyepiece micrometer (1 unit= ≈5 μm at 200X) and a fluorescence microscope equipped with a 12nm BP546 excitation filter and a 590nm barrier filter. Damage was determined visually by the categorization of comets into five classes of DNA migration, from 0 (no tail / no damage) to 4 (maximal tail length). Then, the Damage Index (DI) was obtained by the sum of the individual cell classes, ranging from 0 (no damage: 100 cells x 0) to 400 (maximum damage: 100 cells x 4) (Albertini *et al.*, 2000; Collins *et al.,* 1997; Silva *et al.*, 2000). The damage frequency (DF (%); i.e. the proportion of cells with altered migration), was calculated based on the number of cells with tails versus the number of cells without tails. All the slides were scored blindly on the same day in which they were subjected to electrophoresis and stained.

Micronucleus Test

For each blood sample, duplicate lymphocyte cultures were set up in culture flasks by adding 0.3 ml of whole blood to 5 ml of RPMI 1640 medium (Nutricell, Campinas-SP, Brazil) containing 1% (v/v) phytohemagglutinin and the flasks were incubated at 37°C for 44h before adding 5 μg/ml of cytochalasin B (Sigma), and continuing incubation until the total incubation time reached was 72 h as described by Fenech et al. (1999). After incubation the lymphocytes were harvested by centrifugation at 800 revs/min for 8 min, recentrifuged, fixed in 3:1 (v/v) methanol/acetic acid, placed onto a clean microscope slide and stained with 5% (v/v) Giemsa. For each blood sample, 2000 binucleated lymphocytes (i.e. 1000 from each of the two slides prepared from the duplicate cultures) were scored for both micronuclei presence and nucleoplasmic bridges (NPB) between daughter nuclei, assessment being made using bright-field optical microscopy at a magnification of 200-1000X. All sides were coded to blind analysis.

DNA Isolation and Genotyping

Genomic DNA samples were obtained from whole blood using a salting out method (Lahiri and Nurnberger, 1991).

The *PON*, *XRCC1* and *OGG1* polymorphisms were genotyped by the PCR-restriction fragment length polymorphisms (RFLP)

assay, with the restriction enzymes *Alw* I, *Pvu*II, *Fnu*4HI, respectively (Harris, 1972; Lunn *et al.*, 1999; De Ruyck *et al.*, 2005). After digestion the fragments were separated by electrophoresis in agarose gel (3.0%).

Statistical Analysis

The normality of variables was evaluated by the Kolmogorov-Smirnov test. c^2 and *t*-test were used to compare the basic characteristics of the study populations. The statistical analysis of differences in age between control and exposed workers, being a normal distribution, was tested by Student *t*-test. Differences in smoking and drinking habits, DNA damage and cytogenetic test evaluated, as well as between the different genotypes the control and exposed groups, having a significant deviation from normality, were tested by the non-parametric Mann-Whitney *U* test. The correlations of different variables were determined by Sperman rank correlation test. Two-tailed *p* values are given for significance of differences.

RESULTS

All frequencies in both the exposed group and the control group are in Hardy-Weinberg equilibrium. The allelic frequency of *PON 192Arg* is 0.28 in the studied group, for allele *XRCC1 194Trp* the frequency is 0.08 and for allele *OGG1 326Cys* 0.21.

The results about the effect of individual genotypes on the level of different biomarkers evaluated in control and exposed group are shown in Table 2. In the exposed group homozygote individuals *Gln/Gln* showed higher incidence of MN (8.53±4.94) than *Arg/ –* individuals (6.31±4.55). (p<0.05).

The results for *XRCC1* show the differences for the control group, where the ID (1.88±2.61) and MN (2.44±1.59) are better in individuals *XRCC1 194 Arg/Trp* than individuals *XRCC1 194 Arg/Arg* (5.04±6.25 and 4.35±5.59) (P<0.05).

The *OGG1* heterozygote individuals of the exposed group showed lower incidence of MN (6.60±4.94) comparing to the other genotypes (8.12±4.96, to *Ser/Ser* and 9.00±1.00, to *Cys/Cys*) but the statistical significance is only for the *Cys/Cys* genotype (p<0.05).

Combined analyses with *PON* and *XRCC1* genotypes demonstrated that an effective repair occur in Gln*/Gln* + *Arg/Trp* individuals (p<0.05). Combining the *PON* and *OGG1* genotypes, individuals Gln/Arg + Cys/- presented the lowest frequency of MN (p=0.01).

DISCUSSION & CONCLUSION

The use of pesticides has increased in the last years, leading to increased levels of chemicals being released into the environment, which represent a potential hazard to human health. Individuals occupationally exposed to pesticides (such as field workers, mixers, loaders, applicators, etc.) who are in direct contact with these chemicals may represent a good opportunity to study the adverse health problems caused by such substances.

Pesticides are differentially toxic to humans due to their variability in chemical structure and exposure mode, as well as individual differences in the capacity to metabolize xenobiotics and to repair DNA. Many polymorphisms in genes involved in xenobiotic metabolism and DNA repair were described. Some of these polymorphisms result in different phenotypes concerning xenobiotic metabolism capacity and DNA repair (Villela *et al.*, 2003). Polymorphisms in *PON* gene determine a high or low activ-

ity to paraoxon hydrolyses which determines the intoxication sensitivity to pesticides (Humbert *et al*, 1993).

Our results demonstrate that metabolizing capacity may have an influence on exposure to pesticides, corroborating studies that show the importance of *PON* polymorphisms in response an organophosphate exposure (Poore & Neal, 1972).

Despite the results of genotype *XRCC1* that did not show significant statistical influence in the exposed group, the results were significant when genotype combinations were performed with the PON1 metabolizing gene. The homozygotes *PON Gln/Gln* were the group with the higher risk of DNA damage once they have poor metabolization of organophosphorous substances. Nevertheless, besides this bad genotype for PON, individuals that have the *XRCC1* allele with higher activity seem to be protected against DNA damage. The combination of *OGG1* and *PON* genotype increases the statistical significance of the protective effect of the *OGG1* genotype. These results demonstrate the importance of considering metabolizing and repair pathways together on the individual susceptibility to incorporate DNA damage after exposure to xenobiotics.

TABLE 1. Effect of individual genotype on the level of different biomarkers evaluated in control and exposed group (mean ± S.D.).

	Comet assay (200 leukocytes/subjects)		Cytokinesis-block
Genotypes	Damage Index (0-400)	Damage Frequency (%)	(2000 Cells/subjects)
	Control		
PON Gln/Gln	4.79 ± 6.21 (n=34)	1.97 ± 1.95 (n=34)	3.03 ± 3.10 (n=31)
Gln/Arg or Arg/Arg	4.21 ± 5.61 (n=23)	1.83 ± 2.39 (n=23)	6.35 ± 7.70 (n=20)c
XRCC1 Arg/Arg	5.04 ± 6.25 (n=52)	2.10 ± 2.23 (n=52)	4.35 ± 5.59 (n=52)
Arg/Trp	1.88 ± 2.61 (n=9)	1.00 ± 1.00 (n=9)	2.44 ± 1.59 (n=9)*
OGG1 Ser/Ser	5.13 ± 6.85 (n=39)	2.08 ± 2.44 (n=39)	4.57 ± 6.1 (n=39)
Ser/Cys	3.19 ± 3.10 (n=16)	1.75 ± 1.44 (n=16)	3.36 ± 3.13 (n=16)
Cys/Cys	4.33 ± 1.53 (n=3)	1.67 ± 0.58 (n=3)	3.33 ± 3.21 (n=3)
	Exposed group		
PON Gln/Gln	20.56 ± 9.73 (n=54)	11.04 ± 3.40 (n=54)	8.53 ± 4.94 (n=51)
Gln/Arg or Arg/Arg	20.83 ± 14.55 (n=53)	11.19 ± 4.65 (n=53)	6.31 ± 4.55 (n=52)
XRCC1 Arg/Arg	21.41 ± 12.79 (n=87)	11.32 ± 4.21 (n=87)	7.61 ± 5.03 (n=87)
Arg/Trp	18.11 ± 9.77 (n=18)	10.33 ± 3.20 (n=18)	3.20 ± 0.75 (n=18)
OGG1 Ser/Ser	19.05 ± 11.54 (n=50)	10.40 ± 4.07 (n=50)	8.12 ± 4.96 (n=50)
Ser/Cys	24.31 ± 14.39 (n=35)	12.25 ± 3.99 (n=35)	6.60 ± 4.94 (n=35)*
Cys/Cys	24.33 ± 6.35 (n=3)	15.00 ± 3.61 (n=3)	9.00 ±1.00 (n=3)

*P=0.05

TABLE 2. Effect of combinated genotype (*PON* + *XRCC1* and *PON* + *OGG1*) on the level of different biomarkers evaluated in control and exposed group (mean ± S.D.).

Genotypes	Comet assay (200 leukocytes/subjects) Damage Index (0-400)	Comet assay (200 leukocytes/subjects) Damage Frequency (%)	Cytokinesis-block (2000 Cells/subjects)
Control			
PON Gln/Gln + XRCC1 Arg/Arg	5,17 ± 6,56 (n=29)	2,07 ± 2,05 (n=29)	3,00 ± 3,32 (n=26)
PON Gln/Gln + XRCC1 Arg/Trp	2,75 ± 3,59 (n=4)	1,50 ± 1,29 (n=4)	2,75 ± 1,71 (n=4)
PON Gln/Arg + XRCC1 Arg/Arg	5,50 ± 6,83 (n=12)	2,42 ± 3,00 (n=12)	4,40 ± 2,59 (n=10)
PON Gln/Arg + XRCC1 Arg/Trp	1,25 ± 1,89 (n=4)	0,50 ± 0,58 (n=4)	2,25 ± 1,89 (n=4)
PON Arg/Arg + XRCC1 Arg/Arg	4,17 ± 4,54 (n=6)	1,67 ± 1,63 (n=6)	11,00 ± 13,06 (n=5)
PON Gln/Gln + OGG1 Ser/Ser	5,87 ± 7,09 (n=23)	2,30 ± 2,14 (n=23)	3,29 ± 3,27 (n=21)
PON Gln/Gln + OGG1 Ser/Cys	2,88 ± 3,27 (n=8)	1,38 ± 1,41 (n=8)	2,29 ± 3,25 (n=7)
PON Gln/Gln + OGG1 Cys/Cys	3,00 (n=1)	2,00 (n=1)	2,00 (n=1)
PON Gln/Arg + OGG1 Ser/Ser	4,90 ± 7,77 (n=10)	2,1 ± 3,45 (n=10)	3,67 ± 2,55 (n=9)
PON Gln/Arg + OGG1 Ser/Cys	3,00 ± 2,71 (n=4)	1,75 ± 0,96 (n=4)	4,00 ± 2,65 (n=3)
PON Gln/Arg + OGG1 Cys/Cys	5,00 ± 1,41 (n=2)	1,50 ± 0,71 (n=2)	4,00 ± 4,24 (n=2)
PON Arg/Arg + OGG1 Ser/Ser	3,75 ± 4,79 (n=4)	1,50 ± 1,91 (n=4)	14,00 ± 17,35 (n=3)
Exposed group			
PON Gln/Gln + XRCC1 Arg/Arg	21,07 ± 9,91 (n=46)	11,30 ± 3,51 (n=46)	9,11 ± 4,87 (n=46)
PON Gln/Gln + XRCC1 Arg/Trp	18,86 ± 8,45 (n=7)	10,00 ± 2,00 (n=7)	4,86 ± 3,89 (n=7)
PON Gln/Arg + XRCC1 Arg/Arg	23,41 ± 16,49 (n=34)	11,62 ± 5,23 (n=34)	5,85 ± 4,06 (n=33)
PON Gln/Arg + XRCC1 Arg/Trp	19,25 ± 12,56 (n=8)	10,50 ± 4,54 (n=8)	7,00 ± 4,00 (n=8)
PON Arg/Arg + XRCC1 Arg/Arg	14,00 ± 5,10 (n=7)	10,00 ± 2,89 (n=7)	6,57 ± 7,59 (n=7)
PON Arg/Arg + XRCC1 Arg/Trp	13,33 ± 2,08 (n=3)	10,67 ± 1,53 (n=3)	8,33 ± 4,73 (n=3)
PON Gln/Gln + OGG1 Ser/Ser	20,17 ± 10,71 (n=30)	10,60 ± 3,60 (n=30)	9,50 ± 4,78 (n=28)
PON Gln/Gln + OGG1 Ser/Cys	22,19 ± 10,26 (n=16)	12,25 ± 3,28 (n=16)	8,38 ± 5,24 (n=16)
PON Gln/Arg + OGG1 Ser/Ser	19,05 ± 15,11 (n=20)	9,95 ± 5,39 (n=20)	6,05 ± 3,96 (n=19)
PON Gln/Arg + OGG1 Ser/Cys	28,63 ± 17,56 (n=16)	13,06 ± 4,54 (n=16)	5,38 ± 4,27 (n=16)
PON Gln/Arg + OGG1 Cys/Cys	24,33 ± 6,35 (n=3)	15,00 ± 3,60 (n=3)	9,00 ± 1,00 (n=3)
PON Arg/Arg + OGG1 Ser/Ser	14,29 ± 4,07 (n=7)	11,14 ± 2,12 (n=7)	8,57 ± 7,11 (n=7)
PON Arg/Arg + OGG1 Ser/Cys	12,67 ± 5,51 (n=3)	8,00 ± 2,00 (n=3)	3,67 ± 4,62 (n=3)

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