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Antioxidant effect of haptoglobin phenotypes against DNA damage induced by hydrogen peroxide in human leukocytes

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ABSTRACT. Human haptoglobin is classified into three major phenotypes: Hp1-1, Hp2-1 and Hp2-2; there are two autosomal alleles Hp^{*1} and Hp^{*2}, and the Hp^{*1} allele has two subtypes, Hp^{*1F} and Hp^{*1S}. Haptoglobin acts as an antioxidant, preventing hemoglobin-driven oxidative damage. We used the comet assay to examine oxidative damage to DNA induced by hydrogen peroxide in human leukocytes; we also looked for differences in the antioxidant capacity of haptoglobin subtypes. Haptoglobin genotypes were determined through allele-specific polymerase chain reaction, visualized on a polyacrylamide gel. The Hp1-1 genotype had the least DNA damage; this indicates that *Hp* alleles differ in their protective effects against oxidative damage. Among Hp^{*1} alleles, Hp^{*1F} was the most protective.

Key words: Haptoglobin polymorphism; Antioxidant; Hydrogen peroxide; Comet assay

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INTRODUCTION

Haptoglobin (Hp) is a serum glycoprotein with the ability to bind free hemoglobin (Hb) in the plasma and block Hb-induced oxidative damage. In humans, there are two codominant alleles for Hp, denoted Hp^{*1} and Hp^{*2}, located on chromosome 16q22.1, and there are three different possible phenotypes: Hp1-1, Hp2-1 and Hp2-2 (Smithies, 1955; Smithies and Walker, 1956). On starch gels with urea, the Hp^{*1} allele revealed two subtypes, Hp^{*1S} and Hp^{*1F} (Smithies et al., 1962). Haptoglobin polymorphism is associated with the prevalence and clinical evolution of many inflammatory diseases, including infections, atherosclerosis and autoimmune disorders (Langlois and Delanghe, 1996). Such associations can be explained by functional differences between the subtypes in the binding of Hb and the rate of Hb clearance from the plasma (Langlois and Delanghe, 1996; Wassell, 2000; Carter and Worwood, 2007). However, these functional differences have never been associated with the subtypes Hp^{*1F} and Hp^{*1S}.

The serum levels of Hp are phenotype-dependent; various functional properties of Hp phenotypes have been described as a direct consequence of hemoglobin-binding and protection against oxidative stress. The antioxidant role of haptoglobin to prevent possible oxidative damage induced by free hemoglobin has been widely studied (Langlois and Delanghe, 1996; Wassel, 2000; Sadrzadeh and Bozorgmehr, 2004; Guéye et al., 2006).

It is well known that DNA suffers constant oxidative damage from free radicals generated by reactive oxygen species (ROS). Normal cellular metabolism is well known to be the source of endogenous ROS; these cellular processes account for the background levels of oxidative DNA damage detected in normal tissue (Collins et al., 1995). Single-cell gel electrophoresis (comet assay) provides a very sensitive method for detecting DNA damage at the single cell level (Collins et al., 1995; Brendler-Schwaab et al., 2005). The alkaline comet assay is increasingly used in genotoxicity testing *in vitro* and is also becoming an important tool for evaluating the genotoxic potential of compounds *in vivo* (Hartmann et al., 2003; Brendler-Schwaab et al., 2005). Among the advantages of the comet assay are its sensitivity for detecting low levels of DNA damage, the need for only a small number of cells per sample, and its applicability to any kind of eukaryotic organism and cell type (Silva et al., 2000; Brendler-Schwaab et al., 2005).

We evaluated the genetic polymorphism of Hp and its ability to protect DNA in human leukocytes against oxidative damage induced by exposure to hydrogen peroxide.

MATERIAL AND METHODS

Criteria for selection of donors, description and processing of samples

Peripheral blood samples and clinical data of 61 healthy donors (32 females and 29 males), with ages varying between 17 and 31 years old, were collected from students of the Universidade de Brasília (UnB). Written informed consent was obtained from all participants in the study. This research project was approved by the Ethics Committee of the Universidade de Brasília, number 0.001668/2005-18, following the protocol recommended by the Helsinki Declaration.

Single-cell gel electrophoresis

The comet assay (alkali method) was carried out according to Singh et al. (1988), with a few modifications. Microscope slides were dipped briefly on 1.5% hot (60°C) normal melting agarose prepared in phosphate-buffered saline. The slides were dried overnight at room temperature and then stored at 4°C until use. Subsequently, freshly collected EDTA-treated peripheral blood of each sample (20 µL) was suspended on 120 µL 0.5% low melting point agarose in phosphate-buffered saline (Gibco BRL) at 37°C and pipetted onto eight microscope slides pre-coated with a layer of normal melting point agarose. This mixture was allowed to set at 4°C for 10 min. Treatment was made with 150 µL hydrogen peroxide (H_2O_2) at concentrations of 250 µM, 1.0 and 2.0 mM, for 5 min at 4°C, using two slides for each treatment. The control slides were prepared under the same conditions, but without the H_2O_2. The slides were immersed into a freshly prepared cold (4°C) lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, NaOH, pH 10.0-10.5, 1% lauroyl sarcosine, and 1% Triton X-100 and 10% dimethyl sulfoxide were added immediately before use) at 4°C for 1 h. After lysis, the slides were placed in an electrophoretic tank in 300 mM NaOH and 1 mM EDTA, pH >13.0, for 30 min. Subsequently, electrophoresis run at 25 V and 350 mA for 40 min. Soon after neutralization (3 x 5 min in 0.4 M Tris, pH 7.5, at 4°C), the slides were stained with ethidium-bromide at 20 µg/mL, fixed with 100% ethanol for 5 min and analyzed with a Zeiss Axioskop 2 fluorescence microscope (filter 510-560 nm, barrier filter 590 nm) with a total magnification of 400X. All slides were duplicated. One hundred comets on each slide were scored visually as belonging to one of the five classes proposed by Collins et al. (1995), and the number of comets in each category was counted and the DNA damage was calculated according to Jaloszynski et al. (1997).

Determination of haptoglobin genotypes

Genomic DNA was isolated from peripheral blood samples and collected in Vacutainer tubes containing EDTA using a purification kit GFX (GE Healthcare, Buckinghamshire, England). The samples were stored at -20°C until analysis. Hp^{*1} genotypes were determined by allele-specific polymerase chain reaction (PCR), as described by Yano et al. (1998). PCR primer pairs used for genotyping and identification of alleles Hp^{*1F}, Hp^{*1S} and Hp^{*2} were produced with independent systems, using three PCRs for each DNA sample. The PCR products were visualized by electrophoresis on 6% non-denaturing polyacrylamide gels stained with silver nitrate. The accuracy of haptoglobin genotyping was tested by comparison with the results of haptoglobin protein phenotyping performed with the same plasma samples. There were no discrepancies between genotyping and phenotyping using these two methodologies.

Statistical analysis

Allelic and phenotypic frequencies were calculated by gene counting. Hardy-Weinberg equilibrium was evaluated by means of the chi-square test. Values of P > 0.05 were considered to be in Hardy-Weinberg equilibrium.

In order check for all possible differences among the groups, we used ANOVA from MINITAB® release 14.20. The continuous variables were tested for normal distributions with Shapiro-Wilk. There was heterogeneous variability, so a Box-Cox transformation was used so

that ANOVA could be run. The difference between DNA damage means was analyzed by the Tukey test or by the Fisher test ($\alpha = 0.05$).

RESULTS

The frequencies of haptoglobin phenotypes and genotypes (Table 1) were in Hardy-Weinberg equilibrium (P > 0.05). The allele frequency for Hp^{*1F} was 13.7%; for Hp^{*1S} it was 27.4%, and for Hp^{*2} it was 58.9%.

Table 1. Frequencies of haptoglobin phenotypes and genotypes.

Phenotypes	Genotypes	No. of individuals	Genotype frequencies
1-1	$Hp^{*1S}Hp^{*1S}$	4	0.065
	$Hp^{*1S}Hp^{*1F}$	2	0.032
	$Hp^{*1F}Hp^{*1F}$	3	0.048
2-1	$Hp^{*2}Hp^{*1S}$	24	0.387
	$Hp^{*2}Hp^{*1F}$	9	0.145
2-2	$Hp^{*2}Hp^{*2}$	20	0.323

Allelic and genotypic frequencies were calculated by gene counting. Hardy-Weinberg equilibrium was evaluated by means of the chi-square test.

There was a positive correlation between H_2O_2 concentrations and DNA damage levels (Table 2). The three H_2O_2-treated groups were significantly more damaged than the controls (P = 0.001, ANOVA). The leukocytes treated with 250 μM H_2O_2 were also significantly less damaged than those treated with 1.0 and 2.0 mM (P < 0.05, Tukey test).

Table 2. Results of the comet assay made with peripheral leukocytes of healthy humans.

Phenotypes	μ (score) ± SE Control*	μ (score) ± SE H_2O_2 (250 μM)**	μ (score) ± SE H_2O_2 (1 mM)**	μ (score) ± SE H_2O_2 (2 mM)**	Genotypes	μ (score) ± SE Control*	μ (score) ± SE H_2O_2 (250 μM)**	μ (score) ± SE H_2O_2 (1 mM)**	μ (score) ± SE H_2O_2 (2 mM)**
1-1	36.13 ± 7.05[a]	120.55 ± 26.96[a]	160.39 ± 37.25[a]	241.44 ± 44.20[a]	$Hp^{*1S}Hp^{*1S}$	46.63 ± 14.70	110.63 ± 45.84	147.25 ± 66.93	202.25 ± 81.33
					$Hp^{*1S}Hp^{*1F}$	26.64 ± 1.36	193.70 ± 75.31	253.15 ± 60.85	378.50 ± 3.50
					$Hp^{*1F}Hp^{*1F}$	28.46 ± 5.45[c]	85.00 ± 12.22	116.06 ± 49.93	202.33 ± 46.49
2-1	81.23 ± 11.65[b]	136.81 ± 14.03[b]	204.31 ± 17.46[b]	242.42 ± 18.24[b]	$Hp^{*2}Hp^{*1S}$	91.73 ± 14.91	151.49 ± 17.09	221.06 ± 19.23	260.69 ± 20.38
					$Hp^{*2}Hp^{*1F}$	53.22 ± 12.31[d]	97.68 ± 19.54	159.64 ± 35.97	193.71 ± 35.86
2-2	84.29 ± 16.67	116.47 ± 21.82	169.40 ± 24.44	200.34 ± 26.12	$Hp^{*2}Hp^{*2}$	84.29 ± 16.67	116.47 ± 21.82	169.40 ± 24.44	200.34 ± 26.12

Data are reported as means ± SE (standard error of the mean). Values are presented in arbitrary units for DNA damage in comet assay, considering the control group and the different treatments with H_2O_2, in the different phenotypes and genotypes. *Significantly different from the H_2O_2 groups by the Tukey multiple comparison test**. [a]Significant at P < 0.05 by the Fisher test comparing all treatments[b]. [c]Significant at P < 0.05 by the Fisher comparison test [d]. Values of μ (score) are presented in arbitrary units for DNA damage in comet assay.

The variation in DNA damage among the three Hp phenotypes was significant (P = 0.028, ANOVA). In the 250 μM and 1 mM H_2O_2 treatments, greater DNA damage was observed in Hp2-1, while at 2 mM, less DNA damage was observed in Hp2-2. The Hp1-1 phenotype had the least DNA damage in the untreated group and in the group treated with H_2O_2 at

1.0 mM, while DNA damage was lower for the Hp2-2 phenotype in the treatments with H_2O_2 at 250 μM and 2.0 mM. The only significant differences were found between the phenotypes Hp1-1 and Hp2-1 ($P < 0.05$, Fisher multiple comparisons test), which was associated with Hp1F-1F and Hp1F-2. In fact, in the untreated Hp1-1 group there was less DNA damage in individuals carrying the Hp^{*1F} allele; in the treatments with H_2O_2, only homozygous Hp1F-1F had less DNA damage. The same results were observed for Hp2-1, as Hp2-1F individuals had less DNA damage than did Hp2-1S individuals.

DISCUSSION

The Hp^{*1} allele frequency of 41.1% that we found was similar to those reported from other urban Brazilian populations in São Paulo State (46%) (Wobeto et al., 2007) and Euro-descendants from Porto Alegre (41.4%) (Tondo et al., 1963), and the phenotype frequencies were close to those found by Wobeto et al., 2007. The allele frequencies that we observed were similar to those observed for the European population of Australia (Lai et al., 1986) and among Germans (Kruger and Puschel, 1993). Given that the distribution of haptoglobin alleles in our sample reflects the history of the creation of Brazil's new capital in the 1960s, which was formed by migrants from all regions of the country, this can be considered representative of the Brazilian population (Callegari-Jacques et al., 2003).

Haptoglobin acts as an antioxidant by binding hemoglobin, preventing iron-catalyzation of free radicals (Wassel, 2000). The binding of Hb by Hp is essential for rapid clearance of Hb from the plasma, after which the high-affinity Hp-Hb complex is metabolized by macrophages (Tseng et al., 2004). Exogenous sources of ROS, such as H_2O_2, have been documented as causing ROS-induced damage to cellular macromolecules (DNA, RNA, lipids, and proteins; Franco et al., 2008), and oxidants play an important role in the destabilization of lipid membranes through peroxidation, oxidation and inactivation of proteins, and oxidation of nuclear material (Kirschvink et al., 2008). Sing et al., 1988 developed a simple technique that permits sensitive detection of DNA damage in individual cells. The comet assay has been a useful and effective method for studies of genotoxicology, carcinogenesis and aging. We found that treatments of leukocytes with hydrogen peroxide at concentrations of 250 μM, 1.0 mM and 2.0 mM were effective, causing DNA breaks; this was easily measured with the comet assay.

Guéye et al. (2006) demonstrated that free hemoglobin released by hemolysis is toxic due to oxidative stress and that the protective effect of haptoglobin against this oxidative mechanism is also phenotype dependent. In addition, they showed that subjects with Hp1-1 phenotypes had more protection against oxidative stress than subjects with Hp2-2 phenotypes. Various independent studies have reported the association of Hp phenotypes with increased risk of many chronic degenerative diseases, such as diabetes, retinopathy, nephropathy, and cardiovascular diseases. Moreover, subjects with the Hp2-2 phenotype have an increased risk of developing such diseases compared with those homozygous for the haptoglobin 1 allele, whereas heterozygotes have an intermediate risk (Langlois and Delanghe, 1996; Melamed-Frank et al., 2001; Bernard et al., 2003; Levy, 2003). We also found that there was less DNA damage in the Hp1-1 phenotype than in the other phenotypes. Despite the low frequency of Hp^{*1F} in the Brazilian population and our small sample size, we demonstrated that individuals carrying the Hp^{*1F} allele were more protected against DNA damage than individuals carrying other alleles.

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Various functional properties of Hp phenotypes have been described as a direct consequence of hemoglobin binding and protection against oxidative stress (Wassel, 2000). Hp1-1 is biologically the most effective in binding free hemoglobin, Hp2-2 is biologically the least active and Hp2-1 is moderately active (Sadrzadeh and Bozorgmehr, 2004). This leads us to expect that people possessing the Hp1-1 phenotype should be more protected against DNA oxidative damage than those possessing other phenotypes. Although we did not evaluate the levels of haptoglobin in the sera, it is well known that the Hp phenotype determines the serum levels of the Hp glycoprotein; people with Hp1-1 have the highest plasma concentrations, those with Hp2-2 the lowest plasma concentrations, and those with Hp2-1 have intermediate concentrations (Sadrzadeh and Bozorgmehr, 2004). On the other hand, there are differences in the binding of haptoglobin types by the CD163 receptor (Carter and Worwood, 2007). We suggest that the product of the Hp^{*1F} allele could bind more efficiently to either hemoglobin or the CD163 receptor than the products of the other alleles.

CONCLUSIONS

Phenotype Hp1-1 had greater antioxidant properties than the other phenotypes, which resulted in more efficient protection of leukocytes against oxidative DNA damage. Among Hp^{*1} alleles, Hp^{*1F} was the most protective.

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