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Regulating Redox Balance Gene Expression in Healthy Individuals by Nutraceuticals: A Pilot Study

Francesco Marotta,[1] Keiko Koike,[2] Aldo Lorenzetti,[1] Shalini Jain,[3] Paola Signorelli,[1] Yussef Metugriachuk,[2] Pierre Mantello,[2] and Nicola Locorotondo[4]

Abstract

We tested the effect of a fermented papaya preparation (FPP; ORI, Gifu, Japan) on redox balance gene expression in 11 healthy nonsmoker, teetotaller individuals subjected to a detailed dietary and lifestyle questionnaire who refrained from any multivitamin supplement or fortified food. Redox status was assessed by erythrocyte and plasma parameters together with related leukocyte mRNA (glutathione peroxidase [GPx], superoxide dismutase [SOD], catalase, 8-oxoguanine glycosylase [hOGG1]) before/after 6 grams of FPP supplementation. At 2 and 4 weeks after FPP administration, plasma parameters remained unchanged, whereas FPP significantly upregulated all tested gene expression ($p < 0.05$). Although posttranscriptional/translation protein modifications do occur and larger and longer studies are awaited, these preliminary data suggest that a transcriptomic modification of key redox and DNA repair genes may offer further insights when attempting to interrelate "nutragenomics" to clinical phenomena.

Introduction

One of the most important emerging areas of nutrition science is represented by the area of nutrigenomics, i.e., the effect of diet on gene expression and chromosomal structure, and the extent to which genetic differences between individuals affect response to a specific dietary intake, functional food, or supplement in terms of a defined health outcome. Although there is wide debate to what extent oxidative stress is a causative or epiphenomenic event in aging and in chronic diseases, the question arises as to whether supplemental antioxidants are beneficial.[1–3] Related meta-analyses are often biased by attempting to put together different schedules, dosages, drug associations, risk factor–burdened populations, and the origin itself of the antioxidant employed, whether synthetic or natural, which brings about wide bioavailability and absorption changes. To further complicate the matter, the lack of commonly acknowledged biomarkers to monitor the expected biological changes during nutraceutical interventional trials poses a number of questions to be fully clarified in terms of their effective biological modification at a cellular level. In this study, we used a fermented papaya preparation (FPP; ORI, Gifu, Japan) made under ISO 9001 and ISO 14001 regulations from a patented lengthy biofermentation process of non-GM (genetically modified) *Carica papaya*, which has been demonstrated in *in vitro* and *in vivo* studies to exert significant protective antioxidant properties.[4–7]

Materials and Methods

A detailed lifestyle questionnaire was administered to 11 healthy subjects (7 females, 4 males; mean age, 37; age range, 22–46). Subjects were instructed to refrain from physical exercise, and subjects under overt psychological and physical distress were excluded at the time of selection, as were smokers and habitual drinkers. Moreover, the Web-based version of the National Institutes of Health Diet History Questionnaire (NIH DHQ) was used to assess diet history over the past month and during the study period. Subjects were advised not to use any multivitamin supplements or fortified food while maintaining their usual diet. All subjects were given 6 grams/day FPP sublingually. Blood was collected after an overnight fast at 2 and 4 weeks of observation for biochemical and genomics studies.

[1]ReGenera Research Group, Milano, Italy.
[2]ORI Bioscience Laboratory, Gifu, Japan.
[3]Department of Nutrition, Illinois University, Urbana, Illinois.
[4]Gruppo Locorotondo Laboratories, Palermo, Italy.

Antioxidant enzyme activity, lipid peroxidation, and protein oxidation

The hemolysate was prepared from the red cells and used for the measurement of the activities of the following enzymes: Superoxide dismutase (SOD)[8] catalase (CAT),[9] and glutathione peroxidase (GPx).[10] Serum total protein, albumin, ascorbic acid, and uric acid concentrations were measured spectrophotometrically. Lipid peroxidation in plasma was measured by reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA) according to Ohkawa.[11] The content of MDA was measured spectrofluorometrically using a Jasco (FP-6200) spectrofluorometer (excitation 515 nm, emission 553 nm), whereas the levels of plasma protein carbonyl groups were assessed by a noncompetitive enzyme-linked immunoassay (ELISA)[12] with minor modifications.[13] Protein concentrations were determined by using Coomassie protein assay reagent (Pierce, Rockford, IL).

Isolation of human peripheral blood neutrophils

Fresh heparinized blood was obtained from healthy volunteers after they had given informed consent. Briefly, after sedimentation with 3% dextran (Pharmacia), Ficoll-Paque centrifugation at 400 *g* for 40 min, and hypotonic lysis of red blood cells, the isolated fraction contained more than 90% neutrophils, as estimated by Giemsa stains. To test viability, the cells were cultured in petri dishes under 5% CO_2 at 37°C for up to 24–72 h. Viability by Trypan Blue after isolation was 90%. The neutrophils were washed with phosphate-buffered saline (PBS) (10 mM PBS [137 mM NaCl, 3 mM KCl], pH 7.4) and resuspended at 1×10^6 cells/mL in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 50 units/mL penicillin, and 50 g/mL streptomycin. The cells were used immediately for the experiments or incubated in polypropylene tubes at 37°C in 5% CO_2 in air for 2–10 h.

Semiquantification of antioxidant and DNA repair enzyme mRNA

Total RNA was extracted by TRIzol, following the maker's instruction (Invitrogen, Paisley, UK) and treated with DNase I (Sigma, USA). RNA quality and quantity were confirmed by ultraviolet (UV) spectrophotometry and agarose gel electrophoresis. One microgram of total RNA was reverse-transcribed with random hexamers and Moloney murine leukemia virus reverse transcriptase at a final volume of 20 μL. Specific cDNA amplification of human SOD, CAT, and GPx was carried out with 2 μL of cDNA in a Light Cycler by using FastStart DNA Master SYBR Green I (Roche). The specific primers used were (5′ to 3′): CATCATCAATTTC GAGCAGA and GCCACACCATCTTTGTCAGCAG for SOD; GCGGTCAAGAACTTCACTGA and GCTAAGCTTC GCTGCACAGGT for CAT; and TGGCTTCTTGGACAAT TGCG and GATGCCCAAACTG GTTGCACGGGAA for GPx. Primers for the housekeeping gene porphobilinogen deaminase (*PBGD*) were (5′ to 3′) TCCAAGCGGAGCCA TGTCTG and AGAATCTTGTCCC CTGTGGTGGA.

The quantification was performed by crossing-point extrapolation into a standard curve with known cDNA concentrations by using Light Cycler system software. Relative units represent the ratio of concentration between the specific mRNA and the housekeeping mRNA. As for determination of hOGG1 expression levels, cDNA was synthesized from 0.5–1 gram of total mRNA using a first-strand cDNA synthesis kit. The same sample devoid of reverse transcriptase was used as a negative control. The quality of cDNA was confirmed by PCR amplification of a fragment from the control gene ubiquitin C. Expression of the hOGG1 gene was assessed by a 7500 Real Time PCR System (Applied Biosystems, USA) as total quantification, by using TaqMan Gene Expression Assays from the same manufacturer. Bacterial plasmids containing coding sequences of *hOGG1* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) served as

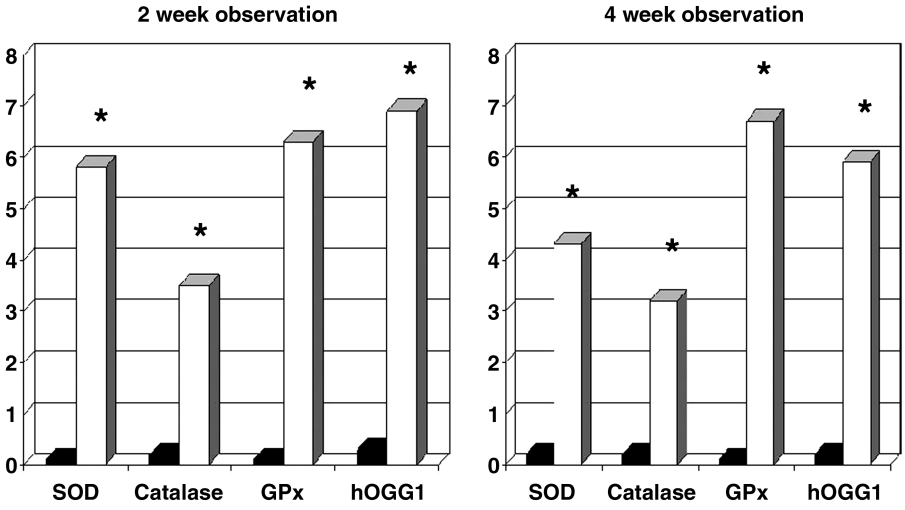

FIG. 1. Semiquantification of antioxidant and DNA repair enzyme mRNA: Effect of fermented papaya preparation. Relative (arbitrary) units represent the ratio of concentration between the specific mRNA and the housekeeping mRNA (see text). (*) $p < 0.01$ versus baseline value (black bars). SOD, Superoxide dismutase; GPx, glutathione peroxidase; hOGG1, 8-oxoguanine glycosylase.

standards for total quantification of gene expression.[14] The expression of *hOGG1* was normalized to the expression of *GAPDH* and expressed in arbitrary units.

Glutathione S-transferase mu 1 *and* hOGG1 *genotype analysis*

PCR analysis was performed in 25 μL of reaction buffer containing 0.5 mmol/L of dNTPs, 2.0 mmol/L of $MgCl_2$, 12.5 pmol of each primer, about 150 ng of DNA, and 1.25 U of thermostable *Taq* DNA polymerase using a programmable thermocycler. The primers used for *GSTM1* (*glutathione S-transferase mu* 1) were 5′-GAACTCCCTGAAAAGCTAAGC and 5′-GTTGGGGCTCAAATATACGGTGG. The PCR protocol included an initial melting temperature set at 94°C for a 5-min period followed by 35 cycles of amplification (with the apparatus set as follows: 2 min at 94°C, 1 min at 59°C, and extension for 1 min at 72°C). A final 10-min extension step processed at 72°C terminated the process. The final PCR product from coamplification of *GSTM1* (215 bp) was visualized on an ethidium bromide-stained 2.0% agarose gel. Either *GSTM-1* or *hOGG1* Ser/Ser genotype frequencies were consistent with the expectations for the Hardy–Weinberg equilibrium (data not shown). Determination of *hOGG1* genotypes was confirmed by random regenotyping, using the TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA). The genotype of DNA samples was identified blindly, and controls were prepared and set up in association with every single PCR operation as a blank control (without DNA template), positive control, and negative control.

Statistical analysis

Comparisons for quantitative variables (enzyme activities, oxidative damage, and enzyme expression) were performed by nonparametric analysis (Mann–Whitney and Kruskall–Wallis tests for nonrelated samples). Comparisons at two different times (T1 and T2) of these quantitative variables were carried out with the Wilcoxon test for related samples. All reported p values represented two-tailed tests, with values $= 0.05$ considered statistically significant.

Results

All subjects fully complied with the study protocol. At either the 2- or 4-week observation points, the plasma level of the tested redox parameter did not change after FPP supplementation, besides a nonsignificant decrease of MDA. On the other hand, starting at the 2-week observation point, FPP brought about a significant upregulation of all gene expression investigated ($p < 0.05$) (Fig. 1), which remained stable at a later testing time. These data applied irrespective of *GSTM-1* and *hOGG1* genotype profiles. No correlation appeared between the erythrocyte level of redox parameters or plasma MDA and related gene expression or genotype profile.

Discussion

Oxidative stress arising as an imbalance between oxidant and antioxidant molecules is likely to contribute to a number of chronic disease-related genotoxic effects, as well aging *per se* and micronutritient deficiencies. Indeed, the guanine moiety of nucleotides represents one of the main targets for hydroxyl radicals and, depending on the molecular context (2-deoxyribonucleotides, ribonucleotides, DNA, RNA), oxidized guanine may follow separate repair pathways leading to different extracellular products.[15] 8-Oxo-7,8-dihydroguanine (8-oxoGua) in DNA is cleaved selectively by specific glycosylases of the base excision repair system, such as the polymorphic 8-oxoguanine DNA *N*-glycosylase 1 (hOGG1).[16] Although some authors have reported that antioxidant enzyme gene expression as determined by quantifying the specific mRNA may show a good correspondence with antioxidant enzyme activity, in our study this did not apply to erythrocyte determination. Indeed, antioxidant enzymes are regulated by multiple factors, and changes in antioxidant enzyme activity do not exactly mirror the changes in the related mRNA levels.[17,18] Moreover, these enzymes are likely to be also regulated at a posttranscriptional level by alterations in the degradation rate of mRNA, protein synthesis (translation), and posttranslational modifications.[19] Nonetheless, while such posttranscriptional/translation protein modifications are known to occur, these preliminary data suggest that our interventional nutraceutical supplementation yielded a transcriptomic modification of key redox genes and DNA repair genes. Larger populations and longer observation periods are required, and they need to be interrelated to clinical phenomena unfolding in healthy and diseased subjects. However, the preliminary data presented here may offer further insights when attempting to follow a "nutragenomic" effect ideally aimed to beneficially modulate the epigenomics of aging *per se*.[20]

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Address correspondence to:
Francesco Marotta
Piazza Firenze, 12
20154 Milano
Italy

E-mail: fmarchimede@libero.it