

Nutraceutical Supplementation: Effect of a Fermented Papaya Preparation on Redox Status and DNA Damage in Healthy Elderly Individuals and Relationship with GSTM1 Genotype

A Randomized, Placebo-Controlled, Cross-Over Study

FRANCESCO MAROTTA,^{a,e} MARK WEKSLER,^b YASUHIRO NAITO,^c CHISATO YOSHIDA,^d MAYUMI YOSHIOKA,^c AND PAOLO MARANDOLA^e

^a*HepatoGastroenterology Unit, S. Giuseppe Hospital, Milano, Italy*

^b*Geriatrics Department, Cornell University Medical Center, New York, USA*

^c*Immunology Research Institute and Clinic, Nagoya, Japan*

^d*GAIA, Age-Management Foundation, Pavia, Italy*

^e*ORI Bioscience Laboratory, Gifu, Japan*

ABSTRACT: Our study group consisted of 54 elderly patients without major invalidating diseases who were randomly divided into two fully matched groups. Group A was given a certified fermented papaya preparation 9 g/day by mouth, while group B received placebo. Treatment was carried out in a cross-over manner with a 3-month supplementation followed by a 6-week washout period. Blood samples were drawn at entry and on a monthly basis to check routine parameters, redox status, and 8-OHdG in circulating leukocyte DNA. Polymorphism analysis of GSTM1 was carried out as well. The glutathione-S transferase M1 (GSTM1) genotype was null (–) in 40% and 46% of groups A and B, respectively. GSTM1 (–) smokers had a significantly higher level of plasma DNA adducts and leukocytes level of 8-OHdG than their GSTM1 (+) counterparts ($P < 0.01$). There was a weak correlation between cigarettes smoked/day and DNA adduct ($r: 0.61, P < 0.05$), which also correlated with antioxidant concentrations, but only in GSTM1 (–) smokers ($P < 0.01$). The fermented papaya preparation (FPP)–supplemented group showed a significant enhancement of the antioxidant protection ($P <$

Address for correspondence: F. Marotta, via Pisanello, 4-20146, Milano, Italy.
e-mail: fmarchimede@libero.it

Ann. N.Y. Acad. Sci. 1067: 400–407 (2006). © 2006 New York Academy of Sciences.
doi: 10.1196/annals.1354.057

0.01 vs. A) within the subgroups with GSTM1 (–) and of plasma DNA adduct, irrespective of the GSTM1 genotype. Only the GSTM1 (–) subgroup was the one that, under FPP treatment, increased lymphocyte 8-OHdG ($P < 0.01$). Such preliminary data show that FPP is a promising nutraceutical for improving antioxidant-defense in elderly patients even without any overt antioxidant-deficiency state while helping explain some inconsistent results of prior interventional studies.

KEYWORDS: GSTM1; redox status; elderly; fermented papaya; 8-OHdG

INTRODUCTION

Reactive oxygen species have been implicated in the pathogenesis of many chronic diseases since they may cause a different degree of damage to DNA other biological molecules. Such DNA damage can account for the genetic changes that take place along with the progression from dysplastic lesions to precancerous lesions and, eventually, to anaplastic cancerous growth and metastatic dissemination. On the other hand, it is known that, even without any overt disease, oxidative damage to DNA, proteins, and lipids accumulates with age and contributes to degenerative diseases and aging phenomena by disrupting cellular homeostasis.¹ Moreover, this population is more prone to depleted antioxidant defenses on account of poor/improper intake, while a number of elderly may concomitantly suffer from a subclinically impaired gut absorption ability. In this respect, a study conducted among 490 geriatric patients has showed that more than 40% had indeed an occult malabsorption.² To make the field of interventional nutrition even more complex, although intriguing, the post-genomic era has opened new avenues in the study of specific genotype-modulated understanding of the interrelationships between food, food components, and xenobiotic exposure with each single individual response. As an example, quite interestingly, Palli *et al.*³ have recently suggested that the effect of dietary antioxidants in reducing DNA adducts is dependent on the detoxifying activity of the GSTM1 isoenzyme. This finding is of great practical relevance and may help explain some contradictory or inconclusive results of studies tackling the issue of antioxidants and genomic abnormalities when considering that GSTM1 gene deficiency has been shown to occur in approximately half of the population of various ethnic origins, mostly Caucasian, Japanese, and white Americans. GSTM1 deficiency has been shown to increase DNA adduct formation⁴ and cytogenetic damage.⁵ Indeed, the glutathione S-transferases (GST) represent a crucial enzymatic system of the cellular mechanism of detoxification by protecting cells against reactive oxygen metabolites by means of the conjugation of glutathione with electrophilic compounds. GST enzymes are involved in the metabolism of xenobiotics that include environmental carcinogens, reactive oxygen species, and chemotherapeutic agents.⁶ Associations of GSTM1 and/or GSTT1 null

genotypes with bladder, lung, and colorectal cancer, as well as head and neck squamous cell carcinoma, have been reported and represent an area of growing intensive research.⁷⁻¹⁰

The aim of the present study was to test in a healthy elderly population the effects of a novel nutraceutical on redox status abnormalities that are likely to take place with advancing age. A number of bench-validated studies of this compound have proved its potent antioxidant and NO-stimulating properties. Moreover, we aimed to get further insights into the role played by GSTM1 genotype status.

PATIENTS AND METHODS

Our study group consisted of 60 generally elderly persons (mean age: 72 years, range 72–84 years; male/female: 36/24). Major invalidating disease that were regarded as exclusion criteria were: prior or ongoing cancer, autoimmune disease, chronic illness requiring steroids or immunosuppressive agents, allopurinol treatment, chronic renal failure, and overt cardio-respiratory abnormality. Thirteen patients (male/female: 9:2) were mild smokers (<10 cigarette/day), 9 were treated for mild hypertension, 10 for osteoporosis, 11 for insomnia, and 4 for mild depression. The patients were randomly divided into two groups matched for age/gender, life style, alcohol/tobacco use, physical activity, and medication. One group was given a GMP-, ISO9001/14000-certified fermented papaya preparation (FPP, Osato Research Institute, Gifu, Japan; 9 g/day) by mouth in the morning, 1 h after breakfast, and fasting for at least a further 30 min, while the control group received the same amount of placebo (flavored powdered sugar). The treatment was carried out in a cross-over manner with a 3-month supplementation period followed by a 6-week washout period between treatments.

As an age-control group for redox status, a group of 10 young/early middle-age healthy nonsmoking patients were also considered.

Diet and Life Style Questionnaire

A detailed life style questionnaire was administered to all patients with particular attention paid to stress factors and physical activity. Moreover, a dietary questionnaire was used, and specific care taken in assessing the daily dietary content of macronutrients and micronutrients. This was re-assessed at the end of the study using the model of 7-day diet history.

Blood Collection and Storage

Blood samples were drawn at entry and on a monthly basis. Studies related to genetic susceptibility were carried out only at entry and at the end of the study.

Assessment of Redox Status

GSH, GSH-Px, and GSSG were measured by means of hemoglobin-catalyzed oxidation of 10-*N*-methylcarmoyl-3,7-dimethylamino-10-*H*-phenothiazione after treatment with phospholipase. Values were read by a fluorescence detector.

Determination of Plasma Malondialdehyde

Malondialdehyde was measured from frozen, EDTA-containing plasma after thiobarbituric acid (TBA) reaction by high-performance liquid chromatography (HPLC) modified by adding 0.01% butylated-hydroxytoluene to the coloring solution to avoid the generation of TBA-reactive molecules during the procedure. The system was heated at 100°C for 45 min. After cooling, the chromophore was extracted with 2 mL of *N*-butanol by vigorous shaking and dried under constant nitrogen flux. The final powder was resuspended in 100 µL of chromatographic solvent A and added to the HPLC system (solvent A: 10 mmol hexate sulfonic acid and H₃PO₄ adjusted to pH 3; solvent B; methanol). Tetra-ethoxy-propane served as a standard source of malonyldialdehyde (MDA), which is a thiobarbituric acid-reactant substance. All samples were processed in duplicate.

Analysis of 8-OHdG in Circulating Leukocyte DNA

DNA was isolated and purified from leukocytes as described by Fraga *et al.*¹¹ with minor modifications. The resulting deoxynucleoside mixture was analyzed by means of a HPLC-electrochemical detection (ECD) system and the amounts of 8-OHdG were referred to the quantities of deoxyguanosine detected, in the same sample, by ultraviolet absorbance. The results are expressed as the ratio of the absorbance peak of 8-oxodGuo adducts to that of dGuo adducts $\times 10^5$.

Polymorphism Analysis

This was conducted by the multiple polymerase chain reaction (PCR) method to check the presence of GSTM1 gene in genomic DNA samples. PCR analysis was performed in 25 µL reaction buffer containing 0.5 mmol/L of dNTPs, 2.0 mmol/L of MgCl₂, 12.5 pmol of each primer, about 150 ng DNA, and 1.25 U of thermostable Taq DNA polymerase, using a programmable thermocycler. The primers used for GSTM1 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 co-amplification of GSTM1 (215 bp) was visualized on an ethidium bromide-stained 2.0% agarose gel. The subjects were accordingly classified as either positive (when at least one copy of the gene was present) or null genotypes. The genotype of DNA samples was identified blindly and controls were prepared and set up in association with every single PCR operation as blank control (without DNA template), positive control, and negative control.

Statistics

Data were evaluated by repeated-measures analysis of variance and independent *t*-tests with a Bonferroni correction for multiple comparisons.

RESULTS

Six participants were excluded during the study for a number of reasons that could theoretically interfere with the understanding of the final data (two participants had flu [complicated in one by transient asthma] and both required drug treatment, one was enrolled in an intensive gym regimen, one moved away, one was hospitalized for an uneventful diverticulitis, and another dropped out spontaneously). As expected, no side effect was reported by participants completing the study beyond a subjective feeling of wellness and mood stabilization. However, such clinical signs were outside the aim of our designed protocol. Elderly patients showed a normal level of the all antioxidants tested, the only abnormalities being a significantly higher level of plasma MDA as well as lower GSH/GSSG ratio ($P < 0.05$ vs. young/middle-age group). At the entry, before the cross-over shift, the two elderly groups proved to be comparable in terms of GSTM1 genotype, which ranged between 40% and 46%. A further finding was that, at baseline assessment, as compared to GSTM1-positive smoker participants, the GSTM1-negative counterpart showed a significantly higher level of DNA adducts (1.8 vs 2.7×10^8 nucleotides, $P < 0.01$) and of 8-OHdG concentration (72 vs 88×10^5 dG, $P < 0.01$) in leukocyte DNA. Moreover, a weak but significant correlation appeared between cigarettes smoked per day and DNA adducts ($r: 0.61$, $P < 0.05$), but the intrinsic limitation of these data is that a larger number of participants are required. Within the GSTM1-negative smoker subgroup, DNA adducts correlated with MDA and GSH/GSSG ratio ($r: 0.78$, $P < 0.01$). FPP brought about a trend in improvement of oxidative/antioxidative balance, but this reached statistical significance only in the GSTM1-negative subgroup, irrespective of smoking ($P < 0.01$). Such results were also confirmed when smokers were excluded from the analysis. Similar protective effects on leukocyte DNA adducts ($P < 0.05$) were obtained when

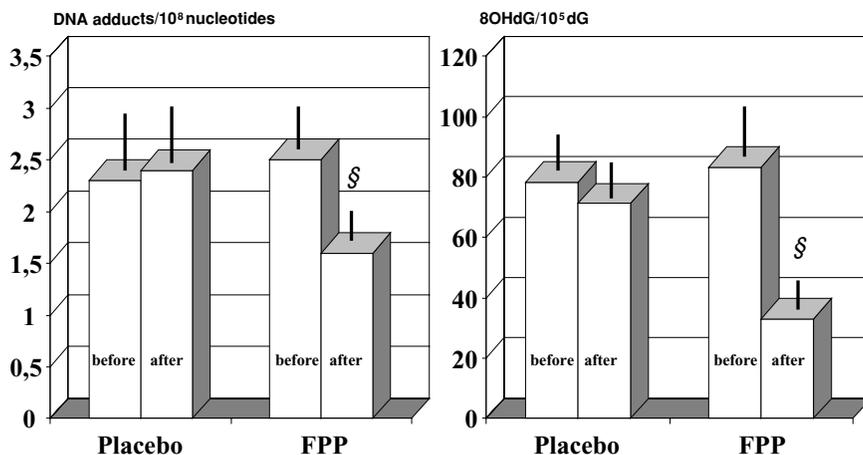


FIGURE 1. *Left:* concentration of DNA adducts in all subjects and of 8-OHdG in circulating leukocytes (only in GSTM1–subjects). *Right:* effect of nutraceutical intervention. §*P* < 0.05 vs. baseline and vs. placebo.

considered subject as a whole (FIG. 1). These data were paralleled by a significant decrease in leukocyte 8-OHdG concentration, but only when considering GSTM1-negative participants (FIG. 1).

DISCUSSION

Although redox status imbalance is well recognized as an adverse factor in a large number of chronic degenerative diseases and aging, the question still remains as to whether antioxidant supplementations are beneficial if they are to be regarded as potential therapeutic tools (nutraceuticals/nutrigenomics). Indeed, one of the major drawbacks in any supplementation study is the limited population and/or observation time. Moreover, a further limitation in evaluating the clinical impact of epidemiologic and/or interventional studies dealing with antioxidants is represented by the questionable appropriateness of suitable markers of oxidative injury *in vivo*.¹² Among the most convincing evidence of the role of oxidative stress and protection by antioxidants in the disease process is provided by studies conducted in patients with heart diseases.¹³ On the other hand, it is becoming all the more important to distinguish the role of oxidants as mediators of disease as well as crucial elements of signal transduction pathways.¹⁴ The post-genomic revolution with the study of polymorphisms thus offers unprecedented opportunities to ideally unfold, tailor, and monitor the impact of diet and dietary components with cell signaling/function in physiological and pathologic situations. As a consequence, the design of nutritional studies becomes even more demanding, but with

far-reaching expectations. In the present study, among the multifaceted scenarios of polymorphisms, we chose GSTM1 because of its high frequency, which may allow a smaller study sample. Having started from an experimentally and clinically supported nutraceutical,^{15–19} we showed that it could significantly improve the oxidative/antioxidative balance that was found to be impaired in elderly people, even in the absence of any overt inflammatory disease. The genetic susceptibility to oxidative stress, as assessed by GSTM1 analysis, further enhanced this result, whereas smokers might prove to get the highest benefit from FPP supplementation. Interestingly, FPP appeared to exert protective effects on leukocyte DNA adducts' formation, irrespective of genotype profile, while also enhancing DNA repair mechanisms against the highly mutagenic base modification, but only in GSTM1-null genotype participants. Although the fundamental epigenetic mechanisms of action of FPP are still a matter of ongoing investigation, and no conclusions can be drawn in the relevance of its beneficial effects on the natural history of the studied population in the long run, the present promising data suggest that there is indeed a role for nutraceutical intervention when supported by proper protocol design and mandatorily bench-validated natural compounds.

REFERENCES

1. SOHAL, R.S. & W.C. ORR. 1995. Is oxidative stress a causal factor in aging? *In* Molecular Aspects of Aging. K. Esser G.M. Martin, Eds.: 109–127. Wiley and Sons. Chichester.
2. HABOUBI, N.Y. & R.D. MONTGOMERY. 1992. Small-bowel bacterial overgrowth in elderly people: clinical significance and response to treatment. *Age Ageing* **21**: 13–19.
3. PALLI, D., G. MASALA, M. PELUSO, *et al.* 2004. The effect of diet on DNA bulky adducts levels are strongly modified by GSTM1 genotype: a study on 634 subjects. *Carcinogenesis* **25**: 1–8.
4. KATO, S., E.D. BOWMAN, A.M. HARRINGTON, *et al.* 1995. Human lung carcinogen-DNA adduct levels mediated by genetic polymorphisms in vivo. *J. Natl. Cancer Inst.* **87**: 902–907.
5. VAN POPPEL, G., N. DE VOGEL, P. VAN BALDEREN, *et al.* 1992. Increased cytogenetic damage in smokers deficient in glutathione S-transferase isozyme mu. *Carcinogenesis (Lond.)* **13**: 303–305.
6. AWASTHI, Y.C., R. SHARMA & S.S. SINGHAL. 1994. Human glutathione S-transferases: minireview. *Int. J. Biochem.* **26**: 295–308.
7. LANDI, S. 2000. Mammalian class theta GST and differential susceptibility to carcinogens: a review. *Mut. Res.* **463**: 247–283.
8. RESZKA, E. & W. WASOWICZ. 2001. Significance of genetic polymorphisms in glutathione S-transferase multigene family and lung cancer risk. *Int. J. Occup. Med. Environ. Health* **14**: 99–113.
9. ENGEL, L.S., E. TAIOLI, R. PFEIFFER, *et al.* 2002. Pooled analysis and meta-analysis of glutathione S-transferase M1 and bladder cancer: a HuGE review. *Am. J. Epidemiol.* **156**: 95–109.

10. COTTON, S.C., L. SHARP, J. LITTLE, *et al.* 2000. Glutathione S-transferase polymorphisms and colorectal cancer: a HuGE review. *Am. J. Epidemiol.* **151**: 7–32.
11. FRAGA, C.G., J. ONUKI, F. LUCESOLI, *et al.* 1994. 5-Aminolevulinic acid mediates the *in vivo* and *in vitro* formation of 8-hydroxy-2'-deoxyguanosine in DNA. *Carcinogenesis* **15**: 2241–2244.
12. WÜNSCH FILHO, V. & G.J.F. GATTÁS. 2001. Molecular biomarkers in cancer: implications for epidemiological research and public health. *Cadernos de Saúde Pública* **17**: 467–480.
13. MCCALL, M.R. & B. FREI. 1999. Can antioxidant vitamins materially reduce oxidative damage in humans? *Free Radic. Biol. Med.* **6**: 1034–1053.
14. ABE, J. & B.C. BERK. 1988. Reactive oxygen species as mediators of signal transduction in cardiovascular disease. *Trends Cardiovasc. Med.* **8**: 59–64.
15. RIMBACH, G., Y.C. PARK, Q. GUO, *et al.* 2000. Nitric oxide synthesis and TNF-alpha secretion in RAW 264.7 macrophages: mode of action of a fermented papaya preparation. *Life Sci.* **67**: 679–694.
16. RIMBACH, G., Q. GUO, T. AKIYAMA, *et al.* 2000. Ferric nitrilotriacetate induced DNA and protein damage: inhibitory effect of a fermented papaya preparation. *Anticancer Res.* **20**: 2907–2014.
17. COLOGNATO, R., I. FONTANA, F. COPPEDÉ, *et al.* Modulation of the hydrogen peroxide induced DNA damage and cell death in PC12 cells by papaya extract and ergothioneine. *Mut. Res.* Accepted for publication.
18. MAROTTA, F., R. BARRETO, H. TAJIRI, *et al.* 2004. The aging/precancerous gastric mucosa: a pilot nutraceutical trial. *Ann. N. Y. Acad. Sci.* **1019**: 195–199.
19. MORI, A., I. YOKOI, Y. NODA & L. J. WILLMORE. 2004. Natural antioxidants may prevent posttraumatic epilepsy; a proposal based on experimental animal studies. *Acta Med. Okayama* **3**: 111–118.