OXIDATIVE-INFLAMMATORY DAMAGE IN CIRRHOSIS. EFFECT OF VITAMIN E AND A FERMENTED PAPAYA PREPARATION

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Abbreviations: ROS: reactive oxygen species; 8-OHdG: 8-hydroxy-deoxy-guanidine; NSAIDs: non-steroidal anti-inflammatory drugs; FPP: fermented papaya preparation; EDTA: ethylenediaminetetra-acetate; GSH: reduced glutathione; GSH-Px: glutathione peroxidase; MDA: Malondialdehyde; GSSG: oxidised glutathione; NO: nitric oxide; HPLC: high performance liquid chromatography; PBS: phosphate bufered solution; TBA: Thiobarbituric acid; BHT: butylated hydroxytoluene; TNF-α: tumor necrosis factor-α.

Abstract

Oxidative DNA damage occurs as an early event in HCV infection and is an indication of the potential for carcinogenesis. The aim of this study was to test a novel antioxidant/immunomodulator in HCV-related cirrhotics. The study group consisted of 50 patients with HCV-related cirrhosis with transaminase values less than twofold increased (ALT<80 IU/l). Patients underwent a standardised food-vitamin composition assessment and were randomly allocated into 2 groups. At the beginning, they were assessed for dietary intake, nutritional status and iron level and then given alpha-tocopherol 900IU/day or given 9g/day of a fermented papaya preparation (FPP, Immun-Age®, Osato Research Institute, Gifu, Japan)(table 1) at bedtime for 6 months. Ten healthy subjects served as controls. Patients were checked monthly for: routine tests, Redox Status (GSH, GSH-Px, GSSG, malondialdehyde), plasma α-tocopherol, 8-OHdG level in circulating leukocyte DNA and serum levels of cytokines. Patients with cirrhosis showed a significant imbalance of redox status (low antioxidants/high oxidative stress markers) ($p<0.005$ vs controls). Both treatment regimens did not affect transaminases as a whole. However, vitamin E supplementation almost normalized ALT only in the limited vitamin-E-deficient subgroup. A significant improvement of redox status was obtained by both regimens. However, only FPP significantly decreased 8-OHdG and the improvement of cytokine balance with FPP was significantly better than with vitamin-E treatment ($p<0.05$). While the present data seem to suggest a potential supportive role of antioxidants/immunomodulators as FPP in HCV patients, more studies are needed to substantiate their effect on the natural history of the disease.

Introduction

The liver is one of the most susceptible organs to oxidative-related cellular damage and DNA mutagenesis and oxidative stress has been implicated as a causative factor in alcoholic and non alcoholic liver disease. In alcoholism there is an understandable link with ethanol metabolism due to the production of ROS such as superoxide and hydroxyl radicals. For non alcoholic liver disease, a complex interplay between malnutrition, trace elements abnormalities, glutathione depletion and several virus-related cellular injuries are indicated (1). A key factor causing oxidative DNA damage is formation of hydroxyl radicals which can alter purine and pyrimidine bases and react with deoxyribose damaging the phosphodiester DNA structure. Oxidative-modification of pyrimidine and/or purine bases occurs through addition of hydroxyl radicals to the π bonds of the bases, to the C5 and C6 of pyrimidines and to C4 and C8 of the purines. Stable oxidative damage products such as 8-OHdG are molecular markers of pathology (2). Oxidative DNA damage, namely 8-OHdG generation, has been indicated as an early event in HCV infection and a marker of liver damage in patients. Persistent genomic changes are factors giving rise to carcinogenesis, as has also been suggested in patients undergoing chronic hemodialysis (3). Therapeutic interventions with antioxidants aiming to curb 8-OHdG as a means to prevent hepatocarcinonegesis have recently been confirmed in an experimental liver cancer model where an herbal preperation, Sho-Saiko-to, appeared to be effective (4). This preparation is not amenable to widespread use given the severe side effects reported in clinical practice (5-7). Theoretically, however, a nutraceutical approach effective in maintaining redox balance and an immunomodulator without adverse effects would be desirable. Experimental studies using a non genetically-modified antioxidant/immuno-stimulating and NO-modulating fermented papaya preparation was described (8-10). Fermented papaya preparations were found to posses highly protective antioxidant properties despite being devoid of any antioxidant vitamin as such (11-14) (table 1). Such studies have been followed by clinical investigations (15-19). In particular, recent gastroenterology studies (19) have demonstrated that FPP was able to significantly decrease the oxidative stress in gastric mucosa affected by

longstanding chronic atrophic gastritis associated with metaplasia and importantly to curb the mucosal concentration of 8-OHdG. Moreover, it has been shown in patients with HCV-related chronic liver disease that high TNF-α levels are associated with the degree and progression of inflammation (20) while the concentration of the soluble TNF p75 receptor seems to be linked to mortality (21). The aim of the present investigation was to test supplementation with vitamin E or the fermented papaya preparation in a group of patients with established HCV-related liver cirrhosis.

Materials and Methods

The study group consisted of fifty patients (29 males/21 females, mean age 62, age range 54-75) with Child A-C, genotype 1 HCV-related cirrhosis without having a history of ethanol consumption for the past 10 years. All patients had abnormal ALT levels but less than 80IU/L. Patients were carefully interviewed with special attention to their dietary-vitamin and iron intake using a standardised food-composition table (22). Exclusion criteria were: hemocromatosis, Wilson's disease, alpha-1-antitrypsin deficiency, autoimmune diseases, alcohol consumption, hepatocellular carcinoma, recent variceal haemorrhage or scheduled endoscopic session of variceal banding, any other malignancy, chronic illness requiring steroids, immunosuppressive agents, allopurinol treatment, antiviral or NSAIDs, concomitant use of vitamin/food supplements, strenuous physical exercise, chronic renal failure, arterial hypertension, overt cardio-respiratory abnormality and high consumption of caffeine-containing food or beverages. Patients were randomly allocated into 2 groups (25 patients each), previously matched with dietary intake, serum iron concentration and iron dietary intake (median: 8.6mg/day, range: 6.9–10.4mg/day) and body mass index. At bedtime, they were supplemented (group A) with alpha-tocopherol 900IU/day for 6 months or (group B) 9g/day of a FPP (Immun-Age®, made under ISO 9001 (production quality) and ISO 14001 (environmental protection) regulations from biofermentation of carica papaya, pennisetum purpureum and sechium edule, Osato Research Institute, Gifu, Japan). Ten healthy subjects who

were not taking any food or vitamin supplements and matched for age and gender with patients' population were used as the control group. After an overnight fast, venous blood samples were taken. A dry tube for serum, a citrate-containing tube and an EDTA-containing tube for plasma were used for clinical chemistry analyses. On the examination day, blood samples were taken for routine testing by automated standardized procedures (Hitachi 911 using commercial kits) and for further studies as described below.

Assessment of Redox Status.

For assessing the variation of the blood redox antioxidant status, plasma was prepared from heparinized whole blood (10ml) by centrifugation at 2000 X g for 15min at 4°C. The following parameters were measured: GSH, GSH-Px and GSSG as described (23) using haemoglobin catalysed oxidation of 10-N-methylcarmoyl-3,7-dimethylamino-10-H-phenothiazione after treatment with phospholipase D. Accordingly, cumene hydroperoxide was used as a standard. Plasma content of α-tocopherol was measured by HPLC analysis. Briefly, (1ml) aliquots of plasma were mixed with 1ml of l00mM sodium dodecylphosphate solution in water, 2ml of absolute ethanol and 1ml of n-heptane and shaken for one minute. After 15 minutes extraction in the dark, the heptane phase was separated by centrifugation, and 50 µl aliquots were used for HPLC assay. Values were read by a fluorescence detector set at 296 nm excitation and 325 nm emission.

Determination of plasma malondialdehyde

Malondialdehyde was measured in frozen, EDTA-containing plasma samples after reaction with TBA using a slight modification of the HPLC method described by Rabl et al (24). Before assay, plasma samples were thawed and 100 mL aliquot was immediately mixed with 100 mL water, 300 mL of 0.15 mol phosphoric acid/L, 10 mL BHT 0.2% methanolic solution), and 100 mL 0.6% TBA and incubated for 60 min at 95°C. The chromogen was extracted with 1.25 mL butanol-1 and analyzed by HPLC with fluorometric detection (emission wavelength: 550 nm; excitation

wavelength: 525 nm;). The malondialdehyde-TBA adduct was calibrated with tetramethoxypropane standard solutions and processed like the plasma samples.

Analysis of 8-OHdG in circulating leukocyte DNA.

Leukocytes were isolated after centrifugation over 100µl Histopaque 1077 at 200 X g for 3 min at 4°C, washed once in PBS (pH7.4) and centrifuged again. Then the leukocyte layer was collected and tested for 8-OHdG by HPLC as follows. DNA was isolated and purified from leukocytes as described (25) with minor modifications. Briefly, after homogenization in 1 ml of ice-cold buffer (0.1 M NaCl, 10 mM EDTA, 10 mM 2-mercaptoethanol, and 0.5% Triton X-100, pH 8.0) and centrifugation for 10 min at 1000*g* at 4°C. The samples were then resuspended in 7.5 ml of lysis solution (120 mM NaCl, 10 mM Tris, 1 mM EDTA, and 0.5% SDS, pH 8.0) with 20% BHT. RNA and protein were digested by incubation with RNase or proteinase K for 30 or 60 min at 55°C, respectively. After extraction, DNA was precipitated by the addition of 5 volumes of ethanol (-20°C). The isolated DNA (100–400 µg) was dissolved in 200 µl of 20 mM sodium acetate (pH 5.0), denatured by heating for 5 to 10 min at 95°C, and cooled on ice. The DNA samples were digested to nucleotides by incubating with 12 units of nuclease P_1 for 30 min at 37°C. Next, after adding 20 µl of 1 M Tris-HCl (pH 8.0) and 4 units of alkaline phosphatase, the samples were incubated for 1 hr at 37°C. The resulting deoxynucleoside mixture was filtered through a Millipore filter (0.22 µm) and analyzed using a HPLC-electrochemical detection (ECD) system equipped with a CoulArray 5600 EC detector. The amount of 8OHdG was compared with the quantity of deoxyguanosine detected, in the same sample, by UV absorbance at 260nm. 8OHdG and 2'-deoxyguanosine were measured using different channels and oxidation potentials of 300 and 900 mV, respectively. The results are expressed as the ratio of the absorbance peak of 8-oxo-deoxyguanosine adducts to that of 2'-deoxyguanosine adducts x 10^5.

Circulating Levels of Cytokines and Cytokine Receptors Serum was collected after centrifugation at 4 °C and samples were stored in aliquots at -20°C before use. Levels of TNF-α and sTNF-Rp75 were measured using commercial sandwich-type enzyme-linked immunosorbent assays kits following the procedure recommended by the manufacturer (Biosource Technologies, Inc., Europe, Nivelles, Belgium and R&D Systems, Minneapolis, MN, USA). Briefly, two diluted serum samples were added to plates coated with antibody and incubated for 2 hours at 37 °C. Afterward, each well was washed five times with washing buffer, then peroxidase-labeled secondary antibody was added to each well and the plate was incubated 1 hour at 37 °C. Then each well was washed in a similar manner and the plate was incubated with tetramethylbenzine for 20 minutes at room temperature. The reaction was stopped by adding 1 N sulfuric acid. Absorbance was measured at 450 nm using a spectrophotometric analyzer. Sample concentration was derived from a standard curve. The following sensitivity limits were achieved in standard curves: TNF-α, 19.86 pg/ml; sTNF-Rp75, 8.5 pg/ml.

Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was performed with the non-parametric Mann-Whitney U-test using the Statistics software package. Statistical significance was set at $p<0.05$. Spearman's correlation analysis was used to examine the correlations between data sets.

Results

Two patients (one from each group) were removed from the study since one underwent flu with a supervening acute bronchitis requiring paracetamol and antibiotic prescription and the other missed FPP supplementation for 1 week, while on holiday and being in good health condition. Altogether, 48 fully complying patients completed the observational study.

No significant weight change was observed. As a whole, routine blood tests were not affected by any of the supplement treatments (data not shown). GSSG serum level in patients with cirrhosis was comparable to healthy subjects (table 2) and remained unchanged by supplementation. However, as compared to healthy controls, reduced GSH and glutathione peroxidase was significantly lower in cirrhotic patients ($p<0.05$) and were comparably improved by either FPP or Vitamin E regimens ($p<0.05$). In patients with cirrhosis, serum MDA levels were significantly higher ($p<0.01$ vs healthy control) and supplementation brought about a comparable partial improvement ($p<0.05$ vs baseline values). Four patients (16.6%) of group A and 5 patients (20.8%) of group B showed a deficiency of vitamin E level (serum levels below 12.5μmol/L as a 5% percentile of healthy controls) and this was reverted to within normal concentration by α-tocopherol supplementation but not by FPP supplementation (data not shown). Three of the four vitamin E-deficient patients who were supplemented with α-tocopherol totally normalized their transaminases level (data not shown). As compared to controls, patients with liver cirrhosis showed significantly higher accumulation of 8OHdG in circulating leukocytes ($p<0.01$, fig. 1). This impairment remained unchanged by α-tocopherol supplementation while it was partially and significantly improved in the FPP-supplemented group ($p<0.05$). Leukocyte DNA damage showed a correlation only with the age of patients, as an independent variable (table 3). Furthermore, patients with liver cirrhosis showed an elevated serum level of TNF-α and of its soluble p75 receptor ($p<0.001$ vs healthy controls, fig. 2), the latter being significantly related to Child-Pugh score (r: +0.67, $p<0.05$, data not shown) among the spectrum of biochemical and clinical parameters tested. While vitamin E supplementation did not affect this abnormality, FPP supplementation significantly lowered their values ($p<0.05$).

Discussion