REDOX BALANCE SIGNALLING IN OCCUPATIONAL STRESS: MODIFICATION BY NUTRACEUTICAL INTERVENTION

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There is increasing evidence that psychosocial stress can be viewed as a system-wide derangement of cellular homeostasis, with heightened oxidative stress and triggered proinflammatory mechanisms. The aim of this study is twofold: a) to replicate findings that psychological stress increases oxidative damage and b) to determine whether a fermented papaya preparation known to exert significant protective antioxidant properties could buffer such increases in nuclear DNA damage while also inducing epigenetic protective mechanisms. Twenty-eight sedentary men and women (age range: 28-52), who reported living a stressful lifestyle but with an overall positive attitude, were recruited for this study. Chronic diseases as well as severe burnout and use of drugs for anxiety constituted exclusion criteria. Subjects were supplemented for 1 month with 9g/day (4.5g twice a day) of a certified fermented papaya preparation. All subjects were given a stress and sleep quality questionnaire together with a diet and lifestyle assessment. Blood was collected at 2 and 4 week, erythrocyte and leukocyte were separated to assess redox balance and heme oxygenase-1 (HO-1) gene expression while bilirubin oxidized metabolites (BOMs) were tested in the urine. Stressed individuals showed a significant abnormality of redox status with increased MDA of erythrocyte and increased level of 8-0HdG in leukocyte and BOMs excretion (p<0.05). Nutraceutical supplementation brought about a normalization of such values already at the 2 week observation (p<0.05) together with a significant upregulation of HO-1 (p<0.01). Taken together, the results of this study confirm that stressful occupational life per se, without any overt psychiatric illness, may be associated to increased oxidative stress. Supplementation with functional food affecting redox regulation may be part of the therapeutic armamentarium to be considered in this clinical setting.

Stress as a concept describes the effects of psychosocial, occupational work and environmental factors on physical or mental well-being as an adaptive response of the organism for a threatening situation. Several studies have shown that mental stress increases oxidative stress as a major damaging factor of cellular homeostasis of aerobic organisms (1). Free radicals are extremely unstable and reactive chemical species, which have been shown in humans to react with proteins, lipids, carbohydrates and

Key words: occupational stress, redox balance, heme-oxygenase, antioxidant, bilirubin oxidized metabolites
nucleic acid (2), as well as leukocytes (3).

The long-term effects of occupational stress and the higher incidences of burnout in high-demand individuals, such as night-shift workers, flight crew and emergency medical staff have been the subjects of several research works (4-5). The first evidence that psychological stress may induce damage to nuclear DNA was shown in the liver cells of rats exposed to a conditioned emotional stimulus (6). Rats subjected to such stimuli exhibited increased 8OHdG in nuclear DNA of the liver after repeated exposure, with a return to baseline levels one hour after stopping the acute stressor. Accordingly, human studies have identified a link between perceived stressful student examinations and increased DNA damage, while no associations were found during low stress periods (7). The observed increases in repair were also used to infer an increase in DNA damage occurring during psychological stress (8). Stress-related changes have been demonstrated by genomic tools in healthy subjects, including up-regulation of a number of cytokines/chemokines and their receptors, but also in individuals reporting post-traumatic stress disorder, including inflammation, apoptosis and stress response (9). Recently, Mancuso (10) highlighted the potential role of heme oxygenase and its products to act as a protective modulator of stress at the endocrine level besides the redox level. Heme oxygenase includes the inducible (HO-1) and the constitutive (HO-2) isoenzymes. Both isoenzymes cleave the tetrapyrrollic ring of cellular heme moieties releasing carbon monoxide and equimolar amounts of free iron and biliverdin (BV). BV is in turn converted into bilirubin by the cytosolic enzyme BV reductase, and together these are molecules with antioxidant, antiapoptotic and immunomodulatory properties (11) (Fig. 1). Bilirubin scavenges nitric oxide (NO) radicals directly and quenches excess oxidants, and the reaction of bilirubin with ROS results in the production of several species of bilirubin oxidative metabolites (BOMs) thus the level of biopyrins reflects the status of NO radicals and ROS in tissues (12). Furthermore, it has also indicated that the concentration of bilirubin and BOMs increases in the cerebrospinal fluid of patients with Alzheimer’s disease (13), supporting the hypothesis that ROS might be involved in the onset of the disease (14).

A number of studies have demonstrated that
a fermented papaya preparation is able to exert significant protective antioxidant properties despite being devoid of any antioxidant vitamin as such (15-17). The aim of this study is to replicate findings that psychological stress increases oxidative damage using different measures of damage and to determine whether the above-mentioned nutraceutical supplementation could buffer such increase in nuclear DNA damage whilst also inducing protective gene expression mechanisms.

MATERIALS AND METHODS

A total of twenty-eight Caucasian men and women between the ages of 28 and 52 were recruited for this study. Participants were eligible for the study if they reported being sedentary. A sedentary adult was defined as one who has not engaged in an exercise regimen exceeding 20 minutes per day, 3 days per week over the previous six months (18). Participants were excluded if they currently smoked or were taking medication that would influence cardiovascular or immune measures, including excessive alcohol (>10 drinks/week) and illegal drugs. Participants with a medical condition, musculoskeletal injuries or psychiatric diagnosis that might affect activity or immune system function were also excluded. This information was collected by self-report and included incidence of cancer, asthma, arthritis, diabetes, heart disease, autoimmune disorders, hypertension, or major depression/anxiety. Subjects suffering at present or in the past from severe burnout (high score for emotional exhaustion or depersonalization) as assessed by a slightly modified Maslach Burnout Inventory (19) were also excluded. Patients were selected who had an overall positive attitude towards their work and personal life, as assessed by Padriini's psychomotional scale, a set of 92 questions aimed to define 5 separate profiles blended by a different extent of overlap between each profile (20). Participants who reported being overweight (BMI>27) or engaged in mind-body relaxation practice were also ineligible to participate. Occupational factors such as qualifications and assigned task, professional record, professional category, working conditions, relationships with other members of the staff, amount of work were recorded (Table 1).

Subjects were supplemented for 1 month with 9g/day (4.5g twice a day) of a certified fermented papaya preparation (ImmunoAge®, Osato Research Institute, Gifu, Japan) made under ISO 9001 (production quality) and ISO 14001 (environmental protection) from a patented biofermentation process of non-GMO carica papaya. During the initial visit the study was explained, compliance with the study reinforced and informed consent obtained prior to participation. A group of age- and gender-matched healthy controls without any reported perceived stress as also assessed by the following anxiety scale (STAI score: 1.4±0.3, see explanation below), served as control for biochemical studies.

Stress questionnaire

Psychological stress was measured by the State Trait Anxiety Inventory (STAI), which is widely used for assessing state or acute anxiety (21). STAI was completed by all participants at the entry and at the completion of the study. The STAI asks the subject to describe how he feels 'right now' by responding to 20 questions with a 4-point response format from 'not at all' (score 1) to 'extremely' (score 4) anxious. The answers are in the form of quadruplicate Likert scale. Total scores ranged from 20 to 80, higher scores indicating greater anxiety. Total scores range from 20 to 80, with higher scores indicating greater anxiety. This measure has been shown to have high reliability and high construct validity.

Sleep Quality

The Pittsburgh Sleep Quality Index (PSQI), which is reported to have high internal validity and an overall reliability coefficient, was used to examine sleep duration, sleep quality, and napping behaviour over the past month, during and after the study period (22).

Diet and lifestyle questionnaire

A detailed lifestyle questionnaire was given to all subject with particular care to stress factor and physical activity. Patients were instructed to refrain from strenuous physical exercise, excess consumption of caffeine-containing beverages. 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 along the study period. The NIH DHQ is a food frequency questionnaire (FFQ) consisting of 124 food items that includes both portion sizes and dietary supplement questions. Patients were advised not to use any multivitamin supplement or fortified food while maintaining their usual diet.

Blood collection and storage

Following completion of the questionnaires, a venous cannula was inserted into the antecubital vein of the nondominant arm and remained in place for fifteen minutes before blood was taken. During this time participants were left to relax sitting position on a comfortable chair in quiet room (24°C, 40% moisture). A total of three 15 ml blood samples were taken from each participant. Samples were placed immediately in EDTA tubes to avoid coagulation. Blood samples were taken at entry, at 2 weeks and at 4 weeks.

Peripheral mononuclear blood cell (PBMC) separation

PBMCs were isolated by centrifugation over Ficoll-Hypaque (ICN, Aurora, OH) in Vacutainer Cell Preparation Tubes (CPT; Vacutainer Systems, Plymouth, UK) according to the manufacturer's instructions and frozen at −80°C in a mixture containing 50% fetal bovine serum (FBS; GibcoRBL, Renfrewshire, UK), 40% culture medium (RPMI 1640; GibcoRBL), and 10% dimethylsulfoxide (DMSO). Part of PBMCs were further fractionated into CD14+ and CD14− cells by magnetic-activated cell sorting using human CD14 MicroBeads (Miltenyi Biotec). Flow cytometric analysis showed that CD14+ cells were >95% pure, while the negatively selected population contained <1% CD14+ cells.

Determination of 8-OH-dG Levels in Leukocytes

The amount of 8-OH-dG was measured by high performance
Table I. Demographic and social characteristics of subjects recruited.

<table>
<thead>
<tr>
<th>Male/female</th>
<th>26/13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (age range)</td>
<td>39 (28-55)</td>
</tr>
<tr>
<td>Occupation</td>
<td></td>
</tr>
<tr>
<td>Call center operators</td>
<td>9</td>
</tr>
<tr>
<td>Executives (CEOs)</td>
<td>6</td>
</tr>
<tr>
<td>Financial promoters</td>
<td>7</td>
</tr>
<tr>
<td>Freelance journalist</td>
<td>8</td>
</tr>
<tr>
<td>Real estate agent</td>
<td>9</td>
</tr>
<tr>
<td>BMI mean(range)</td>
<td>22.5 (20-26)</td>
</tr>
<tr>
<td>Marital status</td>
<td></td>
</tr>
<tr>
<td>married</td>
<td>14</td>
</tr>
<tr>
<td>single (with partner)</td>
<td>19 (11)</td>
</tr>
<tr>
<td>divorced (with partner)</td>
<td>6 (4)</td>
</tr>
<tr>
<td>STAI</td>
<td>3.1 ± 0.7</td>
</tr>
<tr>
<td>PSQI</td>
<td>8.7 ± 4.3</td>
</tr>
</tbody>
</table>

Fig. 2. 8-OhdG level in leukocytes in stressed subject: effect of FPP. Start, baseline value in stressed patients; FPP 2wk and 4wk: values at 2- and 4-week observation during supplementation with fermented papaya preparation; control: age/gender-matched non-stressed healthy individuals. § p<0.01 vs control subjects; * p<0.05 vs baseline values.

Liquid chromatography (Beckman; Ultrasphere-ODS) coupled to an electrochemical detector (ESA Coulochem II; guard cell; 0.35 V, detector 1, 0.15 V, detector 2, 0.30 V). Briefly, the nuclear DNA from peripheral leukocytes was extracted and samples were digested with nuclease P1 and acid phosphatase in a solution of 1 mM EDTA and 10 mM sodium acetate (pH 4.5). After incubation at 37°C for 30 min, iron exchange resin was added to remove the NaI, and the mixture was centrifuged at 15,000 rpm for 5 min. The supernatant was transferred to an Ultrafree filter (Millipore, USA), and was centrifuged at 10,000 rpm for 2 min. The filtered deoxynucleoside was injected onto the HPLC column and aliquots of 20 μl of deoxyguanosine (dG, 0.5 mg/ml) and 8-OH-dG (5 ng/ml) solutions were used as standard samples. The amount of 8-OH-dG was expressed as the number of residues per 10^9 dG.

Assessment of erythrocyte concentration of antioxidative enzyme activity, glutathione peroxidase and malondialdehyde

Red blood cell (RBC) samples were also separated from whole blood, using EDTA as an anticoagulant immediately after blood sampling, followed by centrifugation at 1,000 g for 15 min. The supernatant was separated as plasma and the red cells were then washed three times in 10 vol of 0.9% (w/v) NaCl. These samples were stored at -80°C until assayed. To remove the hemoglobin by precipitation with 0.4 mL of an ethanol chloroform (3:5, v/v) mixture was added to a 1 mL aliquot of the hemolysate cooled in ice. This mixture was stirred constantly for 15 min and then diluted with 0.2 mL of distilled water. After centrifugation for 10 min at 1,600xg, the pale yellow supernatant was separated from the protein precipitate and used to assay the superoxide dismutase (SOD). The hemolysate was diluted 20 times and used as the raw material for the SOD activity measurement. The glutathione peroxidase (GSSGpx) activity was measured by using a reaction mixture containing 1 mmol/l glutathione reductase in a 0.1 mol/l Tris-HCl (pH 7.2) buffer. The reaction was initiated by adding 2.5 mmol/l H_2O_2 and the absorbance was measured at 340 nm for 1 min. A molar extinction coefficient of 6.22 mM/cm was used to determine the activity. The activity was expressed as μmol NADPH/min/g Hb. For erythrocyte reduced glutathione (GSH) assessment, the concentration of nonprotein sulphydryl (NPSH) groups of erythrocytes, which is virtually all GSH, was measured using 5,5’-dithio-bis(2-nitrobenzoic acid) (DTNB). 0.2 mL of a cell suspension (containing 2 mM Hb) was mixed with 2 mL of distilled water, and 0.2 mL of this lysate was used to determine the Hb concentration. Three milliliters of precipitating solution (1.67 g of glacial metaphosphoric acid, 0.2 g of disodium ethylenediaminetetraacetic acid (EDTA) and 30 g of NaCl in 100 ml of distilled water) were added to 2 ml of hemolysate. After
Table II. Erythrocyte concentration of redox status and MDA: effect of supplementation.

<table>
<thead>
<tr>
<th></th>
<th>SOD</th>
<th>GPX</th>
<th>MDA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U/g Hb</td>
<td>µmol NADPH/min/g Hb</td>
<td>µmol/g Hb</td>
</tr>
<tr>
<td>Healthy control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>start</td>
<td>122.6±21.2</td>
<td>40.4±20.1</td>
<td>0.33±0.15</td>
</tr>
<tr>
<td>2 weeks</td>
<td>119.8±25.2</td>
<td>38.8±9.9</td>
<td>0.38±0.08</td>
</tr>
<tr>
<td>4 weeks</td>
<td>127.3±24.3</td>
<td>34.9±12.4</td>
<td>0.27±0.10</td>
</tr>
<tr>
<td>Stressed subjects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>with FPP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>start</td>
<td>166.9±13.7§</td>
<td>25.5±16.4§</td>
<td>0.67±0.23§</td>
</tr>
<tr>
<td>2 weeks</td>
<td>159.7±11.7§</td>
<td>22.9±16.6§</td>
<td>0.39±0.17*</td>
</tr>
<tr>
<td>4 weeks</td>
<td>129.9±16.9*</td>
<td>44.7±18.5*</td>
<td>0.32±0.14*</td>
</tr>
</tbody>
</table>

Start, baseline value in stressed patients; FPP 2wk and 4wk: values at 2- and 4-week observation during supplementation with fermented papaya preparation; control: age/gender-matched non-stressed healthy individuals. § p<0.01 vs control subjects; * p<0.05 vs baseline values.

standing for 5 min, the mixture was centrifuged and NPSH was assayed in the clear supernatant. The reaction cuvette contained 0.2 M Na2HPO4, pH 8.0, 0.5mM DTNB (2 mg in 10 mL of 1%, w/v, sodium citrate solution) and the sample, in a final volume of 2 mL. The absorbance was read at 412 nm against a blank containing 2 mL of 0.2 M Na2HPO4. The concentration of NPSH was expressed as the number of SH per Hb tetramer. A ε 412 of 13.6 m/M/cm was used to calculate the NPSH concentration. Malondialdehyde determination (MDA) levels were measured in erythrocytes. Blood was centrifuged at 2500 g for 10 min and the plasma was then removed as completely as possible. The concentration of MDA was calculated from the area, on the basis of a calibration chromatogram performed with a standard solution of MDA, which was prepared by acid hydrolysis.

HO-1 analysis by reverse-transcription–polymerase chain reaction (RT-PCR) and real-time PCR

Total RNA was isolated from cells with Trizol reagent (Invitrogen, Carlsbad, CA). One microgram of total RNA served as template for single strand cDNA synthesis in a reaction using oligo(dT) primers and SuperScript II (Invitrogen). Quantification of mRNA expression was performed in triplicate by using the Green SuperMix (BioRad, California, USA) and a 2-step PCR reaction procedure, performed on the MyiQ Single Color Real-Time PCR Detection System (BioRad, California, USA). For the PCR, 1 µL of cDNA was incubated with 9.375 µL of deionized distilled water, 2 µL of dNTP, 2.5 µL of 10 X PCR buffer, and 0.125 µL of Taq polymerase and a primer pair for HO-1 (sense 5'-CAGGCAGAGAAGTGCTGAG-3' and antisense 5'-GCTTCACATAGCGGCTGCA-3'), C14 (sense 5'-CGGCGGAGAGATCAGCAAGT-3' and antisense 5'-AGTGCAGTCCCTGGGCTTC-3') and GAPDH (sense 5'-ACAGTCAGGCGCATC-3' and antisense 5'-AGGTGCGGCTCCCTGA3'). After the initial denaturation at 95°C for 3 minutes cycling conditions included 27 cycles of amplification for 30 seconds at 94°C, 30 seconds at 55°C, 1 minute at 72°C, and a final extension phase consisting of 1 cycle of 10 minutes at 72°C. PCR products were run on a 1.5% agarose gel stained with ethidium bromide. Fluorescent emission data were captured, and mRNA levels were quantified using the threshold cycle value. Each realtime PCR assay was repeated twice.

Urinary excretion of bilirubin oxidized metabolites (BOM)

Urine samples taken from 24 h collection were stored at -80°C until assayed. Urinary BOMs were measured in duplicate using ELISA that employs in-house 24G7 anti-bilirubin monoclonal antibody, and the results were corrected for the urinary concentration of creatinine.

Statistics

Group data were distributed non-parametrically and comparisons between groups were made using the Kruskal–Wallis test for multiple group comparisons followed by the Mann–Whitney U test for comparisons between two groups. Correlations were calculated using Spearman’s rank test. p<0.05 was considered statistically significant.

RESULTS

All patients completed the study with thorough compliance with the dietary, lifestyle, and treatment requirements. No significant change was observed and, as a whole, routine blood tests were not affected by FPP supplementation (data not
shown). Anxiety score and sleep quality were not affected by the treatment (data not shown).

**Assessment of 8-OH-dG Levels in Leukocytes in stressed subject when supplemented with FPP**

As compared to controls, stress subjects showed a statistically significant increased level of 8-0HdG (p<0.05) (Fig. 2) while nutraceutical supplementation with FPP brought about a steady state significant decrease within normality (p<0.05). At baseline the level of 8-0HdG was significantly correlated to MDA.

**Assessment of erythrocyte concentration of redox status and MDA**

Compared to unstressed individuals, stressed subjects showed a significant increased level of erythrocyte SOD and MDA and decreased GPX (p<0.05, Table II). FPP supplementation showed a significant decrease of MDA at the second week, while either SOD or GPX abnormalities remained unchanged. However, at the 4-week observation, all parameter returned to normal level under supplementation with the nutraceutical (p<0.01 vs baseline values).

**HO-1 gene expression in PBMC**

When tested against GAPDH and CD14, HO1 gene expression in leukocytes appeared to be significantly up-regulated (p<0.01, Fig. 3). This effect was already clear at the second week observation and remained unchanged two weeks afterwards.

**Urinary excretion of bilirubin oxidized metabolites (BOM)**

Compared to unstressed controls, urinary excretion of BOMs in stressed individuals showed a significant over two-fold increase (p<0.01) (Fig. 4). Under supplementation with functional food FPP, such values were normalized (p<0.05). There was a significant correlation between BOM values and respective HO-1 level (r: 0.78, p<0.01) and MDA (r: 0.64, p<0.5) but no significant correlation appeared with other variables tested.

**DISCUSSION**

There is increasing evidence that psychosocial stress can be viewed as a system-wide derangement of cellular homeostasis, with heightened oxidative stress and triggered proinflammatory mechanisms (23). In a recent report, Cernak et al. (24) demonstrated that chronic psychological stress may lead to an increase of plasma superoxide and malondialdehyde as final products of lipid peroxides.

![HO-1/GAPDH mRNA (AU)](image1)

![HO-1/CD14 mRNA (AU)](image2)

**Fig. 3.** 0: baseline value in stressed patients; FPP 2wk and 4wk: values at 2- and 4-week observation during supplementation with fermented papaya preparation.
decreased SOD level can be found under several conditions of oxidative stress, a most recent work dealing with space-flight training found oxidatively-modified DNA markers concomitantly with elevated concentrations at erythrocyte SOD (32). Indeed, in our study, compared to expected values in healthy subjects, stressed individuals showed an increased level of erythrocyte SOD and this may show an upregulation of antioxidant adaptive response to heightened oxidative stress. This event was paralleled not only with an increase of erithrocyte MDA but also by a concurrent decreased GPX content and oxidative DNA damage as expressed by leukocyte 8OHdG. FPP supplementation showed to normalize red blood cell lipoperoxidation already after two weeks of supplementation and the whole parameters within the fourth week with SOD level comparable to non-stressed subjects, thus suggesting a re-modulation of redox system. We did not address lipid metabolism changes, but these may be worth testing in future studies. Bilirubin synthesis is regulated by the rate-limiting enzyme, HO-1, which is rapidly induced by oxidative stress and inflammatory reactions (33). This appeared also in our subjects in whom it was not surprising to note a significant correlation between BOMs and HO-1 over-expression. When we administered the antioxidant supplementation, we noted a very rapid upregulation of HO-1 which was maintained throughout the 4-week study. This data is of interest when considering that the HO-1 protein contributes to the maintenance of homeostasis (13), and HO-1-deficient mammalian cells are more vulnerable to inflammatory stimuli and oxidative stresses (34). Taken together the results of this study confirm that stressful occupational life per se, without any overt psychiatric illness and in otherwise healthy subjects leading a sedentary life, may be associated with increased oxidative stress. We suggest that FPP supplementation might be potentially associated with epigenetic changes involved in cellular metabolism, oxidative phosphorylation/generation of reactive oxygen species and response to oxidative stress. These changes may serve to ameliorate the negative impact of stress as a part of the therapeutic armamentarium for this clinical setting, although longer studies are awaited to check the possible consequences in the long run. Finally, in a recent

This is in agreement with our findings where stressed but otherwise healthy individuals showed a baseline increased level of both markers, when tested in red blood cells. Stress-associated changes in peripheral blood leukocyte expression of single genes have been already partly identified (25). In this regard, the pro-inflammatory transcription factor NF-kappa B which is activated by psychosocial stress has been identified as a potential link between stress and oxidative cellular activation (26). More recently, shortened telomeres, depressed telomerase activity, decreased anti-oxidant capacity have been found associated with substantial psychosocial stress (27) and higher vulnerability to a number of organic diseases (28). Bilirubin acts as an intrinsic antioxidant that quenches radicals (see Fig. 1), generating several hydrophilic metabolites called biopurrs which are immediately excreted in urine because of their hydrophilic properties, thus reflecting the severity of oxidative stress (29-30). Accordingly, in our study, it appeared that BOMs excretion was significantly elevated in stressed subjects, as reported in patients with overt psychological distress or psychiatric illness (12), and, most recently, in stressed post-menopausal women (31), and this value highly correlated to MDA level or erythrocyte. Although
study conducted by Flint et al. (10), stress hormones such as epinephrine, norepinephrine and cortisol were shown to induce DNA damage in murine 3T3 cells. This aspect was not addressed by our study and may represent a further area to be investigated together with a larger population aiming to search for any gender-related difference.

Conflict of interest. There was no conflict of interest. The study was spontaneously generated by the authors. Osato Research Institute, Gifu, Japan kindly donated the required samples for the trial as an unbiased support.

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