IS THERE A POTENTIAL APPLICATION OF A FERMENTED NUTRACEUTICAL IN ACUTE RESPIRATORY ILLNESSES? AN *IN-VIVO* PLACEBO-CONTROLLED, CROSS-OVER CLINICAL STUDY IN DIFFERENT AGE GROUPS OF HEALTHY SUBJECTS

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The role of oxidants in viral diseases is fairly complex because it includes metabolic regulation both of host metabolism and viral replication. However, a role for reactive oxygen species (ROS) and reactive nitrogen species (RNS) as mediators of virus-induced lung damage is supported by studies and antioxidants can thus be expected to act at many different levels. The aim of the present pilot study was to test an antioxidant nutraceutical approach on some relevant immunological parameters known to be affected in common seasonal respiratory tract infection. The study population consisted of 90 sedentary healthy patients, previously selected as being GSTM1-positive, divided into three groups: A) 20-40 years; B) 41-65 years; B) over 65 years. Each patients was administered a life style and dietary questionnaire. Subjects were supplemented for 6 weeks with either 9g/day (4.5g twice a day sublingually) of a fermented papaya preparation (Osato Research Institute, Gifu, Japan) or placebo. After a further month period of wash out, subjects were treated again in a crossover manner. Parameters checked were as follows: routine blood tests with WBC formula, saliva flow rate and secretary IgA and lysozyme production and redox gene expression of Phase II enzyme and SOD from upper airways cells (from nasal lavage). Salivary secretion rate showed an age-related decline and was significantly increased by FPP supplementation only in the youngest age-group ($p<0.05$). Subjects treated with FPP showed a significantly higher lever of IgA and lisozyme production., irrespective of age group while their baseline production was significantly lower in the oldest age-group as compared to the youngest one (C vs A, $p<0.05$). FPP treatment brought about a significant upregulation of all phase II enzyme and SOD gene expression tested in nasal lavage cells. In conclusion, FPP supplementation during 1 month resulted in higher salivary IgA and increase in phase II and SOD enzyme expression, i.e the most important antioxidant in the respiratory tract. The biological significance of these effects i.e., whether it will help reducing the whole respiratory oxidative stress in the human airway and, hopefully, the incidence and/or severity of URTI remains to be demonstrated in longer clinical trials.

Influenza and flu-like syndromes represent a ubiquitous worldwide health issue causing

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remarkable morbidity and, in some cases, mortality, particularly when affecting infants, elderly people, and frail persons with respiratory and chronic cardiovascular diseases (1). Vaccines can provide only partial benefit in the control of influenza epidemics due also to the constant and abrupt change of the antigenic surface proteins of the virus (2) and thus the available options for the prevention and treatment of this condition have still considerable limitations. On the other hand, some antiviral molecules are not active against influenza B viruses, while their clinical utility is limited by significant adverse side effects and the rapid emergence of resistant strains in the clinical setting (3). Moreover, the usefulness of vaccines in the long-term facilities has been reported to be modest (4). To date, there are only scanty reports in the literature on the efficacy of different therapeutic approaches such as antioxidants during influenza virus infection. Oda et al. (5) documented that administration of pyran polymer conjugated superoxide dismutase protected mice against potentially lethal doses of influenza virus. Indeed, while the role of a proper antioxidant-rich dietary recommendation remains undisputed (6). old home remedies have been found to have a therapeutic rationale *in vitro* (7). Indeed, the role that extracellular ROS and RNS play in the pathogenesis of influenza-induced acute lung injury is not fully unfolded as yet. As a matter of fact, lungs have several potential cellular sources of ROS (8) and an upregulation of xanthine oxidase may contribute to enhanced oxidative stress after viral disease. Indeed, elevated levels of xanthine oxidase, together with increased GSSG and malonildealdehyde have been demonstrated in mouse lung homogenates and BALF after influenza infection (9). One of the crucial defences against oxidative stress is represented by the redox cycling of glutathione. Hennet et al. (10) have shown that murine influenza infection brings about a decrease in total glutathione and in vitamins C and E. This is likely to be due to the development of GSH adducts and S-nitrosylation, as shown by the elevated NO-reactive molecules and nitrotyrosine formation during infection.

The recognized importance of ROS in respiratory inflammation has generated recent interest in therapeutic measures to prevent or reduce ROS-related phenomena. As current standard treatment may not specifically address this issue, additional therapies protecting against ROS may represent a significant step in reducing the morbidity of respiratory disease. These findings have been paralleled by a number of studies tackling the issue of natural approaches to treat acute respiratory seasonal syndromes (11-13). Although the matter is still debated due to the sample limitation, safety issues and great dispersion of data (14), there is a general call to approach the issue under a wider therapeutic perspective (15). A number of studies have demonstrated that a fermented papaya preparations is able to exert a significant protective antioxidant properties despite being devoid of any antioxidant vitamin as such (16, 17). The aim of the present pilot study was to test this compound on some key point immunological parameters known to be affected in common seasonal respiratory tract infection so to provide a scientific rationale for a longer observational study in clinics. In particular, our current investigation intended to explore a strategy to enhance a relevant endogenous cytoprotective response planned to prevent cellular damage from ROS.

MATERIALS AND METHODS

GSTM-1 genotype analysis

PCR analysis was performed in 25 μL reaction buffer containing 0.5mmol/L of dNTPs, 2.0mmol/L of $MgCl_2$, 12.5 pmol of each primer, about 150 ngDNA, and 1.25U of thermostable Taq DNA polymerase, using a programmable thermocycler. The primers used for GSTM1 were 5'-GAACTCCCTGAAAAGCTAAGC and 5'-GTTGGGGCTCAAATATACGGT GG. 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. GSTM-1 genotype frequency was consistent with the expectations for the Hardy–Weinberg equilibrium (data not shown). The genotype of DNA samples was identified blindly and controls were equipped and set up in association with every single PCR operation as blank control (without DNA template), positive control, and negative control. Subjects who were found to be homozygous for *GSTM1 null* were excluded from analysis of GSTM1 expression.

Study group

The study population consisted of 90 sedentary, non-smoker patients without infectious or viral disease, steroid or immunosuppressive therapy, major debilitating diseases and prior or ongoing cancer. Common illnesses such as hypertension, compensated uncomplicated diabetes, dislipidemia did not constitute exclusion from recruitment. The use of inhaled, topical, or systemic corticosteroids or any other immune modulating medications was forbidden during the study period. Patients were divided into three groups, of 30 subjects each, based on different age: A) 20-40 years; B) 41-65 years; B) over 65 years. Each patients was administered a detailed life style questionnaire together with the web-based version of the National Institutes of Health Diet History Questionnaire (food frequency questionnaire consisting of 124 food items that includes both portion sizes and dietary supplement questions) to assess diet history over the past month and along the study period. Data indicate that this instrument provides reasonable nutrient estimates and sufficient reliability and validity (18). Patients were advised not to use any multivitamin supplement or fortified food while maintaining their usual diet.

Subjects were supplemented for 1 month with either 9g/day (4.5g twice a day sublingually) of a certified fermented papaya preparation (Osato Research Institute, Gifu, Japan) made under ISO 9001 (production quality), ISO 14001 (environmental protection) and ISO 22000 (food safety) from a patented biofermentation process of non-GMO charged papaya or placebo (same amount of flavoured sugar finely ground). After a further 6-weeks period of wash out, subjects were treated again in a cross-over manner.

Parameters

Routine blood tests with WBC formula**,** saliva flow rate and secretory IgA and lysozyme production and redox gene expression from upper airways cells (from nasal lavage).

Saliva collection and analysis

Subjects were instructed not to eat, use gum, candy, breath mints, tobacco or drink anything except water for one hour before collection appointments and to remove any lipstick or lip-aid. After an initial swallow to empty the mouth, unstimulated whole saliva was collected by expectoration into a pre-weighed vial (7 ml capacity plastic tubes with screw top) for 2 min with eyes open, head tilted slightly forward and making minimal orofacial movement. All saliva samples were stored frozen in capped test tubes at -20°C for later analysis. Detection of IgA in saliva was performed by sandwich ELISA. In these assays, polystyrene Maxisorb F96 microtitre plates (NUNC, Roskilde, Denmark) were coated overnight at 4ºC with 0.2µg/well of affinity purified rabbit anti-IgA antibodies with alpha chain-specificity in 0.05 M $NaHCO_3$, pH: 9.5. Blocking was performed by use of phosphate buffer containing 0.5% bovine serum albumin (BSA) at room temperature for 90 min. One hundred microliters of saliva samples (in duplicate) and standard samples (in duplicate) were pipetted into the microtitre wells. The plates were incubated for 90 min at 37ºC. The wells were washed 5 times with washing solution. Then, 100 µl of goat anti-human IgA conjugated with hours radish peroxidase were pipetted into each well, and the plates were incubated for 30 min at 37ºC. The wells were washed 5 times with washing solution and tapped dry. A fresh substrate solution, tetramethylbenzidine (100 µl), was added, and the plates were incubated for 15 min at room temperature. The enzyme reaction was stopped with 100 µl of 1 N HCl. Salivary IgA levels were detected by use of a standard curve. The percent coefficient of variation (%CV) for this ELISA was 3.6%. This technique identifies the peak rate of formation of antigen-antibody complexes by measuring their precipitation in the presence of a known concentration of sIgA antigen and expressed as mg/dL. Saliva flow rate (ml $-min^{-1}$) was determined by weighing and its density was assumed to be 1.0 $g \cdot ml^{-1}$. Samples were defrosted and microcentrifuged for 10 minutes before testing to remove any foreign matter, and 200 gL of clear supernatant was aspirated and assayed. Salivary lysozyme concentration was measured by an Elisa method. Briefly, a 96-well microtitre Elisa plate was coated overnight at 4°C with 200µl of rabbit antihuman lysozyme at a concentration of 7.0mg/l in sodium bicarbonate buffer (pH9.6). Then the well was washed with phosphate buffered solution (PBS) containing 0.05% (v/v) Tween 20 (PBS/Tween) and blot dried. The plate was stored at -20°C until assay. A 200µl aliquot of 2% w/v bovine serum albumin in PBS/Tween was added and left at room temperature for 2h. The plate was washed with PBS/Tween 20, 100 µl of sample diluted (1/50) in PBS and 100 µl biotinylated lysozime (1/1000) was added to each well. The plate was incubated at 37°C for 60 minutes. The well was then washed with PBS/Tween 20, 200 µl avidine alkaline phosphatise diluted in 1/3000 in PBS was added and incubated at 37°C for 60 minutes. After washing, 200 µl of enzyme substrate p-nitrophenyl phosphate sodium (0.1% w/v) in dietheanolamine buffer (pH 9.8) was added and incubated in the dark for 30 minutes at room temperature. The reaction was stopped with 50 µl of M NaOH. The absorbance was read with Bio-Rad microplate reader set at 405 nm. Each test included five two-fold dilutions of purified human urine lysozyme from which a standard curve was generated. The lysozyme secretion was

calculated by multiplying the absolute concentration with the absolute saliva flow rate.

Epigenomic in vivo modification of upper airway cells

Nasal lavage. Nasal lavage was carried out using the method described by Noah and Becker (19) by repetitive spraying of sterile normal saline irrigation solution (4 ml total) into the nostril with the subject's head tilted back at an angle of ~70° from vertical, followed by voluntary expelling of the fluid by the subject into a specimen collection cup. Both nostrils were washed in this way, and the resulting nasal lavage fluid (NLF) from both sides was combined. Nasal wash samples were immediately placed on ice and clarified by centrifugation at 14,000 × *g* in an Eppendorf centrifuge. Cytocentrifuge slides were prepared, fixed, and stained using modified Wright stain for differential cell counts by microscopic evaluation of 200 consecutive cells at high magnification. The remainder of the NLF was centrifuged at 500g × 7 min to remove cells and debris, and the cell pellets were isolated from nasal lavage samples, resuspended in 350µl lyses buffer, and stored at −80 °C until RT-PCR analysis.

Semi-quantification assessment of Phase II enzyme gene expression. Gene expression was measured by real-time quantitative reverse transcriptase polymerase chain reaction. Total RNA was extracted by Trizol, following the maker's instruction (Invitrogen, Paisley, UK) and treated with DNAse I (Sigma, USA). 0.3–1.0 µg RNA was routinely extracted from a nasal lavage sampling. RNA quality and quantity was confirmed by UV spectrophotometry and agarose gel electrophoresis. One µg of total RNA was reverse-transcribed with random hexamers and Moloney murine leukaemia virus–reverse transcriptase at a final volume of 20 µl. Specific cDNA amplification of human GSTM1, GSTP1 and HO1 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′GGGACGCTCCTGATTATGAC3′ and 5′GCAAACCATGGCCGCTTCCC3′ for GSTM1; 5′TCCGCTGCAAATACATCTCC3′ and 5′TGTTTCCCGTTGCCATTGAT3′ for GSTP1; 5'-CAGGCAGAGAATGCTGAG-3' and 5'-GCTTCACATAGCGCTGCA-3' for HO-1; (5 '-3'): CATCATCAATTTCGAGCAGA and GCCACACCATCTTTGTCAGCAG for SOD and ACAGTCAGCCGCATC-3' and 5'-AGGTGCGGCTCCCTA- 3' for GAPDH. 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. Quality of cDNA was confirmed by PCR amplification of fragment from the control gene ubiquitin C. Expression of genes was assessed by a 7500 Real Time PCR System (Applied Biosystems, USA) as total quantification, by using TaqMan Gene Expression Assays of the same maker. Bacterial plasmids containing coding sequences of GAPDH served as standard for total quantification of gene expression. PCR measurements was performed in triplicate. The expression was normalized to the expression of GAPDH, a housekeeping gene, and quantified using a PC based densitometric semiquantitative analysis using NIH image software by expressing arbitrary units. For each individual, gene expression at baseline (pre-challenge) was considered the calibrator and was given an arbitrary figure of 1.0 and relative increase in gene expression following FPP treatment was calculated in reference to this value.

RESULTS

All subjects completed the study reporting no side effects. Routine biochemical values were within normal limits at the entry and were unaffected by FPP supplementation (data not shown)

Saliva analysis

Saliva flow rate showed a significant age-related decline ($p<0.05$, Fig. 1). FPP treatment brought about a significant increase of the salivary secretion rate only in the A group ($p<0.05$) while it did not affect the other age groups studied. Salivary IgA but not lysozyme production was significantly lower in the oldest age-group as compared to the youngest one (C *vs* A, $p<0.05$, Fig. 2). FPP treatment brought about a significant increase of these parameters in all age-groups ($p<0.05$) and the final values were comparable among the groups. Lysozyme concentration was unaffected by aging process. There was no significant correlation between salivary flow rate and either sIgA or lysozyme secretion, irrespective of the treatment or age group.

Epigenomic in vivo modification of Phase II enzyme gene expression (GSTM1, GSTP1 and HO1) of upper airway cells

Expression of phase II enzymes and SOD significantly increased in NLF samples of subjects given FPP as compared to baseline values and placebo control. Fig. 4 shows the NLF data for each age group. In contrast, no significant change in phase II enzyme or SOD expression was observed for

subjects completing the protocol with placebo. No significant difference was observed for each enzyme among the different age-group, either at baseline or after treatment.

DISCUSSION

It is known that the presence of local antibody at mucosal surfaces in the form of secretory IgA (s-IgA) correlates with protection against bacterial and viral pathogens in humans and experimental animals (20). Unlike IgG, s-IgA consists of protease-resistant immunoglobulin dimers adapted to function in the external environment of the mucosa in the absence of other immune accessory mechanisms. s-IgA functions to intercept pathogens before they enter the body. These antibodies protect mucosal epithelium by cross-linking and agglutinating microorganisms, thus enhancing their entrapment and clearance in mucus and in some cases by blocking and sterically hindering the microbial surface molecules that mediate epithelial cell attachment (21). It has been reported that the salivary secretion rate may inversely influence the IgA concentration in saliva and salivary IgA levels seem to be higher in elderly subjects as compared to younger controls (22) although this data is debated (23). In our study the concentration of sIgA was unexpectedly lower in the eldest age-group. At the moment ,we do not have a clear explanation but past smoking habit, environmental exposure and possibly a higher perceived stress might have been confounding factors in this group. As a matter of fact FPP supplementation enabled a significant and comparable increase of sIgA in all subjects while also significantly enhancing the salivary flow rate, but only in the youngest group. No significant correlation was found between salivary flow rate and either sIgA or lysozyme concentration. It has been suggested that lysozyme may mitigate the inflammatory response and pathogenesis of chronic bronchitis by providing an accessory antibacterial defense mechanism and indirectly lowering the neutrophil chemotaxis and activation of alveolar macrophages (24). Interestingly, in our study we showed that lysozyme secretion was enhanced by FPP administration. Although at the moment we cannot provide a specific mechanism of action for such findings, based on prior in vitro and very recent animal studies (25, 26) we can envisage and indirect action through the demonstrated monocyte-activating action which may have triggered a higher lysozyme secretion. Interestingly, a recent study shows that low secretion rate of either IgA and lysozyme in elite athletes was associated with an increased risk of of upper respiratory infection (27). A number of experimental evidences suggests that the lethal effect of influenza virus infection is determined by immunopathological consequences of

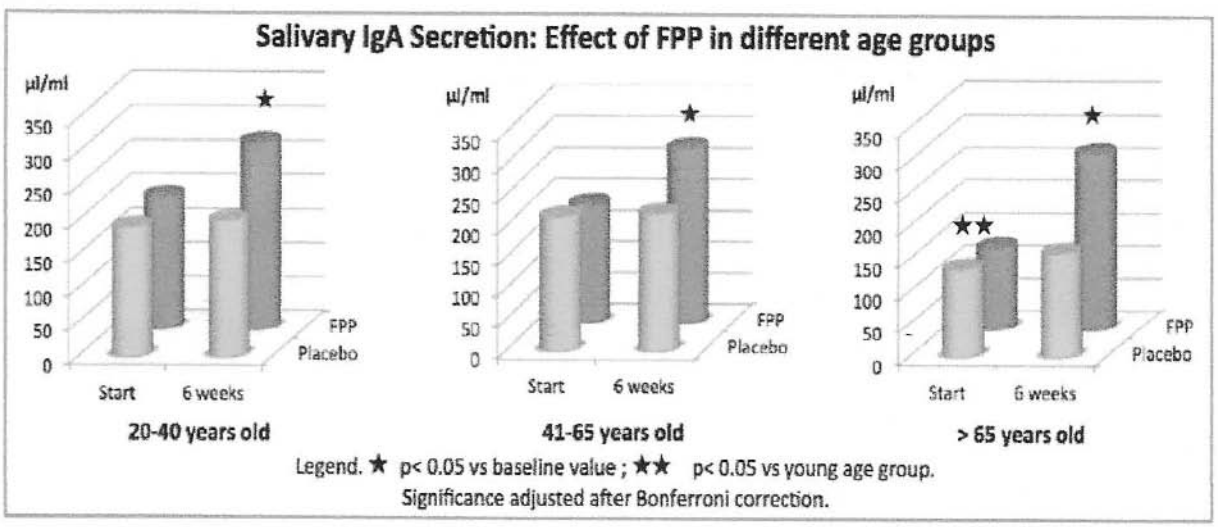

Fig. 1. ** p< 0.05 vs baseline value;. Significance adjusted after Bonferroni correction. Triangled dots: FPP, squared dots: placebo. A significant inverse correlation between age and salivary flow rate occurred (r: 0.71, p<0.05).*

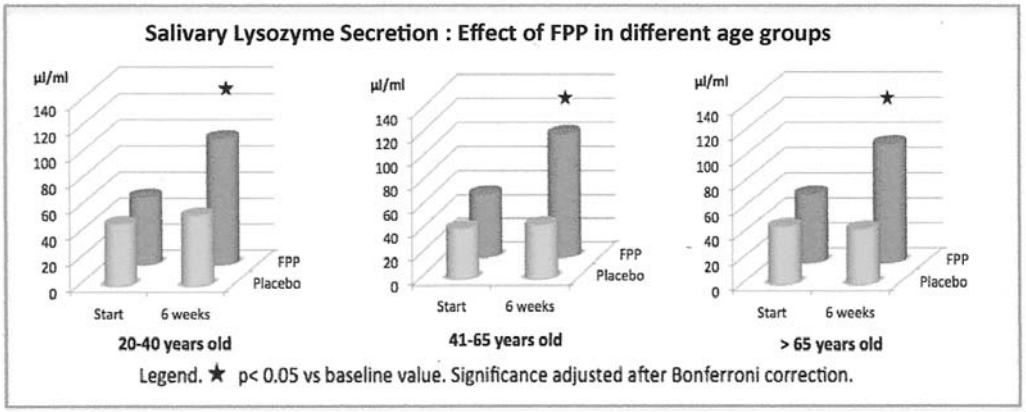

Fig. 2. ** p< 0.05 vs baseline value; ** p<0.05 vs young age group. Significance adjusted after Bonferroni correction. Triangled dots: FPP, squared dots: placebo.*

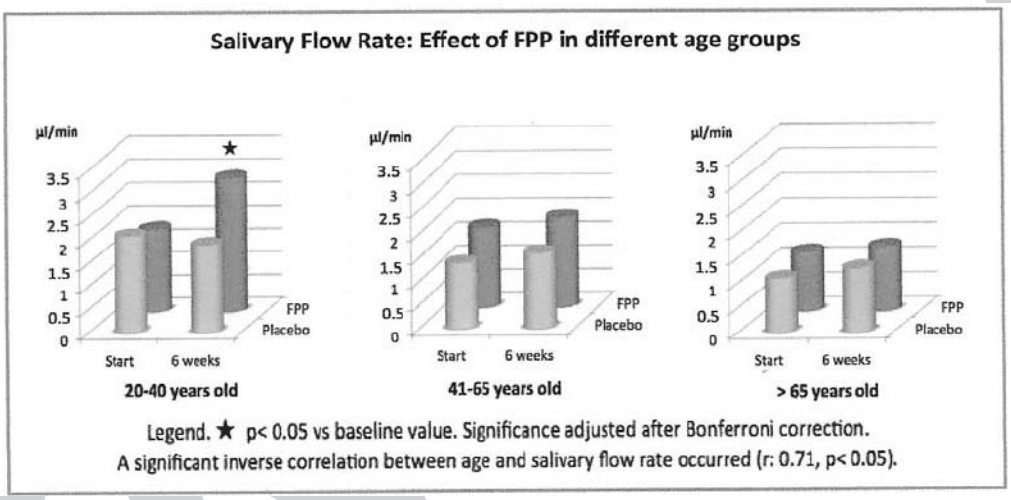

Fig. 3. ** p< 0.05 vs baseline value;. Significance adjusted after Bonferroni correction. Triangled dots: FPP, squared dots: placebo.*

the host rather than by the direct cytopathic effect of viral replication (28). In such a setting, a role for reactive oxygen species (ROS) and reactive nitrogen species (RNS) as mediators of virus-induced lung damage has been suggested by several studies (8, 29, 30) in which exogenous antioxidant treatment has clearly shown to decrease lung damage and mortality in influenza-infected mice. Further, it is likely that ROS may also contribute to an increased viral titer after influenza infection. It has been shown that superoxide may be produced both by influenza-activated leukocytes or by xanthine oxidase, the activity of which is increased in influenza-infected lungs (8) as well as by other local sources such as airway epithelial cells (31). Normal cellular defence mechanisms that protect against free radicals start with the antioxidant enzymes cascade where SODs are a major extracellular antioxidant in the lung especially by the alveolar type II pneumocytes (32). Previous studies (8, 33) on the effects of using exogenous SODs in influenza- induced lung injury have shown protective responses possibly through its inhibitory action against virus-induced inflammatory and oxidant responses in the lung.

Confirming our very recent study analyzing systemic leukocytes (34), we showed that FPP treatment enabled a significant upregulation of SOD also in nasal lavage cells. These data hold some potential relevance when considering that Suliman et al. (35) have envisaged that pharmacological

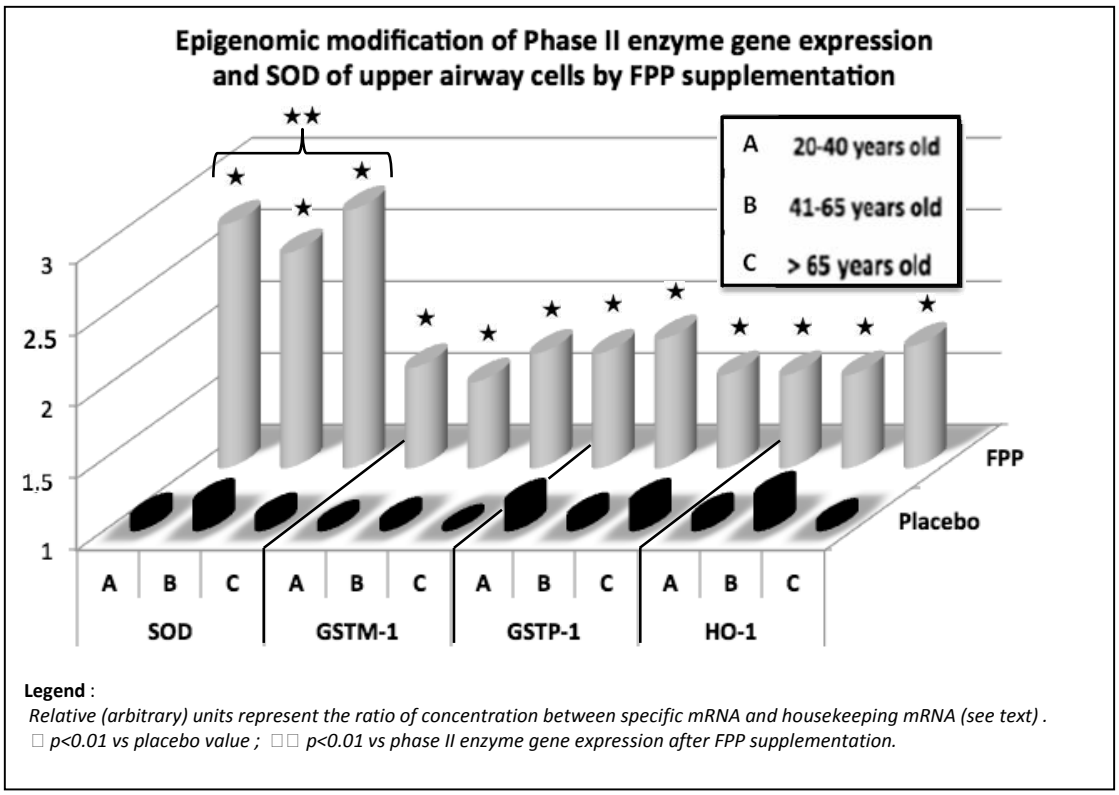

Fig. 4. *Relative (arbitrary) units represent the ratio of concentration between the specific mRNA and the housekeeping mRNA (see text). * p<0.01 vs placebo value (black bars); ** p<0.01 vs phase II enzyme gene expression after FPP supplementation.*

supplementation of airway extra-cellular SOD compounds may provide an opportunity to therapeutically attenuate pathological events that follow influenza virus infection. On the other hand, the effect of cold exposure on the antioxidant defence system is a complex issue and it appear to be both tissue specific and dependent on the duration of the cold exposure experience (36). Indeed, while in some cases compensatory changes may take place in the antioxidant defence system of trained individuals (37), a significant decrease of blood glutathione is a rather constant feature (38). One more relevant finding in our study was the significant gene upregulation of phase II enzyme tested at the nasal lavage cell level. These inducible Phase II enzymes represent an early and sensitive reaction to ROS and act by scavenging them and metabolizing xenobiotics such as air pollutants (39). Although the concomitant measurement of SOD would have added a further value to this data, from our prior study we had shown that FPP could significantly improve SOD plasma status (40). The comparable response of all of the measured PII enzymes, might suggest a common pathway of induction by FFP, i.e. Nrf-2 activation, although we lack specific measurement of it. Indeed, most of the genes for proteins involved in the cellular antioxidant defense and in phase II detoxification share a common regulation via the redox- and electrophile-sensitive transcription factor NF-E2-related factor (41), which binds to the *cis*-acting antioxidant or electrophilic response element (ARE/EpRE) (42) and future studies will be directed to clarify the possible multimodal action of FPP in biological systems.

In conclusion, FPP supplementation during one month resulted in higher concentration of salivary

IgA and lysozyme together with a significant increase in phase II enzyme and SOD expression in the human airway. The biological significance of this effect i.e., whether it will help to reduce respiratory oxidative stress in the human airway and, consequently, the incidence of URTI remains to be demonstrated in longer clinical trials preferably targeted to specific cohorts of patients as well as examining possible genetic polymorphisms affecting Phase II enzymes as a further relevant variable.

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