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CHAPTER 4

Novel Nutrigenomics Avenues in Nutraceuticals Use: The Current Status of Fermented Papaya Preparation

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Abstract: Functional foods present a constantly growing research field, interrelating genomics, epidemiology and clinical investigations, followed by increased interest from the public and food supplement industry. As a matter of fact, the outcome of the implementation of functional foods is now amenable to be assessed by employing many of the most recent diagnostic tools.

Nutrigenomics is a relatively new discipline, which studies the genetic and epigenetic interplay with a nutrient or its functional component(s) in order to bring about a phenotypic modification of key cellular functions, such as, cell metabolism, differentiation or apoptosis. This represents one of the most expanding fields of research to unveil the health benefits of functional foods and their bioactive moieties which cannot be differentiated, as it happens in synthetic molecules devised by pharmaceutical industries.

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M. Victorina Aguilar and Cristina Otero (Eds.)

Within this scenario, a specific functional food, *i.e.* fermented papaya preparation, coming from a controlled bio-fermentation process of papaya, is herewith reported and scientifically backed up by several experimental models and properly-designed clinical protocols.

The promising clinical health benefits provided by fermented papaya preparation are also discussed under the viewpoint of nutrigenomic mechanism understanding, and a significant antioxidant and effective transcriptomic property is described while ongoing investigations are warranted.

In general, this specific fermented papaya preparation represents a functional food, which closely meets the novel criteria of the new nutrigenomic-oriented strategic approach of preventive medicine, aimed to reduce the burden of chronic illnesses, while also offering potential adjuvant benefits within drugs regimens.

Keywords: Fermented papaya preparation, Functional food, Gene expression, Nutraceuticals, Nutrigenomics, Oxidative stress.

INTRODUCTION

The Ever-Changing Nomenclature of Functional Foods

Such a new vision in the last 2 decades has led to continuous modifications in the functional food terminology which in 1999 an expert scientific European panel had defined it as follows "A nutrient can only be easily considered functional if it has been satisfactorily proven that it can beneficially change one or more target functions, besides nutritional effects per se, it can also significantly improve health, well-being while reducing disease risk. A functional food should ideally be a food and should not alter its efficacy when included in a diet, it should not be either a pill or a capsule" [1]. It was then concluded that, from a practical viewpoint, a functional food should meet the following features:

1) a natural food; 2) a food with one added component; 3) a food with no added component; 4) a food which the structure of one or more of its components have been changed; 5) a food which one or more components' availability has been changed; 6) a combination of the previous features. Besides its inner nutritional properties or physiological effects, it was necessary to offer a consistent administration safety profile. Such a condition is nothing but a prerequisite to

further develop any functional food.

The recommendations of such European expert panel led to a definite resolution, stating that, “The design and development of a functional food are key factors, besides the scientific challenge, they should be primarily based on robust understanding of target functions and their potential modulations by nutritional components”. Thus, it was later emphasized that, “functional foods are not universal, thus a conventional nutritional approach would no longer be satisfactory, but rather, a specific scientific approach will only be applicable”. This points towards an innovative nutritional viewpoint regarding the role exerted by “Functional Foods Science”, which now emerges as the leading vision towards deriving effective clinical inferences. An ancient Chinese proverb stated that “medicine and food are isogenic” and this may represent the traditional root of what in 1984, in Japan, a specific national working group had set up, under the patronage of the Ministry of Education, Science and Culture (MESC). This was called to explore the interconnections between nutrition and several aspects of molecular biology. Researchers investigated a number of foods and nutrients which were then officially categorized as “foods to be specifically administered for healthcare” (Food for Specified Health Use, FOSHU) by recognizing their modified nutritional properties, after undergoing a profound bio-fermentation process. A further consensus meeting took place later on reaffirming the above concepts [2].

Such a categorization still has legal implication on improper marketing communication defining natural products, when they are misleading or loosely referring to data in the scientific literature, as a sort of abusive self-endorsement, but not specifically supporting the specific product itself [3]. As a matter of fact, the lack of a coherent terminology between countries has inevitably generated a large mass of publications, dealing with health claims with often incomparable end-points in clinical trials or food production process methodologies from the industries. As an expected counteraction, an overall mistrust on the meaning and real health benefit of “functional foods” is creeping now and then among government officials, public health professionals and consumers.

On the American side, the latest conference organized by the Functional Food

Center held in San Diego in November 2014 [4 and http://functionalfood scenter.net/17th-international-conference.html], gave rise to a revised and more grounded definition of functional food as:

"Foods that are natural or processed which contain known or unknown biologically-active ingredients, which in defined amounts, provide a clinically proven and documented health benefit for the prevention, management, or treatment of chronic disease".

Novel biomarkers and Development Strategy leading to Nutrigenomics. A biochemistry and specific molecular biology research, coupled with advancing biotechnology tools have helped in shedding more light into the mechanisms, explaining how nutrients could indeed regulate relevant body functions involved in health issues, as well as in the risk reduction associated to life style. Such evaluations have to be compliant with reliable biomarker identification, either directly linked (functional factors) to the target biological function or indirectly related (indicators). Suitable marker selection mainly gave rise to the development of genomics. Indeed the post-genomic era originating from the human genome decoding project provided excellent opportunities to the science of functional foods, by availing itself from newest "omics" technologies such as new generation sequencing and some others, leading to nutrigenomics arena [5]. Such a terminology was introduced only in the last decade and constitutes a major step forward as compared with observational studies mainly focused on researching among specific bioactive nutritional components. Nutrigenomics mainly aims at studying genetic and epigenetic interactions with a nutrient determining defined phenotypic modification of cell differentiation, metabolism or modulating apoptosis. Moreover, this has opened the way to try to figure out the minimum "functionally" effective quantity leading to the above-mentioned fundamental biological changes. In fact, several pre-clinical studies supporting biological modifications appear to use dosages of nutritional components at concentrations which are inapplicable in clinical practice.

Some recent papers suggest the remarkable adaptative ability of cells when they are being exposed to overwhelming amounts of nutrients. As mentioned above, it would amount to a weak practical rationale to approach the use of a natural

product: 1) which is only nutrient-specific; 2) and even more, if generally referring to properties simply derived from scientific literature, but with no specific validation or bioavailability study. Furthermore, a few far-sighted companies and food industries are consistently supporting independently-run validation studies on natural compounds, even when they are not required by the regulatory law to do so; 3) considering the negative effect or the variable efficacy of the each nutrient depending on the different formulations (lyophilized products, dehydration processes at low or high temperature, water- or alcohol-extracts, *etc.*) or multi-component associations. Isoflavons and soy proteins represent very good example, where the specific role of each single component has not yet been clarified, as well as the effects of any possible combination among the inner different moieties [6]. However, as for new generation studies, the list of potential interactions between nutrients and host and between nutrients themselves remains largely to be evaluated, while several mechanisms may play an important role all together. Biological response to a functional food could be anti-oxidant (followed by a series of possible genomic sequences mediated by an increased transcriptional rate by: aldehyde-dehydrogenase, glutatione-S- transpherase, NAD(p)H: kinone-reductase, UDP- glucuronosyltranspherase, microsomial hydrolysis, aphta-toxin B1- aldehyde reductase, cytochrome P450s, dihydrodiol-dehydrogenase, glutatione- reductase, *etc.*), supporting the detoxification enzymatic mechanisms, mutagenic transformation, metabolic pathways, hormonal regulation, cell division and apoptosis.

The Fermented Papaya Preparation Case: an Example of the Rationale and Evidence-based Biotechnological Progress. Consequently, it is of interest to further analyze the established and ongoing research process related to fermented papaya preparation (FPP). This is a specific compound derived from a technologically advanced and controlled bio- fermentation process of Carica Papaya Linn, without any genetic modification which has been devised at a Japanese research institute. The above process was carried out in compliance with all quality control and environmental-friendly standards. The anti-oxidant natural papaya properties have been established in the past and they were mainly accounted for by its content in vitamins (A and C) and amino acids. This applies both to the fruit and to the papain enzyme (arginine among all). Papain promotes

digestion activity, but, interestingly enough, such an activity is no longer present in the FPP. A patented several month long yeast-related fermentation, represents the core process enhancing the protection of papaya anti-oxidant properties while expressing significant novel immune-modulating properties. Fermentation profoundly modifies the ratio between complex gluconutrients and proteins, from about 10:1 as it appears in lyophilised papaya accounts for up to 10:0.03 in the final FPP, that is 30 times higher. Unlike what was seen in the fresh fruit, the finally fermented product showed several newly formed oligosaccharides with different polymerisation as well as monomers mimicking the basic structure of (1-3)- β -D-glucan. Such low molecular weight oligosaccharides, showed a wide spectrum of immune-modulating activity. A couple of decades ago, Japanese scientists studying populations living in the Philippines that ate large amounts of papaya on a daily basis, had produced several initial reports on its overall health benefit. Later on, those field of observations, helped to substantiate the study of "functional" properties of a series of specific compounds within fruits- and vegetable-based diet. A peculiar attention was paid to Carica Papaya Linn, which was collected in the Philippines and processed in Japan, with other exotic fruits through a long fermentation process according to traditional ethnopharmacology methods.

Basic Science Research: A mandatory step in the Development of Biotechnologies From the extraction to the final compound. Starting from the above collected data, a series of experimental studies were performed by the Neuro-science Department of the Molecular Biology Institute in Okayama University in Japan, directed by Prof. Mori [7].

Such studies, carried out with updated methodologies such as Electron Spin Resonance, proved that such a product consisting of fermented papaya exhibited a powerful anti-oxidizing activity on *in vitro* cerebral cell cultures [8] as well as on the epilepsy experimental animal model, where the epileptogenic monoamine neutral release was significantly reduced [8] (Fig. **1**). Prof. Mori's group [7] also showed the capacity of fermented papaya to reduce the increase of free radical concentration and superoxide dismutase at the brain level in elderly rats, followed by the reduction of experimentally-induced ischemia-reperfusion cerebral injury. These data were further corroborated by the discovery that FPP could reduce

beta-amyloid-mediated copper neurotoxicity as well [9].

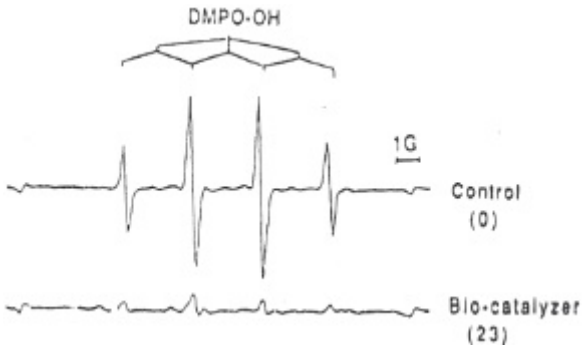

Fig. (1). Effect of FPP termed as Bio-catalyzer on ESR signals on 1,1-diphenyl-2-picryhydrazyl (DPPH) radicals and DMPO spin adducts of superoxide and hydroxyl radicals.

Later on, the significant *in vitro* resistant anti-oxidizing capacity when tested for one hour at high temperatures (100°C) and very acidic pH (pH 1,2) was reported, fact which is a rather unique feature. Moreover, such characteristics were also confirmed after a long-term storage of the compound. The potential neuroprotective effects of FPP are presently the end-points of a clinical study on Parkinson's disease patients, carried out by Prof. Nordera's group in a highly-dedicated neurological in/out-patients clinic located in northern Italy. This study is preliminary(at the early stages) showing some promising results, especially in the aspect of the improvement of rigidity symptoms and redox biochemistry (manuscript in progress).

Recently, Prof. Barbagallo, Chief of Geriatric unit at the University of Palermo published interesting results in Alzheimer disease patients [10]. In this study, the supplementation of FPP enabled an astounding significant decrease of the oxidative marker 8OHdG in the urine of patients.

Indeed, the oxidative stress damage is one of the earliest pathophysiological events in the development of Alzheimer disease (AD) and the brain is characterized by a low content of antioxidant systems despite the fact that it uses up to 30% of all oxygen intake of the body (Fig. **2**). The production of ROS seems to be involved in triggering and maintaining the degeneration cycle of AD, while the alterations of oxidative metabolism may render the brain more vulnerable to

further damage from Aβ.

In addition, the neuroprotective potential as ascertained in an AD cell model showed that the toxicity of the Aβ can be significantly reduced by FPP.

These results confirmed that FPP has the potential to counteract the excessive production of free radicals present in patients with AD, suggesting a role in slowing down the progression of AD.

Then, after thoroughly refining the product and getting its certifications by the governmental body (Table **1**), two significant research studies were performed.

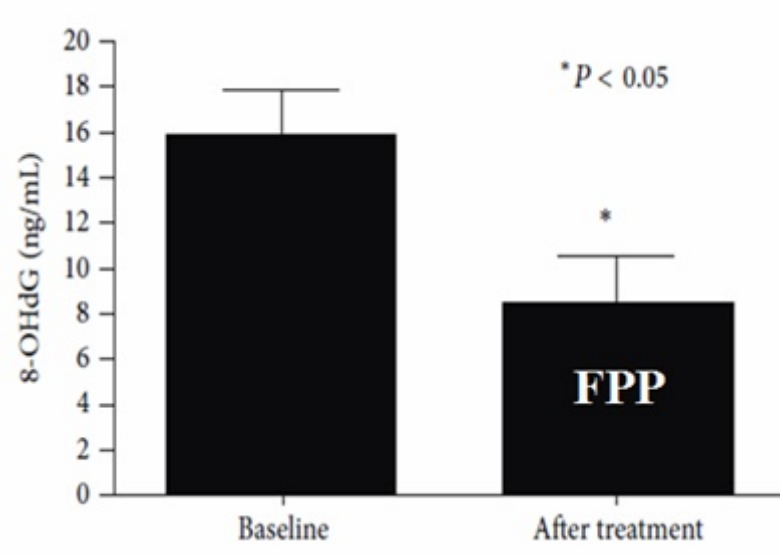

Fig. (2). 8-Hydroxy-2-deoxyguanosine (8-OHdG) level in patients with Alzheimer's disease (group 1) before and after fermented papaya powder (FPP) supplementation. * p<0.01 *vs* baseline value.

Further assessment of the topic of its possible effects on the immune system was investigated together with the Kyoto Pasteur Institute [11], and its effects on the oxidizing stress was probed in co-operation with the Molecular Biology

Department at the UCLA by Prof. Packer, a widely recognized authority in the field, leading to a finer exploration of its action mechanisms. Such successful works, albeit still in progress, led to some extremely interesting *in vitro* and *ex vivo* findings. For example, the group of Pasteur Institute in Kyoto, starting from

the evidence of the positive effects of FPP on the Natural Killer population in a sarcoma experimental model, showed the capacity of FPP to beneficially affect the γ-interferon production in humans. Such data was further paralleled by studies supporting the positive activity of FPP on the macrophage function on rats and humans [12].

Table 1. Fermented Papaya Preparation (100 g). FPP/100 g Composition (Japan Food Res. Lab, Tokyo).

Carbohydrates	90.7 g	Arginine	16 mg
Moist	8.9 g	Lysine	6 mg
Proteins	0.3 g	Histidine	5 mg
Fats	absent	Phenylalanine	11 mg
Ashes	0.1 g	Tyrosine	9 mg
Fiber	absent	Leucine	18 mg
Vitamin B6	17 μg	Isoleucine	9 mg
Folic acid	2 μg	Methionine	5 mg
Niacin	240 μg	Valine	13 mg
Calcium	2.5 mg	Glycine	11 mg
Potassium	16.9 mg	Proline	8 mg
Magnesium	4.6 mg	Glutamic acid	37 mg
Copper	14 μg	Serine	11 mg
Zinc	75 μg	Threonine	8 mg
		Aspartic acid	27 mg
		Tryptophan	2 mg

In the same time period, the research team led by Prof. Mori showed the significant protecting effect by FPP on oxidative stress generated in isolated rat hearts [13], when undertaking a severe ischemia/reperfusion injury, a key epiphenomenon in myocardial infarction (Fig. **3**).

Such data have been confirmed and further enriched by the research work of Aruoma *et al.* [14], who showed the ability of FPP to decrease the oxidative DNA damage in rat pheochromocytoma (PC12) cells as well as protecting the brain of hypertensive rats from oxidative damage. The same Mori' s group also presented significant scientific results showing the role played by the anti- oxidative

properties of FPP in promoting also its immune-modulating activity [8]. In fact on a rat macrophage line, a relevant experimental evidence has demonstrated how FPP can upregulate the interferon-γ-induced nitric acid production (Fig. **4**). By doing so, FPP [15] would thus exhibit a nutrigenomic effect by changing gene expression of inducible nitric acid, TNF-α and of interleukin-1β. Such an activity was further confirmed when two fractions of different molecular weight (cut off: MW 3.000), were separated and tested. This helped also to understand the FPP transcriptional effect on the NF- κB binding to DNA. However, the two different fractions proved a series of dissimilar effects in macrophage stimulation and scavenging properties. It became clear to figure out, for example, that a different degree of immunomodulating activity could depend upon different (1- 3)-β-D-glucan concentration, *i.e.* the most constitutive portion of some peculiar yeasts, used in the FPP bio-fermentation process.

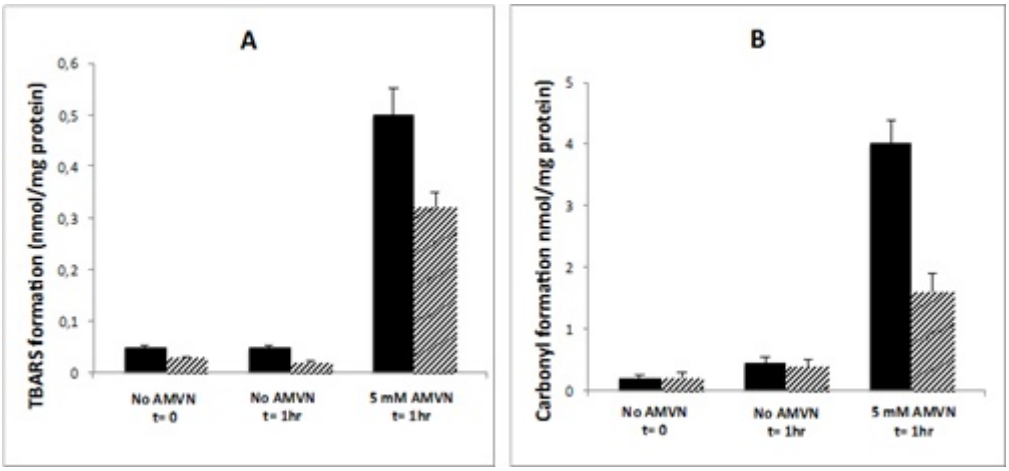

Fig. (3). Effect of FPP supplementation on AMVN-induced accumulation of TBARS and protein carbonylation in rat heart homogenates. Cardiac homogenates (10 mg protein/ml) prepared from animals on control (dark symbols) or FPP supplemented diets (dashed symbols) were exposed to peroxyl radicals generated from 5 mM AMVN at 42°C for the times indicated. TBARS (A) and protein carbonyl derivatives (B) are expressed as mean ± S.E.M of 6 different hearts from each group.

Moving beyond observational studies: Clinical evidences supported by research. Supports offered by experimental evidences and a series of works on humans represented the pre-requisites to plan a series of prospective clinical studies. In 1995, a Russian oncological- haematologic research group [16] showed that FPP

could significantly mitigate the clinical side effect (encephalopathy score based on: anorexia, nausea, vomiting, convulsions, dizziness) and biochemical parameters (change of the redox state due to the erythrocyte gluthatione depletion, increased concentration of leukocyte SOD and, deficit of the monocyte bactericidal activity) in a group of young subjects undergoing radiotherapy for severe mielo- and lympholeukaemia.

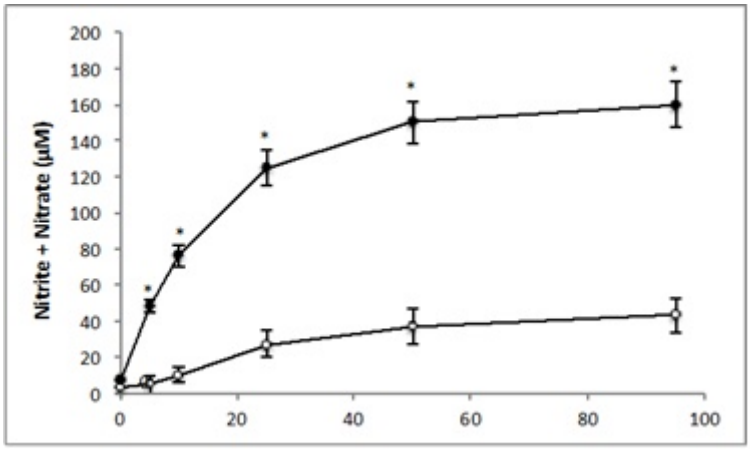

Fig. (4). Efficacy of IFN-γ on NO production by FPP. Macrophages were incubated with different concentrations of IFN-γ in either the absence (open circles) or the presence (closed circles) of 3 mg/ml of FPP for 24 h. All values represent the mean ± SD of three independent experiments. *p<0.05 compared with that of IFN-γ alone treatment.

These results are in agreement with Prof. Mori`s previous experimental studies (7). In the same way, an Israeli group [17] recently presented a study suggesting that, FPP might serve as radioprotector against radiation-induced DNA instability and mutation accumulation, thus envisaging a potential use in prevention of primary and secondary tumors due to radiation exposure (Fig. **5**). Their data showed in in-vitro and in-vivo that FPP reduces: i) radiation-induced toxicity (reduction of intracellular ROS and LIP in human normal foreskin fibroblasts and myeloid leukemia HL60 cells); ii) radiation-induced DNA damage (reduction of DNA oxidation and instability); and iii) the accumulation of somatic mutation (decrease of GPI negative RBC frequency).

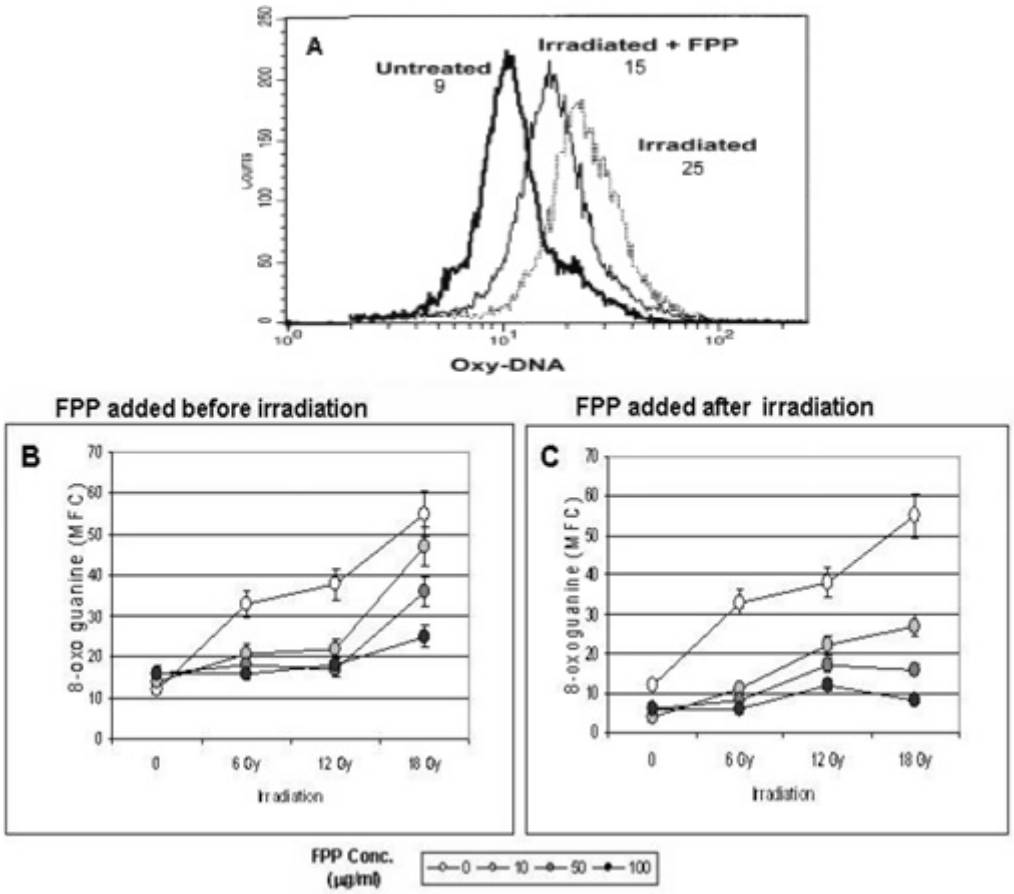

Fig. (5). The *in vitro* effect of FPP on radiation-induced DNA oxidation (8-oxoguanine). (Figure A) Cultured human skin fibroblasts were untreated, irradiated (6 Gy) or irradiated and treated by FPP (100 µg/ml). Two days later, the cells were harvested, washed, fixed, permeabilized and stained with a FITC- labeled protein conjugate specific for 8-oxoguanine. The figure shows the fluorescence cell distribution histograms and their Mean Fluorescence Channels of a representative experiment – indicating 8-oxoguanine content in the DNA (B-C). Human fibroblasts were irradiated at the indicated doses. FPP, at various doses, was added either before (B) or immediately after (C) irradiation. Two days after irradiation, the cells were harvested and stained for 8-oxoguanine. From the results presented, the mean ± SD (N= 5) of the Mean Fluorescence Channel (MFC) showed an increase by irradiation and a decrease by FPP, both in a dose-related fashion.

A group of Italian, French and Japanese scientists coordinated a series of independent studies on ethanol-induced liver disease, proving how FPP enables a reduction of oxidative stress (reduction of plasma and erythrocyte level of malonyldialdehyde as well as of plasma lipoperoxides) [18]. This finding was proven, both during the initial stages of withdrawal when a persistent microsomial

system activation can keep on maintaining a pro-oxidative state, and during the chronic alcoholic abuse. More precisely, taking into account the low compliance in the case of withdrawal, it was shown how the administration of FPP to alcoholics led to the following effects:

1. A significant improvement of haemorheology (reduction of the whole blood viscosity, recovery of the erythrocyte deformability and increase of blood filtration capacity through specific membrane). Indeed, chronic alcoholics showed a consistent increase of malonylaldehyde concentration in the erythrocytes with lipid asymmetry destabilization due to lipoperoxidiation phenomena [19].

Part of these data have been recently confirmed in a small group of healthy elderly individuals too [20]. In HCV-related chronic liver disease, the same research team proved the significant improvement of redox status by both 900 IU/day alpha-tocopherol or 9 g/day of FPP supplementation. However, only FPP significantly decreased 8-OHdG and the achieved improvement of cytokine balance was significantly better in FPP-treated group than with vitamin E [21] (Fig. **6**). Few years later, a similar cohort of patients were further studied [22] and it appeared that patients with liver cirrhosis significantly showed a time-dependent upregulation of TNF- α production from ex-vivo LPS stimulated monocyte. This effect was more pronounced in more advanced stages of the disease together with higher serum level of thioredoxin (Trx) (Fig. **7**). Again, FPP showed the capacity to reach a normalization of Trx and a partial but significant downregulation of TNF-α mRNA.

About gastrointestinal redox status, another recent study [23] pointed out gastrointestinal redox imbalance in young iron-deficient females caused by iron supplementation. The FPP co-supplementation abolished the iron-induced increase of MDA and the depletion of SOD and GPx.

2. The previously mentioned haematological data also proved to interest an authoritative Israeli group led by Prof. Rachmilewitz [24, 25]. These researchers reported that when treating *in vitro* with FPP blood cells from beta-thalassemic subjects, an increase of glutathione concentration of red blood cells occurred, platelets and polymorphonuclear leukocytes and a decrease of oxidative stress,

membrane lipid peroxidation and externalization of phosphatidylserine.

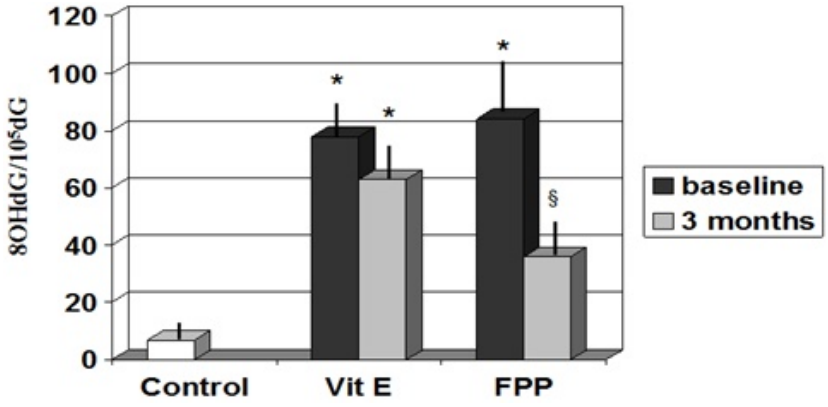

Fig. (6). Effect of fermented papaya preparation (FPP) on concentration of 8-OHdG in circulating leukocytes * P< 0.001 *vs* control; § P< 0.05 *vs* vitamin E group and control.

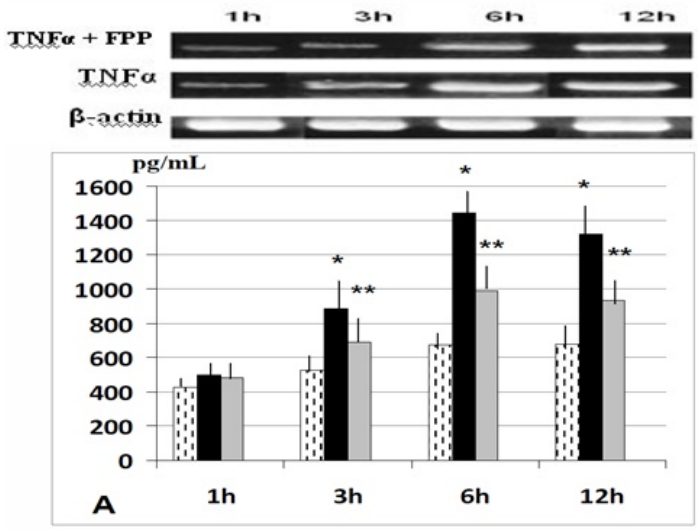

Fig. (7). Ex-vivo LPS-stimulation test of TNFα production from monocytes and PCR-electrophoresis: nutraceutical modulation. Data were obtained at 3 months observation. A) dotted bars: healthy control; black bars: unsupplemented cirrhotics; grey bars: FPP-supplemented cirrhotics. FPP: fermented papaya preparation. Stimulated monocytes from unsupplemented cirrhotics showed a significant time-course increase of TNFα production, *p<0.01 *vs* healthy control. Nutraceutical supplementation partly but significantly decreased such phenomenon, ** p<0.05 *vs* unsupplemented cirrhotics. Top part of figure shows the PCR electrophoresis of TNFα expression in LPS-stimulated cells from FPP-supplemented cirrhotics patients (TNFα + FPP) and in unsupplemented patients (TNFα).

These effects determined a (a) significant reduction of thalassemic RBC sensitivity to hemolysis and phagocytosis by macrophages, (b) enhanced PMN protecting oxidative burst ability and (c) reduced platelet undue activation, as measured by external phosphatidylserine. In conclusion, the administration of FPP to beta-thalassemic mouse model (50 mg/mouse/day for 3 months) and to thalassemic patients (3 g x 3 times/day for 3 months), reduced all the above oxidative stress parameters [26].

Quite recently, Rachmilewitz´s group investigated the effect of FPP on two groups of beta-thalassemic subjects: beta-thal, major and intermedia patients in Israel, and E-beta-thal patients in Singapore. The results indicated that in both groups, FPP treatment increased the content of reduced glutathione in erythrocytes, and decreased free radicals generation, membrane lipid peroxidation and externalization of phosphatidylserine [27] (Fig. **8**).

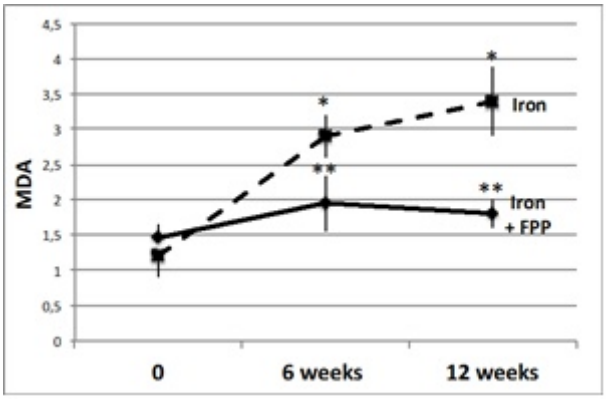

Fig. (8). The study of systemic oxidative stress status revealed that iron supplementation brought about an increasing imbalance of redox status with a significant time-course increase of MDA ($p<0.001$ *vs* baseline values). This phenomenon significantly appeared at 6 weeks and displayed further increase at 12 weeks observation.

Further supporting data came from a concomitant case report of a beneficial administration of FPP to a patient with paroxymal nocturnal haemoglobinuria [28]. A recent study confirmed the beneficial effect of FPP on haemolysis (Fig. **9**) by observing a significant reduction of the rate of haemolysis and accumulation of plasma protein carbonyls in pre-diabetics [29].

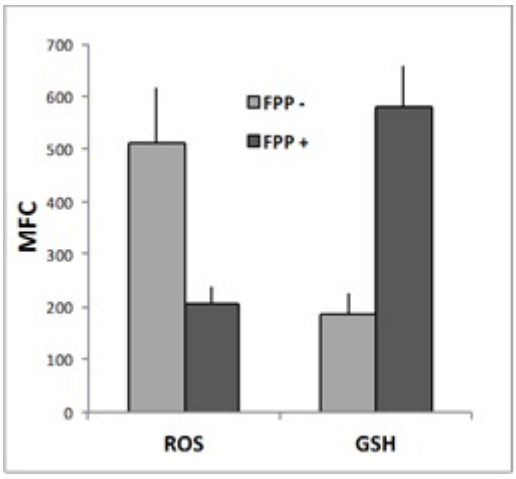

Fig. (9). The in-vitro effect of FPP on oxidative stress and haemolysis of HS-RBC from HS patients were diluted in PBS and incubated for 2 hrs with or without 0.1 mg/ml FPP. The cells were then assayed for ROS and GSH. The results are expressed as the average MFC of 17 patients.

3. A significant recovery of latent alcohol-induced vitamin B12 malabsorption associated to oxidative damage on the gastric mucosa at the binding site between intrinsic factor and cyanocobalamin [18] (Fig. **10**).

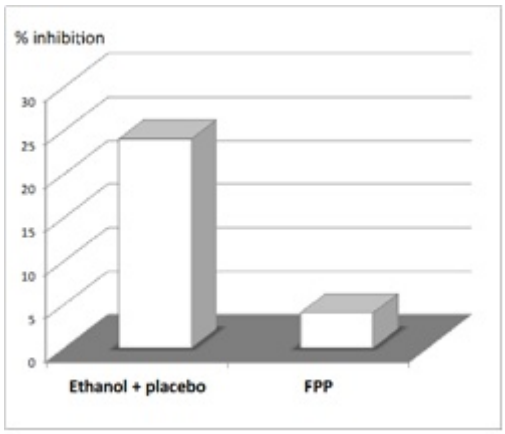

Fig. (10). Vitamin B_{12} binding by IF, by *in vitro* incubation of IF with 57Co-labelled cyanocobalamin.

Such data on the efficacy of FPP defense against alcohol-induced oxidative damage of gastric mucosa was also replicated when healthy subjects were administered a test-dose of ethanol (40 ml 80% ethanol) [30]. According to the

previous findings on the antigenotoxic effect and on the DNA *in vitro* protection by FPP from the group of Prof. Mori (8), and more recently of Prof. Packer's group [31], who highlighted.

The iron chelating effect, a new clinical trial investigated on the gastric mucosa pre- cancerous changes. In fact, a group of Italian and Japanese scientists carried out a 6-months controlled, randomized study in patients with histologically-proven chronic atrophic gastritis without concomitant Helycobacter pilori. This work proved that both, a multivitamin anti-oxidant mixture and high dosage vitamin E and FPP were able to reduce the mucosal level of oxidative stress markers. However, FPP only significantly decreased the two most specific markers of pre-mutagenic changes, namely ornithine decarboxylase and 8-oxoguanine (Fig. **11**).

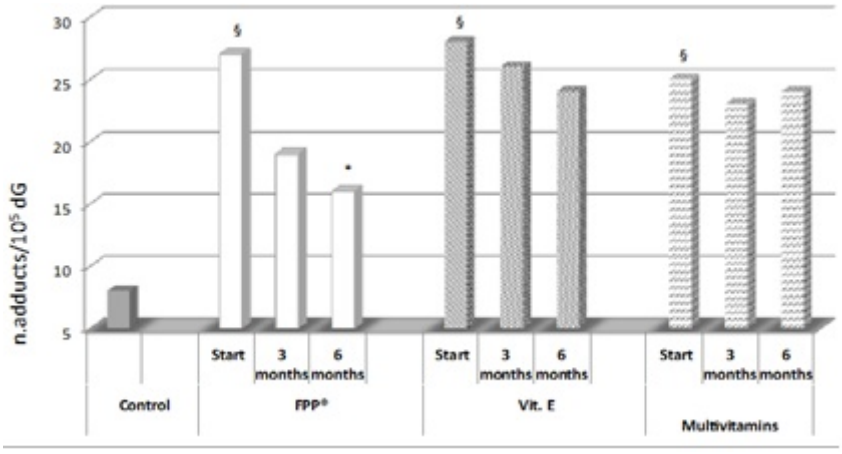

Fig. (11). 8-OHdG rate in mucosa: effect of FPP supplementation. FPP was the only supplementation bringing about a significant decrease of 8-OHdG rate in mucosa. § $p<0.05$ *vs* control, * $p<0.05$ *vs* start.

The latter being a mutated base, is one of the most common biochemical signature linked to DNA oxidative damage and which may be implicated into severe replication errors and anaplastic transformation) [32]. At the time of the first clinical trial on the immuno-modulating FPP effects and related reports (increase of the CD8+ and QOL score), led by Kyoto Pasteur team (Mimaya N. personal communication, Club de France, Paris, 1998), a series of research works were initiated by Prof. M. Weksler of the Cornell University in the USA (personal

communication, Admin. Report Unesco, 2006) and Prof. L. Montagnier, former director of the virology laboratory of the Pasteur Institute in Paris and present chairman of the World AIDS Research and Prevention Foundation.

From a preliminary study, it appeared that the FPP administration for 3 weeks before anti-flu vaccination in 10 hospitalized elderly patients, consistently improved their specific antibody response as compared with a control group, given only the vaccine. Furthermore, Prof. Montagnier's group (personal eCommunication, 2005, unpublished data) performed a study to test whether FPP could be of benefit to poor immunological-responder HIV-positive patients.

Data from the open preliminary work proved that the association of this compound to anti-retroviral treatment, could significantly increase CD4+ concentration as well as hemoglobinaemia, weight increase and overall well-being. Most recently, Dr. Giancarlo Orofino, director of Microbiology and Virology Laboratory of Principe di Savoia hospital in Turin, reported the data of an open trial on 25 HIV patients taking anti-retroviral treatment with good virological response but low immunological benefit, liable to undergo excessive oxidative stress. These were concurrently administered FPP and this proved to significantly increase about 50% of the immunological profile markers together with improved QOL too (personal communication, 2011).

Such overall immune-modulating effect of FPP was shown in another study aimed to ascertain its role in reducing upper respiratory tract infections in healthy subjects of age ranging from 20 to over 60 [33]. The data showed that FPP significantly increased salivary IgA and lysozyme secretion and upregulated phase II enzyme and SOD gene expression in retrieved airways epithelial cells. These data offer a promising application in helping to reduce both the incidence and/or severity of upper respiratory tract infections (Fig. **12**).

Taking into accounts the overall above data, it becomes clearer that either the antioxidant effect of FPP and its beneficial microrheological and macrophage activity-enhancing properties must play a role in the successful study of the Comprehensive Wound Center, Department of Surgery from Ohio State University Medical Center, USA. Indeed, both Dr. Collard and Dr. Roy studied

[34] the effects of FPP on wound healing process in adult obese diabetic (db/db) mice.

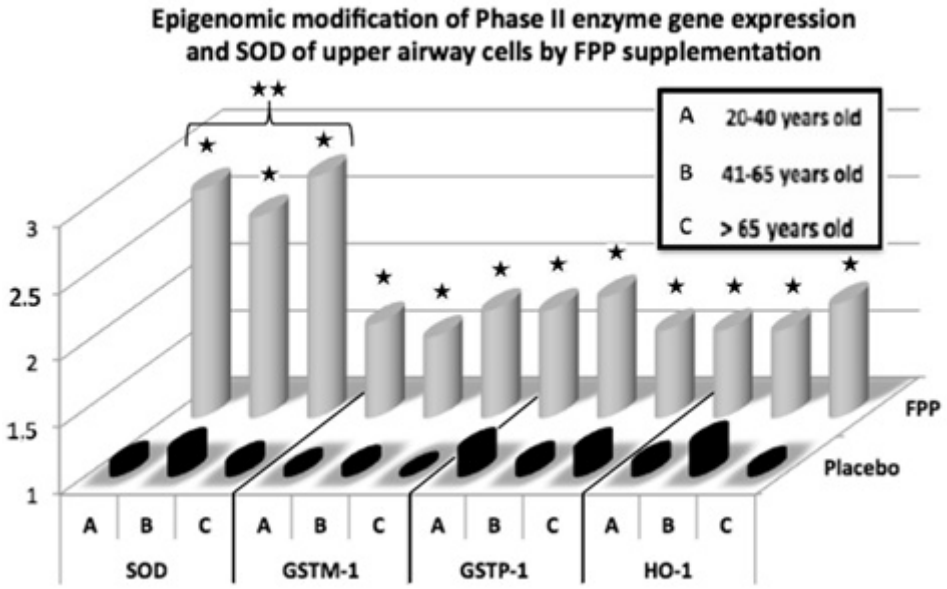

Fig. (12). Epigenomic modification of Phase II enzyme gene expression and SOD of upper airway cell by FPP supplementation. Relative (arbitrary) units represent the ration of concentration between the specific mRNA and the housekeeping mRNA. *p<0.01 *vs* placebo; **p<0.01 *vs* phase II enzyme gene expression after FPP supplementation.

Their work proved that FPP administration significantly improved respiratory-burst function as well as inducible NO production, together with a larger content of CD68 as well as CD31 at the wound site, pointing out a robust recruitment of monocytes and proangiogenic response. Interestingly, FPP blunted blood glucose concentration, and this somehow mirrors the unexpected clinical findings of Danese *et al.* [35] who showed that the daily administration of 3 grams of FPP at lunch, for two months to 25 type-2 diabetics under treatment with glybenclamide and to 25 controls, brought about a significant decrease in plasma glucose concentration in both groups.

Most recently, Dr. Roy's group published a new study confirming their previous pre-clinical findings on the beneficial effect of FPP on wound healing in diabetic patients [36] by highlighting that FPP improved inducible "respiratory burst" ROS

in Type 2 diabetes (Fig. **13**). This finding suggests that FPP may indeed improve diabetic wound outcomes by specifically influencing either systemic and local biological response. However, another very intriguing inference from this latest study is that FPP could indeed enhance mitochondrial efficiency too (Fig. **14**).

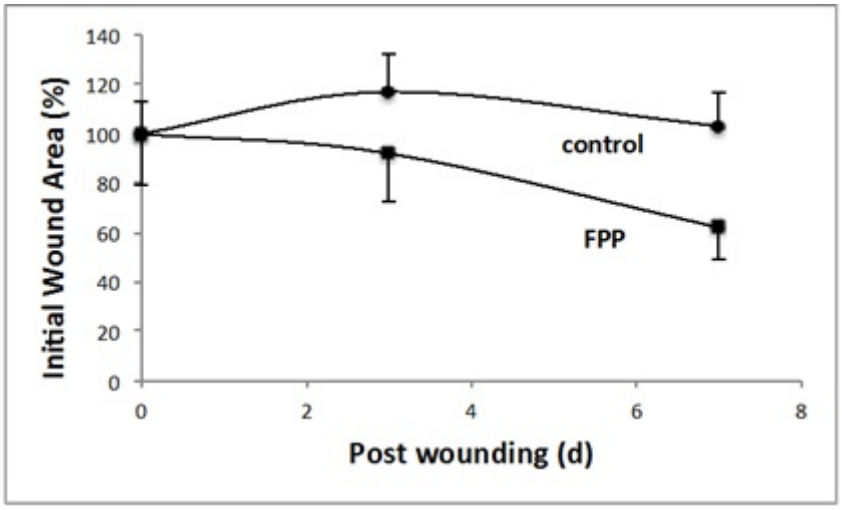

Fig. (13). Improved wound closure in FPP-supplemented diabetic mice. Area measurements of wounds from FPP- or placebo-treated db/db mice. Data are expressed as mean ± SD (n=5). *p < 0.05.

One must say that it remains fundamental to promote a diet rich in organically-grown vegetables, which if correctly enforced, offers proper amounts of micro-nutrients and anti-oxidants.

This diet would be theoretically sufficient to comply with the body requirement in the case of normal health conditions and in the absence of important psychical and physical burdens or other unfavourable environmental conditions. What simply depends on common sense reasoning, was underlined long ago by an authoritative international non- profit institute, which stressed that an healthy diet should not be replaced by a non-controlled diet rich in supplements or food-like compounds as vitamins, extracts or lyophilised products, mainly when the variability of such products in each single batch is uncontrolled or, even worse, when no certified titration is available. However, the absence of specific and referenced studies on each single would-be nutraceutical compound cannot be counterbalanced by referring to general data from literature. Legislative and regulatory issues on

fortified foods supplemented by specific nutrients still pose an uncertain area, which deserves its own discussion.

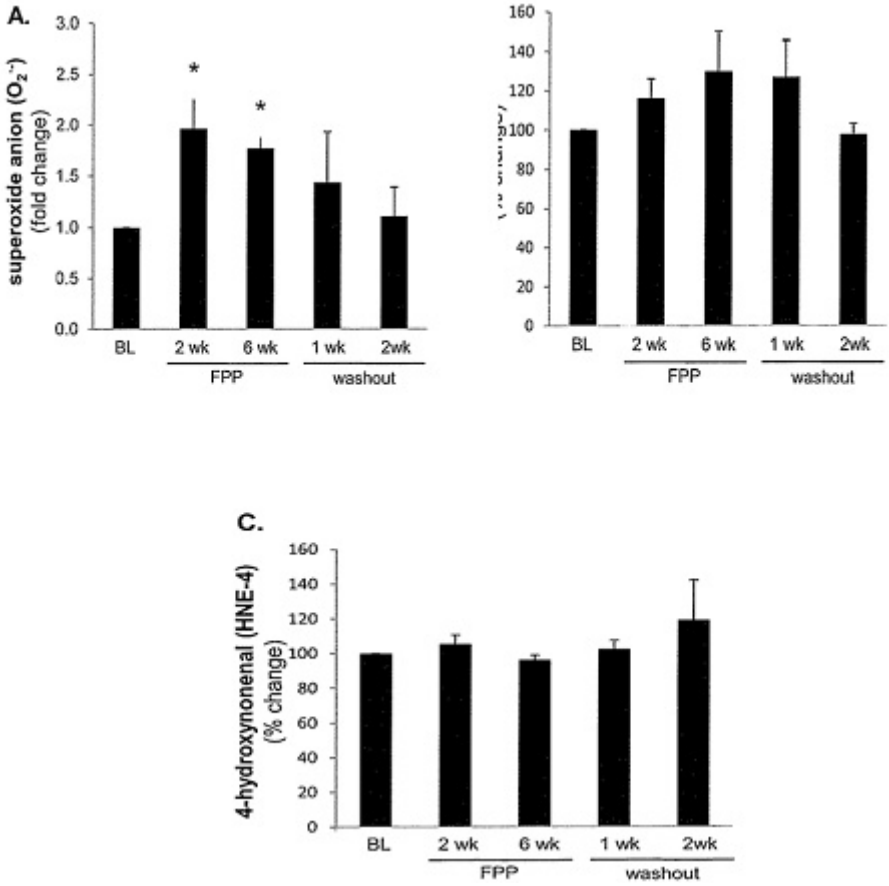

Fig. (14). Oral supplementation of FPP corrected blunted respiratory burst response in PBMC of T2D patients while not affecting the systemic oxidative stress profile. A. Peripheral blood derived monocytes (PBMC) were isolated from T2DM donors. PBMC was collected at baseline (0 wks), at 2 and 6 weeks (wks) of FPP supplementation and 1 and 2 weeks of washout. Superoxide anion production was measured following PMA (1μg/ml) stimulation for 30 minutes. Data were expressed as fold change compared to the baseline (BL). From the data, the value of the mean is ± SEM (n=14). * $p<0.05$. Fresh plasma was flash frozen in liquid nitrogen for storage until the cohort was collected. All samples were measured at the same time. B. Protein carbonyls were measured using a ELISA based assay. Each time point represents the average percentage change for each visit as compared to the baseline (BL) ± SEM (n=10). C. 4-hydroxynonenal (HNE- 4) was measured using a ELISA based assay. Each time point represents the average percent change for each visit as compared to the baseline (0 wks) ± SEM (n=17).

As it was previously underlined by Prof. Packer during an international congress

[OCC conference, USA, 2003], "…we are in front of a consistent evolution of anti-oxidants implying the study on how some of them from a simple scavenger function are instead able to interact in a complex way with the redox balance and immune-modulating network through a genomic adjustment". In this regard, a polymorphism profile designed placebo-controlled study [37] performed in 54 healthy elderly subjects has shown that only those who were GSTM1 (-) were the ones with increased lymphocyte 8-OHdG and who got the main benefit from FPP supplementation.

These data suggest that FPP may be an advisable supplementation for upregulating antioxidant defenses even without any overt antioxidant-deficiency state, and this may help explaining some inconsistent results of prior interventional studies (Fig. **15**).

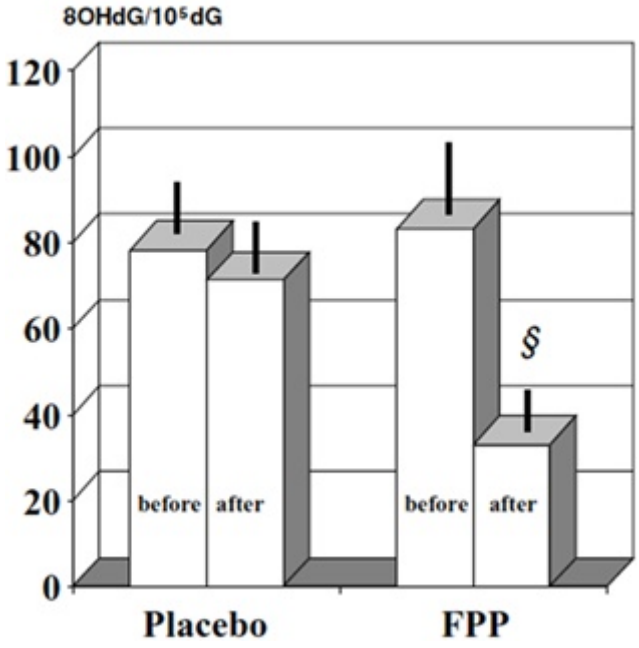

Fig. (15). Left: concentration of DNA adducts in all subjects and of 8-OHdG in circulating leukocytes (only GSTM1(-) subjects). Right: effect of nutraceutical intervention. §P<0.05 *vs.* baseline and *vs.* placebo.

A further study [38] with a similar cohort of patients showed that there may occur a proinflammatory profile acting also as a down regulating factor for inducible Hsp70, particularly in Interleukin-6 promoter -174 G/C-negative subjects, while a

9g/day of sublingually-administered FPP (a preferable route) reverted such phenomena. The unveiling of the complex intracellular/epigenomic mechanisms of FPP still needs further research work such as on posttranscriptional/translation protein changes as by Prof. Migliore's preliminary work at Pisa University (unpublished data). A recent small clinical study, proving FPP- induced up-regulation of SOD, catalase, GPx and hOGG1 gene expression in leukocyte [39], advocated a transcriptomic modification of key redox and DNA repair genes thus offering further insights when attempting to interrelate "nutragenomics" to clinical phenomena (Fig. **16**).

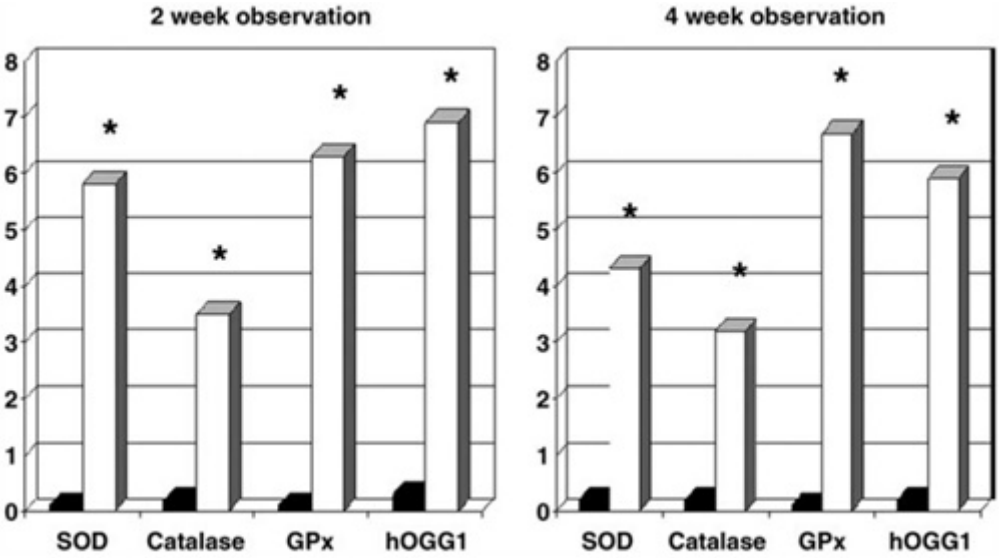

Fig. (16). All subjects fully complied with the study protocol. At either 2 and 4 weeks observation, the plasma level of tested redox parameter showed no variation after FPP administration, besides a non-significant decrease of MDA. On the other hand, starting at 2- weeks observation, FPP brought about a significant up-regulation of all gene expressions checked ($p < 0.05$), which remained stable at later testing time. This data applied irrespective of GSTM-1 and h-OGG-1 genotype profile. No relation appeared between erythrocyte level of redox parameters or plasma MDA and related gene expression or genotype profile.

A recent double-blind, antioxidant cocktail-control study of Bertuccelli J [40] also seems to show that FPP when given to middle-aged people is able to improve redox balance, and NO production at skin level and beneficially modulate skin aging-related genes, by upregulating acquaporin-3 and down-regulating potentially pro-aging/carcinogenetic cyclophilin-A and CD 147 genes, though

without affecting the typical chronoaging gene (progerin) (see below) (Fig. **17**).

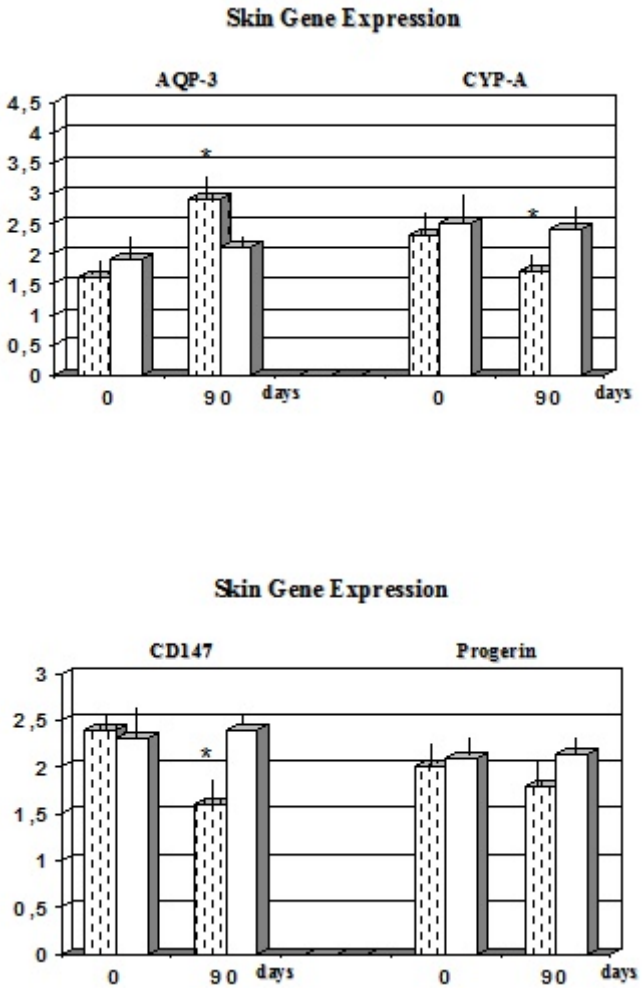

Fig. (17). All subjects fully complied with the study protocol and evaluated at the entry and after 90 days. Dotted line bar: FPP-supplemented; white bar: antioxidant cocktail (trans-resveratrol 10mg, selenium 60mcg, vit E 10mg, vit C 50mg) control. As compared to control, FPP brought about a significant gene expression change ($p<0.05$) of acquaporin-3 (AQP-3), cyclophillin-a (CYP-A) and of cluster of differentiation (CD 147) genes.

Latest suggestions from Osato Research Institute and Foundation's chairman (Y. Hayashi) triggering new stream of studies, led to the assumption that a peculiar high- bioavailability monosaccharide moieties releasing property from FPP might have a preferential blood brain barrier transport and direct energetic protection on

neuronal structures.

Late last year another ongoing RCT double-blind study was performed on 90 subjects ranging from 45 to 65, with impending metabolic syndrome and negative as for ApoE risk gene pattern and was presented at a major functional food congress in Kobe, Japan (http://functionalfoodscenter.net/19th- international-conference.html). FPP 4.5g was given twice a day *vs* common antioxidant cocktail (trans-resveratrol, selenium, vit E, vit C) for 3 months. Then, after a wash out period, a re-treatment was added by using heavy metal chelators (special chabasite-phillipsite-analcine zeolites naturally-occurring mixture), 3gr/day for further 3 months. The preliminary data confirmed that FPP seems to decrease oxidative stress parameters ($p<0.05$ *vs* antioxidant cocktail mixture) and, unlike the control antioxidant, it significantly decreased also oxidised-LDL, although not changing the lipid profile. Moreover, only FPP decreased cyclophilin-A plasma level and plasminogen activator-inhibitor. The addition of a specific oral chelator mixture did not further improve the biochemical and nutrigenomic results obtained by FPP while this specific zeolite remarkably increased the heavy metal clearance unlike other usual zeolites such as clinoptilolite (paper in progress, presented at the 20th International Conference on Functional Foods, Harvard University, Sept 22-23, 2016, USA).

Summing up, FPP certainly stands out as a long-dated, research-based functional food has met the criteria and novel features of the new nutrigenomic-driven preventive medicine strategies, aimed to disease risk reduction and amenable also to successful integration within specific pharmacological approaches.

CONFLICT OF INTEREST

The authors confirm that they have no conflict of interest to declare for this publication.

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