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ORIGINAL ARTICLE

Effect of papaya supplementation on oxidative stress markers in Parkinson's disease

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ABSTRACT

Objective: In Parkinson's disease (PD), oxidative processes occur that are probably involved in the progression of neuronal damage. Recent studies suggest that regular consumption of antioxidant-rich foods or supplementation with functional foods like fermented papaya, may reduce cellular oxidative stress and protect against many age-related diseases by strengthening the physiological antioxidant barrier. Our study aims to assess the antioxidant properties of a commercial fermented papaya preparation (FPP) in PD by evaluation of its effects on a wide panel of oxidative stress markers in blood and urine.

Methods: A group of parkinsonian voluntary patients (T, n = 15) were treated 9 months with FPP and compared with a control group (NT, n = 12). The efficacy of FPP in increasing the physiological antioxidant barrier was evaluated at the times 0, 6 and 9 months with the analysis of 23 oxidative markers including total radical oxygen species, homocysteine, biological antioxidant potential, glutathione, superoxide dismutase, uric acid, total bilirubin, iron, ferritin, coenzyme Q10, 3-nitrotyrosine, total lipoperoxide, 4-hydroxy-nonenal, 8-hydroxydeoxyguanosine (8-OHdG) and 2-deoxyguanosine (2-dG).

Results: Among the considered markers, twenty not showed significant differences at times 0, 6 and 9 months between T and NT patients, demonstrating a consequent non-significant effect of FPP supplementation. Instead, three urinary markers of oxidative stress on nucleic acids, 8-OHdG/2-dG, 2-dG/uric acid and 2-dG/creatinine showed statistical significant interactions between 'time' and 'treatment', highlighting an effective better response to oxidative attack for T than NT.

Conclusions: Papaya supplementation for a medium-long time seems able to affect positively the turnover of oxidized nucleic acids helping to restore the normal nitrogenous bases into nucleotide chains and to replace those altered by radicals' attack.

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INTRODUCTION

The cause of cell death in neurodegenerative diseases remains unknown but the formation of free radicals and the occurrence of oxidative stress may be a common component of many such disorders. In parkinsonian's substantia nigra many key alterations occur, in iron handling, mitochondrial function and antioxidant defenses that can result in oxidative stress. The free radical overproduction mediates a wide pattern of oxidative damages on cellular lipids, proteins and DNA bases [1-16] that do not appear related to levodopa administration [17].

Oxidative damages in basal ganglia are found even in other degenerative disorders such multiple system atrophy, progressive supranuclear palsy and Huntington's disease [18-20]. Moreover, studies on biochemical changes occurring in Alzheimer's disease (AD), motor neuron disease and diabetic neuropathy also suggest the involvement of free radicals in neurodegeneration process [21-24]. It is suggestive to think that, irrespective of the primary cause of individual neurodegenerative disorder, the onset of oxidative stress may be a common mechanism by which neuronal death occurs and contributes to disease progression. Recent studies suggest that regular consumption of antioxidant-rich foods may reduce cellular oxidative stress and protect against age-related diseases by strengthening the physiological antioxidant barrier. Our study aims to assess the antioxidant and neuroprotection properties of a fermented papaya preparation (FPP) in Parkinson's disease (PD) patients. FPP is a supplement food, rich in amino acids and carbohydrates product from yeast fermentation of *Carica papaya*.

The nutritional properties of papaya fruit and the composition of FPP were carefully investigated and described [25-26] but the mechanisms of its antioxidant capacity restoring was not fully understood. Probably, FPP acts both as a direct antioxidant (thanks to its molecular asset) and as an indirect gene up-regulator of enzymes such catalase, glutathione peroxidase and superoxide dismutase and other molecules involved in the strengthening of the antioxidant barrier and repair mechanisms of oxidative damages [27-28]. Many reports on chronic and degenerative diseases such thalassemia, cirrhosis, diabetes, aging and on performance sports showed that FPP favorably modulates immunological, hematological, inflammatory, vascular and oxidative stress damage parameters [29-43]. For these direct and indirect positive effects on health, FPP was therefore proposed as a 'functional food' [44].

With our study, we wanted to verify if FPP can acts as free radical scavenger even in PD and so provide neuroprotection. This observational study, conducted on a group of PD voluntary patients treated long term with

FPP, has evaluated the efficacy of this food in modifying the blood and urine levels of a wide panel of oxidative stress markers [15] and in increasing the physiological antioxidant barrier.

SUBJECTS AND METHODS

The research was conducted according to the principles of the Declaration of Helsinki.

Patients

Serum, plasma and urine samples were obtained from 27 voluntary patients with PD (mean age 64.3 ± 9.1 years), all in stages 1-3 of Hoehn Yahr scale, treated with similar levodopa therapies remained unchanged in course of the study. The patients will be then randomly divided into 2 groups: the first group (15 subjects, 8 males and 7 females) was treated (T) for 9 months, in addition to levodopa, with 9 g/day of a commercial certified papaya preparation administered by mouth as previously reported [26, 34, 45], while the second group (12 subjects, 6 males and 6 females) received levodopa therapy only (NT).

Biological samples

The biological samples were collected from all patients at the times 0, 6 and 9 months. Blood was collected in vacuum sealed tubes without anticoagulant, with EDTA or sodium citrate depending on the analytical method. After sampling, the tubes were immediately centrifuged at 3500 rpm for 10 min at 4°C and the plasma or serum samples frozen at -80°C until analysis. First morning spot urine samples were also collected and frozen at -80°C.

Oxidative stress markers analysis

Plasma homocysteine (Hcy), 4-hydroxynonenal (4-HNE), total and reduced blood glutathion (GSH-tot and GSH-red) were analysed by HPLC and fluorimetric detection using diagnostic kits purchased from Eureka Lab Division (Chiaravalle, Italy); similarly, determinations of plasmatic coenzyme Q10 and 3-nitrotyrosine (3-NT) were performed using Eureka HPLC kits with UV detection.

Superoxide dismutase (SOD) was determined in red blood cells with an automatized colorimetric kit (Ransod, Randox) and the enzymatic activity was expressed as function of hemoglobin concentration (Units/g Hb).

Analysis of urinary 8-hydroxydeoxyguanosine (8-OHdG) and 2-deoxyguanosine (2-dG) were performed with a previously reported HPLC method with electrochemical detection [9]: the values of 8-OHdG and 2-dG were expressed either as reciprocal ratio (8-OHdG/2dG) and as function of the urinary creatinine (8-OHdG/cr and 2-dG/cr) and uric acid (8-OHdG/ua and 2-dG/ua). The oxidizing capacity of the plasma, understood as total content of oxygen free radicals (ROS), the biological antioxidant potential (BAP), understood as total content of antioxidant species and the total lipoperoxides content were determined in serum with the colorimetric assays d-ROMs (derivatives of Reactive Oxygen Metabolites), BAP and LP CHOLOX (level of lipoperoxides in circulation, resulting mainly from cholesterol) test purchased from Diacron (Grosseto, Italy).

The REDOX balance was determined by an experimental algorithm that relates the values of the d-ROMs and BAP between them and with their respective cut-off (BAP/BAP cut-off divided by d-ROMS cut-off/d-ROMs).

Hemoglobin (Hb) was analysed in whole blood by a hematology analyzer (BC5380, Mindray) and serum ferritin (FER) by a fluoroimmunoassay (Vidas, Biomerieux). Serum iron (Fe), uric acid (UA), total bilirubin (BT), urinary uric acid (U-UA) and creatinine (U-CR) were determined with a clinical chemistry multiparametric analyzer (BT-3500, Biotecnica); the urinary concentration of uric acid was expressed as function of the creatinine value (U-UA/cr).

Statistical analysis

Data were analyzed by means of a two-factor repeated measure ANOVA, with one factor between subjects (treatment group, at 2 levels) and one factor within subjects (time factor, at 3 levels). To account for the lack of the 'sphericity' assumption [46], the Box correction [47] was applied at the F test for the main effect of time and at the F test for the interaction between time and treatment group. All the analyses were performed employing R 3.1.1 software [48]; a significance level of 5% was always adopted ($P < 0.05$).

RESULTS

Since most of the analytes studied (Hcy, GSH-tot, GSH-red, Q10, 8-OHdG/cr, 2-dG/cr, 8-OHdG/ua, 2-dG/ua, 8-OHdG/2dG, 3-NT, 4-HNE, SOD, BAP, REDOX balance, BT, FER) showed a very skewed distribution, the corresponding log-transformed values were analyzed (the base 10 was adopted). The remaining variables d-ROMs, CHOLOX, Hb, Fe, UA, U-UA/cr, instead, remained untransformed. Table 1 shows summary statistics (mean, standard deviation and median for original values) for the 27 subjects, separately for NT and T groups. A two-factor repeated measure ANOVA was performed as described in the methods and the results are reported in Table 2.

There were 9 variables (Hcy, GSH-red, GSH-red/tot, Q10, 4-HNE, REDOX balance, UA, Fe, BT) for which none of the three F tests yielded a significant result. Therefore, for these variables, neither a group difference nor a time difference was found. On the other hand, there were 11 variables (GSH-tot, 8-OHdG/cr, 8-OHdG/ua, 3-NT, SOD, BAP, d-ROMs, CHOLOX, Hb, FER, U-UA/cr) for which only the time effect was significant. Therefore, the mean levels of these variables changed along with time, but in a similar manner in T and NT subjects.

In particular, these variables did not show significant differences between two groups and, therefore, it is likely

Table 1. Descriptive statistics for not treated **(NT)** and for FPP treated subjects **(T)** at time 0 (basal), 6 and 9 months. The table shows, according to the biological sample, mean **(m)**, standard deviations **(sd)** and median **(med)** values for each oxidative stress marker; between the brackets are reported the units of measure.

	Basal						After 6 months						After 9 months					
	NT			T			NT			T			NT			T		
	m	sd	med	m	sd	med	m	sd	med	m	sd	med	m	sd	med	m	sd	med
Plasma markers																		
Hcy (µmol/l)	15.1	5.8	13.1	14.2	5.5	15.0	11.7	4.2	11.8	15.4	8.3	14.7	15.2	6.5	14.4	15.3	5.2	15.8
Q10 (µg/l)	866	725	716	734	626	496	491	365	457	627	707	402	504	404	423	519	609	316
3-NT (µg/l)	159	67	156	128	61	124	96	41	97	118	39	111	134	34	122	134	43	136
4-HNE (nmol/l)	41.6	19.9	40.1	33	10.9	33	32	14	32.3	42.1	16.7	41.5	42.8	16.5	44.9	34.5	13.5	38.9
Blood markers																		
GSH-tot (µmol/l)	1640	239	1622	1748	199	1769	1586	197	1554	1447	177	1450	1661	246	1585	1521	189	1457
GSH-red (µmol/l)	943	199	846	1042	246	997	863	169	824	901	210	824	955	177	968	880	112	888
GSH-red/tot (%)	62.9	12.5	60.7	59.7	13.1	55.4	60.5	10.4	59.3	64.6	14.8	58.6	60.3	7.7	58.4	59.9	8.2	57
SOD (U/g Hb)	1856	438	1839	1826	430	1709	1519	400	1469	1510	312	1525	1624	308	1548	1662	245	1671
Hb (g/dl)	13.5	0.9	13.5	13.9	1.3	13.4	13.9	1.1	14.0	14.2	1.0	14.1	14.2	0.8	14.2	14.2	1.3	14
Urine markers																		
8-OHdG/cr (µg/g)	242	576	66	74.1	68.6	68.1	52	76	24	29.7	35.7	17.2	31	31	25	22.5	26.8	10.4
2-dG/cr (µg/g)	6316	9843	2615	7586	21408	1426	820	1129	594	2742	6002	162	131	242	58	1258	2236	397
8-OHdG/2-dG (ng/µg)	51.2	70.5	21.7	150	237	55	183.3	259.4	31.9	231	332	58	868	1817	175	133	230	53
8-OHdG/ua (µg/g)	931	1319	411	1098	1256	726	118	113	91	127	169	72	113	110	92	73	75	38
2-dG/ua (µg/g)	157010	320001	34408	36147	61957	10421	3871	6096	1773	8948	18994	583	774	1874	225	3977	5538	803
U-UA/cr (mg/g)	191	133	222	167	160	94	325	92	335	293	110	297	281	140	283	310	126	318
Serum markers																		
BAP (µEq/l)	1968	348	2096	2052	227	2048	1862	189	1793	1836	202	1801	1892	377	1760	1771	184	1778
d-ROMs (U carr)	356	45	374	365	63	369	326	54	321	332	46	341	331	62	345	346	65	323
CHOLOX (µEq/l)	398	95	393	472	124	461	526	123	519	554	119	555	397	224	329	452	220	509
REDOX balance	0.76	0.14	0.77	0.79	0.14	0.77	0.8	0.12	0.78	0.77	0.12	0.74	0.79	0.14	0.79	0.72	0.17	0.69
UA (mg/dl)	4.46	1.33	4.05	4.2	1.9	3.9	4.62	1.55	4.25	3.88	1.51	3.9	4.69	1.25	4.65	4.19	1.66	4.5
Fe (µg/dl)	88.3	23.3	87.5	87.6	21	86	90.4	30.3	100.5	94.1	19.5	86	111.2	34.8	118.5	88.7	32	79
BT (mg/dl)	0.79	0.26	0.75	0.77	0.41	0.68	0.72	0.29	0.62	0.74	0.24	0.72	0.82	0.37	0.65	0.79	0.33	0.74
FER (µg/l)	174	127	130	155	130	138	174	143	122	171	139	141	164	147	105	135	113	117

that variations in time respect to the basal value could be linked to the progression of the disease, which represents the common condition of both groups. A somewhat regular decrease over time was observed for 8-OHdG/cr, 8-OHdG/ua and BAP, while an opposite pattern was observed for Hb. For GSH-tot, SOD and d-ROMs, average values at 6 and 9 months were lower than those at time 0, while an opposite pattern was observed for U-UA/cr. Mean values of 3-NT were reduced after 6 months, but after 9 months a return to the basal value was found. Similarly, average CHOLOX values increased between 0 and 6 months, but they returned to the basal value after 9 months.

Finally, for the remaining 3 variables (8-OHdG/2-dG, 2-dG/ua and 2-dG/cr), a significant interaction effect was found. Therefore, for these variables some modifications of the mean values among the three considered times were found, and these changes were significantly different between T and NT subjects.

The mean values of 8-OHdG/2-dG, increase progressively in the three times in NT group only, while these remain substantially constant in T. This trend was just perceptible in the analysis for the first two times, but it becomes significant when considering all the three times (Figure 1).

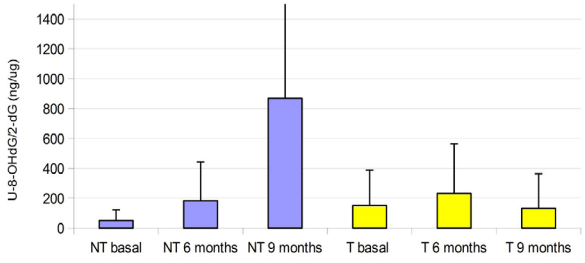

Figure 1. Mean values of 8-OHdG/2-dG ratio in urine in treated (T) and not treated (NT) subjects at basal time, after 6 and 9 months.

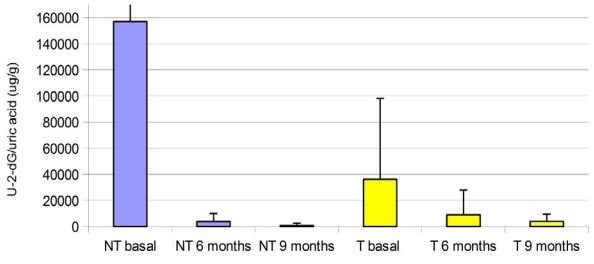

Figure 2. Mean values of the 2-dG/uric acid ratio in urine in treated (T) and not treated (NT) subjects at basal time, after 6 and 9 months.

Table 2. Results of the two-factor repeated measure ANOVA. The results of the F tests for the main effects of time and treatment group, as well as for the interaction are reported. The P values for the time effect and for the interaction are calculated employing the Box correction. Significant interactions are displayed in ***bold italic*** and significant main effect of time is displayed in **bold**.

Marker	Treatment group		Time		Interaction	
	F	P	F	P	F	P
4-HNE	0.11	0.743	0.04	0.843	2.73	0.112
GSH-red/tot	0	0.965	0.29	0.598	0.59	0.449
REDOX balance	0.37	0.551	0.74	0.397	1.5	0.232
UA	0.75	0.395	0.82	0.373	1.12	0.301
BT	0	0.981	1.14	0.297	0.98	0.333
Fe	0.63	0.435	1.83	0.188	3.04	0.093
Hcy	0.22	0.645	2.1	0.161	2.82	0.107
Q10	0	0.989	2.48	0.13	0.14	0.715
GSH-red	0.22	0.647	2.62	0.119	1.25	0.276
8-OHdG/2-dG	0.5	0.486	3.20	0.086	***5.69***	***0.025***
3-NT	0	0.997	**4.32**	**0.048**	3.08	0.092
FER	0.14	0.714	**4.76**	**0.039**	1.85	0.186
SOD	0.01	0.915	**5.03**	**0.034**	0.08	0.782
d-ROMs	0.26	0.616	**5.34**	**0.03**	0.10	0.755
CHOLOX	1.32	0.262	**5.47**	**0.028**	0.17	0.682
GSH-tot	1.3	0.267	**5.69**	**0.027**	2.62	0.121
BAP	0.02	0.883	**5.87**	**0.023**	1.47	0.237
Hb	0.39	0.541	**6.9**	**0.015**	0.88	0.357
U-UA/cr	0.06	0.81	**11.1**	**0.003**	0.59	0.45
2-dG/cr	0.72	0.404	14.84	0.001	***4.36***	***0.048***
8-OHdG/cr	0.66	0.425	**17.85**	**< 0.001**	0.06	0.814
2-dG/ua	0.45	0.507	30.84	< 0.001	***4.30***	***0.049***
8-OHdG/ua	0.18	0.675	**42.57**	**< 0.001**	0.18	0.677

In contrast, the mean values of the variable 2-dG/ua gradually decrease in NT group in the three times while in T, after an initial reduction between 0 and 6 months remain substantially constant (Figure 2). Note that, U-UA/cr ratios increases in the same way in time for both NT and T groups (Figure 3). Therefore, the observation that in the latter the ratio 2-dG/ua is not reduced further between 6 and 9 months, corroborates the demonstration that 2-dG increase over time in T. To confirm this observation, a similar result is obtained for the ratio 2-dG/cr. The mean value of 2-dG/cr decrease gradually in the three times in NT subjects, while in T group, after an initial reduction between 0 and 6 months, remain substantially constant between 6 and 9 months (Figure 4).

DISCUSSION

Oxidative stress contributes to the cascade leading to dopamine cell degeneration in PD. The alteration of the physiological redox balance in PD is intimately linked to other components of the degenerative process, such as mitochondrial dysfunction, excitotoxicity, nitric oxide toxicity and inflammation. It is therefore difficult to determine whether oxidative stress leads to, or is a consequence of, these events. Since previous

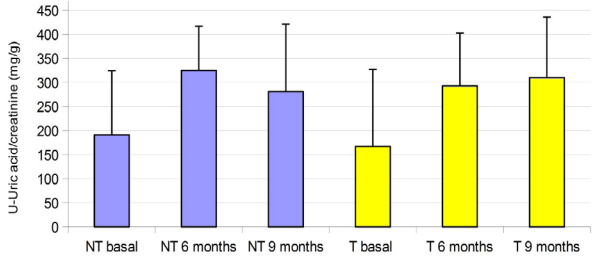

Figure 3. Mean values of the uric acid/creatinine ratio in urine in treated (T) and not treated (NT) subjects at basal time, after 6 and 9 months.

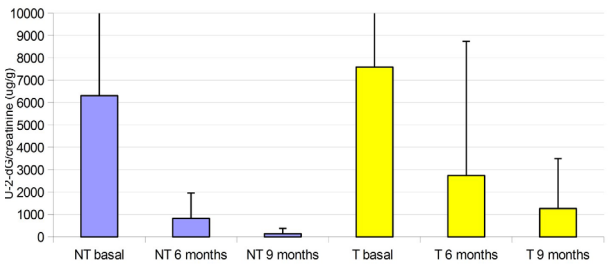

Figure 4. Mean values of the 2-dG/creatinine ratio in urine in treated (T) and not treated (NT) subjects at basal time, after 6 and 9 months.

authors have described some potential benefits of FPP supplementations like anti-inflammatory, antioxidant, immune-stimulatory and mRNA transcription inductor of antioxidant enzymes, we wanted to evaluate the effects of a prolonged assumption of this functional and nutraceutical food in PD. These effects were evaluated by means of a large panel of oxidative stress markers whose variations during time have been studied in relationship with the FPP treatment.

They were part of the panel markers of oxidizing power (d-ROMs and Hcy), antioxidant barrier (BAP, GSH, SOD, UA, BT), mitochondrial function (Q10), peroxidation of proteins (3-NT), lipids (CHOLOX and 4-HNE), and nuclear acids (urinary 8-OHdG, its corresponding not hydroxylated nucleoside 2-dG, the reciprocal respective ratio 8-OHdG/2-dG and their ratios versus creatinine and uric acid as previously reported) [12]. Furthermore, because of the several observations that in PD the iron levels are increased in brain, we have analyzed the plasma concentrations of Fe, FER and blood Hb to evaluate the iron metabolism.

The statistical data processing at the end of our study showed no evidence of significant treatment effects in many biomarkers and several time-trends effects probably attributable simply to the course of disease. Some analytes have not changed over time for either T or NT groups (Hcy, GSH-red, GSH-red/tot ratio, Q10, 4-HNE, REDOX balance, UA, Fe and BT). Statistically significant increases were instead observed for Hb and for U-UA/cr, but not different for T and NT subjects. The increase of urinary uric acid, the end product of purine catabolism, was an expected change in neurodegeneration [1, 18]. The slight increase in hemoglobin levels for both T and NT patients was quite surprising. Indeed, it has been previously suggested that anemia may be a risk factor for PD [49]. Even if the association between anemia experienced early in life and the later development of PD remain uncertain, it seems unlikely that in course of disease may be activated a physiological contrast process.

Even despite to previous observations [28] according to which we expected that the treatment with FPP would result in higher expression of the enzyme GSH peroxidase gene and in a consequent increase of GSH-red and of GSH-red/tot ratio, in our data these markers not change significantly over time. Keeping in mind the contemporaneous decrease in both groups of GSH-tot, statistically significant and compatible with a progressive increase of the oxidative insult in PD, the substantial invariance of GSH-red and GSH-red/tot ratio can be interpreted as a physiological counter-regulatory response mechanism that acts independent from FPP treatment.

During the nine months of observation, in both T and NT groups, SOD and BAP decreased, as expected in the hypothesis of a gradually reduced efficacy of the enzymatic antioxidant response and persistent overproduction of free radials in this degenerative disease. It is however difficult to explain the slight decrease in both groups of the total ROS, not justified by contemporary upgrades of the antioxidant barrier.

To understand the decreased levels of urinary 8-OHdG ratios *vs* creatinine and uric acid in both T and NT patients it is necessary to remember, that in neurodegenerative diseases, nuclear and mitochondrial DNA are probably the most biologically relevant target of oxidative attack. Although more than 20 different oxidative modifications of DNA bases have been identified, the major product of DNA impairment is 8-hydroxyguanine (8-OHGua) and its nucleoside 8-OHdG. As a consequence of the repair systems of DNA *in vivo*, the resulting 8-OHdG is excreted without further metabolism from cells and blood into urine. Thus, the urinary excretion of 8-OHdG may reflect the extent of oxidative DNA damage. Since measures of urinary levels of 8-OHdG mark the total oxidative damage of DNA, a reduction in its excretion rate may be interpreted as a positive event. Nevertheless, this reduction could result not only from a decreased oxidation of DNA bases (likely to be beneficial) but even from a diminished cellular turnover and DNA repair (likely to be detrimental), phenomenon already described in elderly patients with neurodegenerative pathologies.

To overcome the individual efficiency of reparing systems, some authors [50, 51] have previously proposed the expression of 8-OHdG as function of the corresponding not hydroxylated free base 2-dG [12, 15]. In order to obtain more biochemical information, we wanted to express 8-OHdG, not only *vs* 2-dG but also *vs* creatinine and uric acid in the purpose to take into account either the concentration of the urinary samples either the excretion of uric acid, the end result of purine nucleobases catabolism.

Despite to the persistence of oxidative damage on DNA caused by the disease, the observed decreases of 8-OHdG ratios during 9 months either in T and NT patients could show a tendency to a gradual slowdown of DNA turnover in both groups not counteracted by supplementation with FPP. On the contrary, the statistical analysis show that while in the NT patients 2-dG ratios continue to decline and 8-OHdG/2-dG to increase, in T subjects the increase of 2-dG both *vs* creatinine or uric acid undergoes a stabilization of the 8-OHdG/2-dG ratio compared to the baseline value. These significant trends in the two groups of patients are probably explained by the occurrence of two opposite phenomena in T and NT groups related to the FPP treatment.

Indeed, it is possible to hypothesize, that supplementation with FPP, through unclear molecular mechanisms, can strengthen the DNA turnover (understood as the sum of pathways of anabolism, catabolism and anabolic recovery of deoxynucleosides, called 'salvage') otherwise slowed because of the disease. As the treatment increase 2-dG levels and therefore the bioavailability of native nucleosides, FPP seems allow a counter-regulatory response of replacement of the altered nitrogenous base (8-OHdG) with native base (2-dG) through an enhancement of the metabolic pathway of nucleoside salvage.

In conclusion, the oxidative phenomenon in neurodegeneration has been widely documented and is therefore interesting to explore treatments that can prevent or slow its progression. FPP is proposed as a functional food that can modulate the physiological antioxidant response. Our work has shown that FPP

supplementation for medium-long time is able to positively affect the turnover of nucleic acids helping to restore the normal nitrogenous bases into nucleotide chains and to replace those altered by radicals attack. Further studies are however needed to understand if the efficacy of this supplementation can be demonstrated even in other cellular compartments or might results in the enhancement of other metabolic pathways with antioxidant effect.

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