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Molecular effects of fermented papaya preparation on oxidative damage, MAP Kinase activation and modulation of the benzo[a]pyrene mediated genotoxicity

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Abstract. The involvement of oxidative and nitrosative stress mechanisms in several biological and pathological processes including aging, cancer, cardiovascular and neurodegenerative diseases has continued to fuel suggestions that processes can potentially be modulated by treatment with free-radical scavengers and antioxidant. The fermented papaya preparation (FPP) derived from *Carica papaya* Linn was investigated for its ability to modulate oxidative DNA damage due to H_2O_2 in rat pheochromocytoma (PC12) cells and protection of brain oxidative damage in hypertensive rats. Cells pre-treated with FPP (50 μg/ml) prior to incubation with H_2O_2 had significantly increased viability and sustenance of morphology and shape. The human hepatoma (HepG2) cells exposed to H_2O_2 (50 μM) showed an olive tail moment of 10.56 $\pm$ 1.44 compared to 1.37 $\pm$ 0.29 of the solvent control. A significant reduction ($P \leqslant 0.05$) of DNA damage was observed at concentrations $\geqslant$ 10 μg/ml FPP, with 50 μg/ml FPP reducing the genotoxic effect of H_2O_2 by about 1.5-fold compared to only H_2O_2 exposed cells.

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Similarly, concentrations $\geqslant$ 50 μg/ml FPP significantly reduced DNA migration in co-treated cells compared with only the benzo[a]pyrene treated cells with a dose of 100 μg/ml FPP reduced the DNA damage 2-fold. The potential of FPP to regulate the phosphorylation status of ERK 1/2, Akt, and p38 was analyzed by Western blot analysis. FPP showed the potential to modulate the H_2O_2-induced ERK, Akt and p38 activation with the reduction of p38 phosphorylation induced by 250 μM H_2O_2 being more pronounced. Supplementation with FPP significantly inhibited the increased decay rate constant of the MC-PROXYL (a blood brain barrier permeable nitroxyl spin probe) ESR signal in the spontaneously hypertensive rat brain suggesting modulation of oxidative stress. These studies indicate that FPP can modulate oxidative injury supporting the view that prophylactic potentials in neurodegenerative diseases could be facilitated by FPP.

Keywords: Fermented papaya preparation, *Carica papaya* Linn, Alzheimer's disease, Parkinson's disease, oxidative DNA damage, p38 MAPK, inflammation, nutraceuticals, anti-aging supplement, immune-modulator

1. Introduction

Oxidative stress-induced cell damage has been implicated in a variety of cancers, diabetes, cardiovascular dysfunctions and neurodegenerative disorders such as stroke, Alzheimer's disease, and Parkinson's disease prompting the suggestion that the disease pathology can potentially be modulated by treatment with free-radical scavengers and antioxidant [3–5,14,26,39]. Strategies for such intervention and prevention require an understanding of the basic molecular mechanism(s) of prophylactic agents (dietary antioxidant factors from food plants and medicinal plants in this context) that may potentially prevent or reverse the promotion or progression of the diseases. Inflammation, cellular and redox signalling mechanisms play major roles in the pathophysiology of numerous disease states [3–5,14,22,26,39]. Extracts from food plants and medicinal plants continue to be used in herbal medicine practice for the treatment of many chronic or acute diseases, viral pathologies and as immune modulators. Fermented papaya preparation (FPP) is a biotechnology product of yeast fermentation of *Carica papaya Linn*. The principal compositions of FPP are shown in Table 1. The molecular mechanisms of the bioefficacy of FPP are being defined. Earlier studies have indicated that FPP had antioxidant functions *in vitro* and *in vivo* by virtue of its ability to inhibit lipid peroxidation and protect supercoiled plasmid DNA against ferric nitrilotriacetate (Fe-NTA) plus H_2O_2 induced single and double strand breaks and protecting human T-lymphocytes challenged with Fe-NTA/H_2O_2 [30]. That FPP could act as a macrophage activator as a result of its augmentation of nitric oxide synthesis and the secretion of TNF-α (a central regulatory cytokine in macrophage antimicrobial activity) has been suggested [31]. Interestingly, Carica papaya exhibits antibacterial activity that inhibits growth of gram-positive and gram-negative organisms, independent of the stage of the fruits maturity, a property that is highly relevant for the treatment of chronic skin ulcers whereby it promotes wound healing [10]. Marotta et al. [23] have demonstrated that FPP modulates atrophic and metaplastic changes of gastric mucosa in chronic atrophic gastritis patients. In this study, the protective effect of FPP on H_2O_2-induced cytotoxicity in PC12 cells [13] was investigated. PC12 cells have many advantages over primary cultured neuronal cells, including the homogeneity of the cell population. H_2O_2 treatment mimics oxidative stress, which triggers a decrease in cell viability. It was of interest to assess whether the ability of FPP to modulate the oxidative stress could be through the inhibition of the intracellular accumulation of ROS induced by the activation of the MAPKs gene cascade. Further, the genotoxic and antigenotoxic (chemopreventive potency) of FPP was assessed by examining its ability to induce DNA damage in HepG2 hepatoma cells using the DNA migration as a biological endpoint in the alkaline single cell gell electophoresis (SCGE) assay and contrasting with its ability to modulate the benzo[a]pyrene (BaP)-dependent DNA damage in human hepatoma (HepG2)

Table 1
Composition of FPP. FPP® is prepared using a biotechnology process adhering to the ISO 9001 and 14001 using non genetically modified (GMO) papayas. The composition analysis was carried out and authenticated by the Japan Food Research Laboratories, Nagoya, Japan

Component	Level of component per 100 g FPP
Moisture [vacuum oven method]	8.4 g
Protein[a]	0.3 g
Fat	< 0.1 g
Ash	< 0.1 g
Carbohydrates[b]	91.3 g
Energy[c]	366 kcal
Sodium	0.5 mg
Amino Acids	
Arginine	16 mg
Lysine	6 mg
Histidine	6 mg
Phenylalanine	12 mg
Tyrosine	8 mg
Leucine	18 mg
Isoleucine	10 mg
Methioneine	5 mg
Valine	14 mg
Alanine	13 mg
Glycine	11 mg
Proline	12 mg
Glutamic acid	40 mg
Serine	11 mg
Threonine	8 mg
Aspartic acid	23 mg
Tryptophan	2 mg
Cysteine	Not detected

[a]The nitrogen to protein conversion factor was 6.25.
[b]The formula used was 100-(Moisture + Protein + Fat + Ash).
[c]Energy conversion factors were in accordance with Notification No. 176 (2003) Standards for Nutrition Labeling, Ministry of Health, Labour and Welfare, Japan.

cells [17]. Increased oxidative stress burden in the brain can arise given that the brain has low levels of antioxidant enzymes, a high and constant oxygen requirement, contains membrane lipids rich in oxidizable polyunsaturated fatty acids and non-protein-bound Fe^{3+} in the cerebrospinal fluid and in brain tissues. Indeed earlier studies of Aruoma and colleagues reported elevated catalytic metal ions particularly copper ions in the cerebellum, median putamen and substantia nigra of post mortem PD brains [35]. The normal vs. PD values of 21 ± 10 vs. 29 ± 7 nmol/g tissue (cerebellum), 21 ± 16 vs. 56 ± 30 nmol/g tissue (median putamen) and 53 ± 48 vs. 63 ± 48 nmol/g tissue (substantia nigra) based on the assessment of 20–30 mg of post-mortem human brain tissues from the various regions were reported. Indeed the increased iron loading in the substantia nigra may participate in free radical production and neuronal dagmage [26,28,35].

Nitroxyl radicals are very useful as exogenous spin probes for measuring free radical distribution, oxygen concentration, and redox metabolism by *in vivo* ESR in biological systems. Given that the nitroxyl radicals lose their paramagnetism through a redox reaction when exposed to a reducing agent in biological systems, the signal decay rate of the nitroxyl radical gives evidence of free radical generation and changes of redox status in biological systems. This has led to the description of the technique

involving the blood brain barrier (BBB)-permeable nitroxyl spin probe 3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (MC-PROXYL) for the assessment of oxidative stress in the brain [19,24]. The spontaneously hypertensive rat (SHR), a model of essential hypertension, has several characteristics of increased oxidative stress. The ability of FPP to modulate oxidative stress in the brain of spontaneously hypertensive rats (SHR) was assessed using the MC-PROXYL -L-band ESR technique [19,24].

2. Materials and methods

2.1. Chemicals

Normal melting point agarose (NMA) was purchased from Merck (Darmstadt, Germany). Low melting point agarose (LMA) was from Boehringer Mannheim, Germany. Dulbeccos Minimal Essential Medium (DMEM) and fetal calf serum (FCS) were from PAA (Pasching, Austria). Hydrogen peroxide (p.a.) was obtained from Merck (Darmstadt, Germany). Gentamycin and benzo[a]pyrene ($>$ 98%) were from Sigma Aldrich (Steinheim, Germany). Fermented papaya preparation (FPP) (Osato Research Institute, Gifu, Japan) was dissolved in sterilized and filtered ($<$ 0.22 μm) double distilled water or in the appropriate tissue culture media prior to use.

2.2. Cell culture

The PC12 cells were a kind gift from one of the co-authors Dr. S. Coecke of JRC, ECVAM Unit, Ispra, Italy. Monolayer culture were grown in RPMI 1640 medium added with 10% of fetal horse serum, 5% fetal bovine serum, with both 100 U/ml of penicillin and streptomicyn (GIBCO BRL, Italy). The cells were grown at 37°C in an atmosphere of 5% CO_2 with more than 95% humidity [7]. HepG2 cells were kindly provided by Dr. Firouz Darroudi, University of Leiden Medical Centre, The Netherlands. The HepG2 cells were cultured in DMEM supplemented with 15% FCS and Gentamycin (50 μg/ml) in a 5% CO_2-atmosphere at 37°C.

2.3. Chemical treatment of cells and viability assay

The treatment of cells was performed in two different incubation periods: 1 h of pre-treatment with FPP and 23 h of incubation with H_2O_2 and 23 h of pre-treatment with FPP and 1 h of incubation with H_2O_2. Before every treatment old medium was replaced with fresh pre-warmed media. The MTT assay [3(4,5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide] assay was used to assess cell viability essentially as described in [7].

2.4. Cell area analysis

The cell area analysis was performed using the UTHSCSA Image Tool Kit on 3 independent experiments. 0.5×10^6 cells were plated onto a 25 cm^2 culture flask and allow to adhere and recovery from the trypsinization procedure for 24 h. Subsequently the cells were treated as described in the results section and photos were taken random from all flasks. Data were obtained by counting cell area from 100 cells for each replicates. A total of 5 random photos were analyzed.

2.5. Comet assay

The comet assay was performed as described in Singh et al. [34] and Tice et al. [38] with slight modifications as described in Colgnato et al. [7]. Fifty randomly selected cells (25 cells from each replicate slides were scored) per experimental point, and analyzed using a Comet Image Analysis System, Version 5.5 (Kinetic Imaging Ltd., Andor Bioimaging Division, Nottingham, UK). Results were reported as % of Tail DNA, which is indicative of the presence of DNA damage, expressed as mean of the 50 cells scored. Four independent experiments were performed. For the FPP/benzo[a] pyrene combination assays, the cells were treated with FPP solution (5–100 μg/ml) for 24 h prior to their exposure to benzo[a]pyrene (50 μM) for another 24 h. For co-treatment experiments with FPP and hydrogen peroxide, the cells were exposed to FPP (5–100 μg/ml) for 24 h and subsequently treated with hydrogen peroxide in a concentration of 50 μM for 30 min. DNA migration as an indicator of genotoxicity was evaluated as Olive tail moment (OTM) using the Optilas® Comet image analysing system. The viability of the cells was determined by the Erytrosin B method and only cultures with a viability of more than 80% were used for analysis (10^2 systematically screened cells per concentration were used). The reported OTM are mean $\pm$ standard deviation (S.D.) of three independent experiments. The effect of chemical treatment in comparison to the negative control (distilled water) on DNA migration was analysed using the student's t-test ($P \leqslant 0.05$).

2.6. Immunoblotting

After the treatment period the medium was removed from the flasks and the cells were rinsed with ice cold PBS. Cells were then lysed in ice-cold lysis buffer pH 7.5 (20 mM Tris pH 7.5, 150 mM NaCl, 10 mM EDTA, 10 mM EGTA, 1% Triton X-100, 2.5 mM NaPP, 1 mM β-glycerolphosphoate, 10 mM Na_2VO_3, 100 mM, and 1 mM PMSF). Western blot analysis on some MAPKs protein cascade genes: ERK 1/2, Akt and p38 essentially as described in [24]. All results are expressed as mean standard error of the mean (S.E.M.). Statistical analysis, where reported, are performed by χ^2 square statistical analysis for the viability test assay and by one-way ANOVA for the cell area analysis. Differences were considered statistically significant at $P < 0.05$.

2.7. Effect of FPP on SHR rats

The spontaneously hypertensive rat (SHR), a well characterized high oxidative stress animal model [32, 37], was used to study the effect of FPP on oxidative damage in the brain. Rats were fed standard diet or diet containing FPP for 12 weeks. The study involved use of isolated brain of the spontaneously hypertensive rats (SHR) and 3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine-1-oxyl (MC-PROXYL), a blood brain barrier (BBB)-permeable nitroxyl spin probe with application of the L-band ESR technique essentially as described in [19,24] to assess the effect of FPP. Protective effect against oxidative stress was assessed using an L-band ESR spectrometer and measuring the decay rate constant of MC-PROXYL in the isolated brain of SHR [19,24] fed with the standard feed and that of SHR fed with the FPP supplemented feed (FPP-SHR).

3. Results

3.1. Effect of FPP on H_2O_2-mediated oxidative damage in PC12 cells

The cytoprotective effects of FPP was analyzed in PC12 by pre-incubating the cells for a total period of time, including the H_2O_2 treatment, of 24 h: 23 h with FPP followed by 1 h of H_2O_2 incubation

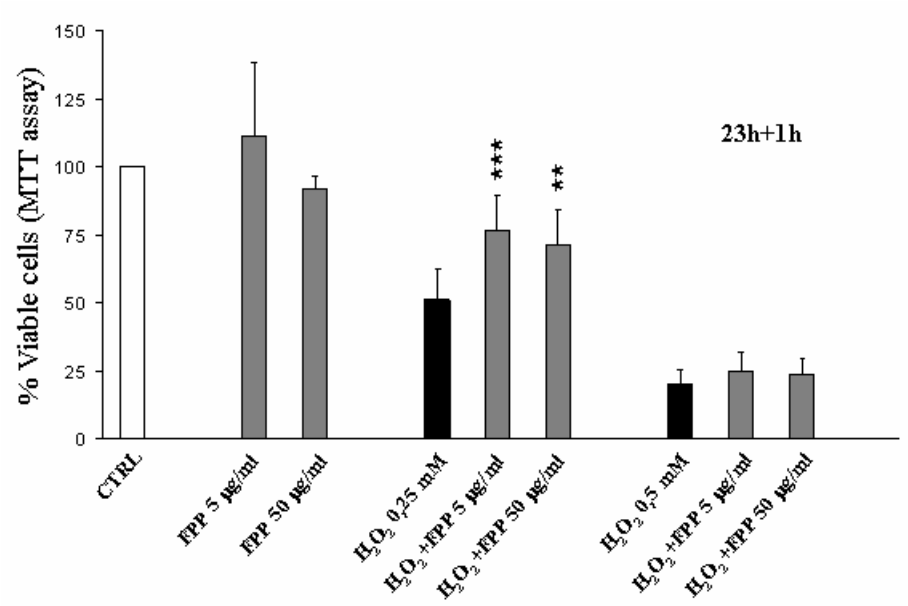


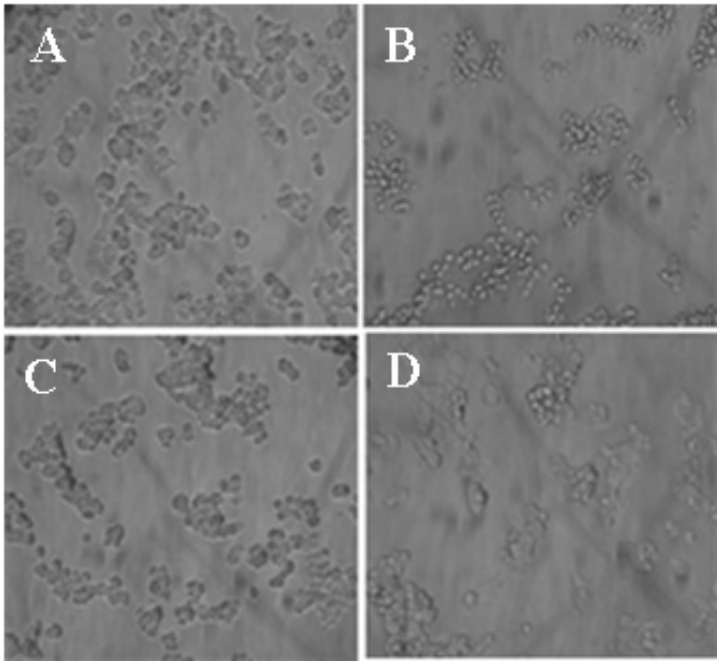
Fig. 1. Protective effects of FPP on H_2O_2-induced cytotoxicity in PC12 cells. PC12 cells were treated with 5 and 50 μg/ml of FPP for 23 h followed by 1 h H_2O_2 incubation with both 250 μM and 500 μM concentrations (23 h + 1 h total incubation time). Viable cells were determined using the MTT reduction assay for n = 4 independent experiments. Values are expressed by means ± S.E.M. *** indicates $P < 0.001$ performed by χ^2 square statistical test.

(23 h+1 h) (Fig. 1). The FPP afforded an increase of 25% following the 1 h incubation with 250 μM H_2O_2 for 1 h and 23 h of pre-treatment with 5 and 50 μg/ml of FPP (Fig. 1), which was statistically significant. There was a clear difference both in the cell shape and in the percentage of the adhering cells between samples treated with H_2O_2 alone and the samples where FPP was added (Fig. 2A). The difference in the respective cell area (μm^2) (Fig. 2B) was calculated using the UTHSCSA Image Tool. The Comet assay was used to assess the effect of FPP on the H_2O_2-induced oxidative DNA damage. Pre-incubation of PC12 cells with FPP decreased the extent of DNA damage at both the concentration of H_2O_2 tested but this did not reach statistical significance, a point that is dependent on the dose and the time of use of the prepared sample (data not shown).

3.2. Effect of FPP on benzo[a]pyrene and H_2O_2 -mediated DNA migration on human HepG2 hepatoma cells

No genotoxic effect was observed in experiments with only FPP exposed HepG2 cells in a concentration range of 5–100 μg/ml. Figure 3(A) and (B) show the OTM of FPP/B[a]P and FPP/ H_2O_2 co-treated cells as a measure of DNA damage in the SCGE/HepG2 model. In Fig. 3(A), the OTM of B[a]P-treated (positive control, 50 μM) HepG2 cells was 5.11 ± 0.74; the corresponding OTM of cells exposed to the solvent control was 1.28 ± 0.54. A significant ($P \leqslant 0.05$) reduction of DNA migration in co-treated cells could be observed in concentrations $\geqslant$ 50 μg/ml FPP compared to only B[a]P treated cells. A dose of 100 μg/ml FPP reduced the DNA damage 2-fold compared to only B[a]P-treated cells. The H_2O_2 (50 μM) exposed HepG2 cells showed an OTM of 10.56 ± 1.44 compared to 1.37 ± 0.29 of the solvent control (Fig. 3B). A significant reduction ($P \leqslant 0.05$) of DNA damage was observed in concentrations

(A)



(B)

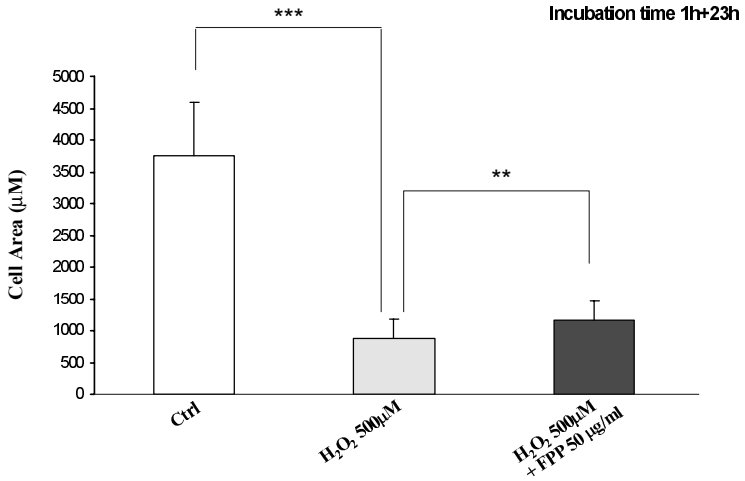


Fig. 2. (**A**) Protective effect FPP on the H_2O_2-induced change in cell morphology. (A) PC12 control batch; (B) 23 hr incubation with H_2O_2 (500 μM) (C) 23 hr incubation with H_2O_2 (500 μM) followed by 1 h of FPP treatment (50 μg/ml), (D) 23 hr incubation with H_2O_2 (500 μM) followed by 1h with ergothioneine (500 μM) as a positive control. Ergothioneine inhibit cell death caused by H_2O_2 (Aruoma et al. [2]). (**B**) Graphical representation of FPP protective effect on the H_2O_2-induced change in cell morphology. 0.5×10^6 cells were treated with H_2O_2 (500 μM) for 23 h preceded by 1 h of FPP treatment (50 μg/ml). Data were obtained by counting cell area (μm^2), using the UTHSCSA Image Tool Kit, from 100 cells for each replicates. A total of 5 random photos were analyzed. Cell area was determined from n = 3 independent experiments. Values are expressed by means ± S.E.M. *** $P < 0.001$; ** $P < 0.01$ by ANOVA test.

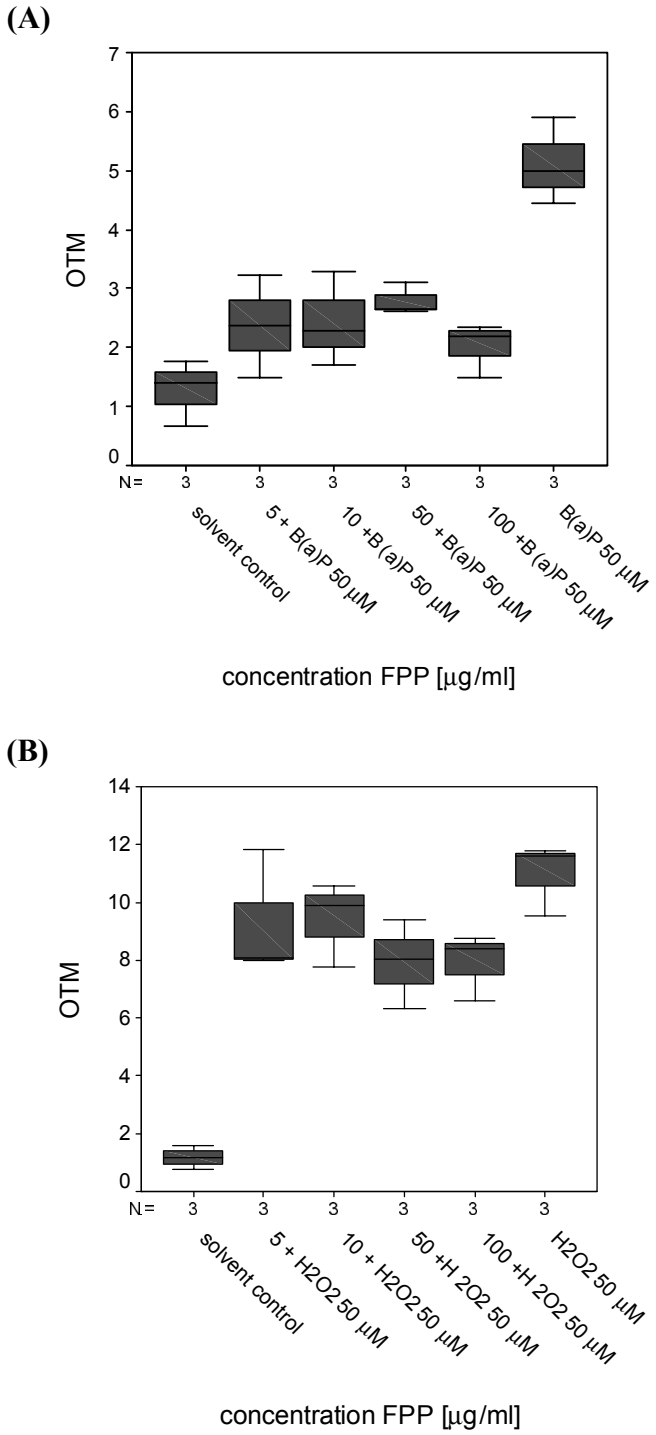


Fig. 3. (**A**) OTM of HepG2 cells exposed to FPP (5–100 μg/ml) for 24 h and Benzo[a]pyrene (50 μM) for another 24 h (solvent control = double distilled and sterilized water). (**B**) OTM of HepG2 cells exposed to FPP (5–100 μg/ml) for 24 h and H_2O_2 (50 μM) for another 30 min (solvent control = double distilled and sterilized water).

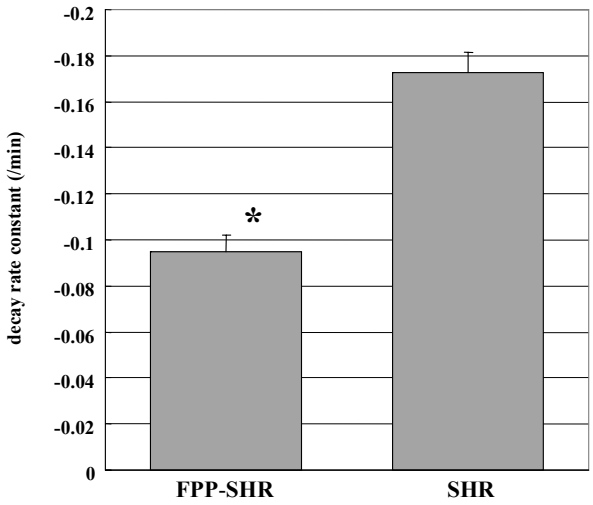


Fig. 4. Effects of FPP on L-band ESR signal decay rate constant of MC-PROXYL in the isolated brain of SHR after i.v. injection of MC-PROXYL [19,24]. Each column represents the mean (n = 4–6) ± S.D. *$p < 0.05$ vs. the corresponding value for the SHR group.

$\geqslant$ 10 μg/ml FPP. A concentration of 50 μg/ml FPP reduced the genotoxic effect of H_2O_2 about 1.5-fold compared to only H_2O_2 exposed cells. The FPP concentrations tested exhibited no relevant cytotoxicity and cell viability was always $\geqslant$ 80%. These data are consistent with the observations in studies with the PC12 cells.

3.3. Effect of FPP on oxidative stress in the isolated brain of SHR

The effect of FPP on SHR-induced oxidative stress in the brain was investigated using the spin probe MC-PROXYL with the resulting spectra analyzed with an L-band ESR spectrometer. Supplementation of SHR rats with FPP significantly inhibited the increased decay rate constants of MC-PROXYL in the isolated SHR brain, suggesting that FPP reduced the oxidative stress in the SHR brain (Fig. 4).

3.4. Effect of FPP on MAP kinase activation

Oxidative stress clearly induces cell death in a variety of cell types and this involves the modulation of intracellular signalling pathway. Here the role of the mitogen-activated protein kinase subfamilies in the H_2O_2-induced cell death in PC12 cells was investigated with the view of establishing the protective effects of FPP. Western blot analysis on some MAPKs protein cascade genes: ERK 1/2, Akt and p38. Although a clear upregulation of the ERK phosphorylation was observed for H_2O_2 (1h incubation), the 23 hr pre-treatment with FPP did not affect ERK nor Akt and p38 compared with the controls. Treatment with 500 μM H_2O_2 clearly increased the phosphorylation of Akt and p38 which was inhibited by FFP (Fig. 5A and 5B respectively).

4. Discussion

Links continue to be made between oxidative stress and increased risk of chronic diseases such as cancer, cardiovascular diseases, diabetes, hypertension, immune and neurodegenerative disorders as well

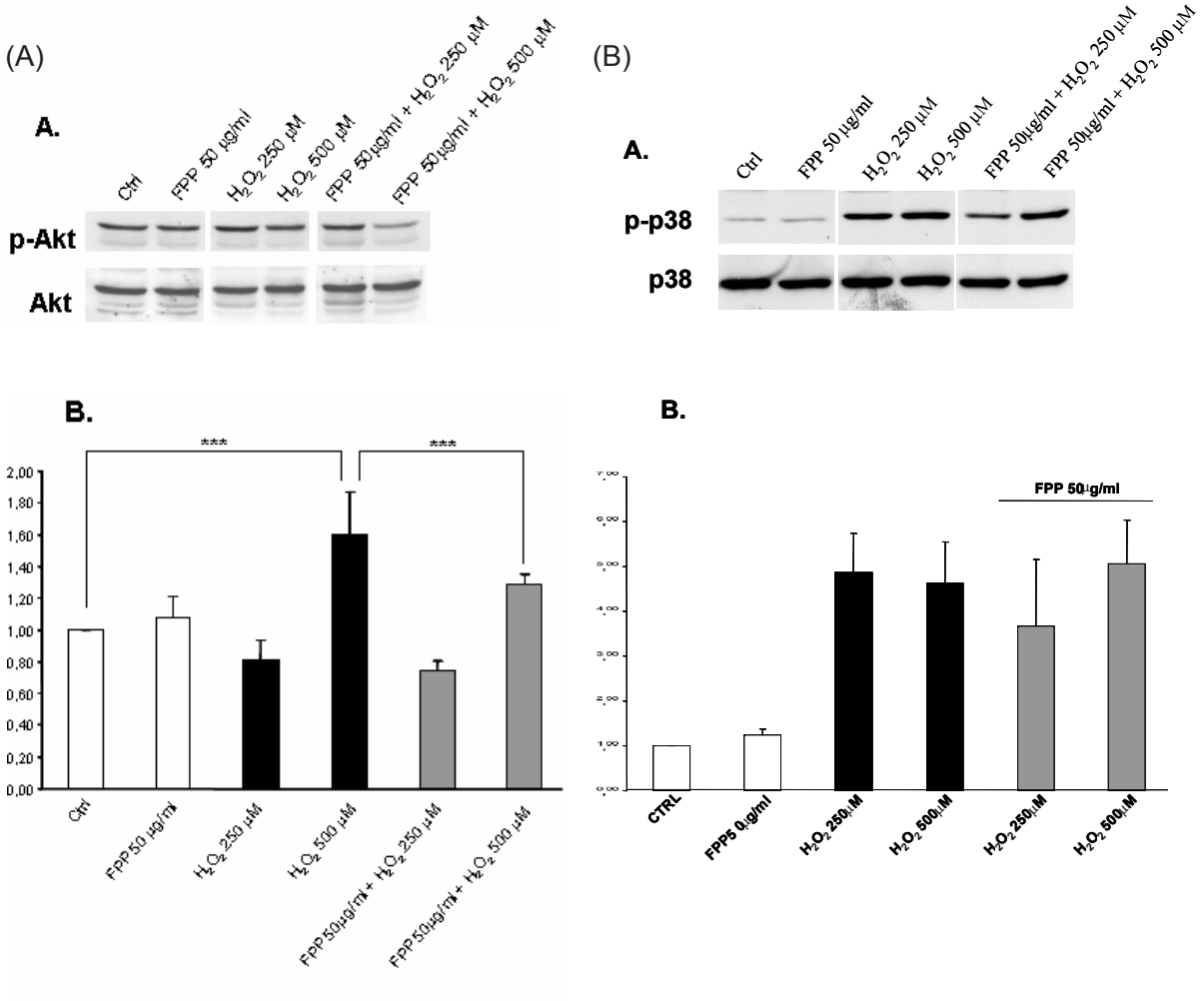


Fig. 5. (**A**) Western blot analysis of Akt phosphorylation. (A) Western blots of Akt phosphorylation. (B) Graphical representation of the inhibitory effect of a 23 h pre-treatment with FPP (50 μg/ml) followed by a 1 hr H_2O_2 (250 μM and 500 μM) induced on Akt phosphorylation. (**B**) Western blot analysis of p38 phosphorylation. (A) Western blots of p38 phosphorylation. (B) Graphical representation of the inhibitory effect of a 23 h pre-treatment with FPP (50 μg/ml) followed by a 1hr H_2O_2 (250 μM and 500 μM) treatment on p38 phosphorylation. Data represent the O.D. normalized to control of n = 4 independent experiments. Values are expressed by means $\pm$ S.E.M; ***indicates $P < 0.001$.

as for the general ageing process [3–5,14,22,26,39]. Indeed dietary antioxidants and components of fruit and vegetable extracts are increasingly suggested to have the capacity to significantly contribute to the modulation of the complex mechanisms of these diseases. The view being that they may be essential in optimizing in vivo antioxidant defenses. Defining if the presence of oxidative stress in various diseases and how these could be attenuated by the administration of antioxidant compounds in food plants coupled with establishing the relationship to the presence of particular genetic polymorphism and modulation of the complex cell signalling cascades involving gene transcription remains a major future scientific challenge [3,4]. Marchetti and Abbracchio [22] have argued that "novel therapeutic approaches must rely on potentiation of endogenous anti-inflammatory pathways and a combination of treatment involving

immune modulation and anti-inflammatory therapies" It was then of interest to assess the ability of FPP to modulate the oxidative stress, to attest if this could be through the inhibition of the intracellular accumulation of ROS induced by the activation of the MAPKs gene cascade and including the assessment of its genotoxic and antigenotoxic (chemopreventive potency) potentials.

FPP rescued the apoptotic cell death in PC12 cells induced by H_2O_2 at the 250 μM and 500 μM concentrations tested but the effect was more pronounced at the lower concentration of H_2O_2. The FPP at the 23 h of pre-treatment and 1h incubation with 250 μM of H_2O_2 was able to rescue the change in the cell morphology and shape. The high S.E.M. in the control cell batch and the samples where FPP was added, suggests the presence of a wide heterogeneity of cell shapes and areas. However, the low S.E.M. in the positive control suggests homogeneity of the cell morphology. The partial recovery suggests the involvement of signalling mechanisms which may be due to the activation of specific proliferative or mitogen-activated protein kinases (MAPKs) pathways, by itself, or through the inhibition of the H_2O_2 activated apoptotic/necrotic pathways. The family of mitogen-activated protein kinases (MAPK) play central roles in the signalling pathways of cell proliferation, differentiation, survival, and apoptosis [36]. The major enzymes belonging to this family are the extracellular signal regulating kinase 1/2 (ERK1/2 or p44/42 MAPK), c-Jun N-terminal kinase (JNK), and p38 MAPK, which are activated in response to a variety of extracellular stimuli. p44/42 MAPK is predominantly activated by mitogens through a Ras/Raf/MEK signalling cascade leading to cell growth and survival [11]. The JNK and p38 MAPK are preferentially activated by pro-inflammatory cytokines and oxidative stress resulting in cell differentiation and apoptosis [6,27]. The inflammation mechanisms in Alzheimer's disease and stroke have been postulated to be regulated in part by activation of the p38 pathway [8,16,18,33]. The potential of FPP to regulate the phosphorylation status of ERK 1/2, Akt, and p38 was analyzed by Western blot analysis. FPP showed the potential to modulate the H_2O_2-induced ERK, Akt and p38 activation with the reduction of p38 phosphorylation induced by 250 μM H_2O_2 being more pronounced. However, studies are continuing in order to clarify the concentration dependence of the effect of FPP.

Attack of $^{\cdot}OH$ radicals on DNA lead to fragmentation, base loss, and strand breakage with a terminal sugar residue fragment [1,12,14,29]. FPP was able to reduce the extent of the H_2O_2-induced DNA damage and was able to reduce the extent of DNA migration in the B[a]P-treated cells. No genotoxic effect was observed in experiments with only FPP exposed to HepG2 cells nor was FPP toxic to the PC12 cells. Indeed the LD50 for FPP has been estimated to be 29.24 ± 0.64 g/kg of adult body mass. An acute toxicity testing for FPP on Himedaka fish (measuring 1.9 cm and weighing 0.2 g) where the fish swam for 96 hr in 1 g/L FPP water showed no sign of adverse effects, attesting to the safety of FPP.

The spontaneously hypertensive rat (SHR) is a model of essential hypertension that show several characteristics of increased oxidative stress [32,37]. The decay rate constant of MC-PROXYL in the isolated brains of SHR has been shown to increase in the brain of normal male Wistar Kyoto rats (WKY). Indeed supplementation of SHR animals with FPP significantly inhibited the increased decay rate constant of the MC-PROXYL ESR in the SHR brain (Fig. 4) indicating clearly that oxidative stress level in the SHR brain was reduced by FPP. An accepted hypothesis of the mechanism of hypertension is that excess superoxide radical ($O_2^{\cdot -}$), by interacting with endothelial nitric oxide ($^{\cdot}NO$), could contribute to increased vascular smooth muscle contraction (in a reaction mediated by peroxynitrite, $ONOO^-$) and hence cause the elevated total peripheral resistance [9,15,20,21,25]. It is widely suggested that $O_2^{\cdot -}$ contributes to increased systemic vascular tone in SHR [32,37]. Thus FPP reduced oxidative stress in the brain (Fig. 4), however the rate of increased mean arterial blood pressure was not significantly different between SHR and FPP-SHR groups suggesting that the involvement of complex mechanisms such as the signaling pathways. These studies indicate that FPP can modulate oxidative injury supporting the view that prophylactic potentials in neurodegenerative diseases and in particular diseases of overt inflammation.

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