Ferric Nitrilotriacetate Induced DNA and Protein Damage: Inhibitory Effect of a Fermented Papaya Preparation

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Abstract. The carcinogen Fe-NTA catalyzes the hydrogen peroxide-derived production of free radicals and possibly acts through a mechanism involving oxidative stress. Fermented papaya preparation (FPP) has been reported as a natural antioxidant able to prevent lipid peroxidation in vitro and in vivo. However, little is known about the antioxidant properties of FPP regarding iron-mediated oxidative damage to DNA and proteins. In the present study FPP protected supercoiled plasmid DNA against Fe-NTA plus H2O2 induced single and double strand breaks. Similar protective effects of FPP were evident when human T-lymphocytes were challenged with Fe-NTA/H2O2 and DNA damage was determined using the Comet assay. Fe-NTA/H2O2 also induced fragmentation of bovine serum albumin (BSA) in vitro and depleted cellular GSH levels in lymphocytes. BSA fragmentation and GSH depletion were dose dependently counteracted by FPP. EPR spin trapping studies demonstrated that antioxidant properties of FPP are related to both hydroxyl scavenging as well as iron chelating properties.

Redox cycling is a characteristic of transition metals including iron, which is centrally involved in the generation of reactive oxygen species. Redox reactive iron is generally present in a weakly chelated form (1). Nitrilotriacetic acid (NTA) is a synthetic aminotriacetic acid, which forms water-soluble complexes with iron at neutral pH. NTA is a constituent of various domestic and hospital detergents and is a common water contaminant (2). A high incidence of renal cell carcinoma is prevalent in mice following repeated intraperitoneal Fe-NTA injections (3). The carcinogenicity of Fe-NTA seems to be associated with the interaction of NTA with Fe3+, since no tumor formation was observed by administra
tion of NTA alone (3). Fe-NTA has also been reported to induce severe hemochromatosis, diabetes as well as adenocarcinoma in laboratory animals (4). It is that the mechanism of Fe-NTA toxicity is mediated via the production of free radicals which in turn induce oxidative damage to lipids, proteins and DNA. Additionally, other reports have shown that Fe-NTA increases both hepatic ornithine decarboxylase and 3H thymidine incorporation possibly through the generation of oxidative stress (5, 6). It has been previously shown that Fe-NTA decreased antioxidant enzyme activities with the concomitant increase in the production of lipid peroxides and hydrogen peroxide (7). Antioxidants such as vitamin E and butylated hydroxyanisol, as well as the iron chelator desferrioxamine have been reported to partially prevent the toxic effect of Fe-NTA (8, 9). There is a growing interest in the utilization of plant extracts as dietary food supplements. A wide spectrum of beneficial activity for the human health has been advocated for such supplements due, at least in part to their antioxidant activity (10). More recently the ability of antioxidant nutrients to affect cell response and gene expression has been reported in vitro, providing a novel and different mechanistic perspective underlying the biological activity of plant derived nutriceuticals (11, 12, 13). Fermented papaya preparation (FPP) is made by yeast fermentation of Carica papaya Linn., Pennisetum purpureum Schum. and Schism edulis Swartz and is used as a natural food health supplement in different parts of the world. FPP has been shown to upregulate phorbol ester or zymosan-induced superoxide production in rat peritoneal macrophages (14), natural killer cell activity (15), and the level of IFN-γ in human blood (16). Recent studies of our laboratory demonstrated that FPP affects NO and hydrogen peroxide production as well as tumor necrosis factor alpha secretion in RAW 264.7 macrophages (17). Such evidence suggests a role of FPP as an immunomodulator. It has also been reported that FPP protects the brain of aged rodents in vivo challenged either by oxidative stress (18) or by ischemia-reperfusion injury (19). Furthermore, the accumulation of thiobarbituric acid reactive substances were found to be lower in heart homogenates from FPP supplemented rats exposed to peroxyl radicals as compared to non-supplemented.

Key Words: Ferric nitrilotriacetate, free radicals, DNA and protein damage, glutathione, fermented papaya preparation, Jurkat cells.
controls (20). From these reports it has been proposed that 
beside immuno-modulating FPP possess also antioxidant 
activities. However, the underlying mechanisms by which FPP 
acts as an antioxidant are largely unknown. In the present 
study both hydroxyl radical scavenging and iron chelating 
properties of FPP have been evaluated. Furthermore, the 
ability of FPP to combat Fe-NTA oxidative damage to DNA 
and proteins has been investigated both in vitro as well as in 
cultured cells.

Materials and Methods

Cell culture. Jurkat cells, a human acute leukemia T-cell line (clone E6-1, 
American Type Culture Collection, ATCC, Manassas, VA) were grown 
in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD) supplemented 
with 10% FCS, 100 μg/ml penicillin and 100 μg/ml streptomycin, 110 
mg/ml sodium pyruvate and 2 mM L-glutamine (Cell culture facility, 
University of California, San Francisco). Cells were maintained in a 
standard culture incubator with humidified air containing 5% CO2 at 
37°C. FPP (BionormizerTM). Osato Research Foundation, Japan) was 
dissolved in PBS (pH 7.4) and added to the Jurkat culture medium at the 
indicated concentrations.

Preparation of Fe3+.NTA solution. Fe-NTA solution was prepared 
according to the method of Awai et al. (21). Briefly, a solution of ferric 
nitrate dissolved in 1.0 N HCl was mixed with a 4-fold molar excess 
of NTA and the pH was adjusted to 7.4, using sodium bicarbonate.

Single and double DNA strand breaks. The reaction mixture (15 μl final 
volume, adjusted with distilled water) for assaying Fe-NTA plus H2O2 
induced plasmid DNA damage contained 0.1 μg superhelical pUC18 
DNA, 100 μM Fe-NTA and 100 μM H2O2 with and without various 
concentrations of FPP, in phosphate buffered saline, pH 7.4. 
The reaction was performed at 37°C for 30 min, and stopped by adding 10 
mM desferrioxamine. After addition of 3.0 μl 5% detergent solution 
to the reaction mixture, the sample was applied to a slot of a 0.8% agarose gel containing 0.5 μg/ml ethidium bromide, and 
electrophoresed for conformational analysis of DNA.

Comet assay. The reaction mixture (1 ml final volume, adjusted with 
PBS) containing 1×10⁶ Jurkat cells, 50 μM Fe-NTA, 50 μM H2O2 and various concentrations of FPP was incubated at 37°C for 60 min. 
The reaction was stopped by chilling the samples to 0°C and centrifugation at 
5,000 g for 5 min. Comet assay formation was determined with a test kit 
(Trevigen, Inc., MD) according to the manufactures protocol. Comets 
were evaluated by fluorescence microscopy. Cells were graded according to 
their DNA damage into 5 classes from undamaged (class 0), to 
maximally damaged, (class 4). A total damage score for the slide was 
derived by multiplying the number of cells assigned to each grade of 
damage by the numeric value of the grade and summing over all grades. 
Thus total score or 100 randomly selected cells could range from 0 (no 
damage) to 400 (corresponding to 100 cells of damage grade 4).

Assay for protein fragmentation. The ability of FPP to inhibit protein 
fragmentation was tested by incubation 1 mg/ml bovine serum albumin 
(Sigma) with 50 μM Fe-NTA, in phosphate buffer (pH 7.4) in the 
presence of increasing concentration of FPP. Reaction was started by the 
addition of hydrogen peroxide (2.5 mM final concentration). After 24 
hours of incubation at 37°C, portions of reaction mixtures were loaded on a 10% SDS-PAGE according to Hunt et al. (22). Protein fragments 
were detected by staining with Coomassie brilliant blue.

HPLC determination of glutathione. Cells were pelleted (125 x g for 5 
min) and deproteinized with 5% monochloroacetic acid. 

Following the acid treatment, the mixtures were snap-frozen in liquid 
nitrogen at -80°C for the HPLC determination of GSH. Samples were 
clutured from an Altima C18 250 mm, 4.6 ID, 5 μM column (Alttech, 
Dearfield, IL) with 100 mM monochloroacetic acid (pH 3.0) containing 
5% methanol and detected with a thin-column gold mercury column 
(23).

Electron paramagnetic resonance spectroscopy spin trapping measurements.

The antiradical activity of FPP was measured by the diphenylpyrydylhydrayl (DPPH) assay as described previously (24). 50 μM DPPH 
(Sigma Chemical Co., St. Louis, MO) was mixed with the indicated 
concentrations of FPP dissolved in phosphate buffer (50 mM, pH = 7.4). 
Hydroxyl radical scavenging activity of FPP was evaluated both in an 
iron independent (NP-III/UV) as well as in iron-dependent systems (Fe-
NTA plus H2O2, Fenton reaction). For the iron independent system 20 
mM NP-III (N,N-bis(2-hydroperoxy-2-methylethyl)-1,4,5,8, 
naphthalene-tetracarboxylic diimide) were irradiated by an UV lamp at 365 
nm for 1 min according to the procedure of Matsugo and co-workers 
(25). The reaction mixture for the Fe-NTA/H2O2 system contained 10 
μM Fe-NTA and 80 μM H2O2. The reaction mixture for the Fenton 
systems contained 25 μM FeSO4 and 50 μM H2O2. In all three systems 
the spin trap 5,5 dimethyl-1-pyrroline-N-oxide (DMPO) dissolved in 
phosphate buffer was used at a concentration of 200 mM. EPR spectra 
were recorded using an IBM ER 2000-SRC EPR spectroscopy 
(Danbury, CT). EPR spectrometer settings were as follows: central field 
3475 G; modulation frequency 100 KHz; modulation amplitude 2.0 G; 
microwave power 10 mW; scan width 200 G, gain 6.3 x 10⁶; temperature 
298 K.

Nuclear magnetic resonance spectroscopy. The 1H and 13C NMR spectra 
of FPP were recorded at room temperature on a Bruker Avance 400 
ICON NMR spectrometer (1H: 500 MHz, 13C: 100 MHz). FPP (10 mg) 
was dissolved in 700 μl of D2O and transferred to 5 mm phai NMR tube. 
Accumulation was carried out 16 times for 1H and 1024 times for 13C 
respectively.

Data presentation. Data in figures are the mean ± standard deviation 
(SD) of three different experiments performed in triplicate. One 
representative picture out of at least three is present regarding DNA 
single and double strand breaks, comet assay and BSA fragmentation.

Results

DNA damage in vitro and in T-lymphocytes. Untreated pUC18 
DNA was composed of abundant superhelical DNA (form I) 
and small amounts of nicked open circular DNA (form II). 
No linear form DNA (form III) was present. Plasmid DNA 
remained intact after incubation either with Fe-NTA, H2O2 or 
FPP alone (data not shown). The treatment of pUC18 
DNA with a mixture of Fe-NTA and H2O2 increased nicked 
open circular DNA with single strand breaks as well as the 
linear form with double strand breaks significantly. However 
FPP dose-dependently inhibited Fe-NTA plus H2O2 induced 
DNA damage (Figure 1A).

The effect of FPP on Fe-NTA plus H2O2 induced DNA 
damage was also investigated in a cellular system using the 
comet assay. In agreement with the plasmid DNA data, 
exposure of human T-lymphocytes to Fe-NTA plus H2O2 induced 
DNA damage as indicated by the comet formation (Figure 1B). Pretreatment of Jurkat cells with FPP 
decreased Fe-NTA plus H2O2 induced DNA damage as
Figure 1. A) Effect of increasing concentrations of FPP on DNA single and double strand breaks of superhelical pUC18 plasmid DNA induced by Fe-NTA/H₂O₂; B) Representative microphotographs of microgel electrophoresed T-lymphocytes treated without and with Fe-NTA/H₂O₂ in the absence and presence of FPP, DNA damage was measured with the comet assay; C) Relative score of damaged DNA in T-lymphocytes induced by Fe-NTA/H₂O₂ in the absence and presence of FPP as measured with the comet assay.
shown in Figure 1B. The corresponding DNA damage scores are given in Figure 1C.

Protein fragmentation in vitro and cellular GSH levels. Representative SDS-PAGE of BSA samples treated with Fe-NTA plus \( H_2O_2 \) in the absence and presence of FPP are shown in Figure 2A. Hydrogen peroxide or Fe-NTA alone resulted in no apparent change in the original BSA band (data not shown). However, in the presence of Fe-NTA plus \( H_2O_2 \) a marked fragmentation of the original BSA band was evident. FPP, at the highest concentration tested, partially attenuated Fe-NTA/\( H_2O_2 \) induced BSA damage.

Treatment of Jurkat cells with Fe-NTA plus \( H_2O_2 \) significantly depleted cellular GSH levels to about 40\% as compared to untreated control cells (absolute GSH concentration=2.20 nmol/10^6 cells). However, the supplementation of human T-lymphocytes with increasing concentrations of FPP dose-dependently counteracted Fe-NTA plus \( H_2O_2 \) induced GSH depletion (Figure 2B).

Radical scavenging activity of FPP. First, the ability of FPP to scavenge DPPH radicals was investigated. DPPH is a stable free radical (inset of Figure 3A) which can be detected by EPR. As shown in Figure 3A, DPPH radical formation was dose-dependently inhibited by FPP. A concentration as high as 25 mg/ml resulted in a 50\% (IC_{50}) inhibition of the DPHH signal.

In the next step hydroxyl radical scavenging activity of FPP has been studied in both iron independent (NP-III/UV) as well as iron-dependent systems (Fe-NTA/H_2O_2, Fenton) in order to discriminate between antioxidant and iron chelating properties of FPP. The typical EPR spectrum of the DMPO-OH spin adduct was detected in all three systems used (inset of Figure 3B). Concentrations of FPP scavenging 50\% of the hydroxyl radicals were 8.0 mg/ml (Fenton) and 45.0 mg/mL (Fe-NTA plus \( H_2O_2 \)) respectively. However, in the NP-III/UV system the highest concentration of FPP (60 mg/ml) resulted only in a 30\% inhibition of hydroxyl formation.

Discussion

A large number of dietary factors afford protection against carcinogenesis. Although the exact underlying mechanisms are not completely elucidated, many of the beneficial properties of chemopreventive molecules have been attributed to their ability to upregulate carcinogen metabolizing enzymes and/or to bind to carcinogens. Alternatively, various constituents of the human diet act as antioxidants thereby countering the increased formation of free radicals generated by the carcinogens (7). In this regard, the term nutraceutical has been recently established in order to describe nutrients able to significantly and positively affect human health (26).

In the present study Fe-NTA caused marked elevations of antioxidative damaged DNA and protein both in vitro as well as in human T-lymphocytes. It is believed that the toxicity of NTA is due to the binding of Fe^{3+} to form complexes, which in turn generate free radicals. The nature of reactive oxygen species, which induced DNA and protein modifications, was studied by EPR using DMPO as a spin trap. In accordance to previous finding by Ogino and Okada (1) no detectable EPR signal was evident in the absence of \( H_2O_2 \) (data not shown). However, in the presence of \( H_2O_2 \), Fe-NTA showed a typical four line spectrum with a 1:2:2:1 hyperfine splitting. Its hyperfine splitting constants (\( a_{HH} = a_{VV} \approx 14.9\) G) are the same as reported in the literature for the DMPO-OH spin adduct (27), suggesting that hydroxyl radicals are possible candidates for the Fe-NTA induced damage in the presence of \( H_2O_2 \). Furthermore, in this regard it has been demonstrated that hydroxyl radical scavengers such as DMSO, D-mannitol and ethanol (4) significantly counteracted oxidative damage due to Fe-NTA/\( H_2O_2 \) in vitro as well as in cultured cells. These findings in conjunction with the present results strongly support the hypothesis that DNA damage was mainly caused by hydroxyl radicals, generated by the reaction of Fe-NTA with \( H_2O_2 \). Protein molecules also can be targets of reactive oxygen species. Metal catalyzed oxidation of proteins is supposed to be a site-specific reaction and iron-binding sites have been postulated on protein molecules, where the metal-catalyzed protein damage is likely to occur (28). So far little is known about interactions between Fe-NTA and proteins. Under the conditions investigated Fe-NTA plus \( H_2O_2 \) caused significant BSA fragmentation. In a previous investigation it has been shown that Fe-NTA plus \( H_2O_2 \) caused bi-tyrosine formation and tryptophan decrease of BSA, suggesting that this damage is mainly related to free radical formation (1). Since cystolic peptides are candidates for iron mediated damage the effect of Fe-NTA plus \( H_2O_2 \) on GSH levels in T-lymphocytes has also been investigated. Fe-NTA plus \( H_2O_2 \) significantly depleted levels of GSH in Jurkat cells to about 40\% possibly due to an increased formation of oxidized glutathione. In addition to the present findings in Jurkat cells, it has been demonstrated that when injected to laboratory rats Fe-NTA lead a dose-dependent decrease in glutathione reductase and glutathione-S-transferase (29), enzymes, which are important for GSH synthesis. In contrast, \( \gamma \)-glutamyl transpeptidase activity was significantly increased by Fe-NTA. This enzyme catalyzes the degradation of GSH, which may lead to a higher accumulation of cysteiny1, glycine and cysteine. Elevated levels of these GSH metabolites have been suggested to enhance reduction of Fe-NTA to its ferrous complex, which in turn enhances oxidative cellular damage (30). Overall, present data as well as reports from the literature suggest that the depletion in cellular GSH levels induced by Fe-NTA/\( H_2O_2 \) might be related to both direct oxidation of GSH, to GSSG as well as modulation of key enzymes centrally involved in GSH homeostasis and turnover.

In the current study it has been clearly shown that FPP protected both in vitro as well as in human T-lymphocytes against Fe-NTA/\( H_2O_2 \) induced DNA and protein damage. The underlying mechanisms by which FPP combats oxidative stress in biological systems are not completely understood. Antioxidant action can be exerted through different
Figure 2. A) Representative SDS-PAGE of BSA samples treated with Fe-NTA/H₂O₂ in the absence and presence of increasing concentrations of FPP; B) Effects of FPP and Fe-NTA/H₂O₂ on glutathione levels in human T-lymphocytes.
Figure 3. A) *DPPH* radical scavenging activity of FPP; B) Effect of increasing concentrations of FPP on DMPO-OH spin adducts formed by iron independent as well as iron dependent hydroxyl radical generating systems.
mechanisms, e.g. metal chelation, radical scavenging as well as
electron donation (31). In order to get further insights into
FPP’s mode of action it was desirable to generate hydroxyl
radicals both with iron dependent as well iron independent
systems. In the present investigation the photosensitive
organic hydroperoxide NP-III has been used to generate
hydroxyl radicals in the absence of iron. NP-III is a novel
compound developed by our laboratory that generates
hydroxyl radicals upon irradiation with UV under controllable
experimental conditions (32). In combination with the Fenton
reaction it is thereby possible to distinguish between metal
chelating as well as radical scavenging activities of compounds
of interest. Under the conditions investigated, FPP acted as
an antioxidant due to both hydroxyl scavenging as well as iron
chelating properties. By comparing hydroxyl radical scavenging
activity of FPP between the Fenton and NP-III system (Figure 3) it can be concluded that iron chelating is an
important mechanism, which significantly contributes to the
antioxidant mode of action of FPP. Iron binding affinity of
FPP might prevent that Fe$^{3+}$ is being reduced back to Fe$^{2+}$,
which in turn promotes the formation of hydroxyl radicals via
Fenton reaction. Removal of Fe$^{2+}$ by FPP would thus further
reduce hydroxyl radical generation (33). However it should be
mentioned that relatively high concentrations of FPP were
necessary to combat Fe-NTA induced oxidative damage.
Moreover, it has not been clarified yet which particular
constituents of FPP are mainly responsible for its antioxidant
activity. NMR spectroscopy indicated that FPP contained no
aromatic protons or carbons (data not shown) thus anti-
oxidant properties of FPP seem not to be related to the
presence of flavonoids or flavonoid-like compounds. Further
studies are necessary to better characterize the active prin-
cipal(s) of FPP in terms of its antioxidant properties.

Collectively, present data demonstrate that Fe-NTA plus
H$_2$O$_2$ induce oxidative DNA and protein damage. Fur-
thermore Fe-NTA/H$_2$O$_2$ decreases antioxidant defenses, as
indicated by a significant fall in GSH levels in Jurkat cells.
FPP significantly blocked oxidative damage to DNA and
proteins probably both due hydroxyl radical scavenging as
well as iron chelating properties.

References

1 Ogino T and Okada S: Oxidative damage of bovine serum albumin
and other enzymes by iron-chelate complexes. Biochim
2 Anderson RL, Bishop WE and Campbell RL: A review of the environ-
mental and mammalian toxicity of nitrotriazocetic acid Critical
3 Li JL, Okada S, Hamazaki S, Ebina Y and Midoriwaka O: Subacute
nephrotoxicity and induction of renal cell carcinoma in mice treated
4 Sarker AH, Watanabe S, Seki S, Akiyama T and Okada S: Oxygen
radical-induced single-strand DNA breaks and repair of the damage
5 Iqbal M, Giri U and Athar M: Ferric nitrotriazocetate (Fe-NTA) is a
potent hepatic tumor promoter and acts through the generation of
6 Iqbal M, Sharma SD, Zadeh HR, Hassan N, Abdulla M and Athar M:
Glutathione metabolizing enzymes and oxidative stress in ferric
nitrotriazocetate (Fe-NTA) mediated hepatic injury. Redox Rep 2:
385-91, 1996.
7 Iqbal M and Athar M: Attenuation of iron-nitrotriazocetate (Fe-
NTA)-mediated renal oxidative stress, toxicity and hyperproliferative
response by the prophylactic treatment of rats with garlic oil, Food
8 Okada S, Hamazaki S, Ebina Y, Li JL and Midoriwaka O: Nephro-
toxicity and its prevention by vitamin E in ferric nitrotriazocetate-
9 Goddard JG, Basford D and Sweeney GD: Lipid peroxidation stimu-
lated by iron nitrotriazocetate in rat liver, Biochem Pharmacol 35:
2381-7, 1986.
10 Rice-Evans CA and Miller N: Antioxidant activities of flavonoids as
11 Virgili F, Kobuchi H and Packer L: Procyanidins extracted from
Pinus maritima (Pycnogenol): scavengers of free radical species and
modulators of nitrogen monoxide metabolism in activated murine
12 Pradja N, Singhal RL, Yeh YA, Olah E, Look KY and Weber G:
Linkage of reduction in 1-phosphatidylinositol 4-kinase activity and
inositol 1,4,5-trisphosphate concentration in human ovarian carci-
13 Csoyka B, Pradja N, Weber G and Olah E: Molecular mechanisms in
14 Osato JA, Korkina EG, Santiago LA and Afanas’ev IB: Effects of bio-
normalizer (a food supplementation) on free radical production by
human blood neutrophils, erythrocytes, and rat peritoneal macro-
15 Okada D, Omiami H, Zhou A, Osato A and Santiago LA: Studies on
16 Santiago LA, Uno K, Kishida T, Miyagawa F, Osato JA and Santiago
LA: Effect of BioNormalizer on serum components and immunol-
17 Kobuchi H and Packer L: Bio-normalizer modulates interfer-
gamma-induced nitric oxide production in the mouse macrophage cell
18 Santiago LA, Osato JA, Liu J and Mori A: Age-related increases in
superoxide dismutase activity and thiobarbituric acid-reactive sub-
stances: effect of bio-catalyzer in aged rat brain. Neurochem Res 18:
19 Santiago LA, Osato JA, Ogawa N and Mori A: Antioxidant protec-
tion of bio-normalizer in cerebral ischaemia-reperfusion injury in the
20 Marcocci L, D’Anna R, Yan JJ, Haramaki N and Packer L: Efficacy of
Bio-Catalyzer alpha,rho no.11 (Bio-Normalizer) supplementation
against peroxyl radical-induced oxidative damage in rat organ homo-
21 Awai M, Narasaki M, Yamani Y and Seno S: Induction of diabetes
in animals by parenteral administration of ferric nitrotriazocetate. A
22 Hunt JV, Simpson JA and Dean RT: Hydroperoxide-mediated
23 Allison LA and Shoup RE: Dual electrode liquid chromatography

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