Volume 12, No 2 | International Journal of Radiation Research, April 2014

Protective effect of ferulic acid on ionizing radiation induced damage in bovine serum albumin

K. Mishra[#], H. Ojha[#], S. Kallepalli, A. Alok, N. Kumar Chaudhury[*]

Chemical Radioprotector and Radiation Dosimetry Research Group, Division of Radiation Biosciences, Institute of Nuclear Medicine and Allied Sciences, Delhi-110054, India

[#] Contributed equally

► ***Original article***

**** Corresponding author:***
Dr. N. Kumar Chaudhury,
Fax: *+91 11 23919509*
E-mail:
nkcinmas@rediffmail.com

Received: Feb. 2013
Accepted: May 2013

Int. J. Radiat. Res., April 2014; 12(2): 113-121

ABSTRACT

Background: Ionizing radiation causes deleterious effects on living system mainly due to oxidative damages of macromolecules and protein is the major target due to its abundance. The aim of this study was to investigate the effects of ionizing radiation induced changes in the molecular properties of bovine serum albumin (BSA); its secondary and tertiary structures, degradation, cross linking and radioprotective role of ferulic acid, a natural antioxidant on these radiation induced changes. ***Materials and Methods***: This study was carried out to investigate the gamma radiation induced oxidative, structural damage of BSA and radioprotective efficacy of ferulic acid through SDS-PAGE, DTNB assay, DNPH assay, FOX assay methods. Hydroxyl radical scavenging capacity of ferulic acid was estimated using 2-deoxy ribose assay. Further, radiation induced changes in the anisotropy and excitation state lifetimes of BSA were examined. ***Results***: SDS –PAGE data suggested that the loss of protein was linearly dependent on the radiation dose. Gamma-irradiation of BSA caused the formation of protein carbonyls, hydroperoxides and loss of thiols . Ferulic acid protected the radiation induced loss of protein as well as reduced various oxidative damages. Ferulic acid protected the protein from radiation induced damages in a concentration dependent manner. ***Conclusion***: The results provide insight into radiation induced molecular changes in the protein. Ferulic acid protected the BSA from oxidative modification caused by radiation suggesting that ferulic acid possesses strong antiradical properties. Ferulic acid is known to protect DNA, the prime target of radiation and further its ability to protect protein suggesting its ability to protect different biomolecules and therefore can be a good candidate for development radioprotector.

Keywords: *BSA, protein carbonyl, radioprotection, ferulic acid.*

INTRODUCTION

Ionizing radiation generates free radicals by a variety of mechanism through radiolysis of water in cells and target all macromolecules DNA, proteins and lipids. As a result a plethora of damages, collectively called as oxidative damages in these biomolecules occur. Accumulation of radiation induced such oxidative damages can lead to cell death, tissue damages, organ dysfunction and systemic failures. Oxidative damages to biomolecules have been implicated in the pathogenesis of various diseases such as cancer, atherosclerosis, rheumatoid arthritis, infectious diseases and diabetes mellitus. It is believed that in many cases higher oxidative stress and occurrence of disease are correlated [1, 2]. Free radicals mediated oxidative damages of proteins result in their structural and functional impairment and increases proteolytic susceptibility [3].Oxidation

of amino acids in protein, formation of carbonyls and hydroperoxides etc. lead to structural changes, aggregation, cross linking and fragmentation in proteins [4-6]. Therefore, protein damage may implicate in initiation and propagation of several pathological conditions.

Although many synthetic and natural compounds were investigated for radioprotection considering DNA as a critical biomolecules in living cells [7, 8] but very few studies were attempted for protein damage. Several studies have suggested the use of natural dietary antioxidants as therapeutic or prophylactic agents against various diseases and also as a countermeasure to radiation induced effects on living system [7, 9, 10].

Ferulic acid (4- hydroxyl-3- methoxy cinnamic acid) (FA) is a natural phenolic compound produced from metabolism of amino acid (phenyl alanine and tyrosine) through shikimic pathway. Presence of extended conjugated aromatic structure along with electron donating ability of hydroxyl and methoxy substituted groups, carboxylic group with unsaturated C-C double bond provides strong antioxidant properties to ferulic acid [11]. Ogiwara *et al.* reported that ferulic acid scavenges free radicals viz., hydroxyl radical, superoxide and various oxygen centered and nitrogen centered radicals [12]. Khanduja *et al* reported antiapoptotic potential of ferulic acid in normal human peripheral blood mono nuclear cells [13]. In murine peripheral blood leukocytes both in pre-irradiation and post radiation administration of ferulic acid lowers gamma irradiation induced dicentric aberration on bone marrow and lipid peroxidation in cultured lymphocytes [14-18]. Therefore, above mentioned studies suggested the potential of ferulic acid as therapeutic agent against various ailments such as diabetes, cancer, alzheimer and parkinson [19] as well as in radioprotection.

The aim of the present study was to investigate the radioprotective efficacy of ferulic acid against gamma radiation induced oxidative damage of bovine serum albumin (BSA) using SDS PAGE and biochemical assays viz., 2, 4-dinitrophenylhydrazine (DNPH) 5, 5'-Dithio-bis (2-nitrobenzoic acid (DTNB) and FOX assays.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA, fraction V, approximately 99%; protease free and essentially g-Globumin free), Guanidine hydrochloride (GuHCl), trichloroacetic acid, xylenol orange (o-cresosulphononaphalene-3,3-bis-(sodiummethyliminodiacetate)), tris (hydroxymethyl) aminomethane (TRIS), acrylamide, tetramethylethylenediamine (TEMED), b-mercaptoethanol, coomassie brilliant blue, bromophenol blue, 2, 4-dinitro phenyl-hydrazine (DNPH) and 5, 5'-dithio-bis (2-nitrobenzoic acid), were purchased from Sigma chemical company (USA). Ferulic acid was from Acros Organics. Methanol, sodium dihydrogen phosphate dihydrate, anhydrous disodium hydrogen phosphate, sodium chloride (NaCl), hydrogen peroxide, ethanol, ethyl acetate, butyrate hydroxyl toluene (BHT) and ferrous sulphate were purchased from E Merck Germany. 2-deoxy ribose was purchased from Himedia (Mumbai, India). Milli Q grade (18 Mega ohm) water was used for the preparation of different reagents. (Millipore Corp USA, model Elix 3).

Sample preparation

BSA stock solutions (2mg/ml and 10 mg/ml) were prepared in 0.1M phosphate buffer (pH 7.6) and concentrations were quantified spectrophotometrically at 278 nm using extinction coefficient, $e^{1\%}$ 6.8. Stock solution of ferulic acid (10 mM) was prepared in phosphate buffer of pH 7.6. The molar extinction coefficient of ferulic acid in phosphate buffer at 310nm was determined as 18000 $M^{-1}cm^{-1}$ spectrophotometrically [20] and used for estimation of concentration. The working concentrations of ferulic acid were prepared by further dilution in 0.1 M phosphate buffer.

Biochemical assays viz., carbonyl and thiol determination, the working concentration of BSA was 5 mg/ml while for hydroperoxide assay it was 1 mg/ml. The samples were prepared by mixing BSA (10mg/ml and 2mg/ml) and phosphate buffer (pH 7.6) in the ratio 1:1 (v/v). Ferulic acid working solutions 20

μM and 200 μM were prepared and mixed in 1:1 volume ratio with BSA to obtain final concentrations of 10 μM and 100 μM respectively.

Gamma irradiation

BSA solution was prepared in borosilicate glass vials and irradiated at different doses upto to a maximum of 1200 Gy (dose rate, 1.4 k Gy/h) using ^{60}Co gamma ray irradiator, Model GC 5000, BRIT, Mumbai, India,). Protein samples containing 10 μM and 100 μM ferulic acid were irradiated at 400 Gy and 1000 Gy.

SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli [21]. Equal volume of the protein samples (1mg/ml) were loaded in each lane, resolved on a 12 % separation and 5% stacking gel and stained with Coomassie Brilliant Blue. Image was taken using Gel DOC system (Model GelDoc XR, Biorad, USA). Bands were quantitated by intensity densitometry using (quantity one) software. The D_0 doses were calculated from the semi log plot of radiation dose versus fraction of BSA remained intact. The equation for linear regression was $y = a\ e^{-bx}$. where y is equal to the fraction of BSA remain intact a and b are intercept and slope respectively. D_0 dose is defined as dose responsible for 37% damage in BSA of its initial concentration. [8].

Protein carbonylation assay

Carbonyl content of protein was analysed spectrophotometrically as described elsewhere [22]. Briefly 200μl of each sample was incubated with 200 μl of 10 mM of DNPH (prepared in 2N HCl) and incubated in dark for 1hour at room temperature and vortexed after every 10 minutes. The samples were precipitated by ice cold 20 % solution of TCA and centrifuges at 10000 rpm for 10 minutes. The pellets were washed three times with ethanol/ethyl acetate (1/1; v/v) to remove excess of DNPH reagent. The resulting protein pellet was dissolved in 600 μl of 6M GuHCl. Samples were mixed and the absorbance was measured at 370 nm against blank (GuHCl) as reference. The molar absorption coefficient of 22,000 $M^{-1}cm^{-1}$ was used to quantify the level of protein carbonyls. Protein of the samples was quantified by measuring absorbance at 278 nm. The amount of protein carbonyl groups were expressed in μ moles carbonyl per mg of BSA.

DTNB assay

The cystein/thiol content of proteins was determined by Ellman's reagent as described earlier [22]. Briefly 2 ml of each sample was mixed with 100 μl of DTNB (5mM) and incubated for 30 minutes at room temperature. Absorbance of each sample was measured at 412 nm against blank. Concentration of thiols was determined using extinction co-efficient of 13600 M^{-1} cm^{-1}.

FOX assay

Protein hydroperoxides of the samples were determined by FOX reagent as described earlier [22]. Fox reagents were prepared by mixing acidified ferrous sulphate (5 mM), water and xylene orange (5 mM) (1:2:2). Briefly, 200 μl of each sample was mixed with 100 μl of 10% trichloro acetic acid(TCA). Samples were kept at ice temperature for 30 minutes. The samples were stored at 0°C overnight. Samples were then centrifuged at 10000 rpm for 5 minutes. Protein pellets were washed thrice with 3 mM BHT (prepared in methanol). Samples were then dissolved in 500 μl of guanidium hydrochloride (6 M). 25 μl of above mentioned fox reagent was added to the sample and left for 30 minutes at room temperature. Absorbance was measured at 560 nm against blank containing guanidium hydrochloride. The concentrations of the hydroperoxides equivalent to the hydrogen peroxide were determined using the standard plot of hydrogen peroxide (0-50 μM) prepared in guanidium hydrochloride [22].

Determination of hydroxyl radical scavenging by ferulic acid

Hydroxyl radical estimation was performed using 2-deoxyriobose assay. 2 mM solution of 2-deoxyribose was prepared in 0.1 M phosphate buffer (pH 7.4). Different concentrations of ferulic acid10μM and 100μM were mixed and solutions were irradiated at 400 and 1000Gy. 1

ml each of TBA (1% prepared in 0.5 N NaOH) and TCA (10%) was added to 1 ml of the sample. The samples were heated at 95°C for 30 min in water bath. The samples were cooled to room temperature and absorbance was measured at 531 nm. Extinction co-efficient of MDA (1.56×10^5 $M^{-1}cm^{-1}$) was used to calculate the MDA concentration in each sample [10].

Statistical analysis

Origin 7.0 statistical software was used for statistical analysis. All the values were reported as mean ± SD of three or more experiments. These mean values were compared using ONE WAY ANOVA using tukey test and significant values of < 0.05 was considered significant.

RESULTS AND DISCUSSION

Gel Electrophoresis of BSA

SDS-PAGE is a sensitive method and has been used to monitor gamma irradiation mediated protein damage. Gamma irradiation may cause breakage of the polypeptide chains through oxidative damage of amino acids in peptide chains and protein damage can be visualized by the formation of fragmented bands and smearing of gel [23, 24]. Fig1a shows radiation induced damage of BSA up to 1200 Gy using SDS PAGE. Densitometry of bands indicates linearity in the loss of protein with increasing dose of radiation. Radiation doses of 1000 Gy and 1200 Gy caused about 60 % damage in protein (figure 1a). The D_0 dose for the BSA damage was calculated from the slope of semi log plot and the value obtained as 1250 Gy (figure 1b). The SDS profile in figure 1c showed protective effect of ferulic acid at two concentrations (10 and 100 µM) against gamma radiation doses of 400 and 1000 Gy. Therefore, it is inferred that ferulic acid has ability to reduce radiation induced protein degradation/loss.

SDS-PAGE profile of irradiated BSA was found to be in agreement with reported observations [24, 25]. Radiation causes breakage of covalent bonds, the effect of which is manifested in the SDS profile by the gradual disappearance of major band. Ferulic acid prevents the radiation induced BSA degradation by scavenging free radicals as evident from SDS-PAGE by the presence of intact band. The extent of degradation is dependent on both the radiation dose and concentration of protein [25]. Cho *et al* has showed that at 0.13 % and 0.41 % BSA concentration, the radiation effect was significant and clear aggregated pattern of protein bands appeared [26]. In the present study, 1mg/ml BSA (0.1 %) the SDS profile shows radiation effect of FA.

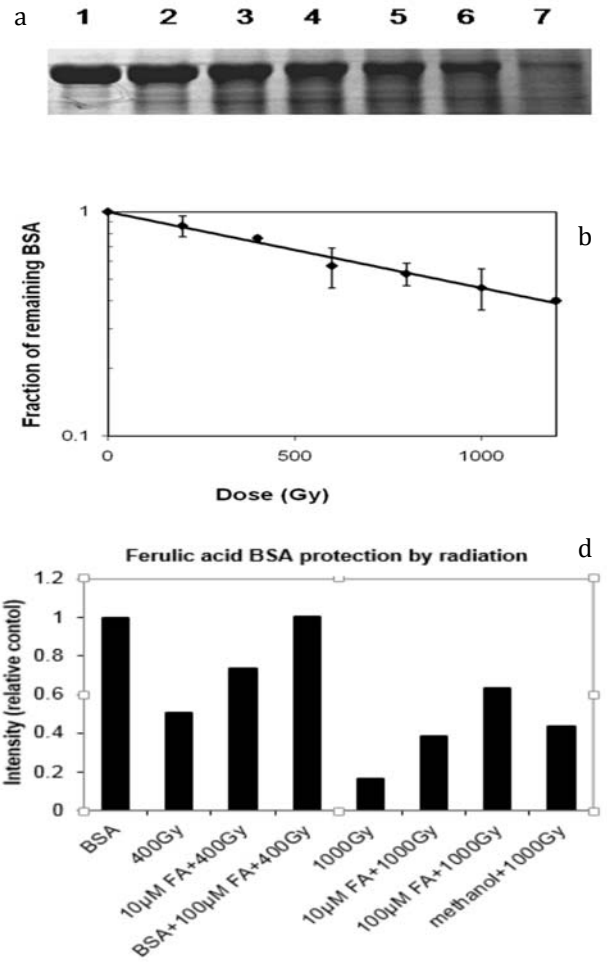
Protein carbonylation

Carbonyl content of protein was measured using spectrophotometer in all sample by dinitrophenylhydrazine (DNPH) labelling (figure 2). Radiation enhances the oxidation of proteins and causes formation of carbonyl groups. Protein carbonylation decreased in the presence of ferulic acid in irradiated solutions. Gamma irradiation of BSA at 400 Gy increased the carbonyl content from 1.05 µmol CO/mg to 2.5 µmol CO/mg of BSA while at 1000 Gy carbonyl content increased further to 4.21 µmol CO/mg of BSA .In presence of ferulic acid (10 µM) protein carbonyl formation decreased to 2.13 µmol CO/mg in samples irradiated at 400 Gy and to 2.76 µmol CO/mg in samples irradiated at 1000 Gy. Similarly ferulic acid (100 µM) decreased the carbonyl content to 2.4 and 2 µmol CO/mg of BSA at 400 Gy and 1000 Gy respectively.

Gamma-irradiation caused increase in the carbonyl content of BSA, [27] in irradiated sample carbonyl content increased upto 140 and 300 % at 400 and 1000 Gy respectively. Ferulic acid 100 µM decreased carbonyls level to almost un-irradiated control . While this decrease was relatively less at 10 µM FA, viz., 120 and 160% at 400 Gy and 1000 Gy respectively. Decrease in carbonyl content suggesting radioprotective effects of FA.

DTNB assay

BSA contains thiol groups in the form of cysteine residues; oxidation of these thiol groups is used as the indicator of protein modification. DTNB reacts with reduced thiols resulting in for-mation of 5-thio-2-nitrobenzoic acid which is



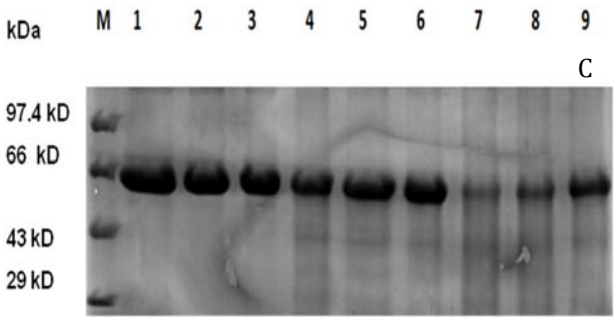


Figure 1. Representative SDS-PAGE profile of BSA irradiated at different dose of radiation with or without ferulic acid. The concentration of BSA was 1mg/ml. **a)** PAGE profile of BSA irradiated at different doses without ferulic acid. Lane 1 to 7 represents radiation dose 0, 200, 400, 600, 800, 1000 and 1200 Gy respectively.
b) Semilog plot of densitometric data obtained from figure 1a.
c) Representative gel image of BSA irradiated at 400 and 1000 Gy in presence of ferulic acid10 µM and 100 µM respectively. The lane descriptions are: Lane1, BSA (control); lane 2, BSA+ ferulic acid (10 µM); lane 3, BSA + ferulic acid (100 µM); lane 4, BSA+ 400 Gy; lane 5, BSA+ ferulic acid (10µM) + 400Gy; lane 6, BSA+ ferulic acid (100µM)+ 400Gy ; lane 7, BSA + 1000 Gy ; lane 8, BSA+ ferulic acid (10µM) + 1000Gy ; lane 9, BSA+ ferulic acid (100µM) + 1000Gy. M denotes molecular weight markers (Medium range protein marker, Bangalore Genie, India).
d) Relative quantitation of remaining BSA in each well using gel image shown in figure 1c.

quantified at 412 nm spectrophotomerically. Figure 3 shows the changes in the thiol content of irradiated and combination (irradiation with different concentrations ferulic acid) samples. The thiol content of protein decreased from 14.7 ± 1 µM to 1.5 ± 0.3 µM in irradiated solution. Ferulic acid at 10 µM and 100 µM protected thiol content of BSA from radiation induced damages in a concentration dependent manner and protected the level of thiols upto 3 ± 0.2 µM and 4.1 ± 0.4 µM respectively. Radiation dose (1000 Gy) did not further decrease the thiol content. Ferulic acid on the other hand protected thiol damage even at 1000Gy.

It is reported that thiol loss can be correlated to loss of activity of protein [27]. In the present study, ferulic acid provided significant protection against radiation induced oxidation of thiols groups in a concentration dependent manner.

FOX assay

FOX assay was performed to assess the radiation induced protein peroxide generation. Carbon centered radicals of protein participate in the formation of peroxyl radical upon irradiation. Once peroxyl radical is formed from side chain amino acid, it propagates further and damages other amino acids resulting in formation of hydroperoxides. Figure 4 shows increase in the yield of protein hydroperoxides when BSA solution was irradiated at 400 and 1000 Gy. Radiation dose of 400 Gy produced about 4.5 µM of hydroperoxides. Ferulic acid decreased the production of hydroperoxides to 3.7 and 3.3 µM at 10 µM and 100 µM respectively. Radiation dose of 1000 Gy produced around 6 µM of hydroperoxides. Ferulic acid decreased the production of hydroperoxides to 5.4 and 4.3 µM at 10 µM and

100 µM respectively. Ferulic acid decreased the formation of hydroperoxides in concentration dependent manner signifying the role of FA as potential antioxidant.

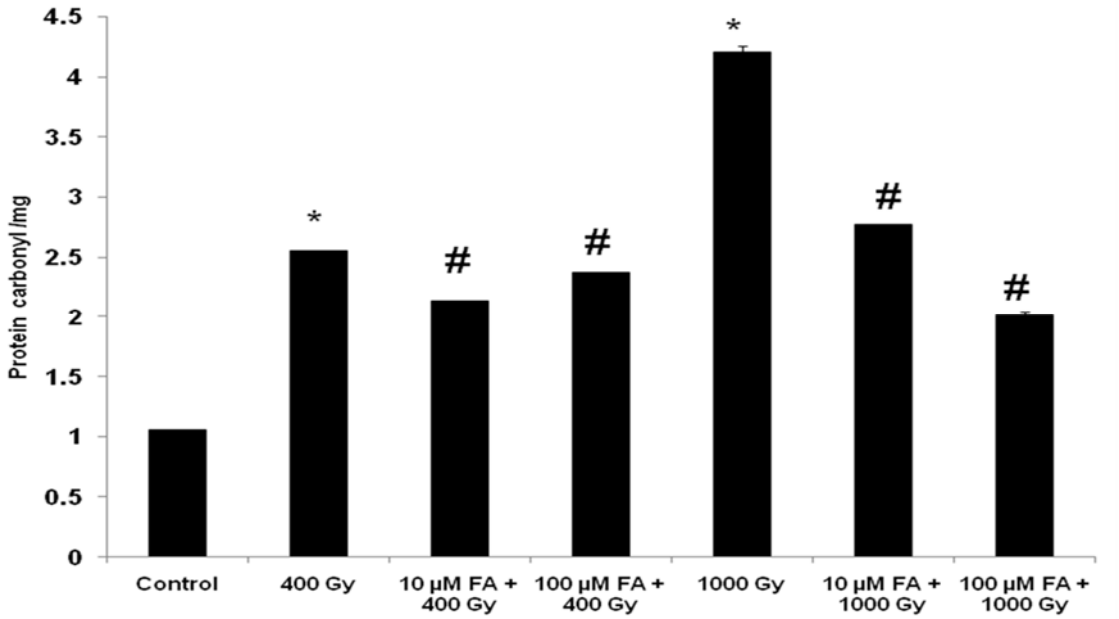


Figure 2. Irradiation (400 Gy and 100 Gy) induced changes in the carbonyl formation of BSA. Two different concentration 10 µM and 100 µM of ferulic acid were used. ferulic acid decreased the radiation induced carbonyl formation in concentration dependent manner. For details see material and methods. The data is the mean ± SD of three independent experiments. *control vs radiation, # radiation vs ferulic acid treated group. The data was significant at the $p < 0.05$.

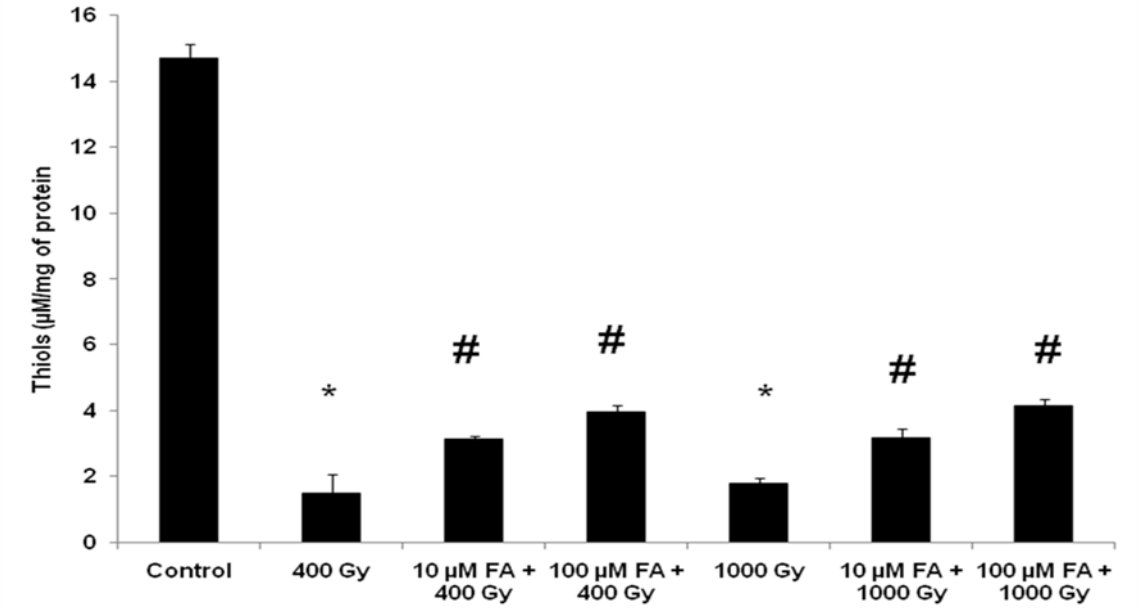


Figure 3. Irradiation (400 Gy and 1000 Gy) induced loss of protein thiols. Two different concentration of ferulic acid, 10 and 100 µM were used. Ionizing radiation caused decrease in the thiols formation. Ferulic acid protected the loss of thiole moieties. The data is the mean ± SD of three independent experiments. *control vs radiation, # radiation vs ferulic acid treated group. The data was significant at the p value of < 0.05.

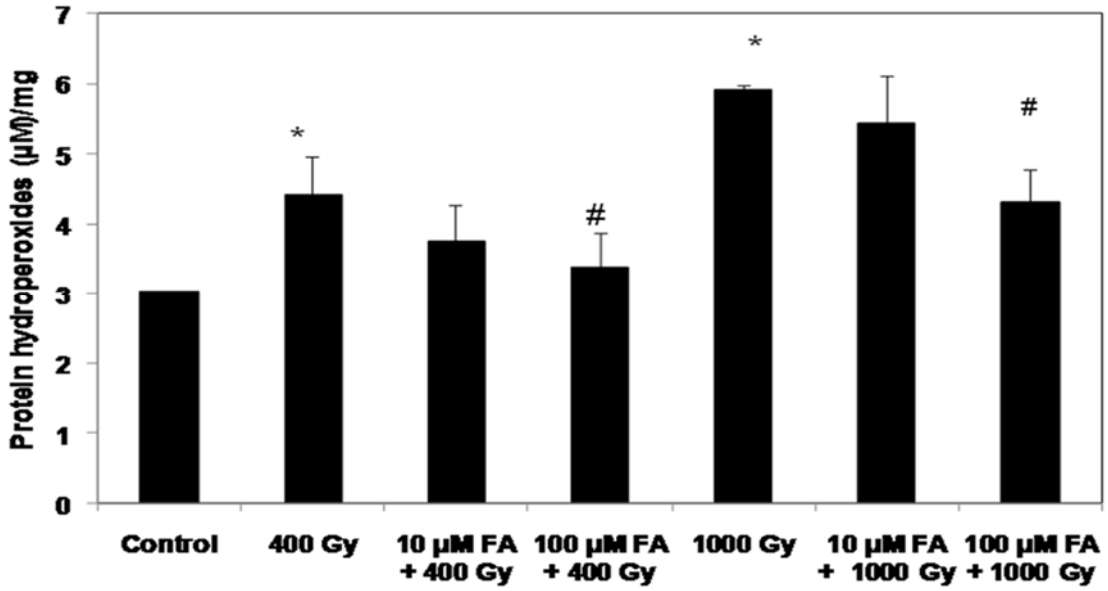


Figure 4. Irradiation (400 Gy and 1000 Gy) induced formation of protein hydroperoxides. All other experimental details were similar to figure 5. The data is the mean ± SD of three independent experiments. *control vs radiation, # radiation vs ferulic acid treated group. The data was significant at the p value of <0.05

Hydroxyl radical scavenging assay

Hydroxyl radical (˙OH) scavenging potential of ferulic acid was investigated by using 2-deoxy ribose degradation assay (figure 5). Hydroxyl radicals generated by radiolysis of water by ionizing radiation is the primary damaging agent for all biomolecules in living system. Antioxidant scavenges various free radicals including hydroxyl radicals. Ionizing radiation enhances generation of melonaldehyde (MDA) production in dose dependent manner. Radiation dose of 400 Gy and 1000 Gy produced 13 μM and 19 μM MDA respectively. Presence of ferulic acid decreased the production of MDA to 23% and 54 % at 10uM and 100uM respectively at 400 Gy. Similarly, ferulic acid in decreased the MDA concentration to 18 and 11 μM at 10 μM and 100 μM respectively in BSA irradiated at 1000 Gy.

Hydroxyl radical (˙OH)-mediated protein oxidation has received most attention due to its importance as hydroxyl radicals are highly reactive free radicals to protein. Hydroxyl radical upon reacting with protein may lead to formation of reactive carbonyl groups. Tryptophan, histidine and cystein are most susceptible to the hydroxyl radical mediated damage. Protein carbonyl derivatives may also be generated through oxidative cleavage of protein backbone at the site of a-carbon peroxyl radicals via amidation pathway or through oxidation of glutamine side chains, resulting in the formation of a peptide in which the N-terminal amino acids is blocked by an α-ketoacyl derivative [27, 28]. Similarly protein peroxide and breakage of thiol bonds are also related to reaction of OH radicals with protein, thereby inhibiting the OH radicals FA may reduce irradiation mediated protein damage. The OH radical scavenging data is supplemented with our another recently reported study where it was observed that ferulic acid scavenges DPPH free radical quite significantly with EC_{50} value of 22 ± 2.2 μM [29].

Binding of ferulic acid with serum protein

Besides radical scavenging, binding of antioxidant with protein may affect the stability of protein. It is reported in our earlier study that ferulic acid binds non-covalently to bovine serum albumin with binding constant (K_a) 40.15 ± 0.02 x10^4 M (30). Hydrogen bonding and electrostatic forces play a vital role in stabilization of ferulic acid-BSA complex. It was further observed through circular dichroism and thermal melting study that in the presence of ferulic acid melting temperature (T_m) of BSA

increased from 341 K for native BSA to 348 K in the presence of ferulic acid (10 μM). Therefore, ferulic acid on binding with BSA increases the protein stability. Similarly Kapoor *et al.* [31] have reported that non-covalent binding of another antioxidant curcumin with bovine serum albumin has shown inhibition of radiation induced protein damage. Since ferulic acid also interacts with BSA, binding of ferulic acid with BSA may be implicated in case of ferulic acid mediated radioprotection. Salvi *et al.* [32] reported structural damage to proteins such as lysozyme, human serum albumin (HSA) and beta-lactoglobulin A induced by free radicals. Authors have reported that out of four well-known antioxidants (quercetin, melatonin, Trolox, and chlorogenic acid), melatonin and chlorogenic acid, which did not bind to any of the three proteins under study, showed scavenging and protective activities well correlated with the amount of free radicals generated. Trolox, which binds only to HSA, was a better protector of HSA than of the two other proteins, indicating that its antioxidant capacity is increased by a shielding effect. These results demonstrate that binding of an antioxidant to a protein may potentiate protection or damage depending on the properties of the antioxidant.

CONCLUSION

Role of oxidized products of proteins as the source of causing toxicity or cell death in the system has been implicated. Further this study provides insight into the ferulic acid mediated radioprotection and antiradical efficacy of ferulic acid. This study suggested that both efficient ROS scavenging ability and binding interaction with BSA played an important role in the radioprotective efficacy of ferulic acid. The results can help in designing the applications for ferulic acid as a radiation protector even as for food preservation.

ACKNOWLEDEGEMENT

K. Mishra is thankful to UGC, Delhi for the senior research fellowship. The work was funded through a DRDO NBC project (1.29), Govt. of India.

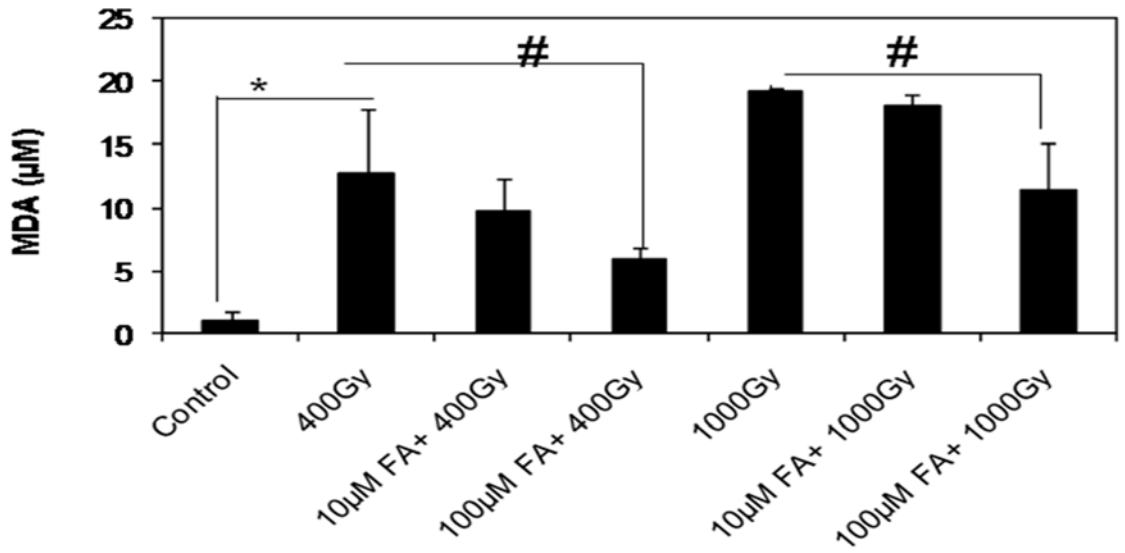


Figure 5. Radiation induced generation of hydroxyl radical and scavenging by ferulic acid. Ferulic acid decreased the radiation induced generation of hydroxyl radical in concentration dependent manner. 2-deoxyribose degradation assay were used to estimate the hydroxyl radical scavenging by ferulic acid. The data is the mean ± SD of three independent experiments. *control vs radiation, # radiation vs ferulic acid treated group. The data was significant at the $p < 0.05$.

REFERENCES

1. Finkel T and Holbrook NJ (2000) Oxidants, oxidative stress and the biology of ageing. *Nature,* ***408****:239-247.*
2. Taysi S, Polat F, Gul M, Sari RA, Bakan E (2002) Lipid peroxidation, some extracellular antioxidants and antioxidant enzymes in serum of patients with rheumatoid arthritis*. Int J Rheumatol,* ***21****: 200–204.*
3. Davies MJ (2005) The oxidative environment and protein damage*. Biochim Biophys Acta,* ***1703****: 93–109.*
4. Mayo JC, Tan DX, Sainz RM, Natarajan M, Lopez-Burillo S, Reiter RJ (2003) Protection against oxidative protein damage induced by metal-catalyzed reaction or alkylperoxyl radicals: comparative effects of melatonin and other antioxidants. *Biochim Biophys Acta,* ***1620****: 139-150.*
5. Pachala M and Schessler H (1993) Oxygen effect in the radiolysis of proteins. *Int J Radiat Biol,* ***64****:149-156.*
6. Kempner ES (1993) Damage to proteins due to the direct action of ionizing radiation. Q Rev Biophys, **26**:27-48.
7. Hosseinimehr SJ (2007)Trends in the development of radioprotective agents. *Drug Discovery Today,* ***12****:794-805.*
8. Mishra K, Srivastav PS, Chaudhury NK (2011) Sesamol as a potential radioprotective agent: an *in-vitro* study*. Radiat Res,* ***176****: 613-623.*
9. Ames BN, Shigenaga MK, Hagen TM (1993) Oxidants, antioxidants and the degenerative diseases of ageing. *Proc Natl Acad Sci USA,* ***90****: 7915-7922.*
10. Halliwell B and Gutteridge JMC (2007) Free radicals in biology and medicine.4th ed. UK, Clarendon Press, Oxford.
11. Graf E (2000) Antioxidant potential of ferulic acid. *Free Radic Biol Med,* ***28****: 1249–1256.*
12. Ogiwara T, Satoh K, Kadoma Y, Murakami Y, Unten S, Atsumi T, Sakagami H, Fujisawa S (2002) Radical scavenging activity and cytotoxicity of ferulic acid. *Anticancer Res,* ***22****:2711-2717.*
13. Khanduja KL , Avti PK, Kumar S, Mittal N, Sohi KK, Pathak CM (2006) Anti-apoptotic activity of caffeic acid, ellagic acid and FAin normal human peripheral blood mononuclear cells: A Bcl-2 independent mechanism. *Biochim Biophys Acta,* ***1760****: 283-289.*
14. Srinivasan M, Sudheer AR, Menon VP (2007) Ferulic acid: therapeutic potential through its antioxidant property. *J Clin Biochem Nutr,* ***40****: 92–100.*
15. 15. Prasad NR, Srinivasan M, Pugalendi KV, Menon VP (2006) Protective effect of ferulic acid on γ-radiation induced micronuclei, dicentric aberration and lipid peroxidation in human lymphocytes. *Mutat Res,* ***603****: 129–134.*
16. Maurya DK, Salvi VP, Nair CK (2005) Radiation protection of DNA by FA under *in-vitro* and *in-vivo* conditions. *Mol Cell Biochem,* ***280****: 209-217.*
17. Baskaran N, Manoharanm S, Balakrishnan S, Pugalendhi P (2010) Chemopreventive potential of FAin 7, 12-dimethylbenz[a]anthracene-induced mammary carcinogenesis in Sprague-Dawley rats. *Eur J Pharmacol,* ***637****: 22-29.*
18. Itagaki S, Kurokawa T, Nakata C, Saito Y, Oikawa S, Kobayashi M, Hirano T, Iseki K (2009) *In-vitro* and *in-vivo* antioxidant properties of ferulic acid: a comparative study with other natural oxidation inhibitors. *Food Chem,* ***114:*** *466-471.*
19. Middleton E, Kandaswami C, Theoharides TC (2000) The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease and cancer. *Pharmacol Rev,* ***52****:673-839****.***
20. Ojha H, Murari BM, Anand S, Hassan MI, Ahmad F, Chaudhury NK (2009) Interaction of DNA minor groove binder Hoechst 33258 with bovine serum albumin. *Chem Pharm Bull,* ***57****: 481-486.*
21. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* ***227****: 680–685.*
22. Hawkins CL, Morgan PE, Davies MJ (2009) Quantification of protein modification by oxidants. *Free Radic Biol Med,* ***46****: 965-88.*
23. Maire LM, Thauvette LD, Foresta B, Beauregard V, Potier AGM (1990) Effects of ionizing radiations on proteins. *Biochem J,* ***267****: 431-439.*
24. Lee Y and Song KB (2003) Effect of g-Irradiation on the Molecular Properties of Myoglobin. *J Biochem Mol Biol,* ***35*** *590-594.*
25. Silvester JA, Timmins GS, Davies MJ (1998) Photodynamically generated bovine serum albumin radicals: evidence for damage transfer and oxidation at cysteine and tryptophan residues*. Free Radic Biol Med,* ***24:*** *754-766.*
26. Cho Y, Yang JS, Song B (1999) Effect of ascorbic acid and protein concentration on the molecular weight profile of bovine serum albumin and b-lactoglobulin by g-irradiation. *Food Res Int,* ***32****: 515-519.*
27. Perricone N, Nagy K, Horváth F, Dajkó G, Uray I, Zs, Nagy I (1999) Alpha lipoic acid (ALA) protects proteins against the hydroxyl free radical-induced alterations: rationale for its geriatric topical application. *Arch Gerontol Geriatrics,* ***29****, 45–56.*
28. Stadtman ER (2006) Protein oxidation and aging. *Free Radical Res,* ***40****:1250-1258.*
29. Mishra K, Ojha H, Chaudhury NK (2012) Estimation of antiradical properties of antioxidants using DPPH assay: A critical review and results. *Food Chem,* ***130****: 1036-1043.*
30. Ojha H, Mishra K, Hassan MI, Chaudhury NK (2012) Spectroscopic and isothermal titration calorimetry studies of binding interaction of ferulic acid with bovine serum albumin. *Thermochim Acta,* ***548****:56-64.*
31. Kapoor S and Priyadarsini KI (2001) Protection of radiation-induced protein damage by curcumin. *Biophys Chem,* ***30;****119-126.*
32. Salvi A, Carrupt P , Tillement J Testa B (2001) Structural damage to proteins caused by free radicals: assessment, protection by antioxidants, and influence of protein binding. *Biochem Pharmacol,* ***61:****1237-1242.*