Luteolin ameliorates ferric nitrilotriacetic acid induced renal toxicity and tumor promotional response in rat

Sarwat Sultana*, Lakshmi Prasad & Tamanna Jahangir
Section of Chemoprevention and Nutrition Toxicology, Department of Medical Elementology and Toxicology
Jamia Hamdard (Hamdard University), Hamdard Nagar, New Delhi 110 062, India

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Ferric nitrilotriacetic acid (Fe-NTA) (9 mg Fe/kg body weight, ip) caused significant depletion in the detoxification and antioxidant enzyme armory with concomitant elevation in renal lipid peroxidation, serum toxicity markers viz. creatinine, blood urea nitrogen, hydrogen peroxide generation, ornithine decarboxylase activity and [3H] thymidine incorporation into renal DNA in wistar rats. However, pretreatment of animals with luteolin (10 and 20 µmol/kg body weight) for 7 consecutive days resulted in significant decrease in above parameters level. Renal glutathione content, glutathione metabolizing enzymes and antioxidant enzymes were also recovered to significant level. The enhanced reduced glutathione level and enzyme activities involved in xenobiotic metabolism and maintaining antioxidant status of cells is suggestive of a chemopreventive efficacy of luteolin against Fe-NTA mediated oxidative stress, toxicity and cell proliferation response in rats.

Keywords: Cell proliferation response, Chemoprevention, Ferric nitrilotriacetate, Luteolin

Natural polyphenols can range from simple molecules (phenolic acids, phenylpropanoids, and flavonoids) to highly polymerised compounds (lignins, melanins, tannins), with flavonoids representing the most common and widely distributed sub-group. Flavonoids have been the objective of various studies as far as their biological, pharmacological and chemical properties are concerned. Luteolin, a 3′, 4′, and 5,7-tetrahydroxyflavone is a polyphenolic compound available in foods of plant origin. It belongs to the flavones subclass of flavonoids, usually occurring as glycosylated forms in celery, green pepper, perilla leaf and camomile tea. Luteolin is reported to have anti-inflammatory properties and mediates its action by inhibition of nitric oxide production. It has also been found to inhibit the release of TNFα from neutrophils, and to inhibit matrix metalloproteinases. It also inhibits the effects of tyrosine kinase; an enzyme involved in tumor cell proliferation, and therefore may have potential as a dietary anticarcinogenic agent.

Oxidative lesions result from imbalance in oxidant-antioxidant equilibrium. Fe-NTA, being one of the most potent iron pro-oxidant compounds, was used in the present study to induce oxidative stress. Nitrilotriacetic acid is a synthetic tricarboxylic acid, which forms water-soluble chelate complexes with various metal ions including iron at neutral pH and has been used as a potential substitute for polyphosphates in household detergents. Its iron complex, Fe-NTA is a potent nephrotoxic agent. The renal toxicity is assumed to be caused by the elevation of serum free iron concentration following its reduction at the luminal side of proximal tubule generating reactive oxygen species leading to enhancement in lipid peroxidation with a concomitant decrease in tissue glutathione level. 8-hydroxydeoxyguanosine, a marker product of oxidative DNA damage has been observed following exposure of animals to Fe-NTA. Administration of exogenous antioxidant may be beneficial so as to combat the deleterious effect of Fe-NTA. The present study has been designed to screen the efficacy of luteolin against ferric nitrilotriacetic acid (Fe-NTA), induced oxidative stress and tumor promotional response.

Materials and Methods

Animals—Four to six weeks old male Wistar rats (130-150 g) obtained from Central Animal House of Hamdard University, New Delhi, India, were housed...
in polypropylene cages in groups of six rats per cage and were kept in a room maintained at 25°C±2°C with a 12 hr light/dark cycle. They were allowed to acclimatize for one week before the experiments and were given free access to standard laboratory feed (Aashirwad Industries, House No. 1544, Sector-38, Chandigarh India). Animals are given enough food to permit normal growth and maintenance of age-appropriate body weight. Food was stored in designated restricted areas that are cool, clean, dry and free of vermin. Animal facilities and other areas in contact with laboratory animals were cleaned and disinfected often to keep them free of dirt, debris, and biological or chemical contaminants. Animals received mild anaesthesia (di-ethylether) before sacrifice. All procedures using animals were reviewed and approved by the Institutional Animal Ethical Committee that is fully accredited by the Committee for Purpose of Control and Supervision on Experiments on Animals (CPCSEA) Chennai, India.

Chemical—Reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (GR), bovine serum albumin (BSA), 1,2-dithio-bis-nitrobenzoic acid (DTNB), 1-chloro-2, 4-dinitro benzene (CDNB), nitrotriacetic acid (NTA), reduced nicotinamide adenine dinucleotide phosphate (NADPH), glucose-6-phosphate, tween-20, 2,6-dichlorophenolindophenol, thiobarbituric acid (TBA) and luteolin were obtained from Sigma Chemicals Co (St Louis, MO, USA). Diacetyl monoxide, urea, picric acid, sodium tungstate, sodium hydroxide, ferric nitrate, trichloroacetic acid and perchloric acid (PCA) were purchased from BDH, India. DL [14C] ornithine (sp. Activity 56 mCi/mmol) and [3H] thymidine (sp. activity 82 mCi/mmol) were purchased from Amersham Corporation (Little Chalfort, UK). All other chemicals were of the highest purity and commercially available.

Preparation of Fe-NTA solution—Fe-NTA was prepared fresh immediately before its use as per Awai et al. To prepare Fe-NTA, ferric nitrate (0.16 mmol/kg body weight) solution was mixed with 4-fold molar excess of disodium salt of NTA (0.64 mmol/kg body weight) and the pH was adjusted to 7.4 with sodium bicarbonate solution.

Treatment regimen—To study the effect of pretreatment of animals with luteolin on Fe-NTA induced renal oxidative stress and ornithine decarboxylase induction, 30 male Wistar rats were randomly allocated to 5 groups of 6 rats each. The animals of group I served as control and received normal saline (0.85% NaCl). The animals of group II received only a single ip injection of Fe-NTA (9 mg Fe/kg body weight). Group III received pretreatment with luteolin by gavage once daily for 7 days (10 µmol/kg body weight). Groups IV and V received pretreatment with luteolin extract once daily for 7 consecutive days (20 µmol/kg body weight). One hour after the last treatment with luteolin the animals of group III and IV received single intraperitoneal injection of Fe-NTA (9 mg Fe/kg body weight). All the animals were sacrificed by decapitation 12 hr after last treatment. Kidneys were quickly removed and washed with ice-cold saline. Immediately before sacrifice, blood was collected from the retro-orbital sinus for the estimation of serum creatinine and blood urea nitrogen (BUN).

To study the effect of pretreatment with luteolin on Fe-NTA mediated [3H] thymidine incorporation into renal DNA, the grouping of animals and schedules for prophylaxis were same as described above. After one hour of the last treatment with luteolin, the animals of group II, III and IV received only a single ip injection of Fe-NTA at a dose level of 9 mg Fe/kg body weight. After 18 hr of the treatment with Fe-NTA the rats were given [3H] thymidine (30 µCi/animal) by ip injection. Two hours later, they were sacrificed by cervical dislocation and their kidneys were quickly removed.

Tissue preparation—After the desired time, control and treated animals were killed by cervical dislocation. Animals received mild anaesthesia (di-ethylether) before sacrifice. For biochemical studies, a known amount of tissue was minced and homogenized in a polytron homogenizer (Kinematica AG) and subjected to subcellular fractionation, which was prepared by filtering through a muslin cloth and was centrifuged at 800 g for 5 min at 4°C to separate the nuclear debris. The aliquot so obtained was centrifuged in an Altec refrigerator centrifuged (Model RC 4100 D) at 105,000 g for 20 min at 4°C to get post mitochondrial supernatant (PMS) which was used as a source of enzymes. A portion of the PMS was centrifuged in an ultracentrifuge (Beckmann L7-55) at 105,000 g for 60 min at 4°C. This pellet was considered to be the microsomal fraction and was suspended in phosphate buffer (0.1 M, pH 7.4) containing KCl (1.17%).

Reduced glutathione (GSH) was determined as per Jollow et al. Activities of glutathione

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S-transferase, glutathione reductase and lipid peroxidation were measured. Blood urea nitrogen and creatinine were estimated. Assay for hydrogen peroxide, glucose-6-phosphate dehydrogenase, glutathione peroxidase, ornithine decarboxylase, DNA synthesis were done. The amount of DNA in the filtrate was estimated by diphenylamine method.

Protein was determined following Lowry’s method.

Statistical analysis — Differences between groups were analyzed using analysis of variance (ANOVA) followed by Dunnet’s multiple comparison tests. All data points are presented as the treatment group ± SE.

Results and Discussion

Treatment of rats with Fe-NTA (9 mg Fe/kg body weight) caused overproduction of cellular oxidants and modulation of antioxidant defense system. In the present study, several doses of luteolin were tested and maximum efficacy was found at 10 and 20 µmol/kg body weight. Treatment with Fe-NTA alone resulted in the depletion of renal glutathione and reduction in the activities of glutathione-S-transferase and glutathione reductase that of saline-treated control group (Table 1). However, pretreatment of animals with luteolin at 10 and 20 µmol/kg body weight resulted in the recovery as compared with Fe-NTA treated group. Fe-NTA alone treatment caused reduction in the activities of renal antioxidant enzymes such as catalase, glutathione peroxidase, and glucose-6-phosphate dehydrogenase compared to saline-treated control group. Treatment with luteolin at both the doses caused recovery of the above enzymes as compared with Fe-NTA treated control group. Only luteolin at higher dose of 20 µmol/kg body weight shows a slight increase in all these parameters when compared with control group.

Fe-NTA treatment enhanced the activity of xanthine oxidase susceptibility of renal microsomal membrane for iron-ascorbate induced lipid peroxidation and H₂O₂ with simultaneous reduction in the activity of quinone reductase as compared with saline-treated controls (Table 2). Luteolin treatment (10 and 20 µmol/kg body weight) caused reduction in activity of xanthine oxidase and renal microsomal lipid peroxidation and increases in the level of quinone reductase. Only luteolin dose caused slight alterations in these parameters.

Fe-NTA treatment leads enhancement in the values of blood urea nitrogen, serum creatinine respectively, as compared with saline-treated controls (Fig. 1). Prophylaxis with luteolin at both doses resulted in reduction in the values of blood urea nitrogen, serum creatinine and H₂O₂ respectively as compared with Fe-NTA treated group.

The effect of pretreatment of animals with luteolin on Fe-NTA mediated induction of renal ODC activity and enhancement in the incorporation of [³H] thymidine into renal DNA is shown in Fig. 2. Treatment with Fe-NTA caused induction in the ODC activity and increase in the incorporation of [³H] thymidine into renal DNA. Only luteolin dose caused slight alterations in these parameters.

Table I — Effect of pretreatment with luteolin on Fe-NTA-mediated depletion of renal glutathione content and decrease in the activities of glutathione metabolizing enzymes and antioxidant enzymes in rats

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Reduced glutathione (nmol GSH/g tissue)</th>
<th>Glutathione-S-transferase (nmol CDNB conjugate formed/min/mg protein)</th>
<th>Glutathione reductase (nmol NADPH oxidized/min/mg protein)</th>
<th>Catalase [nmol H₂O₂ consumed/min/mg protein]</th>
<th>Glutathione peroxidase [nmol NADPH oxidized/min/mg protein]</th>
<th>Glucose-6-phosphate dehydrogenase [nmol NADP reduced/min/mg protein]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (control)</td>
<td>0.67±0.01</td>
<td>350.8±1.2</td>
<td>375.3±4.6</td>
<td>70.5±0.5</td>
<td>273.5±6.9</td>
<td>40.3±1.1</td>
</tr>
<tr>
<td>Fe-NTA (9 mg Fe/kg body weight)</td>
<td>0.48±0.01c (-28.3)</td>
<td>185.6±1.1c (-47)</td>
<td>239.0±7.5c (-36.3)</td>
<td>30.4±0.74c (-56.8)</td>
<td>154.8±1.6c (-43.4)</td>
<td>15.4±1.4c (-61.7)</td>
</tr>
<tr>
<td>Luteolin (10 µmol/kg body wt) + Fe-NTA (9 mg Fe/kg body weight)</td>
<td>0.58±0.02c (+20.8)</td>
<td>264.2±9.7c (+42.3)</td>
<td>293.4±1.42c (+22.7)</td>
<td>46.8±0.35c (+53.9)</td>
<td>191.0±8.3c (+23.3)</td>
<td>23.3±1.0c (+51.2)</td>
</tr>
<tr>
<td>Luteolin (20 µmol/kg body wt) + Fe-NTA (9 mg Fe/kg body weight)</td>
<td>0.62±0.01c (+29)</td>
<td>336.3±6.0c (+81.1)</td>
<td>345.2±7.0c (+44.4)</td>
<td>66.3±0.82c (+118)</td>
<td>224.2±8.8c (+44.8)</td>
<td>30.2±1.3c (+96)</td>
</tr>
<tr>
<td>Only Luteolin (20 µmol/kg body wt)</td>
<td>0.69±0.01 (+2.9)</td>
<td>354.5±1.6 (+1)</td>
<td>376.3±2.5 (+0.2)</td>
<td>74.8±0.88 (+6)</td>
<td>290.7±4.9 (+6.2)</td>
<td>46.2±1.9 (+14.6)</td>
</tr>
</tbody>
</table>

P values: a<0.05; b<0.01; c<0.001 compared with Fe NTA treated group; d<0.001 compared with saline treated control group.
thymidine into renal DNA as compared with saline-treated control. The pretreatment of rats with luteolin at two doses caused inhibition in the elevation of ODC activity with concomitant reduction in the enhancement of DNA synthesis as compared with Fe-NTA treated control group. Chemoprevention strategies are very remarkable and have earned serious consideration as potential means of controlling the incidences of cancer. The demand for natural antioxidants for use in foods has increased recently because they affect a wide range of cellular, biological functions by virtue of their radical scavenging properties. The multifunctional effect of luteolin is very intimately connected with its structure and hydroxyl as its functional group which participates in electron delocalization and is, therefore, an important determinant for its antioxidative potential.

The findings of present investigation are based on an examination of tumor promotion response, induction of enzymes involved in xenobiotics metabolism and maintaining the antioxidant status of the cell. Luteolin at both doses showed protection in Fe-NTA mediated renal oxidative stress in rats, which is manifested by decrease in the susceptibility of renal microsomal membrane for LPO, H$_2$O$_2$ generation. Lipid peroxidation is a major mechanism of reactive oxygen species in which they attack the cellular organelles and membrane-bound enzymes. Lipid peroxidation can arise as a consequence of disease states and may contribute to the tissue injury. Fe-NTA-induced lipid peroxidation was associated with impaired renal function, as evidenced by increased serum creatinine.
and urea. The protective effects of many naturally occurring chemopreventive agents against carcinogenesis have been ascribed to decreased bioavailability of potential DNA-damaging entities and their destruction into excretable metabolites, facilitated through induction of GST. GST is a critical detoxification enzyme that functions primarily in conjugating "functionalized P450 metabolites" with endogenous ligand (GSH), favoring their elimination from the organism. There are convincing evidences to support induction of GST and protection against a wide spectrum of cytotoxic, mutagenic and carcinogenic chemicals. The specific activity was the sum of all its isoforms, as CDNB was used as a non-specific substrate for GST. Induction of GST activity in luteolin pretreated groups support the detoxification capacity of the compound. The present investigation also revealed that luteolin can attenuate oxidative stress significantly by modulating cellular enzymatic and non-enzymatic antioxidant defense systems. The depletion of GSH below its basal level by Fe-NTA treatment promotes the generation of reactive oxygen species and oxidative stress with a cascade of effects on the functional and structural integrity of cells and organelle membranes. The elevated level of GSH induced by luteolin protects cellular proteins against oxidation via redox cycle and also directly detoxifies reactive oxygen species and/or neutralizes reactive intermediate species generated from exposure to xenobiotics, including chemical carcinogens. The attenuated GR level observed in the present investigation plays a significant role in the reduction of oxidized GSH to GSH at the expense of NADPH and regulates the GSH-GSSG cycle in the cell. Luteolin also ameliorated Fe-NTA mediated inhibition of the activities of antioxidant enzyme like glucose-6-phosphate dehydrogenase which is a secondary antioxidant enzyme and helps in the detoxification of reactive oxygen species by decreasing peroxide level or by maintaining a steady supply of metabolic intermediates like GSH and NADPH for primary antioxidant enzymes. Glutathione peroxidase and catalase, which are of central importance in the detoxification of peroxides and hydro peroxides, were also recovered in luteolin pretreatment groups. Luteolin pretreatment also elevated renal QR activity and it has been reported to have correlation with the prevention of cancer. There was concomitant significant reduction in the elevated levels of marker parameters of kidney toxicity, viz., BUN and serum creatinine in luteolin pretreated groups. This shows the efficacy of luteolin in regulating renal functions.

ODC activity and [3H] thymidine incorporation have been widely used as biochemical markers to evaluate tumor-promoting potential of an agent. In the present study both were inhibited dose dependently by luteolin, suggesting its anti-proliferative and antitumor potential. Inhibition of ODC activity and DNA synthesis reveals that luteolin may intercept the tumor promoting and damaging functions of polyamines and arachidonic acid.

The major mechanism of luteolin in protection against toxic effects of Fe-NTA is the modification of cellular detoxification enzymes. The increase in the activities of these enzymes may be due to the ability of luteolin to directly react with various reactive oxygen species as well as to interfere with oxidation processes in the lipid and cellular compartment. Treatment of rats with luteolin also counteracted the reduced catalase activity, which is found to be a major determinant of cellular resistance to hydrogen peroxide toxicity.

A combination of these events contributes to luteolin’s ability in inhibiting Fe-NTA-induced oxidative stress, cell proliferation response and toxicity in rat kidney and hence, it can be concluded that luteolin possibly has chemopreventive effect through interference with pathways of reactive oxidants and by modulation of tumor promoting markers.

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