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Inositol hexa phosphoric acid (phytic acid), a nutraceuticals, attenuates iron-induced oxidative stress and alleviates liver injury in iron overloaded mice

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ABSTRACT

Inositol hexa phosphoric acid (IP6) or Phytic acid, a natural antioxidant of some leguminous plants, known to act as a protective agent for seed storage in plants by suppressing iron catalyzed oxidative process. Following the same mechanism, we have tested the effect of IP6 on iron overloaded *in vitro* oxidative stress, and studied it's *in vivo* hepatoprotective ability in iron-dextran (injection)-induced iron overloaded liver injury in mice (intraperitoneal). Our results showed that IP6 had *in vitro* iron chelation (IC_{50} 38.4 μg/ml) activity, with the inhibition of iron-induced lipid peroxidation (IC_{50} 552 μg/ml), and deoxyribose sugar degrading hydroxyl radicals (IC_{50} 448.6 μg/ml). Oral administration of IP6 (0–200 mg/kg) revealed significant decrease in biochemical markers such as serum iron, total iron binding, serum ferritin and serum enzymes. Histopathology of liver stained with hematoxylin-eosin and Prussian blue showed reduced hepatocellular necrosis, ballooning and inflammation, indicating the restoration of normal cellular integrity. Interestingly, the IP6 was found to down-regulate the mRNA expression of tumor necrosis factor (TNF)-α, Interleukin (IL)-1β, and IL-6 in iron overloaded liver tissues. Thus, we provide an insight that IP6, a natural food component, can serve as an iron chelator against iron overload diseases like Thalassemia, and also as a dietary hepatoprotective supplement.

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1. Introduction

Iron is one of the most essential nutrients for normal cellular and physiological functions of living system [1], but in excess it is highly toxic. Iron overload may lead to cell injury, and when accumulate in liver, heart and spleen it results organ damage with morbidity to mortality [2,3]. One such disease associated with iron overload is Thalassemia, an inherited autosomal recessive disorder. Thalassemic patients (Thalassemia major) are only managed by long-term multiple blood transfusions, and had to fight with consequences of iron overload. In Thalassemia the iron overload leads to iron toxicity with deterioration of organs, which often lead to death [4]. Iron being a pro-oxidant can induce liver toxicity through the generation of free radicals and oxidative stress of biomolecules [5]. Being a major iron storage organ, liver is highly labile for iron-induced tissue injury. Further, the oxidative tissue injury leads to membrane lipid peroxidation, protein oxidation and inflammatory responses with histological alteration of liver tissues [6]; while lipid peroxidation leads to the damage of bio-membrane of cell organelles, including mitochondria and lysosome. Collectively, it contributes to the hepatocellular apoptosis and necrosis that eventually leads to the hepatocellular fibrosis, cirrhosis and carcinoma [5,7].

The excess iron is usually deposited in the body as Ferritin, a soluble globular protein complex consisting of 24 subunits, as a main intracellular non-toxic iron storage protein of the body. Ferritin can be detected in patient's serum when present in higher concentration as evident with patients received repeated

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transfusion [8]. Thus, it is important to eliminate the excess iron before it can cause any damage, and the excess iron is removed by iron chelator, through the chelation therapy [9,10]. The commonly available iron chelating agents are deferoxamine, deferiprone and deferasirox. However, these agents are inadequate in one hand and disadvantageous in another, as they have several side effects including toxic manifestations in long-term use [11,12].

Neutraceuticals or nutritional molecules of food, especially the antioxidants are believed to be less toxic with minimum side effects [13]. In living system, they interfere with the cellular oxidation process by reacting with free radicals, chelating catalytic metals and scavenging oxygen [14]. External supply of such dietary antioxidant molecules, especially through food, help to overcome the effect of free radicals in the body, and thereby prevent many diseases [15]. The early introduction of chelating agent to Thalassemia patients is reported to control iron overload, inhibit reactive oxygen species (ROS)-generation and regulate lipid peroxidation (LPO) processes leading to the improved life expectancy [16]. Inositol hexaphosphoric acid (Synonym Phytic acid) or IP6, a natural antioxidant presents in food and medicinal plants of leguminous family, serve as a protective agent in seeds for long-term storage. However, IP6 is reported to have several beneficial effects on human body, including chelation of ferric ion, resistance to oxidation and apoptosis, improved immunity and anti-inflammatory activity [17–19]. Moreover, IP6 has neuro-protective, lipid lowering and cholesterol reduction activity, thereby serve as a disease preventing natural molecule [20,21]. A recent study revealed that IP6 inhibits IL-1β induced cancer cell migration and invasion along with anti-inflammatory activity in epithelial intestinal and peripheral blood mononuclear cells [22]. The antioxidant and lipid lowering potential of IP6 is reported to be due to its protective effect by inhibition of ROS production [23]. However, its antiinflammatory and anticancer potential is probably due to inhibition of nuclear translocation of NF-κB in Caco-2 cells stimulated with IL-1β [24], ability to stimulate AP-1 DNA-binding activity, p38 ligand-activated and ligand-induced Akt phosphorylation [25]. However, IP6 has not yet been investigated for its hepatoprotective effect against iron overload liver injury. Thus, the current study was aimed to assess the *in vitro* antioxidant and hepatoprotective role of IP6 against iron overload liver injury in mouse model with safety profile to propose this dietary ingredient as food supplement for the management of iron overload diseases like Thalassemia.

2. Materials and methods

2.1. Chemicals

Inositol hexaphosphoric acid (IP6) sodium salt and Thiobarbituric acid (TBA) were obtained from Sigma, USA; KH_2PO_4 –KOH buffer was procured from SRL; 2-deoxy-D-ribose and Ferrozine from HiMedia Laboratories Pvt. Ltd., India; Deferoxamine (DFO) from Novartis India Ltd., India; and Desirox (DFX) dispersible tablets (500 mg) from Cipla Ltd., India. Other analytical grade reagents were obtained from various commercial suppliers. The TIBC ferrozine kit was procured from Crest Biosystems, India; while serum ferritin assay kit (Pathozyme ferritin kit) was from the Omega Diagnostics Ltd, UK. The serum bilirubin was from the Medsource Ozone Biomedicals Pvt. Ltd.; while Alanine aminotransferase (ALT) is purchased from the Merck Specialities Private Limited. The aspartase aminotransferase (AST), Alkaline Phosphatase (ALP), creatinine and urea were procured from the Span Diagnostics Ltd., India. All the parameters were measured by the commercially available kits from respective manufacturers, following manufacturer's instructions.

2.2. Animals

Seven week old male and female BALB/c mice (18–20 g) were acclimatized in polypropylene cages in the Animal House facility, with standard food and water *ad libitum* for 15 days. Animal experiments were carried out following the OECD guidelines, accepted by the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), India, and as approved by the Institutional Animal Care and Use Committee (IACUC) of Jadavpur University, Kolkata (Approval No: 0367/01/C/CPCSEA). When needed, the surgical procedures were conducted under Ketamine hydrochloride (100 mg/kg i.m.) anesthesia, to minimize the suffering of the animals.

2.3. In vitro *antioxidant activity*

2.3.1. Ferrous ions chelation assay

The ferrous ion chelating activity of IP6 was evaluated by the method described by Dinis et al., 1994 [26]. Briefly, the IP6 (pH 7) at various concentrations (0–160 μg/ml) in water (0.4 ml) was added to the solution of 2 mM $FeCl_2$ (0.05 ml). The reaction was initiated by adding 0.2 ml of 5 mM Ferrozine in the above solution, and the total volume of the mixture was adjusted to 4 ml with ethanol. The mixture was shaken vigorously and left to stand for 10 min at room temperature. The absorbance was read at 562 nm, using DFO as positive control. All tests were performed three times. The percentage of inhibition of Ferrozine-Fe(II) complex formation was calculated by the formula: Metal chelating effect (%) = $[(A_0 - A_1)/A_0] \times 100$; Where A_0 is the absorbance of control and A_1 is the absorbance in presence of IP6. The control contained $FeCl_2$ and Ferrozine. The IC_{50} (μg/mL), concentration of the test agent/standard that chelate 50% of the ferrous ion, was calculated by linear interpolation between above and below 50% activity.

2.3.2. Lipid peroxidation assay

Thiobarbituric acid (TBA) reaction assay was used to measure the lipid peroxide formation using egg yolk homogenate as lipid source [27]. The egg yolk lipids undergo rapid non-enzymatic peroxidation when incubated in the presence of ferrous sulphate, with subsequent formation of malonodialdehyde (MDA) and other aldehydes that form pink chromogen in presence of TBA, generated a peak at 532 nm. Egg homogenate (0.5 ml of 10%, v/v) was added with 0.1 ml of different concentration of either IP6 (0–800 μg/ml) or DFO (0–200 μg/ml) in a test tube and the volume was made up to 1.0 ml with distilled water. Then 0.05 ml of ferrous sulphate (0.07 M) was added to induce lipid peroxidation by incubating the mixture for 30 min at room temperature. The mixture was then added with 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% (w/v) thiobarbituric acid in 1.1% Sodium Dodecyl Sulphate (SDS). The resulting mixture was vortexed and then heated at 95 °C for 1 h. After cooling the mixture was added with 5.0 ml of butanol to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532 nm, and the inhibition of lipid peroxidation percent (%) by the test agent was calculated as: 100- $[(A1/A2) \times 100]$; where A1 is the absorbance in IP6 or DFO treated set and A2 is the absorbance of fully oxidized control set.

2.3.3. Deoxyribose assay

To estimate the reduction of substances reactive to TBA from deoxyribose sugar degradation by hydroxyl radical, generated by the Fenton reaction Deoxyribose assay was used [28]. Test tubes containing Ascorbic acid (10 μl of 100 μM), KH_2PO_4-KOH buffer (20 mM, pH 7.4), and 50 μl of different concentrations of IP6 (0–800 μg/ml) or DFO (0–100 μg/ml), along with 10 μl each of Deoxyribose (2.8 mM), H_2O_2 (1 mM), $FeCl_3 \cdot 6H_2O$ (50 μM) and

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