PROTECTIVE EFFECT OF CHRYSIN AGAINST CISPLATIN INDUCED PULMONARY TOXICITY IN WISTER RATS

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Abstract – Objective: *Cisplatin is one of the most potent antitumor agents known, with clinical activity against a wide variety of solid tumours. Its cytotoxic action is DNA mediated and forms DNA adducts. High incidences of pulmonary drug toxicity have been associated with Cisplatin treatment in cancer patients. Chrysin possesses antioxidant, anti-inflammatory and anti-cancer properties. This study investigated the protective efficacy of chrysin against cisplatin-induced pulmonary toxicity.*

***Materials and Methods:** Chrysin was administered orally (10 ml/kg body weight in corn oil) once daily for 14 consecutive days. On day 14, a single dose of Cisplatin at 7.5 mg/kg body weight was administered intraperitoneally. Reduced glutathione (GSH), glutathione reductase (GR), glutathione-S-transferase (GST), glutathione peroxidase (GPx) and catalase (CAT) activities were evaluated in lung tissues.*

***Results:** The results showed effective protection of Chrysin against Cisplatin-induced oxidative stress, cytotoxicity and inflammation on the lungs. Chemopreventive treatment with Chrysin showed a significantly positive modulation of altered activities of GSH, GR, GST, GPx and CAT in lung tissues. The gel electrophoresis of genomic DNA especially revealed a reduction in the intensity of the change in the DNA status in Chrysin pre-treatment groups as compared with Cisplatin administered group. The histo-pathological findings correlate positively with biochemical and cellular parameters of the lungs, thus validating the protective role of Chrysin against Cisplatin-induced lung inflammation.*

***Conclusions:** This study suggests that Chrysin plays a decisive role in protecting the lung against Cisplatin-induced lung injuries following chemotherapeutic administration.*

KEYWORDS: *Chrysin, Cisplantin, Pulmonary, Rats, Toxicity.*

INTRODUCTION

Cisplatin (cis-diamminediachloroplatium) and its derivatives are among the most effective anticancer drugs used clinically in the treatment of solid tumors, including ovarian, testicular, cervical, and small cell lung cancers[1,2]. The anticancer effect of cisplatin mainly depends on its DNA-damaging activity; via its direct interaction with DNA forming DNA adducts[3]. In both cancer and normal cells, Cisplatin forms DNA adducts, resulting in generation of reactive oxygen species (ROS), which induce programmed cell death (apoptosis)[2]. The mechanisms by which cisplatin induces ROS formation and how this response affects normal and cancer cell sensitivity to the drug remains unclear[4]. Although the formation of mitochondrial DNA (mtDNA) adducts with cisplatin have been demonstrated, inhibition of all the mitochondrial respiratory chain complexes in isolated mitochondrial has been reported, and increased mitochondrial ROS scavenging reduces cellular sensitivity to cisplatin has also been shown[4-6] but the toxicological relevance of such adducts remains poorly investi-

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gated. Previous studies reported that Cisplatin is known to be cytotoxic to organs by promoting an imbalance in oxidative stress and antioxidant reserves[7-9]; however, the mechanism by which this drug causes lung damage is poorly understood. The drug's therapeutic effectiveness is limited by the severity of the numerous undesirable side effects, and the potential progression of tumour cells to a cisplatin-resistant state. Therefore, combining cisplatin with other drugs has been highly considered to overcome drug resistance and reduce toxicity[2,10]. Combination therapies with a similar class of drugs also come with challenges, such as increased pulmonary toxicity e.g. bleomycin and cisplatin[7,11]. Hence, combining chrysin with other compounds such as flavonoids presents a potential for improved effectiveness and reduced toxicity of the chemotherapy. Many studies have reported that flavonoids enhance cisplatin chemosensitivity and protect against cisplatin-induced organ toxicity, by modulating several gene transcription factors, and inducing cell death through apoptosis[12-18]. Oxidative stress and DNA damage appear to be important in cisplatin tissue and organ toxicity; hence, any agent that did not induce oxidative stress and exhibited anticancer activity would reduce cisplatin-related pulmonary toxicity. Chrysin, a flavanone in various plants, has been used as a traditional medicine from ancient[19,20]. It is the main ingredient of some medicinal plants, such as *Radix scutellariae* (Baikal skullcap)[21], *Passiflora incarnate*, wild apricot and *Passiflora caerulea* (blue passionflower)[22], propolis (bee glue) and honey[23]. Other plants that contain chrysin are mushrooms such as *Lactarius deliciosus* (red pine mushroom), *Suillus bellinii* (the Champagne bolete)[24] and *Pleurotus ostreatus* (oyster mushroom)[25], *Oroxylum indicum* (Indian trumpet tree)[26], and *Alpinia oxyphylla* (Black Cardamom)[27] respectively. Common foods sources of flavones include broccoli, chili peppers, celery and rosemary[28,29]. Chrysin is a potent antioxidant[30], hepatoprotective agent[31], cardioprotective agent[32], with anti-inflammatory effect[30-33], anticancer activity[30-34], renoprotective capacity[35], and anti-metastatic potentials[36]. In view of these facts, pre-treatment with chrysin was done prior to cisplatin administration to evaluate the possible protective potentials of chrysin against cisplatin induced toxicities.

MATERIALS AND METHODS

Chemicals and reagents

The chemicals used for the study such as: Nicotinamide adenine dinucleotide phosphate reduced (NADPH), reduced glutathione (GSH), oxidised glutathione (GSSH), 1-chloro-2, 4-dinitrobenzene (CDNB), nicotinamide adenine dinucleotide reduced (NADH), bovine serum albumin (BSA), 1,2-dithiol-bis-nitrobenzoic acid (DTNB), trichloroacetic acid (TCA), glutathione reductase (GR), Hydrogen peroxide (H_2O_2), were obtained from Sigma Chemical (St. Louis, MO, USA). Proteinase K and hydrochloric acid (HCl), were obtained from Himedia Lab Pvt Ltd (Maharashtra, India). Tris, boric acid, ammonium acetate, isoamyl alcohol (IAA), phenol, sodium dodecyl sulphate (SDS), agarose gel, glycerol, ethidium bromide, bromophenol blue, were obtained from Sisco Research Lab Ltd (New Delhi, India). Di-Sodium hydrogen phosphate, sodium dihydrogen phosphate, sulphur salicylic acid (SSA), ethylenediamine tetraacetate (EDTA), sodium azide, sodium hydroxide (NaOH), sodium carbonate (Na2CO3), sodium/potassium (Na+/K+) tartrate, copper sulphate (CuSo4), chloroform, folin ciocalteu reagent (FCR) and chrysin were purchased from E. Merck (Maharashtra, India) and cisplatin from Dr Reddy's Laboratories Ltd (Hyderabad, India). All the chemicals were of the highest grade.

Animals

Male rats of Wistar strain weighing 192.8 ± 18.6 g were used for this study. Animals were obtained from the Central Animal House Facility of Hamdard University, New Delhi, India. They were housed in large spacious polypropylene cages of six rats per cage, kept in a room maintained at 25 ± 10°C and relative humidity of 50 ± 15%. Lighting was regulated to provide equal hours of light and dark. Animals were allowed to acclimatise to standard laboratory conditions for one week before the experiments commenced. They were provided with standard food pellets processed by Hindustan Lever Ltd., India and clean water was provided ad libitum.

Treatment regimen

Thirty (30) male Wistar rats were randomly allocated to five groups, each comprising six animals. Groups I and II animals were administered corn oil (10 ml/kg BW) while groups III, IV and V animals were administered Chrysin at the doses of 25, 50 and 50 mg/kg body weight, respectively. All the treatments were given orally once daily for 14 consecutive days. On the last day the animals in groups II, III, and IV, were administered a single dose of Cisplatin at a dose of 7.5 mg/kg body weight intraperitoneally. Twenty four hours

post Cisplatin administration, the animals were sacrificed and the lungs harvested immediately, washed in ice-cold saline (0.9%), kept in ice and processed for subcellular fractionation for the determination of biochemical parameters. Some tissues were stored in 10% buffered formalin for histopathology.

Harvesting of Postmitochondrial Supernatant (PMS)

Lung tissues from the sacrificed animals of each group were quickly excised, washed in ice-cold saline (0.9%) and kept in ice. A 10% homogenate was prepared in chilled phosphate buffer (0.1 M, pH 7.4) using a Potter Elvehjem homogeniser. Homogenate was then centrifuged at 12000 g for 20 mins at 4°C to obtain post-mitochondrial supernatant (PMS), which was used as a source of enzyme. The harvested PMS was divided into different aliquots and stored at -20°C for biochemical assays.

BIOCHEMICAL ASSAYS

Glutathione-S-transferase estimation

The glutathione-S-transferase activity was estimated by the method of Habig et al[37]. The reaction mixture consisted of 197 µl of sodium phosphate buffer (0.1 M, pH 7.4), 25 µl reduced glutathione (1 mM), 3 µl CDNB (1 mM) and 25 µml PMS (10 % w/v) in a total volume of 250 µl. The changes in the absorbance were recorded at 340 nm, and enzymes activity was calculated as nmol CDNB conjugate formed min-1mg-1 protein using a molar coefficient of $9.6 \times 10^3 M^{-1} cm^{-1}$.

Assay for Catalase activity

Catalase activity was assayed by the method of Claiborne[38]. The reaction mixture consisted of 1.95 ml phosphate buffer (0.1 M, pH 7.4), 1.0 ml hydrogen peroxide (50 mM) and 0.05 ml 10% PMS in a final volume of 3 ml. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated as nmol H_2O_2 consumed/min/mg protein.

Assay for glutathione reductase activity

Glutathione reductase activity was determined by the method of Carlberg and Mannervik[39]. The reaction mixture consisted of 204 µl phosphate buffer (0.1 M, pH 7.6), 13 µl EDTA (1 mM), 7

µl oxidized glutathione (1 mM), 13 µl NADPH (0.1 Mm) and 13 µl PMS (10%) in a total volume of 250 µl. Enzyme activity was quantitated at 25°C by measuring the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidised/min/mg protein using a molar extinction coefficient of $6.22 \times 10^3 M^{-1} cm^{-1}$.

Assay for glutathione peroxidase activity

Glutathione peroxidase activity was determined by the method of Mohandas et al[40]. The reaction mixture consisted of 17 0µl phosphate buffer (0.1 M, pH 7.4), 13 µl EDTA (1 mM), 13 µl sodium azide (1 mM), 7 µl glutathione reductase (1 IU/ml), 13 µl GSH (1 mM), 13 µl NADPH (0.1 Mm), 2 µl H_2O_2 (50 mM) and 13 µl PMS (10%) in a total volume of 250 µl. Enzyme activity was quantitated at 25°C by measuring the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidised/min/mg protein using a molar extinction coefficient of $6.22 \times 10^3 M^{-1} cm^{-1}$.

Assay for reduced glutathione activity

GSH was determined by the method of Jollow et al[41]. One millilitre of PMS (10%) was mixed with 1.0 ml of SSA (4%). The samples were incubated at 4°C for at least 1 hour and then centrifuged at 1200 g for 15 mins at 4°C. The reaction mixture contained 0.4 ml supernatant, 2.2 ml phosphate buffer (0.1 M, pH 7.4), 0.4 ml DTNB (4 mg/ml) in a total volume of 3.0 ml. The yellow colour developed was read immediately at 412 nm on a spectrophotometer (Perkin Elmer, Lambda EZ 201). The reduced glutathione concentration was calculated as nmole GSH conjugated/g tissue.

Protein estimation

Protein concentration in all samples was determined by the method of Lowry et al[42].

DNA Extraction

DNA extraction was carried out by Phenol Chloroform Isoamylalcohol (PCI) method. Two millilitre of DNA extraction buffer (1 M Tris HCL, 0.5 M EDTA, 5% SDS, pH 8.0) was added to 200 mg tissue and incubated at room temperature for one hour. 100 µl Proteinase K (10 mg/ml stock) was added and incubate overnight in a mixing water bath at 50-55°C. 2.4 ml of phenol (pH 8.0) was

added, mixed gently for 5-10 min and centrifuge at 5000 rpm for 10 min. The upper layer containing DNA was taken in a new tube and then 1.2 ml of chloroform-isoamyl alcohol mixture (24:1) was added. This was mixed gently for 5-10 min and centrifuged at 3000 rpm for 10 min. The supernatant was taken, and the previous step repeated. 200 µl of ammonium acetate was added and mixed for 5 min, and then 5 ml of chilled ethanol was added; mixed gently until DNA precipitate. DNA strands were hooked with a pipette and washed with 1.5 ml of 70% ethanol twice. DNA was then dried and re-suspended in 100 µl of Tris-EDTA buffer. The purity of the DNA was checked by taking the ratio of $O.D._{260}$ /$O.D._{280}$, which had a range of 1.8-2.0.

Gel Electrophoresis

Agarose gel electrophoresis was performed on the extracted DNA. This was carried out using 1.2% agarose gel which was heated at 100°C for 5 mins and allowed to cool to 60°C; then 5µl Ethidium bromide (10 mg/ml) was added, mixed and allowed to cool to 37°C before plating. The comb was placed immediately, and the gel was allowed to solidify, and the comb removed, forming the well for loading. 1× Tris borate EDTA (TBE) buffer was used as a loading buffer at pH 8.0. This was poured into the electrophoresis tank allowing the gel to be submerged in the buffer; 3 µl of loading dye was mixed with 7 µl of the DNA sample before loading into the wells. The wells were loaded with DNA samples, and the gel was run for $2^1/_2$ hours at 60 V. The gel was then viewed using a UV illuminator.

Histopathological Evaluation

The lungs were fixed in 10% formalin and embedded in paraffin. Sections (5 µm) were cut from the middle lobes of the lungs of each group more or less from similar positions. Using xylene and ethanol, the paraffin-embedded lung tissue sections (5 µm) were deparaffinised. The slides were washed with phosphate-buffered saline (PBS) and permeabilised with permeabilization solution (0.1 M citrate, 0.1% TritonX-100). The deparaffinised sections were stained with haematoxylin and eosin (H&O). Analysis was done on 400X enlargement.

Statistical Analysis

The data generated were expressed as means ± SEM. Differences between the groups were ana-lysed using two-way analysis of variance (ANO-VA) followed by Dunnett's multiple comparison test, and the statistical significance was detected at $p<0.05$.

RESULTS

Antioxidant assay

The intra-peritoneal administration of Cisplatin (7.5 mg/kg b.wt) caused a significant decrease ($p<0.05$) in the activities of glutathione and its metabolising enzymes such as GST, GPx, GR and catalase activity when compared with control group. No significant change was observed in GSH, GPx and GR activity in the Chrysin only group (50 mg/kg b.wt) compared to control group. Also chrysin significantly induced GST and CAT activities in the lung. The administration of chrysin and Cisplatin showed a significant increase in CAT activity ($p<0.05$) as compared to cisplatin treated animals. Higher dose Chrysin (50 mg/kg b.wt) + Cisplatin showed significant increase ($p<0.05$) in the activities of GR, GST, GSH, and GPx as compared to Cisplatin treated group (Table 1).

DNA fragmentation

Gel electrophoresis of isolated genomic DNA reveals that the administration of Cisplatin (7.5 mg/kg b.wt) caused a significant change in the DNA status as compared with the control group. There was a reduction in the intensity of the difference in the DNA status in both Chrysin pre-treatment groups compared with the Cisplatin only group (Figure 1).

Histopathological findings

Figure 2 shows the lung micrographs observed under a microscope (X400) which revealed marked interstitial oedema and alveolar wall thickening in the cisplatin only treated group (Figure 2 B) when compared to the other groups (Figure 2 A C D E). Inflammatory cell infiltration was observed in Figure 2 B while both inflammatory response and oedema were moderately reduced in the chrysin administered group (Figure 2 C). The higher dose of chrysin offered protection to the lungs with micrographs similar to the control group. However, chrysin only treated group offered protection better than the group B C D.

TABLE 1. Effect of pre-treatment of Chrysin on Cisplatin mediated depletion of glutathione content and its metabolising enzymes in the lung tissue of Wistar rats.

Groups	Reduced glutathione (nM GSH/g tissue)	Glutathione reductase (nM NADPH oxidized/ min/mg protein)	Glutathione peroxidase (nM NADPH oxidized/ min/mg protein)	Catalase (nmol H_2O_2 consumed/ min/mg protein)	Glutathione-S-transferase (nmol CDNB conjugate formed/min/mg protein)
I (Only Vehicle)	121.67±1.97	464.84±32.74	517.77±2.0	337.96±21.68	926.68±3.00
II (Toxicants)	110.89±0.41**	244.54±4.67**	154.45±24.18**	177.83±7.47**	767.56±25.86**
III (D1 + Toxicants)	115.55±1.65	299.87±19.90	224.46±20.57	227.32±3.10*	870.05±33.83
IV (D2 + Toxicants)	120.46±1.70*	338.69±39.83*	428.84±30.40*	155.96±9.18	1039.47±16.15*
V (Only D2)	114.36±2.13	431.67±9.57	511.85±33.63	153.62±4.66**	1115.58±36.87**

Results represent mean ± S.D (n = 6). Significant differences are indicated by **p<0.05 when compared with control animals (group I) and *p<0.05 when compared with the Chrysin treated animals (group II). D1 = low dose modulator (Chrysin), D2 = high dose modulator, Toxicant =Cisplatin.
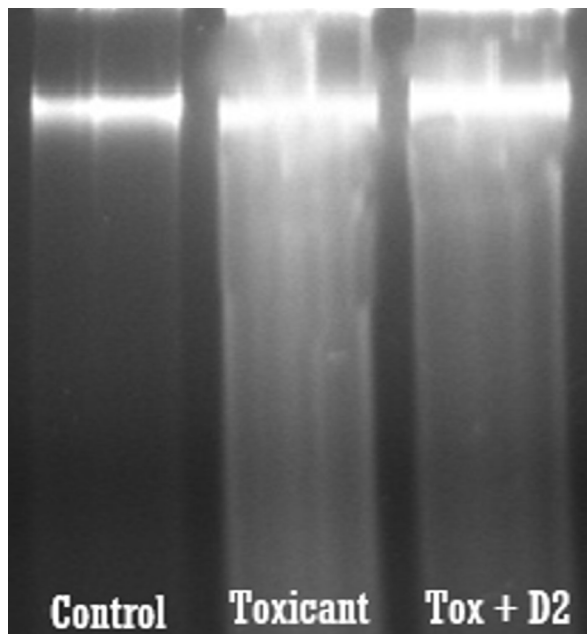
Fig. 1. Agarose Gel electrophoresis showing the effect of Chrysin on Cisplatin-induced genomic DNA damage in lung tissues of Wistar rats. Tox+D2 = Cisplatin + high dose modulator.

DISCUSSION

Cytotoxicity (organ toxicity) or resistance is a significant problem in antineoplastic therapy, resulting from both abortive attempts to repair the DNA lesions and loss of the mismatch repair activity. The lung has significant susceptibility to injury from a variety of chemotherapeutic agents. This injury is due to exposure to oxidative stress, which disrupts normal biological functions, characterised by the concentration of these stress markers and the duration of exposure[7-9,43,44]. According to the present findings, Cisplatin caused a significant decrease in both enzymatic (catalase, glutathione and its metabolising enzymes) and non-enzymatic antioxidants (reduced glutathione), increasing oxidative stress. The thiol group (-SH) containing molecules maintains intracellular redox homeostasis. Under certain conditions, a thiol group may lead to the formation of thiyl radicals (RS) that can interact with molecular oxygen, therefore generating ROS, which may deplete antioxidant reserves, hence creating an imbalance in the system[7,45]. Chrysin
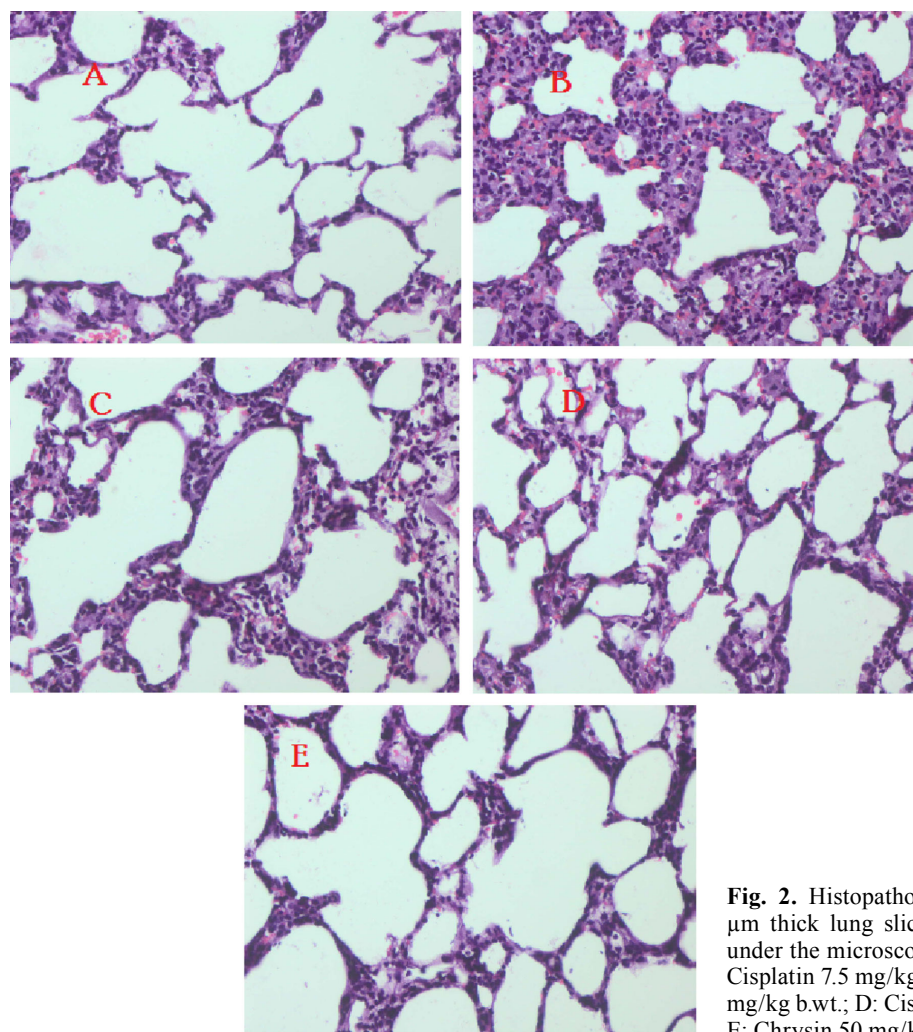

Fig. 2. Histopathological micrographs showing 5 μm thick lung slices stained with H&E observed under the microscope (X400). A: Control group; B: Cisplatin 7.5 mg/kg b.wt.; C: Cisplatin + Chrysin 25 mg/kg b.wt.; D: Cisplatin + Chrysin 50 mg/kg b.wt.; E: Chrysin 50 mg/kg b.wt.

restored the redox cycle components (GR, GPx and GSH) in the experimental lung tissue. It also restored catalase levels, an enzymatic antioxidant responsible for the breakdown of hydrogen peroxide into water and oxygen. Chrysin potentiated endogenous defence enzymes in rat lungs against cisplatin-induced oxidative stress. Similar findings were reported for renal, jejunum and lung induced oxidative stress in rats[46-49]. Induction of GST activity in chrysin pre-treated animals supports the detoxifying capacity of the flavonoid. This is because GST is a phase II detoxifying enzyme. Chrysin is a nonpolar compound; hence, it is insoluble in water, poorly absorbed in the intestine, has poor bioavailability and rapid systemic elimination. Therefore, serum concentration is affected[24,50,51]. This implies that a higher dose will be required to attain an effective serum concentration as observed in this study. The increased amount of chrysin (50 mg/kg bwt) showed an effective attenuation of cisplatin effects in glutathione and its metabolising enzymes such as GSH, GR, and GPx activities. The activity of GST is known to be affected by single nucleotide polymorphisms as recorded in several reports[52-54] but in this study Genotyping analyses of the GST gene were not assessed. Cisplatin and Low dose Chrysin (25 mg/kg bwt) influenced CAT activity. Catalase, a free radical scavenging enzyme, is beneficial in mopping up ROS generated due to oxidative stress[7,45]. Expression of the significant amount of catalase activity seen in the low dose Chrysin group compared to the group B is evident of an oxidative stress induced by cisplatin with insufficient chrysin. The isolated genomic DNA of lung tissues in this study, by the gel electrophoresis showed a significant change in the DNA status of the Cisplatin treated group compared with the control group (Figure 1). This study agrees with the report indicating that anticancer effect of cisplatin mainly depends on its DNA-damaging activity via its direct interaction with DNA to form DNA adducts[3]. It is also established that its ability to induce programmed cell death (apoptosis) in both cancer and normal cells has been associated with the formation of reactive oxygen species (ROS)[2,4-6]. Hence this change in DNA status could result from either form of DNA adduct or reactive oxygen species (ROS). Chrysin shows an effective protective potential against Cisplatin-induced DNA damage at a high dose. In this study, chrysin reduced the intense inflammatory responses and oedema induced by Cisplatin and micrographs reveal its anti-inflammatory activity in the lung. Studies have shown that Chrysin induces an anti-inflammatory effect[30,33], either by inhibiting the damaging of alveolar epithelial cells[55] or by inhibiting the prostaglandin-E2 formation or cyclooxygenase inflam-mation pathways[24,56]. The cisplatin group (Figure 2 B), with low antioxidants, was characterised by extensive alveolar oedema and alveolar septal fibrosis, severe alveolar damage, infiltration of polymorphic nuclear leukocytes, and haemorrhagic foci in the lung tissue. Studies show that lung injury results in loss of alveolar epithelial cells, which results in inflammation[57] (Figure 2). Low antioxidants are associated with oxidative stress, as seen in this group, agreeing with reports by Daniil et al[58] and Kliment et al[59] indicating that oxidative stress can also be related to the dysregulation of alveolar epithelial cells. The lung tissue of the control group (Figure 2 A) was histopathologically similar to that observed in the chrysin only group (Figure 2 E). Figure 2 D showed only slight oedema and congestion in the high dose chrysin group, indicating that the biochemical results agree with the histopathological findings. Previous reports have shown that cisplatin chemotherapy-induced structural pulmonary damage is associated with interstitial inflammation, fibrosis, and obliterative bronchiolitis[60,61]. These histopathological findings demonstrate that chrysin protects against pulmonary oxidative stress by preventing decreases in antioxidants and promoting anti-inflammatory activity. These findings suggest that Chrysin plays a significant role against Cisplatin-induced pulmonary toxicity in Wister rats.

CONCLUSIONS

Chrysin has shown protective effects on rat lungs against oxidative damage caused by Cisplatin administration. It may have similar effects on Cisplatin administration either used for chemoprevention or for chemotherapeutic purposes in human lungs. The present investigation gives a path to investigate the effects of Chrysin and other flavonoids more deeply on lung injuries caused by Cisplatin as a combination therapy or on lung injuries caused by other chemotherapeutic drugs.

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