American-Eurasian Journal of Toxicological Sciences 7 (4): 292-299, 2015
ISSN 2079-2050

DOI: 10.5829/idosi.aejts.2015.7.4.96233

Whey Protein: A Novel Protective Agent against Oto-Toxicity Induced by Cis-Platin in Male Rat

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Abstract: *Background:* Cis-platin is a widely used chemotherapeutic drug to treat many malignant disorders including head and neck malignancies. Oto-nephrotoxicity is an important and dose-limiting side effect of cis-platin therapy. Nowadays, more attention had been paid to ototoxicity caused with cis-platin. Aim of The Work: This study was designed to investigate the potential protective effect of whey protein (WP) against cis-platin induced ototoxicity compared to the effect of *N*-acetylcysteine (NAC) in rats. Methodology: Male albino rats were randomly divided into 6 groups: untreated rats (control), rats orally treated with whey protein(1g/kg b.w/day)for seven executive days, rats treated orally with N-acetylcysteine(500 mg/kgb.w /day) for seven executive days,rats intoxicated intraperitoneal (ip) with cis-platin(10 mg/kgb.w. once),rats treated with whey protein (1g/kgb.w./day) for seven executive days) followed by one injection (ip) of cis-platin(10 mg/kg b.w.) one hour after the last oral administration of whey protein, rats treated with N-acetylcysteine(for seven executive days followed by one injection (ip) of cis-platin(10 mg/kgb.w)one hour after the last oral administration of N-acetylcysteine. The Organ of Corti, the stria vascularis and spiral ganglia were visualized by light microscopy at different magnifications. Results: Cis-platin intoxicated animals showed a significant decrease in serum level of total antioxidant capacity (TAC), with inhibition in the activity of serum glutathione-s-transferase (GST) and paraoxonase-1 (PON-1) in comparison with control. Group treated with either NAC or WP with cis-platin showed significant elevation in the activity of both GST and PON-1 with increased serum level of TAC when compared with cis-platin intoxicated rats. Animals treated with NAC or WP with cis-platin compared to those treated with cis-platin alone showed marked degree of improvement towards control rats as there was a significant drop in the serum level of cortisol, nitric oxide(NO) and melandialdehyde (MDA). Histopathologic, in NAC pre-treated group there was no changes in stria vascularis or spiral ganglia. In group pre-treated with WPC, there was no histopathologic alteration detected in the Organ of Corti and Reissers membrane but oedema and haemorrhage were founded in the stria vascularis in minimal focal manner. Conclusion: These findings showed that whey protein is a natural dietary supplement product proved its ability of protection of anti-oxidant system and the Cochlea against cis-platin induced ototoxicity.

Key words: Anti-oxidant · Cis-platin · N-Acetylcysteine · Ototoxicity · Whey Protein

INTRODUCTION

Cis-platin is a well known platinum-based chemotherapeutic agent used for the treatment of various malignant tumours. A frequent side effect of cis-platin therapy is ototoxicity which is dose-limiting [1]. Cis-platin induced-hearing loss is usually bilateral and irreversible and is particularly serious in the pediatric population, age 6 months and onwards [2]. Although ototoxicity caused by cis-platin may occur within hours to days after

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drug administration, delayed ototoxicity from cis-platin may occur in children. Loss of hearing at this developmental stage hampers the speech, cognitive and social development of the child [3]. Laboratory animal and *in vitro* studies showed that cis-platin ototoxicity has been shown to have at least three major tissue targets in the cochlea: Organ of Corti, spiral ganglion cells and lateral wall (stria vascularis and spiral ligament) [4]. Cis-platin undergoes hydrolysis in blood to form cis-diamineaquachloroplatinum (II), the major aqueous metabolite, responsible for its cytotoxic actions [5]. Cis-platin ototoxicity proceeds via the formation of reactive oxygen species (ROS) in cochlear tissue, with apoptotic cell death as a consequence in addition to DNA damage [6]. Increased ROS generation has been demonstrated in all three major tissue targets of the cochlea. The cochlea is practically a closed system and therefore is unable to flush out these ROS overload causing depletion of the cochlear antioxidant enzyme system and leading to cell injury and apoptosis. [3].Oxidative stress occurs due to an imbalance between the production of ROS and the protection by cellular antioxidants [7]; thus, exogenous administrations of antioxidants have been the primary focus for devising the treatment strategy against cis-platin-induced ototoxicity [8].Numerous studies have been reported on the protective effects of various antioxidants on cis-platin induced ototoxicity. The protective effects of N-acetylcysteine (NAC) have been demonstrated experimentally [9, 10] and clinically [11, 12].

Whey protein (WP) is a natural product that was able to reduce the effects of oxygen radicals and inhibits lipid peroxidation by increasing the antioxidant glutathione and thus stimulating the early normal events of the healing process [13]. Moreover, Haraguchi *et al.* [14] concluded that WP has a protective effect against oxidative stress, mainly in the liver and a beneficial effect on renal function in rats supplemented with WP. Whey protein concentrate (WPC) is milk serum protein that provides all essential amino acids and branched-chain amino acids, which are important factors in tissue growth and repair [13]. Whey proteins concentrate, "fast proteins" do not coagulate in acidic conditions. They reach the jejunum quickly after entering the gastrointestinal tract, where its hydrolysis is slow, allowing for greater absorption all over the length of the small intestine [15]. There is a great need to find safe and natural protective agents against cis-platin ototoxicity. This would eliminate one of the dose-limiting side effects of its therapy and improve the quality of life for many patients. Consequently, the present study hypothesized that oto-protection in cis-platin treated animals could be improved by supplementing them with whey protein concentrate (WPC). This action will be compared to the effect of NAC, as a pharmaceutical protective agent, against cis-platin ototoxicity.

MATERIALS AND METHODS

Experimental Design: Male Sprague Dawley rats (200-250g) were obtained from the Animal House Colony of National Research Centre, Cairo, Egypt. The environmental conditions were standardized with respect to temperature, humidity and light. All animals received human care in compliance with the standard institution's criteria for the care and use of experimental animals. The experimental protocol was approved by local ethical committee. Rats were randomly divided into six groups (n=8 for each group): (1) sham or control untreated normal rats; (2) normal rats orally treated with Whey protein concentrate (WPC) dissolved in distilled water (1g/kg b.w./ day) for seven executive days [16]; (3) normal rats treated orally with *N*-acetylcysteine (NAC) (obtained from El-Nasr Co., Egypt) alone (500 mg/kg b.w./day) for seven executive days [17]; (4) normal rats intoxicated with intraperetoneally (ip) with cis-platin (obtained from Pfizer Co., Egypt) dissolved in saline (10 mg/kg b.w once); (5) rats pre-treated with NAC (650 mg/kg b.w/day) for seven executive days, followed by once injection (ip) of cis-platin (10 mg/kg b.w) one hour after the last oral administration of NAC; and (6) rats orally pre-treated with WPC 80% (purchased from USA, through Light Food Co., Egypt) (1g/kg b.w/day) for seven executive days followed by one injection (ip) of cis-platin (10 mg/kg b.w) one hour after the last oral administration of WPC.

Blood Sample Collection: After 24 hours of cis-platin injection, animals were fasted overnight; and under diethyl ether anaesthesia, blood samples were collected from retro-orbital venous plexus of the rats of all groups, then centrifuged and the sera were separated into aliquots and stored at-20°C for later biochemical analyses.

Cochleae Tissues Harvest: After blood collection, all animals were rapidly decapitated and the cochlear bone capsules were dislocated and removed by micro-dissection using an operating microscope. Samples were fixed with 10% buffered formaldehyde-saline for 3 days and decalcification was performed using 10% EDTA solution (pH 7.4). After 7 days, running water was used to wash away the decalcification solution and the tissue was

soaked again for 2 more days in formaldehyde-saline buffer. An automated tissue tracking device was used for routine processing, 13h post-fixation, with two containers of formaldehyde-saline buffer, four containers of alcohol, two containers of xylene and two vessels of paraffin. After processing, the sections were embedded in paraffin by the basal turn apex axis, parallel to the cochlea. Paraffin block serial sections of the mid-modiolar area (up to 50 um thick) were prepared. Mid-modiolar area sections (4um thick) were stained with hematoxylin and eosin and examined using light microscopy by a pathologist blinded to the treatment groups. Scoring was performed similar to previous studies. The stria vascularis, the Organ of Corti and spiral ganglia were visualized by light microscopy at different magnifications and photographed.

Biochemical Analyses: Serum total antioxidant capacity (TAC) and serum glutathione-S-transferase (GST) activity were determined according to the colorimetric methods described by Koracevic [18] and Habig *et al*. [19] respectively, using reagent kits obtained from Bio-diagnostic Co, Egypt. Serum nitric oxide (NO) level was estimated according to the method of Moshage *et al.* [20] using kits of R and D System GmbH (Germany). Serum paraoxonase-1 (PON-1) activity was determined by kinetic spectrophotometric method described by Eckerson *et al.* [21], using paraoxon (Sigma, USA) as substrate. PON-1 can hydrolyze paraoxone to *p*-nitrophenol and diethylphosphate. The rate of paraoxone hydrolysis can be monitored spectrophotometrically at λ405 nm at 37 °C by measuring the increase of absorbance at zero time and each two minutes interval for 10 minutes. Serum level of lipid peroxidation as malondialdehyde (MDA) was determined by chemical method described by Ruiz-Larnea *et al.* [22] which is based on its reaction with thiobarbituric acid (TBA) that forms a pink complex can be measured photometrically at λ 535 nm. Serum cortisol level was determined according to Bondy [23] using ELISA kit purchased from immunospect, Canoga Park, USA.

Statistical Analysis: All values were expressed as mean ± standard deviation (SD). The obtained data were subjected to one way analysis of variance (ANOVA) using statistical analysis system (SAS) program software; copyright (c) 1998 by SAS institute Inc., Cary, NC, USA. Duncan's multiple range tests were used to clarify the significance between the individual groups at level $p \leq 0.05$. Means with the different superscript letters are significantly different at ($p \leq 0.05$).

RESULTS

Biochemical Analyses: The effects of cis-platin, WPC and NAC, either alone or in combination with cis-platin on serum level of total antioxidant capacity (TAC) as well as the activities of serum paraoxonase-1 (PON-1) and glutathione-S-transferase (GST) are summarized in table (1). The data showed that animals given orally either NAC or WPC showed no inverse effects on serum level of total TAC, the activities of serum PON-1 and GST; while animals group injected ip with cis-platin alone showed a significant ($p<0.05$) decrease in the serum level of TAC, matched with inhibition in the activity of both serum PON-1 and GST when all were compared to the normal (sham) control group or animal groups treated with NAC or WPC alone. The effect of NAC and WPC on TAC, PON-I and GST in animals groups intoxicated previously with cis-platin showed significant ($P \leq 0.05$) elevations when both were compared to the cis-platin intoxicated group. In addition, regarding animal's groups treated with NAC or WPC in combination with cis-platin, a remarkable degree of improvement was monitored from the significant ameliorations in the serum level of cortisol, NO and MDA towards normal controls when both were compared to the cis-platin intoxicated group.

Histological Examination: The normal histological architecture of control cochlea detected in the spiral ganglion, hair cells, Reissners membrane, stria vascularis,

Table 1: Serum levels of TAC, GST, PON-1, Cortisol, NO and MDA of WPC, NAC and cis-platin treated adult male albino rats as compared to normal controls

	TAC	GST	PON-I	Cortisol	NO	MDA
Control	1.31±0.04^{A}	2289±57^{A}	645±9.7^{A}	11.53±0.8^{A}	82.5±5.6^{A}	12.87±0.6^{A}
NAC	1.30±0.07^{A}	2163±64^{A}	669±22.7^{A}	9.3±0.87^{A}	77.9±7.6^{A}	12.79±0.9^{A}
WPC	1.31±0.06^{A}	2292±63^{A}	652±32^{A}	9.88±1.44^{A}	74.8±12.2^{A}	12.45±0.8^{A}
Cis	o.56 ±0.1^{B}	638±100^{B}	468±17.3^{B}	16.86±0.8^{B}	154±17.56^{B}	20.13±1.4^{B}
Cis+NAC	1.19 ±0.02^{A}	2001±12^{A}	544.5±12.2^{C}	12.67±0.6^{C}	106.7±12.2^{C}	14.74±0.6^{C}
Cis+WPC	0.89±0.21^{C}	1641±129^{A}	552.2±19^{C}	12.1±1.9^{C}	129.8±10.1^{C}	15.82±0.7^{C}

All data are expressed as mean ± standard deviation.

Means with the different superscript letters are significantly different at $p \leq 0.05$.

NAC (*N*-acetylcysteine), WPC (whey protein concentrate), Cis (Cis-platin).

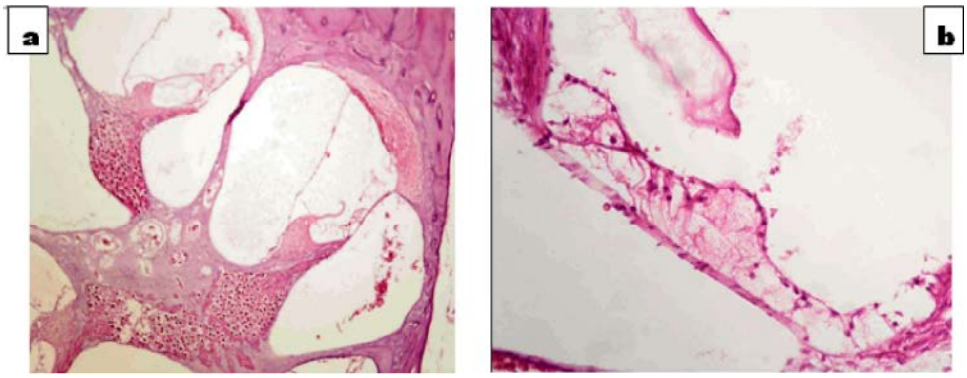

Fig. 1: (a): showed no histopathological alterations were detected in the spiral ganglion, hair cells, Reissners membrane, or stria vascularis; while (b): illustrated a preserved architecture of Organ of Corti of control group.

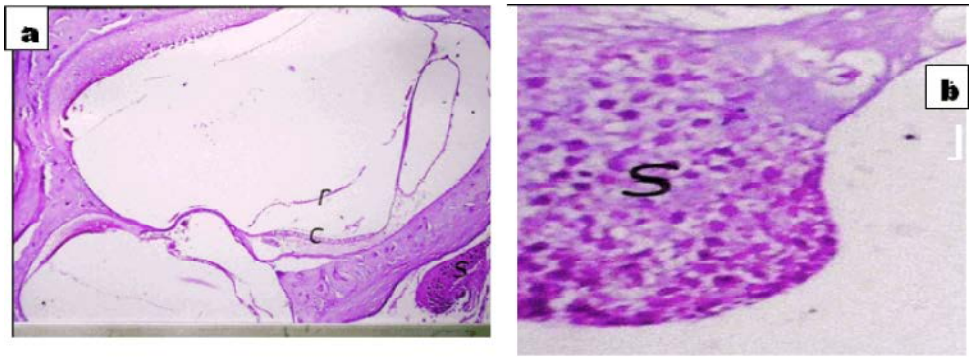

Fig. 2: (a) showed a complete alteration in histology of architecture of Organ of corti; while (b) the lumen showed inflammatory cells infiltration associated with eosinophilic spots replacing the degenerated spinal ganglionic cells(s).in cis-platin intoxicated group.

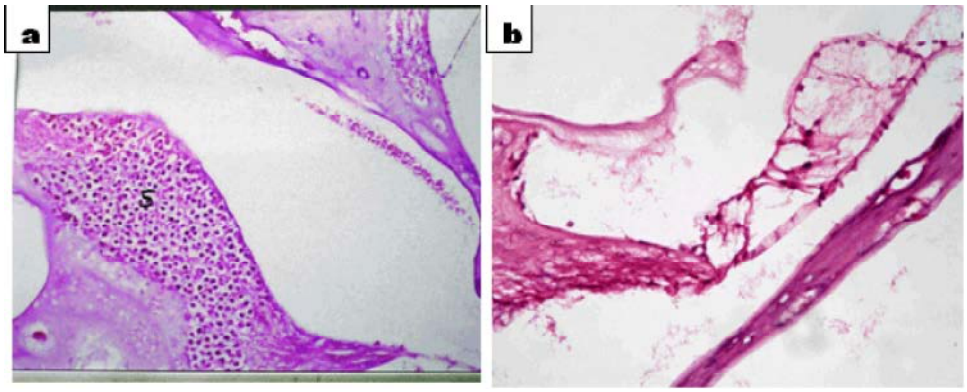

Fig. 3: (a and b) showed a preserved normal architecture with no histopathological alteration in the spiral ganglion, stria vascularis and hair cells of animals group treated with NAC in combination with cis-platin intoxication..

(Fig. 1a) and Organ of Corti (Fig. 1b). In cis-platin intoxicated group complete histologic alteration with extensive loss of the normal micro-architecture of the Organ of Corti, outer and inner hair cells without definition. No tectorial membrane in rat group treated with cis-platin (Fig. 2a); moreover demyelination and degeneration of spinal ganglionic cells are seen (Fig. 2b).

In NAC pre-treated animal group, there was no histopathological alteration in the spiral ganglion, stria vascularis as showed in (Fig.3a and b); while in WPC pre-treated animal group, there was no histopathological alteration detected in the Organ of Corti and Reissners membrane (Fig.4a), but minimal oedema and haemorrhages were detected in the stria vascularis in focal manner (Fig. 4b).

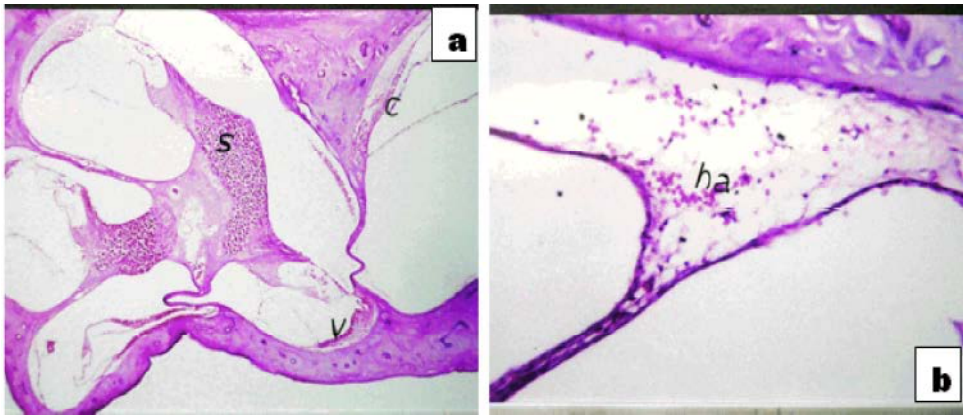

Fig. 4: (a and b) showed a preserved normal architecture with no histopathological alteration in the spiral ganglion, stria vascularis and hair cells of animals group treated with WPC in combination with cis-platin intoxication.

DISCUSSION

Cis-platin is a highly successful and widely used chemotherapy for the treatment of various solid malignancies in both adult and pediatric patients. Side effects of cis-platin treatment include nephrotoxicity and ototoxicity. Hearing loss has primarily been associated with the loss of inner ear hairy and spiral ganglion cells due to oxidative stress [24]. In our study, we investigated the protective potential of WPC as a novel supplement, antioxidant against cis-platin-induced ototoxicity in compare to pharmaceutical antioxidant NAC in experimental animals. The obtained results of this study demonstrated that, oral administration of WPC or NAC exerts an oto-protective effect in cis-platin-intoxicated animal models, evidenced by biochemical and histopathological findings. Previous study suggests that cis-platin mediated hearing loss essentially involves a robust generation of reactive oxygen species (ROS) in the cochlea, outer hair cells, spiral ganglia, stria vascularis and the spiral ligament. Despite the presence of various endogenous antioxidant, cyto-protective mechanisms like glutathione and other antioxidant enzymes as well as heat shock proteins, the damage seems to be inevitable as these mechanisms become overwhelmed (over time and cumulative dosage) and succumb to the lethal/cytotoxic effects of cis-platin Rybak *et al.* [4].

Glutathione is a water soluble antioxidant tripeptide compound, consisting of glycine, glutamic acid and cysteine molecules those were synthesized de novo in mammalian cells. Its conjugation is considered to be an innate protective mechanism, developed to protect the body from potentially damaging electrophilic compounds. GST is a complex group of iso-enzymes which catalyze the conjugation of potentially damaging electrophiles with glutathione. Compounds metabolized by GSTs include environmental pollutants, pesticides, carcinogens, drugs, drug metabolites and byproducts of oxidative stress, all of which represent electrophilic threats to the body [25].

Paraoxonases are a family of three enzymes called PON1, PON2 and PON3. They have multifunctional roles in various biochemical pathways such as protection against oxidative damage and lipid peroxidation, contribution to innate immunity, detoxification of reactive molecules, bioactivation of drugs and modulation of endoplasmic reticulum stress and regulation of cell proliferation/apoptosis. Since they are able to perform multiple autonomous and often unrelated functions, they are considered as “moonlighting proteins” [26]. PON-1 is the most studied enzyme of the family. It is synthesized primarily in the liver and appears mainly in serum, where is associated to high-density lipoproteins (HDL) [27].

Initially the interest on this enzyme arose from the toxicological point of view, by its protective role from poisoning by organophosphate derivates. But more recently research has been focused on other clinical aspects such as protective role in vascular disease as well as its use as biomarker of diseases involving mainly in oxidative stress, since PON-1 protects against oxidation [28]. About cortisol, its level increased in severe trauma and stressful events can elevate cortisol levels in the blood for prolonged periods [29].

In the current study, cis-platin intoxicated animals showed a significant decrease in the serum level of TAC, inhibition in the activity of serum PON-1 and reduction in the activity of GST when were compared to control rats; these results are in conformity with work done by Rybak *et al*. [30] who stated a significant depletion of glutathione and decrease of the antioxidant enzymes activities in cis-platin induced-ototoxicity in rats.

The decrease in the serum PON-I activity was in agreement with the study carried out by Khaled *et al*.[31]. While there were significant elevations in the serum TAC as well as in the activity of serum PON-1 and in the activity of serum GST in both groups treated with either WPC or NAC in combination with cis-platin compared to these of cis-platin alone intoxicated animals. Such results indicate that administration of WPC or NAC could attenuate ototoxicity induced by cis-platin therapy, via increasing activity of PON-I, serum level of TAC and activity of GST. These results indicated that WPC can reduce the degree of oxidative stress in the cochlea of rats. Previously, some researchers revealed that, NAC displayed important effects as antioxidant and free-radical scavenging, especially the otoprotective effect [8, 32]. Also, it was reported that the cis-platin-induced ototoxicity is attributed to the increased ROS and the altered antioxidant defense system in the cochlea [3]; moreover, the ototoxic effect of cis-platin is a dose dependent [33] and involves multiple targets: the Organ of Corti, the stria vascularis and the spiral ganglion [34, 35].

Cis-platin, after injection, promotes the generation of ROS, which in turn were known to have diverse effects on different cellular function and induced inner ear injury [36, 37]. Membrane-associated polyunsaturated fatty acids are attached by ROS in a process that results in the peroxidation of lipid, that explain the significant increase in serum level of MDA, which is an end product of lipid peroxidation in cis-platin-intoxicated group. This finding is in agreement with previous study carried out by Rybak *et al*. [30]. Additionally, cis-platin has three important tissue targets in the cochlea, including Organ of Corti, spiral ganglion cells and lateral wall (stria vascularis and spiral ligament). In guinea pigs that received consecutive cis-platin applications, destruction of outer hair cells and myelin sheath detachment of spiral ganglion cells were observed [38]. Furthermore depletion of glutathione and antioxidant enzymes (superoxide dismutase, glutathione peroxidase and glutathione reductase) with an increase in MDA levels, were demonstrated in cochlear tissue samples from animals receiving cis-platin [30]. These results were in agreement with the current work.

Nitric oxide (NO) was an important molecule mediator in inner ear injury with cis-platin therapy [39]. Redundant NO in pathological condition was harmful, since NO could interact with superoxide anion radical to produce peroxynitrite, a potent membrane oxidant [40], as well as inhibit those enzymatic system that participated in mitochondrial respiration [41]. In the current study we record that oral administration of either NAC or WPC didn't adverse the serum levels of cortisol, NO and MDA in comparison to the control group. Contrarily, intraperitoneal intoxication of rats with cis-platin alone induced a significant increase in the serum level of cortisol, NO and MDA when compared to the normal control. These results were in accordance with the previous study of Watanabe *et al*. [39]. Also, similar results were observed by Kelly *et al*. [42] Who stated that: increased level of NO has been found in rat whole cochlea extract after treatment with cis-platin. In consistently, Ryback *et al*. [4] in their study found that there were an elevation in the level of MDA of cis-platin intoxicated animals. In the same time, a similar result was obtained by Campbell *et al.* [43] who reported that MDA level increased in the cochlea of rats treated with cis-platin.

Regarding animal's groups pre-treated with NAC or WPC in combination with cis-platin, a remarkable degree of improvement of serum level towards normal controls; this was monitored from the significant amelioration in serum level of cortisol, NO and MDA when both were compared to the cis-platin intoxicated group. In the current study the improvement recorded in animals pre-treated with WPC could be explained by its free radical scavenging properties and antioxidant activity that was in accordance with Gad *et al.* [16] who reported antioxidant protective effects in rat liver treated by WPC.

Light microscopic examination of modiolar longitudinal sections makes it possible not only to observe changes in outer hair cells, but also to identify stria vascularis and spiral ganglion damage [44, 45]. Histological damage in chocleas of cis-platin-intoxicated animals showed hemorrhage, vacuolization in stria vascularis and demyelination and degeneration in spinal ganglionic cells. This result contrary to one study [34] and in another study reported that histological effects were not observed in the stria vascularis which cannot be detected by light microscop; however detachment of the myelin sheaths of type-I SGCs in cis-platin-intoxicated animals [46]. Antioxidant protective effects of WPC could be confirmed by the histological examination for cochlea of animal pre-treated with WPC in combination with cis-platin that showed no histopathological alteration detected in outer hair cells and Reissner`s membrane, but minimal edema and hemorrhage were detected in the stria vascularis in focal manner. Histopathological differences among the six groups are remarkable and significant.

The present work demonstrates an inner ear injury induced by cis-platin in a rat model, which is evidenced by both biochemical indices and morphological examinations. WPC ameliorated the ototoxicity induced by cis-platin probably through its ant-oxidative and with its efficiency as a free radical scavenger agent. Finally, WPC

as an antioxidant can be considered as a novel therapeutic natural supplement for otoprotective with or before cis-platin therapy.

ecommendation: Further studies are needed by using higher doses of WPC to ameliorate completely the side effects of cis-platin therapy on ear toxicity.

ACKNOWLEDGMENT

To prof. Adel Baker prof. of pathology Cairo University for his help in this study by processing tissues of choclea of animals studied in the present work and comments on the histopathologic slides of the different animal groups at his private lab.

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