

Silymarin Modulates Cisplatin-Induced Oxidative Stress and Hepatotoxicity in Rats

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Cisplatin (CDDP) is a widely used anticancer drug, but at high dose, it can produce undesirable side effects such as hepatotoxicity. Because silymarin has been used to treat liver disorders, the protective effect of silymarin on CDDP-induced hepatotoxicity was evaluated in rats. Hepatotoxicity was determined by changes in serum alanine aminotransferase [ALT] and aspartate aminotransferase [AST], nitric oxide [NO] levels, albumin and calcium levels, and superoxide dismutase [SOD], glutathione peroxidase [GSHPx] activities, glutathione content, malondialdehyde [MDA] and nitric oxide [NO] levels in liver tissue of rats. Male albino rats were divided into four groups, 10 rats in each. In the control group, rats were injected i.p. with 0.2 ml of propylene glycol in saline 75/25 (v/v) for 5 consecutive days [Silymarin was dissolved in 0.2 ml of propylene glycol in saline 75/25 v/v]. The second group were injected with CDDP (7.5 mg /kg, I.P.), whereas animals in the third group were i.p. injected with silymarin at a dose of 100 mg/kg/day for 5 consecutive days. The Fourth group received a daily i.p. injection of silymarin (100 mg/kg/day for 5 days) 1 hr before a single i.p. injection of CDDP (7.5 mg/kg). CDDP hepatotoxicity was manifested biochemically by an increase in serum ALT and AST, elevation of MDA and NO in liver tissues as well as a decrease in GSH and the activities of antioxidant enzymes, including SOD, GSHPx in liver tissues. In addition, marked decrease in serum NO, albumin and calcium levels were observed. Serum ALT, AST, liver NO level, MDA was found to decrease in the combination group in comparison with the CDDP group. The activities of SOD, GSHPx, GSH and serum NO were lower in CDDP group than both the control and CDDP pretreated with silymarin groups. The results obtained suggested that silymarin significantly

attenuated the hepatotoxicity as an indirect target of CDDP in an animal model of CDDP-induced nephrotoxicity.

Keywords: Cisplatin, Hepatotoxicity and nitric oxide, Silymarin

Introduction

Cisplatin [cis-diamminechloroplatinum (II)] (CDDP) is a potent antineoplastic agent used for the treatment of a wide range of cancers (Saad *et al.*, 2004; Wang *et al.*, 2004). Nevertheless, this drug has severe toxic effects that interfere with its therapeutical efficacy, namely nephrotoxicity and hepatotoxicity. Although the nephrotoxicity of CDDP has been recognized as the most important dose-limiting factor, little is known about CDDP-induced liver injury. Hepatotoxicity is not considered as a dose-limiting toxicity for CDDP, but liver toxicity can occur when the antineoplastic drug is administered at high doses (Zicca *et al.*, 2004). Oxidative stress is one of the most important mechanisms involved in CDDP-induced toxicity. The mitochondrion is the primary target for CDDP-induced oxidative stress, resulting in loss of mitochondrial protein-SH, inhibition of calcium uptake and a reduction in the mitochondrial membrane potential (Saad *et al.*, 2004).

Silymarin, an antioxidant flavonoid complex derived from the herb milk thistle (*Silybum marianum*), has long been used in the treatment of liver diseases (Naveau, 2001; Laekeman *et al.*, 2003). These properties seem to be due to their ability to scavenge free radicals and to chelate metal ions (Borsari *et al.*, 2001). Silymarin is frequently used in the treatment of liver diseases where it is capable of protecting liver cells directly by stabilizing the membrane permeability through inhibiting lipid peroxidation (Mira *et al.*, 1994) and preventing liver glutathione depletion (Valenzuela *et al.*, 1989).

Thus the aim of the present work was to study the effect of CDDP on liver tissue and the effect of silymarin, an antioxidant agent, to ameliorate the hepatotoxicity of CDDP.

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Materials and Methods

Animals. Adult male Sprague-Dawley rats, weighing 120-150 g were obtained from the experimental animal house of the National Cancer Institute (NCI), Cairo University. Animals were kept under standard conditions and had access to a standard diet and clean drinking water.

Materials. Cisplatin (Cisplatyl 50 mg, Laboratoire Roger Bellon) was a generous gift from NCI drug store. Silymarin was purchased from Aldrich Chemical Co. The required dose was dissolved in 0.2 ml of propylene glycol in saline 75/25 (v/v) (Mereish *et al.*, 1991).

Experimental design. Cisplatin treatment protocol used in this study to develop liver toxicity has been previously reported (Ramadan *et al.*, 2001). Silymarin treatment protocol used in this study to treat liver toxicity has been previously reported (Crocenzi *et al.*, 2003).

Animals were divided at random into 4 groups of 10 animals each. The first group: rats were administered propylene glycol in saline 75/25 (v/v), this group served as a control. The second group: rats were i.p. injected with CDDP 7.5 mg/kg. The third group: rats were i.p. injected with silymarin at a dose of 100 mg/kg/day for 5 consecutive days. The Fourth group: rats received silymarin by i.p. injection of 100 mg/kg/day for 5 days, 1 hr after the last injection, animals were injected with a single i.p. injection of CDDP (7.5 mg/kg).

Twenty-four hrs after the last dose of the specific treatment, animals were anesthetized with ether, and blood samples were obtained by heart puncture. Serum samples were separated for the measurement of indices of hepatotoxicity and calcium concentration. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were estimated according to the method of Reitman and Frankel (Reitman and Frankel, 1957). Colorimetric determination of ALT or AST was estimated by measuring the amount of pyruvate or oxaloacetate produced by forming 2, 4-dinitrophenylhydrazine. The color of which was measured at 546 nm.

Albumin level was measured according to the method of Wrenn and Feichtmeir (Wrenn *et al.*, 1956) using. Serum calcium levels were determined using Randox Kits (Randox Laboratories).

Animals were then sacrificed by decapitation after exposure to ether in a dessicator kept in a well-functioning hood. Livers were removed and washed with ice-cold saline, blotted with a piece of filter paper and homogenized using a Branson sonifier (250, VWR Scientific).

In the liver homogenate, reduced glutathione (GSH) was determined according to the methods of Ellman (Ellman, 1959), The method is based on the reduction of Ellman's reagent [5,5'-dithio-bis-(2-nitrobenzoic acid)] by SH groups to form 1mole of 2-nitro-5-mercaptobenzoic acid per mole of SH. The nitro-mercaptobenzoic acid has an intense yellow color and can be determined spectrophotometrically. To 0.5 ml of 10% trichloroacetic acid. 6 mM disodium ethylene diamine tetraacetic acid, 0.5 ml of homogenate was added and shaken gently for 10-15 minutes. This was followed by centrifugation at 2,000 rpm for 5 minutes. 0.2 ml of the supernatant was mixed with 1.7 ml of 0.1 M potassium phosphate buffer (pH 8). At least a duplicate was made for each sample. 0.1 ml of Ellman's reagent was added to each tube. After 5 minutes the

optical density was measured at 412 nm against a reagent blank. The results were expressed as $\mu\text{mol/g}$ tissue.

Glutathione peroxidase (GSH-Px) was determined in liver homogenate according to the method of Lawrence and Burk (Lawrence and Burk, 1976). This method is based on measuring the oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) using hydrogen peroxide as the substrate. A reaction mixture of 1 ml contained 50 mM potassium phosphate buffer (pH 7), 1 mM EDTA, 1 mM NaN_3 , 0.2 mM NADPH, 1 unit/ml oxidized glutathione reductase and 1 mM GSH was prepared. The homogenate was centrifuged t 105,000 for 15 minutes at 4°C. 0.1 ml of the supernatant was added to 0.8 ml of the reaction mixture and the solution was incubated for 5 minutes at 25°C. 0.1 ml of 0.25 mM hydrogen peroxide solution was added to initiate the reaction. Absorbance was measured at 340 nm for 5 minutes, and an extinction coefficient of 6.22×10^{-3} was used for calculation. The results were expressed as $\mu\text{mol/min/gm}$ tissue. The changes in the absorbance at 340 nm were recorded at 1-min interval for 5 min.

Superoxide dismutase (SOD) activity in liver homogenate was determined according to the method of Minami and Yoshikawa (Minami and Yoshikawa, 1979). This method is based on the generation of superoxide anions by pyrogallol autoxidation, detection of generated superoxide anions by nitro blue tetrazolium (NBT) formazan color development and measurement of the amount of generated superoxide anions scavenged by SOD (the inhibitory level of formazan color development). The homogenate was centrifuged t 105,000 for 15 minutes at 4°C. To 0.25 ml of supernatant, 0.5 ml of tris cacodylic buffer, 0.1 ml of 16% triton x-100 and 0.25 ml NBT were added. The reaction was started by the addition of 0.01 ml diluted pyrogallol. Incubation was maintained for 5 minutes at 37°C. The reaction was stopped by the addition of 0.3 ml of 2 M formic acid. The formazan color developed was determined spectrophotometrically (Spectronic 501, Shimadzu). Enzymatic activity was expressed as $\mu\text{g/gm}$ of tissue.

Malondialdehyde (MDA) levels in liver tissue homogenates were determined spectrophotometrically using the method of Buege and Aust (Buege and Aust, 1978). 0.5 ml of tissue homogenate was shaken with 2.5 ml of 20% trichloroacetic acid in a 10 ml centrifuge tube. To the mixture, 1 ml of 0.67% thiobarbituric acid was added, shaken and warmed for 30 minutes in a boiling water bath followed by rapid cooling. Then 4 ml of n-butyl-alcohol was added and shaken. The mixture was centrifuged at 3,000 rpm for 10 minutes. The resultant n-butyl-alcohol layer was taken and MDA content was determined from the absorbance at 535 nm. The results were expressed as nmol/g tissue.

Nitric oxide (NO) levels in serum and liver tissue homogenates were determined according to the method of Ignarro *et al.* (1987). The assay is based on the diazotization of sulfanilic acid with nitric oxide at acidic pH and subsequent coupling with N-(10-naphthyl)-ethylenediamine to yield an intensely pink colored product that is measured spectrophotometrically at 540 nm. A nitrate standard solution (100 μl) was serially diluted in duplicate in a 96-well, flat-bottomed, micro titer plate. After loading the plate with samples (100 μl), addition of vanadium (III) chloride (VCl_3) (100l) to each well was rapidly followed by the addition of the Griess reagents, sulfanilamide (50 μl) and N-(1-Naphthyl)ethylenediamine dihydrochloride (50 μl). The absorbance at 540 nm was measured using a plate reader following incubation (usually 30 minutes).

Table 1. Effect of cisplatin, silymarin and their combination on serum ALT, AST, albumin and calcium in male albino rats

Groups	ALT (U/min)	AST (U/min)	Albumin (g/dL)	Calcium (mmol/L)
Control	53.86 ± 2.16	85.68 ± 1.44 ^a	3.8 ± 0.27	15.81 ± 0.94
CDDP	100.9 ± 1.21	162.47 ± 3.21	1.83 ± 0.1 ^a	11.04 ± 1.8 ^a
Silymarin	60.55 ± 1.51	81.05 ± 1.17	2.72 ± 0.07	7.85 ± 0.54
Silymarin + CDDP	85.06 ± 1.55	86.16 ± 2.94 ^a	2.05 ± 0.1 ^a	10.97 ± 0.95 ^a

All values are the mean ± SD (n = 10). Statistical significance between means was performed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer as a post ANOVA test ($p < 0.05$). Means with the same letter within the same parameter are not significantly different.

Table 2. Effect of cisplatin, silymarin and their combination on the levels of malondialdehyde (MDA), superoxide dismutase activity (SOD), glutathione peroxidase activity (GSHPx) and reduced glutathione (GSH) in rat liver tissue

Groups	MDA (nmol/gm tissue)	SOD (μ g/gm tissue)	GSHPx (mole/gm tissue)	GSH (μ mol/gm tissue)
Control	18.92 ± 1.37 ^a	107.6 ± 3.18 ^a	0.47 ± 0.012 ^a	0.16 ± 0.027 ^a
CDDP	43.05 ± 2.04	68.65 ± 5.19	0.40 ± 0.001	0.07 ± 0.012
Silymarin	16.64 ± 2.41 ^a	105.6 ± 1.49 ^a	0.50 ± 0.029 ^a	0.14 ± 0.007 ^a
Silymarin + CDDP	21.83 ± 2.99 ^a	104.8 ± 2.27 ^a	0.45 ± 0.006 ^a	0.15 ± 0.017 ^a

Statistical analysis. Differences between obtained values (mean ± SD, n = 10) were carried out by one way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test. A p-value of 0.05 or less was taken as a criterion for a statistically significant difference.

Results

Administration of 7.5 mg/kg CDDP resulted in a significant increase of 87% and 90% in the level of serum ALT and AST ($p < 0.001$) and significant decrease of 30% and 52% in serum calcium and albumin levels ($p < 0.001$); respectively; compared to control. Administration of silymarin (100 mg/kg/day for 5 days) induced significant increase of 12% in serum ALT and significant decrease of 5%, 50% and 28% in serum AST, calcium and albumin, respectively compared to control. Prior treatment with silymarin before CDDP significantly reduced the level of ALT and AST in serum by 16% and 47%; respectively; and non-significant increase in the level of calcium and albumin ($p > 0.05$) compared to CDDP group (Table 1).

Table (2) shows the effects of cisplatin, silymarin and their combination on levels of GSH, MDA, and the activity of SOD and GSHPx in liver tissues. A single dose of CDDP resulted in significant decrease of 57%, 36% and 14% in GSH, SOD and GSHPx ($p < 0.001$) and significant increase of 127% in MDA ($p < 0.001$), respectively, as compared with the control group. Administration of silymarin for 5 consecutive days induced no significant change on levels of GSH, MDA, and the activity of SOD and GSHPx in liver tissues as compared to controls. Treatment with silymarin for 5 consecutive days prior to a single injection of CDDP resulted in a significant increase of 125%, 53% and 12% in the level of

Table 3. Effect of cisplatin, silymarin and their combination on the level of nitric oxide in serum and liver tissue

Treatment	Nitric Oxide	
	nmol/gm tissue	nmol/ml
Control	58.46 ± 5.48 ^a	4.09 ± 0.23 ^a
CDDP	78.08 ± 3.95	3.09 ± 0.29
Silymarin	62.72 ± 4.35 ^a	4.09 ± 0.26 ^a
Silymarin + CDDP	55.89 ± 3.59 ^a	4.19 ± 0.43 ^a

GSH ($p < 0.001$), SOD ($p < 0.001$) and GSHPx ($p < 0.001$), respectively, as compared with CDDP alone, and significant decrease of 49% in MDA ($p < 0.001$) compared with CDDP alone.

Administration of 7.5 mg/kg CDDP resulted in significant increase of 34% in NO ($p < 0.001$) in liver tissues and significant decrease by 24% in serum NO as compared to the control group. Treatment of rats with silymarin for 5 days did not lead to any change in the level of NO in both serum and liver tissues. Whereas, pretreatment with silymarin resulted in a significant decrease of 28% in NO level in liver tissue and a significant increase in serum NO level compared with CDDP alone ($p < 0.001$) (Table 3).

Discussion

Cisplatin, one of the most active cytotoxic agents against cancer, has several toxicities. Hepatotoxicity is one of them occurred during high doses treatment (Zicca *et al.*, 2004; Kim *et al.*, 2004; Koc *et al.*, 2005; Pratibha *et al.*, 2006).

In the present study, a single dose of CDDP (7.5 mg/kg) induced hepatotoxicity as manifested by increase in serum

ALT and AST and a decrease in serum levels of NO, albumin and calcium compared to control animals. Our results confirm those previously reported by Dubskaia *et al.* (1994), Saad *et al.* (2001; 2002; 2004), Kadikoylu *et al.* (2004), and Klukowska *et al.* (2001), who reported that, CDDP administration induced significant increase in serum ALT and AST and significant decrease in serum NO, albumin and calcium levels. The ability of cisplatin to cause alterations in the activity of these enzymes could be a secondary event following CDDP-induced liver damage with the consequent leakage from hepatocytes. High dose CDDP chemotherapy for cancer patients induces an acute decrease of serum total calcium and serum ionized calcium (Grau *et al.*, 1996).

Zhang and Lindup (1996) postulated that, exposure of rat kidney cortical slices to CDDP significantly increased the concentration of cytosolic Ca^{2+} . They suggested that the increase in cytosolic Ca^{2+} is related to decrease in Ca^{2+} uptake, depletion of SH-groups, but independent of lipid peroxidation. Interestingly, administration of silymarin prior to CDDP significantly prevented the increase in serum ALT and AST decrease in nitric oxide compared to untreated control rats. Silymarin significantly restores the changes of ALT and AST due to its antioxidant effect and its ability to act as a free radical scavenger, thereby protecting membrane permeability (Hakova *et al.*, 1996).

Administration of silymarin, 1 hr before CDDP administration, did not significantly change the indices of CDDP -induced hepatotoxicity in comparison with those animals treated with cisplatin alone. Ahmed *et al.*, (Ahmed *et al.*, 2003) reported that, silymarin has antihepatotoxic activity against carbon tetrachloride induced hepatotoxicity in albino rats. Silymarin protects against increase in serum ALT, AST and alkaline phosphates and a decrease in total protein and total albumin.

Chavez and Bravo (Chavez and Bravo, 1988) reported that silymarin induced mitochondrial Ca^{2+} release. It is proposed that due to its hydrophobic character, silymarin produces an alteration in the lipidic medium of the inner membrane which is conducive to an inhibition of the electron transport in the respiratory chain, as well as to the loss of the energy dependent accumulated Ca^{2+} .

Reports obtained from our study indicate that cisplatin increases lipid peroxidation and nitric oxide in the treated tissue of rat. The drug is also involved in altering the thiol status of the tissue with concomitant alterations in the enzymatic antioxidants. Glutathione, superoxide dismutase and glutathione peroxidase levels were significantly decreased after cisplatin therapy. This effect may be a secondary event following the CDDP-induced increase in free radical generation and/or decrease in lipid peroxidation protecting enzymes. CDDP can cause the generation of oxygen free radicals, such as hydrogen peroxide, superoxide anions and hydroxyl radicals. The hydroxyl radical is capable of abstracting a hydrogen atom from polyunsaturated fatty acids in membrane lipids to initiate lipid peroxidation. These radicals can evoke extensive tissue damage, reacting with

macromolecules, such as membrane lipids, proteins and nucleic acids (Antunes *et al.*, 2000; Emerit *et al.*, 2001). Moreover, depletion of glutathione may contribute to CDDP -induced lipid peroxidation (Antunes *et al.*, 2000). Thus, an alteration in enzymatic antioxidant status with increase in lipid peroxidation and nitric oxide indicates that the enzymes play an important role in combating free radical induced oxidative stress on the tissue.

This speculation agreed with previous studies which have demonstrated the involvement of oxidative stress, lipid peroxidation, and mitochondria dysfunction in CDDP -induced liver toxicity (Ramadan *et al.*, 2001; Yilmaz *et al.*, 2005). Oxidative stress is a common pathogenetic mechanism contributing to initiation and progression of hepatic damage in a variety of liver disorders. Cell damage occurs when there is an excess of reactive species derived from oxygen and nitrogen, or a defect of antioxidant molecules (Medina and Moreno-Otero, 2005). This observed increase in both reactive oxygen and nitrogen species after CDDP treatment was parallel to the increase in NO, MDA and the decrease in SOD, GSHPx and GSH. Also, the contribution of NO in the cytotoxicity and organ toxicity of anticancer drugs have been previously reported (Wink *et al.*, 1997; Sayed-Ahmed *et al.*, 2004). Many authors reported that increased NO production after CDDP and interferon-gamma was a secondary event following increase in inducible nitric oxide synthase (iNOS) (Curran *et al.*, 1991). Furthermore, NO reacts spontaneously with the available superoxide radical to form the more potent and versatile oxidant peroxynitrite. This highly toxic species reacts with GSH, lipids, proteins and DNA. Moreover, NO production inactivates GSHPx activity via modification of a cysteine-like essential residue on GSHPx (Rhoden *et al.*, 2001).

In this study, the observed decrease in GSHPx and SOD suggests an oxidative type of injury with CDDP-induced hepatotoxicity. Hasegawa *et al.* (1992) reported that the decrease in GSHPx is potentially ascribable to inactivation by the increase in reactive oxygen species (ROS) or lipid peroxides when oxidative damage is extreme.

In contrary to our results, Pratibha *et al.* (2006) reported that glutathione peroxidase activity, gamma-glutamyl transpeptidase and catalase showed a significant increase after cisplatin therapy.

In the current study, administration of silymarin one hour before CDDP significantly attenuated CDDP -induced increase in both ROS and nitrogen species. The protective action of silymarin is associated with its antioxidant properties, as it possibly acts as a free radical scavenger, an inhibitor of lipid peroxidation and a plasma membrane stabilizer (Ramadan *et al.*, 2002). It acts as a preservative of liver GSH content and prevents lipid peroxidation. This effect may be due to its antioxidant activity associated with less increase in inducible nitric oxide synthase (iNOS) protein content (Son and Kim, 1995; Son, 1997).

Soto *et al.* (2003) reported that, the protective effect of

silymarin, a free radical scavenger, on pancreatic damage induced by alloxan may be due to an increase in the activity of antioxidant enzymes “superoxide dismutase and glutathione peroxidase” that, in addition to the glutathione system, constitute the more important defense mechanisms against damage by free radicals.

Silymarin is known to have hepatoprotective and anticarcinogenic effects (Kang *et al.*, 2004). Silymarin possesses a hydroxyl group at C₅ in addition to the carbonyl group at C₄, which may form a chelate with ferrous iron. This chelation can raise the activity to the level of most active scavengers, possibly by site specific scavenging (Abu Ghadeer *et al.*, 2001). The free hydroxyl groups at C₅ and C₇ on the silymarin structure may also favour the inhibition of lipid peroxidation by reacting with peroxy radicals. This ability of silymarin leads to a significant increase in the cellular antioxidant defense machinery by ameliorating the deleterious effects of free radical reaction and by the increase in GSH content, which is important in maintaining the ferrous state (Abu Ghadeer *et al.*, 2001; Ramadan *et al.*, 2002).

Singh and Agarwal (2002) reported that, silymarin strongly prevents both photocarcinogenesis and skin tumor promotion in mice, by scavenging free radicals and reactive oxygen species and strengthening the antioxidant system. Lee *et al.*, (2003) reported that, administration of silymarin (100 mg/kg) by gastrogavage twice a day for 2 consecutive days resulted in an elevation of hepatic superoxide dismutase levels. It also resulted in a decrease in hepatic inducible nitric oxide protein in liver homogenate after 24 hr after CCl₄ intoxication. This effect may be due to its antioxidant activity associated with less increase in hepatic iNOS protein content.

Conclusion

Administration of silymarin caused a generally protective and ameliorative effect against CDDP-induced hepatotoxicity. The protective effect of silymarin is associated with its antioxidant properties, as it possibly acts as a free radical scavenger, lipid peroxidation inhibitor and glutathione levels preservation

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