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Protective Effect of Silymarin on Cisplatin-induced Nephrotoxicity in Rats

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Abstract: Cisplatin (CDDP) is a potent anticancer agents used for the treatment of solid tumors. However, its clinical use is often limited by its adverse effects including nephrotoxicity. The present study was designed to estimate if silymarin, a bioflavonoid with antioxidant potential can inhibit or at least ameliorate the alteration in some renal structures induced by cisplatin in rats or not. Five equal-sized groups (18 rats each) of male Sprague Dawley rats [Control, vehicle; cisplatin (5 mg/kg); silymarin (50 mg/kg) 2 h after cisplatin injection; and silymarin (50 mg/kg) 2 h before cisplatin injection] were used. Results revealed that cisplatin produced animal behavioral and morphological changes, as well as cellular and subcellular changes in kidneys. The most important changes were: decreased body weight, increased kidney wet weight, atrophied glomeruli, dilated urinary space, loss of PCT brush borders, hypertrophied podocyte pedicels, thickened glomerular basement membrane as well as tubular cell vacuolization. Post-treatment of silymarin 2 h after cisplatin however, significantly increase the body weight returning it to normal value, yet it failed in complete protection against the pathological alteration caused by cisplatin. Pre-treatment with silymarin 2 h before cisplatin significantly decreased the histological and ultrastructural changes induced by cisplatin and appear highly protective. These results suggested that the effects of cisplatin on glomerular and renal tubular cells morphology could be totally or to a great extent inhibited by silymarin.

Key words: Cisplatin, histopathology, kidney, silymarin, ultrastructure

INTRODUCTION

The human kidneys are primarily involved in filtering and concentrating various substances and chemical agents that may reach a high concentration and become toxic (Loh and Cohen, 2009). Nephrotoxicity is an inherent adverse side effect of the anticancer drugs for solid and hematologic malignancy (Kintzel, 2001). Antimetabolites, alkylating agents and anthracyclines are commonly used anticancer drugs resulting in nephrotoxicity (Erkut *et al*., 2008). Renal tubular damage is a well-known renal complication induced by anticancer drugs (Kakihara *et al*., 2003). The rate of glomerular damage may have been underestimated because tubular dysfunction can mask glomerular dysfunction (Ikarashi *et al*., 2004). It was reported that the mechanisms of chemotherapy-induced renal dysfunction generally include damage to vasculature or structures of the kidneys (Kintzel, 2001). He also added that, patients with cancer are frequently at risk of renal impairment secondary to disease-related and iatrogenic causes.

The anticancer drug cisplatin (CDDP) is a very effective platinum compound in the treatment of a variety of cancer (Kintzel, 2001). He also added that its clinical use is associated with severe side-effects; including renal impairments of which nephrotoxicity the most common side effect (Kintzel, 2001). Nephrotoxicity of CDDP has been recognized as the most important dose-limiting factor (Mora Lde *et al*., 2003). Although wide investigations have been conducted on the general organ toxicity of this anticancer drug (Pal *et al*., 2008), the exact mechanisms of nephrotoxicity induced by CDDP are still not fully elucidated. Stewart *et al*. (1982) reported that cisplatin is preferentially taken up and accumulated in the kidney cells. Nevertheless the major site of renal injury is the proximal convoluted tubule as reported by Kuhlmann *et al*. (1998). Therefore, the enhanced production of Reactive Oxygen Species (ROS) (Saad *et al*., 2004), oxidative stress, (Saad *et al*., 2004) and the decrease in antioxidant enzymes (Mora Lde *et al*., 2003) in kidneys have been implicated in the pathogenesis of cisplatin induced renal injury (Yilmaz *et al*., 2004). However, the involvement of oxidative stress in cisplatin induced toxicity is further supported by the fact that many antioxidants prevent cisplatin induced nephrotoxicity (Ajith *et al*., 2002; Lee *et al*., 2007).

Cytoprotective agents can be applied in therapy to ameliorate functional renal disorders (Behling *et al*., 2006). Behling *et al*. (2006) also added that

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cytoprotection is also considered as a suitable tool to elucidate the pathogenesis of chemically induced injury. Silymarin is a flavonoid extracted from *Silybum marianum*, that has already successfully been applied as a protective agent in various clinical and both *in-vivo* and *in-vitro* experimental models of hepatotoxicity (Laekeman *et al*., 2003; Eminzade *et al*., 2008) and nephrotoxicity to a certain extent (Karimi *et al*., 2005). Silymarin possesses antioxidant property that seems to be due to their ability to scavenge free radicals and to chelate metal ions (Borsari *et al*., 2001). Silymarin has been shown to be safe in animal models and no significant adverse reactions are reported in human studies (Hogan *et al*., 2007). The study presented here attempted to evaluate nephroprotective effects of the flavonoid silymarin if present on acute CDDP toxicity.

MATERIALS AND METHODS

Male Sprague Dawley rats (*Rattus norvegicus*), 150 g each were used in this work. Animals were housed in cages (4 animals/cage), under standardized laboratory conditions with controlled light-dark cycle, temperature of 23±2°C and relative moisture 60-70%. Animals had free access to tap water and to standard food diet *ad libitum*. All animals were adapted to handling and cages repeatedly during a 5-day period prior to experiment.

Cisplatin [(CDDP) or *cis*-Dichlorodiammine Platinum (II)] was obtained as yellowish crystalline powder, soluble in physiological saline solution and purchased from Sigma-Aldrich, CAS Number 15663-27-1 (P4394 Sigma). Silymarin was purchased from Sigma-Aldrich Chemical Co. (S0292).

Experimental design and procedures: The animals were divided into five equal sized groups (12 rats/each). In the first group (GIa) animals received no chemical treatment. The second group of animals (GIb) served also, as controls and were dosed with vehicle solutions only (propylene glycol and saline; 75/25 v/v). In the third group (GIIa) Animals were i.p injected with single dose of cisplatin dissolved in normal saline (5 mg/kg) at the beginning of the experiment (Mansour *et al*., 2006). In the fourth group (GIIb) animals were i.p injected with single dose of cisplatin (5 mg/kg), followed after 2 h by i.p. injection of silymarin (50 mg/kg/day) dissolved in vehicle solution as in the second group (Karimi *et al*., 2005). In the fifth group (GIIc) animals were i.p injected with silymarin (50 mg/kg/day) dissolved in vehicle solution as in the second group, 2 h before CDDP injection.

Animal behavior and body weight of all rats were recorded 4 times weekly. For histological evaluation, randomly selected rats were killed by decapitation after 2 weeks and the kidney tissues were immediately removed, weighed and washed with saline, cut into small pieces then dropped in 10% buffered formalin and dehydrated in ascending grades of ethanol concentration, cleared in xylene and processed in paraffin wax for embedding. Histological sections (5-6 µm thick) were stained with hematoxylin and eosin method and examined with light microscope.

For ultrastructural study, anaesthetized rats from all groups were rapidly dissected and then perfusion fixation with formalin-glutaraldehyde fixative ($_4F_1G$) in phosphate buffer was performed. Kidney tissues (1 mm^3) were removed and dropped into $_4F_1G$ buffered with 0.1 M phosphate (pH = 7.4) at 4°C. Samples were post-fixed in 2% OsO4 for 2 h at 4°C in the same buffer. The specimens were washed and dehydrated at 4°C through a graded series of ethanol. Tissues were then treated with propylene oxide solution and embedded in a mixture of 1:1 of Epon-Araldite. Specimens were embedded for 1 h. Polymerization was done in the oven at 65°C for 24 h. Ultrathin sections (50 µm) were cut on LKB ultratome, then mounted on copper grids, double stained with uranyl acetate and lead citrate and investigated on JEOL 100CX TEM.

The statistical significance was evaluated by one-way ANOVA using SPSS Version 16 and the individual comparison were obtained by LSD method. Values were considered statistically significant when $p<0.05$.

RESULTS

Effects of chemical used on behavior, body weight and kidney wet weight: Animals of all groups showed no obvious symptoms or signs of toxicity throughout the experiment. Moreover; they did not exhibit any case of mortality or death. Rats dosed with single injection of cisplatin (GIIa) (5 mg/kg) also, showed no adverse effects and remained alert as control groups until the 10th day of the experiment, where they became slightly nervous, less active, with minimal loss of furring. However, rats treated either with silymarin 2 h after cisplatin injection (GIIb), or received the same dose of silymarin 2 h before cisplatin injection (GIIc); exhibited no behavioral changes or clinical symptoms apart from increasing activity especially after the 7th day of the experiment.

It was noticed that in cisplatin treated rats, water and pellet diet consumption was decreased, if compared to normal. Also, a significant decrease in the body weight gain after 2 weeks (186.08±5.08 g) as compared to control (299.02±6.68 g) was noticed. However, the body weight of rats exposed to cisplatin and silymarin (GIIb and GIIc) were found to be equal 219.3±3.47 g and 218.86±4.88 g respectively, i.e. similar to that of controls. Moreover, a significant increase in cisplatin treated groups and significant depression in both groups receiving silymarin before and after cisplatin in kidney wet weight as well as relative weight to body weight was detected, i.e. returned value to normal (Table 1).

Table 1: Effects of cisplatin and silymarin on kidney wet weight and relative kidney to body weight ration among control and experimental rats[1]

Group	Kidney wet weight		Relative kidney to body weight ratio	
	0 Week[2]	2 Weeks	0 Week[2]	2 Weeks
Control GIa	2.30±0.31[a]	2.32±0.13[a]	0.010±0.005[a]	0.0094±0.007[a]
Vehicle GIb	2.33±0.23[a]	2.16±0.10[a]	0.010±0.004[a]	0.008±0.006[a]
Cisplatin GIIa	2.37±0.34[a]	2.68±0.12[b]	0.013±0.007[a]	0.0144±0.008[b]
Silymarin 2 h after cisplatin GIIb	2.36±0.30[a]	2.43±0.04[a]	0.011±0.004[a]	0.010±0.0017[a]
Silymarin 2 h before cisplatin GIIc	2.34±0.29[a]	2.39±0.06[a]	0.011±0.004[a]	0.010±0.002[a]
Results of one way ANOVA (Dose)	F = 0.04	F = 3.5	F = 0.04	F = 3.5
	p>0.05	p<0.05*	p>0.05	p<0.05*

[1]Data are means±SE (n = 6). Means in the same column with different superscript letters are significantly different, p<0.05 (one-way ANOVA followed by post-hoc LSD)

Table 2: Morphometric measurements of thickness of renal filtration barrier, pedicel length and filtration slit width (µm) among control and experimental group[1]

Groups	Thickness of renal filtration barrier (µm)	Pedicel length (µm)	Filtration slits width (µm)
Control GIa	0.26±0.01[a]	0.58±0.02[a,c]	0.02±0.003[a,d]
Vehicle GIb	0.22±0.01[a]	0.46±0.04[a,d]	0.01±0.0003[a,d]
Cisplatin GIIa	0.48±0.03[b]	0.67±0.05[a,c]	0.06±0.01[b,c]
Silymarin 2 h after cisplatin GIIb	0.32±0.02[a]	0.52±0.04[a,d]	0.03±0.001[a,d]
Silymarin 2 h before cisplatin GIIc	0.28±0.05[a]	0.51±0.04[a,d]	0.04±0.007[a,c]
Results of one way ANOVA	F = 8.56	F = 3.28	F = 6.91
	p<0.05*	p<0.05*	p<0.05*

[1]Data are means±SE (n = 15). Means in the same column with different superscript letters are significantly different, p<0.05 (one-way ANOVA followed by post-hoc LSD)

Histopathological and ultrastructural findings: Examination of hematoxylin and eosin stained kidney sections of control rats after 2 weeks from the beginning of the experiment, revealed normal basic structure. Kidney sections showed a large number of renal corpuscles and numerous urinefrous tubules within the cortex (Fig. 1a). Renal corpuscles appeared morphologically normal with double walled Bowman's capsule surrounding the glomerulus. However, between the two layers of Bowman's capsule is preserved a narrow urinary space. Ultrastructurally, it was noticed that the visceral layer of Bowman's capsule of renal corpuscle consists of podocytes (Fig. 1b) with large eccentric kidney shaped nuclei, fairly dense cytoplasm and numerous small bell shaped pedicels that appeared in direct contact with the renal filtration barrier and were separated by filtration slits (Table 2). However, glomeruli possessed numerous capillary loops that were lined with flattened fenestrated endothelial cells resting on the glomerular basement membrane. The capillaries loops were supported by mesangial cells having small densely stained nuclei with electron dense mesangial matrix (Fig. 1b). Moreover, light microscopic preparations showed that Proximal Convoluted Tubules (PCT) of normal kidneys have narrow lumen (Fig. 1a); occupied by striated brush borders and a regular basal lamina lined by a single layer of pyramidal shaped cells (Table 3). Ultrastructurally these cells attained numerous closely packed and regularly oriented microvilli (Fig. 1c) and possessed large spherical centrally or basally located nuclei with evenly distributed chromatin. Mitochondria were numerous apically; exhibiting rounded shapes with dense matrices and transverse cristae (Fig. 1c; Table 4). Poorly developed RER and few free ribosomes scattered within the cytoplasm were observed. On the other side, light microscopical observation revealed that Distal Convoluted Tubule (DCT) attained a wide lumen (Fig. 1a), numerous smaller sized cuboidal cells. Besides, measurements showed that DCT attained larger dimensions than those recorded in PCT (Table 3). Ultrastructurally these cells attained few short apical microvilli within the lumen. Nuclei were spherical euchromatic and basally located. Mitochondria were small in size (Table 4) located within basal infoldings that appeared pronounced in most cells (Fig. 1d).

Worth mentioning that vehicle administration (GIb) did not affect significantly any of the cellular investigated in kidneys sections in control group (GIa) (Fig. 2 a-d). At the ultrastructural level also, vehicle group revealed normal subcellular structures including elongated mitochondria that were regularly arranged within basal infoldings in most PCT cells (Fig. 2b) (Table 4).

In the current study, kidney sections of rats injected with cisplatin (GIIa); showed more intense characteristics of chronic nephropathy when compared to controls after 2 weeks. These changes included: hypertrophied renal corpuscles (5.73±0.17 µm), with reduced glomerular cellularity (Fig. 3a) and maximally and significantly dilated urinary space (0.68±0.10 µm). Significant

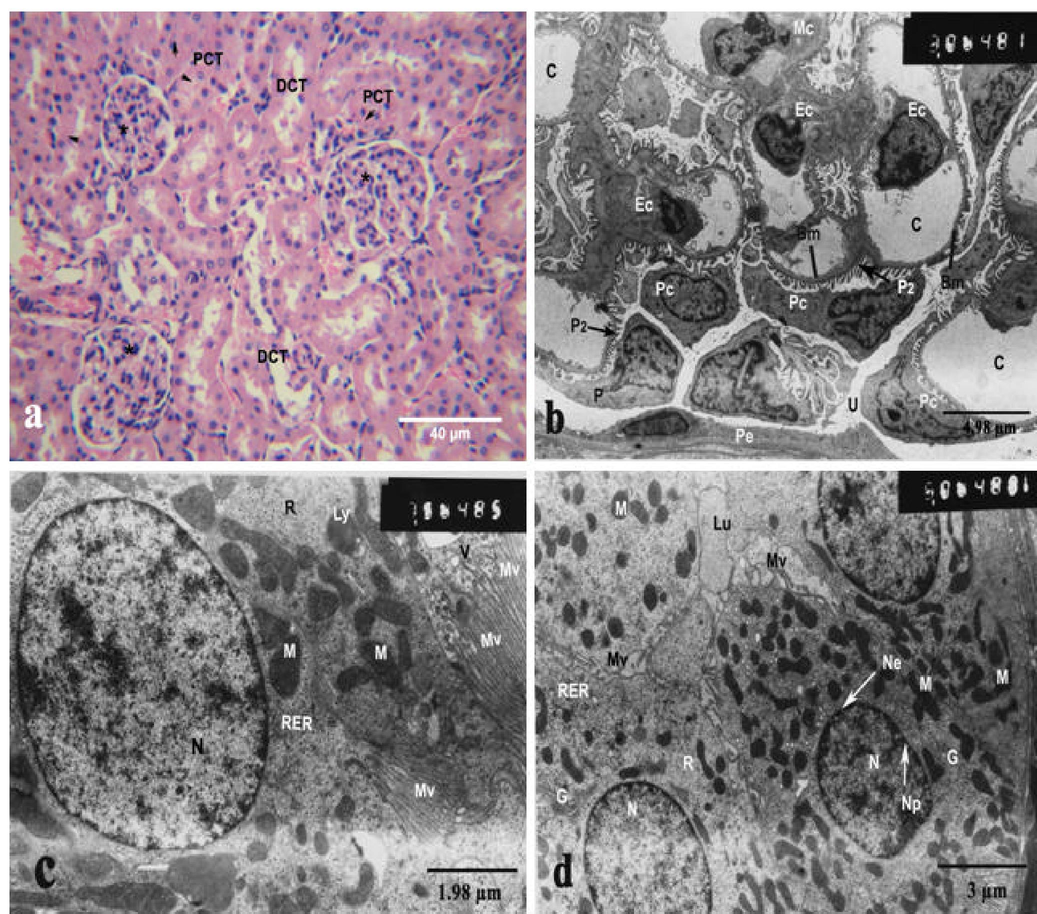

Fig. 1 (a-d): a) Light micrographs. Sections of normal rat kidneys after 2 weeks. Showing, Renal corpuscle (asterisk), brush borders of PCT (arrow head), distal convoluted tubule DCT (Hematoxylin and eosin stained sections). b-d) Electron micrographs. Control group after 2 weeks. b) Showing parts of renal corpuscle, Parietal epithelium (Pe), Urinary space (U), Podocyte (Pc) with secondary foot processes (P2), Endothelial cells (Ec), renal filtration barrier Basement membrane (Bm), Capillary loops (C), Mesangial cells (Mc). c) Part of PCT with numerous Microvilli (Mv), oval basal Nucleus (N); organized Mitochondria (M), RER, Ribosomes (R), Vesicles (V); Lysosomes (Ly). d) Part of DCT: spherical apical Nucleus (N), Nuclear envelope (Ne), Nuclear pores (Np), Mitochondria (M), RER, Golgi bodies (G), few short Microvilli (Mv), Lumen (Lu)

ultrastructural changes were greatly detected among renal structures after 2 weeks (Fig. 3b-d) including atrophied endothelial cells. Most of the podocytes revealed swollen and fused pedicels in parallel with significant increase in filtration slit width as compared to those of control (Table 2). However, slight thickening in renal filtration barrier that appeared highly irregular was detected (Table 2). Moreover, mesangial cells decreased in their number and some of them showed dense pyknotic nuclei and matrices as well as reduced dimensions (Fig. 3b). Moreover, it was observed that cisplatin treatment revealed focal and severe PCT tubular degenerative features including, significant reduction in the mean number of cells/tubules (11.00±0.57) accompanied with a significant increase in their mean width compared to controls (Table 3). Their tubular lumen appeared wide and contained cellular debris and most of them showed swollen outlines. Light preparations showed also, mononuclear cellular infiltration among renal tubules. Ultrastructurally, PCT revealed severed signs of tubular necrosis after cisplatin administration including, harshly fragmented and elongated microvilli, apical cytoplasmic blabbing and vacuolization (Fig. 3c) and thickened basement membrane. In addition, nuclei of most PCT cells attained corrugated nuclear envelope with numerous nuclear pores (Fig. 3c). These preparations showed rounded mitochondria with increased diameter (compared to control) possessing obscure cristae and located at the apical cellular part (Table 4). Also

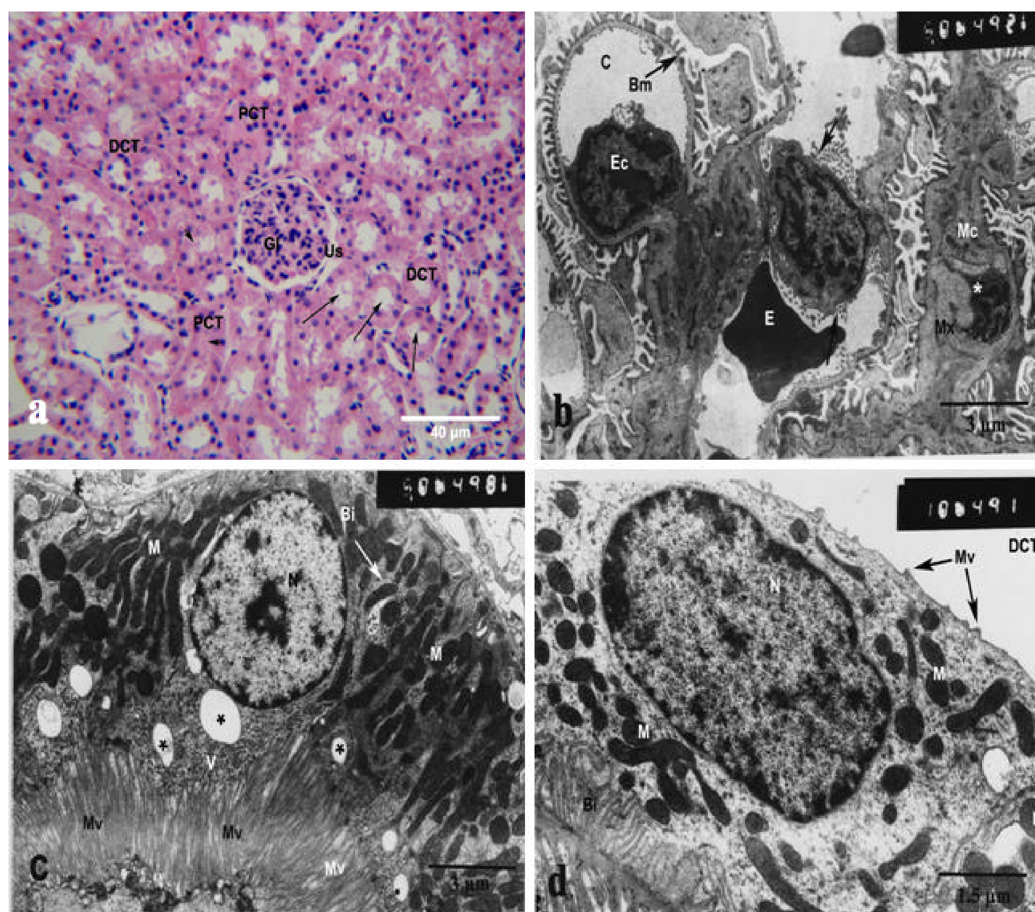

Fig. 2 (a-c): a) Light micrographs of sections of rat kidneys in vehicle administrated groups after 2 weeks; showing Glomerulus (Gl), with narrow Urinary space (Us), Proximal Convoluted Tubule (PCT), with brush borders (arrow head), Distal Convoluted Tubule (DCT) with wide lumen (arrow). (Hematoxylin and eosin stained section). b-d) Electron micrographs, sections of kidneys of male Sprague Dawley rats in vehicle administrated group after 2 weeks. b) Parts of renal corpuscle; Capillary (C): Endothelial cells (Ec), Erythrocyte (E), Mesangial cell (Mc), dense mesangial Matrix (Mx) and numerous processes (arrow); pyknotic nucleus (asterisk); regular Basement membrane (Bm). c) Part of PCT: rounded basal Nucleus (N); numerous condensed Mitochondria (M), numerous long Microvilli (Mv), Vesicles (V); lipid droplet (asterisk), numerous Basal infoldings (Bi). d) Part of DCT: oval apical Nuclei (N), with even chromatin distribution, dense Mitochondria (M), few short Microvilli (Mv), Basal infoldings (Bi)

numerous lysosomes were commonly detected. Light microscopy however, showed that most DCT showed few changes and revealed significant decrease in cellular number and moderate change in dimensions (Table 3). Ultrastructurally, few tubular changes were pronounced in DCT cells where their nuclei appeared euchromatic attaining different sizes with uneven arrangement and highly corrugated nuclear envelope (Fig. 3d). Mitochondria were pleomorphic in shape, with electron dense matrices and were highly disorganized and scattered irregularly throughout the cytoplasm (Table 4). Basal infoldings appeared greatly disrupted and irregular (Fig. 3d), whereas numerous desmosomes were greatly pronounced apically.

Further light microscopical examinations revealed that after 2 weeks of experiment, silymarin when administrated 2 h after cisplatin (GIIb), showed little pathological changes, if compared to those observed in rats administrated cisplatin alone (Fig. 4a), where renal corpuscle significantly showed normal dimensions (4.71±0.17 µm) and appeared more regular with moderate glomerular cellularity, minimal blood congestion and slightly narrow urinary space (0.31±0.10 µm). Rare tubular necrosis and inflammatory cells among renal tubules were detected (Fig. 4a). Ultrastructurally post treatment of silymarin revealed resemblance to those recorded in control group (GIa) (Fig. 4b-d). Glomerular capillary loops occupied with

Table 3: PCT and DCT tubules (µm) changes in response to treatment among control and experimental group[1]

	PCT		DCT	
Groups	Length (µm)	Width (µm)	Length (µm)	Width (µm)
Control GIa	1.66±0.14[a]	6.40±0.19[a]	1.74±0.1[a]	7.23±0.15[a]
Vehicle GIb	1.64±0.15[a]	6.33±0.19[a]	1.62±0.1[a]	7.92±0.4[a]
Cisplatin GIIa	2.64±0.09[b]	7.63±0.29[b]	2.66±0.1[b]	7.90±0.4[a]
Silymarin 2 h after cisplatin GIIb	1.71±0.07[a]	7.12±0.2[b]	2.31±0.1[b]	7.61±0.2[a]
Silymarin 2 h before cisplatin GIIc	1.52±0.07[a]	5.76±0.21[c]	2.25±0.1[b]	7.52±0.2[a]
Results of one way ANOVA	p<0.05*	p<0.05*	p>0.345	p>0.05
	F = 9.8	F = 29.7	F = 1.112	F = 11.26

[1]Data are means±SE (n = 15). Means in the same column with different superscript letters are significantly different, p<0.05 (one-way ANOVA followed by post-hoc LSD)

Table 4: Morphometric measurements of mitochondrial dimensions of proximal tubular cells of kidneys (µm) among control and experimental group[1]

	Elongated mitochondria		Rounded mitochondria
Groups	Length (µm)	Width (µm)	Diameter (µm)
Control GIa	3.16± 0.19[a]	0.34±0.02[a]	0.48±0.02[a]
Vehicle GIb	2.95±0.17[a]	0.38±0.01[a]	0.55±0.01[a]
Cisplatin GIIa	2.64±0.27[a]	0.37±0.8[a]	0.75±0.04[b]
Silymarin 2 h after cisplatin GIIb	2.75±0.12[a]	0.58±0.02[a]	0.81±0.07[b]
Silymarin 2 h before cisplatin GIIc	2.74± 0.24[a]	0.47±0.0[a]	0.84±0.04[b]
Results of one way ANOVA	F = 0.99	F = 1.95	F = 13.71
	p>0.05	p>0.05	p<0.05*

[1]Data are means±SE (n = 15). Means in the same column with different superscript letters are significantly different, p<0.05 (one-way ANOVA followed by post-hoc LSD)

normal endothelial cells and few erythrocytes. It is of interest that atrophied endothelial cells were minimally detected. Podocytes showed normal cellular and nuclear outlines and their pedicels, filtration slits, renal filtration barrier thickness returned significantly to normal values (Table 2). In addition, mesangial cells remained irregular with dense nuclei and matrices (Fig. 4b). Besides, pyknotic nuclei were detected among few mesangial cells (Fig. 4b). On the other sides, mild to moderate tubular necrosis were detected among PCT and DCT of this group especially at the level of light microscope. PCT cells/tubules increased significantly (15±0.88) in number accompanied with a significant increase in their mean tubular width (Table 2), (returning values to normal). PCT appeared slightly organized with regular lumen occupied by evident brush borders; a large number of epithelial cells were less hypertrophied (compared to cisplatin group) (Fig. 4a). Mononuclear cellular infiltrates was less common among most renal tubules. Ultrastructurally, most PCT cells showed as normal numerous elongated microvilli, attained minimal apical vacuolization and endocytotic vesicles (Fig. 4c). Nuclei appeared euchromatic, oval in shape with segregated and centric nucleoli. Mitochondria appeared rounded in shape and distinct transverse cristae with either dense or light matrices and showed normal dimension (Table 4). Basal infoldings appeared regular possessing numerous elongated mitochondria in between (Fig. 4c). Numerous free ribosomes and moderate number of lysosomes were scattered throughout the cytoplasm (Fig. 4c). In addition, hematoxylin and eosin stained kidney section of this group revealed that DCT did not show significant tubular damage (Fig. 4a) and their dimensions returned significantly to normal values (Table 2). Moreover, most DCT cells revealed higher cellular and nuclear organization with great reduction of cytoplasmic vacuolization. Ultrastructurally, silymarin posttreatment showed that most DCT preserved regular cellular and nuclear arrangement and structures, few cells showed reduced microvilli, rounded heterochromatic nuclei and organized cytoplasmic organelles (Fig. 4d).

Similarly, pretreatment with silymarin 2h before cisplatin (GIIc) exhibited few changes compared to GIIb (Fig. 5 a-d) after 2 weeks. At the level of light microscopy, majority of renal corpuscles returned to normal dimensions (4.48±0.16 µm) along with narrow urinary space (0.30±0.08 µm), regular glomerular cellularity and minimal erythrocytes leakage (Fig. 5a). Electron microscopic observation showed well preserved cell structures and organelles in most kidney samples of this group. However, some minor alterations were observed among renal corpuscles including few glomerular capillary loops with mild congestion. Podocytes achieved significant recovery ultrastructural features as they appeared normal (Fig. 5b) with less swollen pedicels and more regular renal filtration barrier basement membrane (Table 2). In addition to the well preserved cell structures and organelles already recorded in group Iib, pretreatment with silymarin

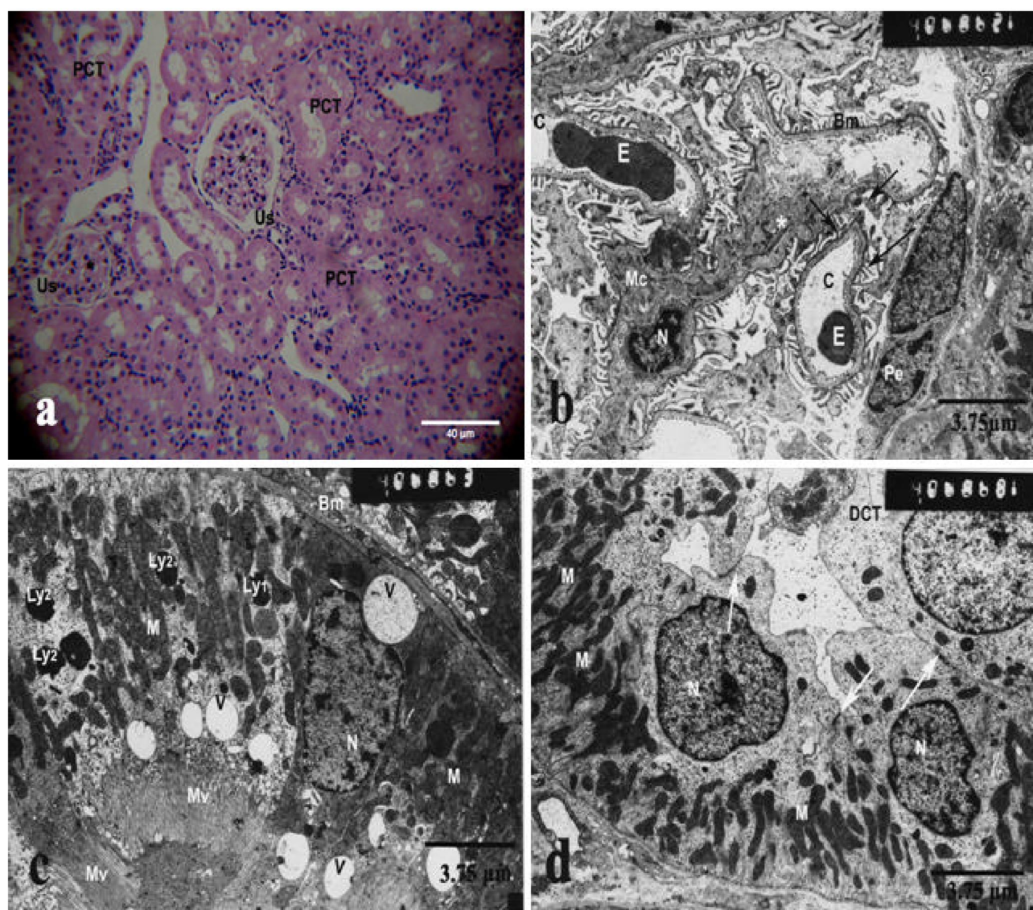

Fig. 3 (a-c): a) Light micrograph of sections of rat kidneys in cisplatin treated group after 2 weeks; showing shrunk glomerulus (asterisk), wide Urinary space (Us) (Hematoxylin and eosin stained sections). b-d) Electron micrographs, sections of kidneys of cisplatin injected male Sprague Dawley rats after 2 weeks. b) Parts of renal corpuscle: Capillaries (C) with deformed Erythrocytes (E). Mesangial cell (Mc): small irregular Nucleus (N). Ballooned secondary foot processes (arrow), thickened Basement membrane (Bm) with disruption (asterisk); cleaved nucleus of Parietal cell (Pe) c) PCT cells with few Microvilli (Mv), increased apical and basal Vacuoles (V), irregular Nucleus (N); numerous disorganized Mitochondria (M), numerous primary (Ly1) and secondary (Ly2) lysosomes, thickened Basement membrane (Bm). d) Part of DCT: absence of microvilli, increased tight junctions (arrow); severe cellular blabbing with irregular Nuclear outlines (N) of different sizes, pleomorphic Mitochondria (M) with swelling profiles

exhibited the highest minimal frequency of pyknotic nuclei among mesangial cells (Fig. 5b).

Pretreatment with silymarin treatment succeeded in keeping PCT dimensions as well as number of cell/tubules as normal values (15±0.82) when observed at the level of light microscope. Tubular necrosis was completely absent among a large number of PCT examined; lumen appeared narrow with brush border arising from a well organized pyramidal shaped PCT cells (Fig. 5c). On the other side, silymarin pretreatment revealed normal histological features including significantly elevated PCT dimensions when compared to their corresponding in cisplatin group (Table 3). Ultrastructurally, PCT cells appeared highly organized with minimal loss of microvilli, complete absence of cytoplasmic vacuolization and regular basal infoldings (Fig. 5c). PCT cells illustrated normal rounded and basally located nuclei. Most mitochondria appeared normal with regular dimensions (Table 4), attaining dense matrices and transverse cristae and distributed apically above and basally within basal infoldings (Fig. 5c).

Moreover, light microscopic observation showed that DCT maintained normal number of cells and their dimensions significantly approaches those of controls (Fig. 5a) and most of them exhibited regular

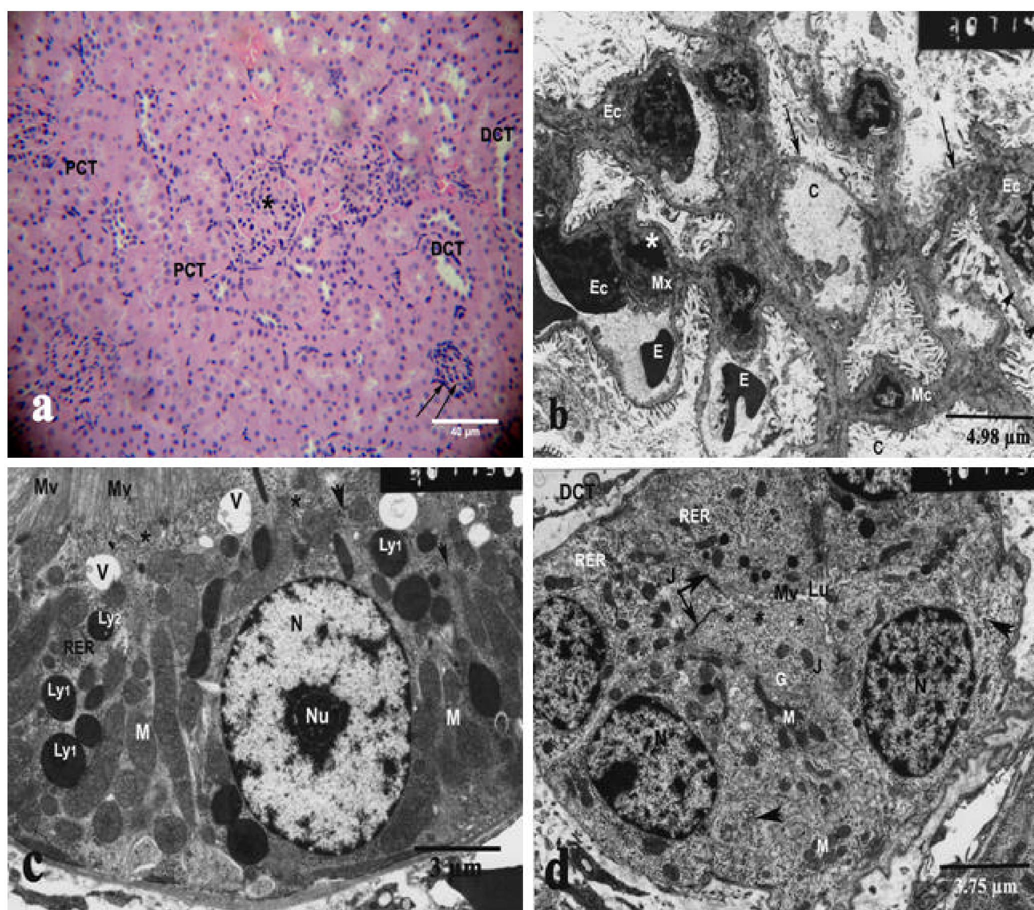

Fig. 4 (a-c): a) Light micrograph of sections of rat kidneys treated with silymarin 2 h after cisplatin of male Sprague Dawley rats after 2 weeks; showing normal renal corpuscles (asterisk), tubules (PCT and DCT), few mononuclear infiltration (double arrow) (Hematoxylin and eosin stained sections). b-d) Electron micrographs, sections of kidney treated with silymarin 2 h after cisplatin of male Sprague Dawley rats after 2 weeks. b) Parts of renal corpuscle: Capillary (C) lumen congested with few Erythrocytes (E), Endothelial cell (Ec); Mesangial cell (Mc) with dense mesangial Matrix (Mx): pyknotic nucleus (asterisk); reduced secondary processes (arrow); regular basement membrane (arrow head). c) PCT cell: numerous Microvilli (Mv), pinocytotic vesicles (*), apical Vacuoles (V), ribosomes (arrow head), oval basally located Nucleus (N) with centric and segregated Nucleolus (Nu); numerous organized Mitochondria (M) with transverse cristae; RER, moderate number primary (Ly1) and secondary lysosomes (Ly2). d) Part of DCT: Microvilli (Mv), apical vesicles (asterisk), tight Junctions (J), oval Nuclei (N), more organized dense Mitochondria (M), RER, Golgi bodies (G).

architecture with wide lumen. DCT attained normal cellular and tubular appearance (Table 3). Ultrastructurally, pretreatment of silymarin inhibited the hazardous effect of cisplatin and resulted in minimal changes in DCT where most cells appeared normal cellular and cytoplasmic appearance similar to those recorded in control group (GIa) Most cytopalsmic organelles and mitochondria appeared organized (Fig. 5d).

DISCUSSION

In the current study, the proposed plan aimed to assess and examine the possibility of silymarin to prevent the alterations induced by cisplatin in kidney tissues of male Sprague Dawley rats, which were used as biological test animals.

Cisplatin is an effective chemotherapeutic agent for a wide variety of tumors (Park *et al*., 2009). Nevertheless, it has several side effects including hepatotoxicity (Mansour *et al*., 2006; Pratibha *et al*., 2006) and nephrotoxicity (Park *et al*., 2009). Mora Lde *et al*. (2003) showed that, a decrease in antioxidant enzymes resulted from cisplatin induced tissue toxicity. They added also that the development of therapies to prevent the appearance of cisplatin-induced tissue toxicities has focused on administration of antioxidants along with

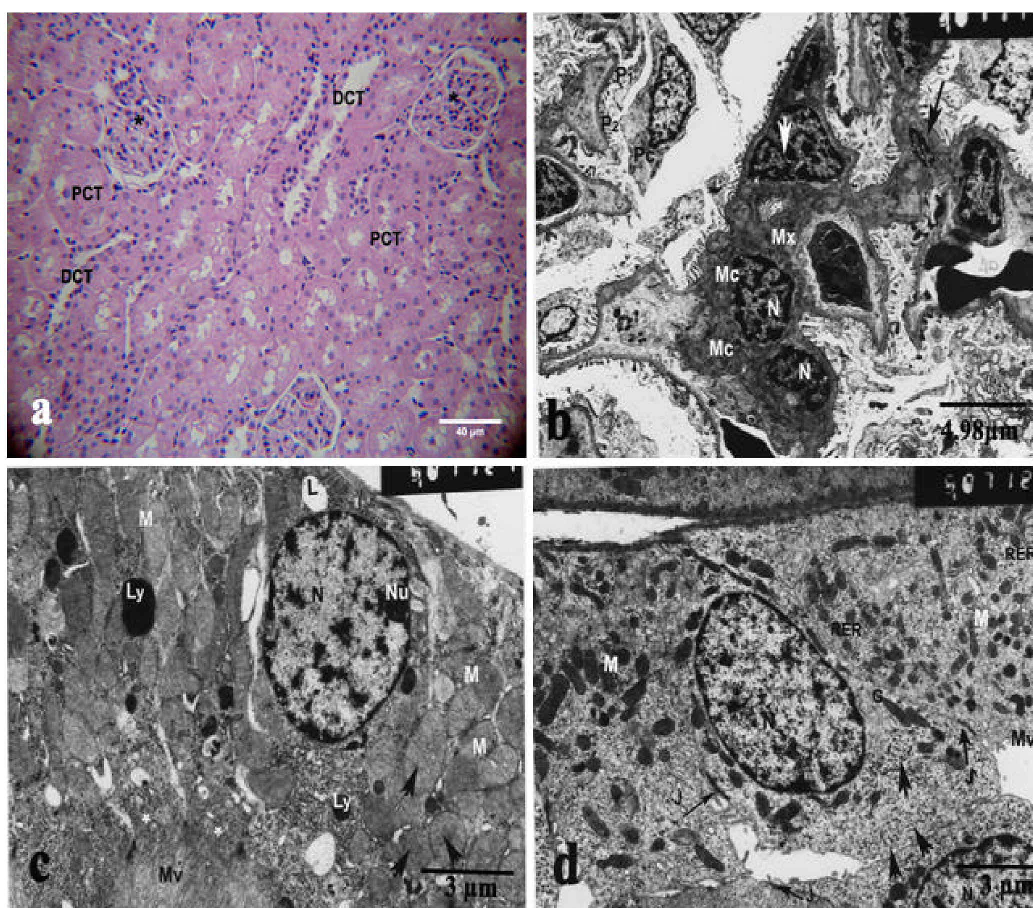

Fig. 5 (a-c): a) Light micrograph of sections of rat kidneys treated with silymarin 2 h before cisplatin of male Sprague Dawley rats after 2 weeks; showing regular renal corpuscles (asterisk) and tubules (PCT, DCT) (Hematoxylin and eosin stained sections). b-d) Electron micrographs, sections of kidneys treated with silymarin 2 h before cisplatin of male Sprague Dawley rats after 2 weeks. b) Parts of renal corpuscle: regular mesangial cell (Mc) with dense matrix (Mx): cleaved nucleus (arrow head), pyknotic nucleus (arrow); podocytec (Pc) with organized outline and processes (P1 and P2). c) PCT cell: numerous Microvilli (Mv) and vesicles (asterisk), rounded basally located Nucleus (N) with marginated Nucleolus (Nu); numerous organized Mitochondria (M) with transverse cristae (arrow head), Lipid droplet (L), Lysosomes (Ly). d) Organized part of DCT: Microvilli (Mv), tight Junctions (J), euchromatic Nucleus (N), numerous well oriented dense Mitochondria (M), RER, Golgi bodies (G), ribosomes (arrow head)

cisplatin treatment. Thus, many studies dealing with the protective effects using extracts of natural products and dietary antioxidant against cisplatin induced tissue toxicities have been reported (Behling *et al*., 2006; Mansour *et al*., 2006).

Silymarin, the root extract from *Silybum marianum*, is known to have hepatoprotective effect against numerous liver diseases (Eminzade *et al*., 2008). Karimi *et al*. (2005) reported that silymarin has antinephrotoxic activity against cisplatin induced nephrotoxicity in albino rats.

In the present investigation, a single dose of cisplatin (5 mg/kg), in male rats resulted in significant body weight reduction and decreased food intake that was most pronounced after 2 weeks. In accordance with our results, Chirino *et al*. (2004) suggested that i.p. administration of a single dose of cisplatin to male Wistar rats (7.5 mg/kg) after 3 days significantly decreased their body weight. Confirming our results, Shimeda *et al*. (2005) stated that cisplatin has been shown to decrease total body weight in male rats. During the course of the present study, post-treatment and pre-treatment of silymarin (50 mg/kg) remarkably prevented the reduction in body weight induced by cispaltin, thus leading to normal body weight. Our results were in agreement with the results of Gaedeke *et al*. (1996), who noticed that daily i.v. injections of silibinin (active compound of silymarin) (200 mg/kg) to female Wistar rats succeeded in the complete inhibition of the hazardous effect of cisplatin on the experimental animals' body weight over a period of 11 days.

Contradicting our results, Shimeda *et al*. (2005) stated that daily oral administration of capsaicin antioxidant (10 mg/kg) for 6 consecutive days, increased male Sprague Dawley body weight, yet it failed in the complete recovery, i.e. return to normal body weight.

In the present study, our results showed that cisplatin induced kidney damage characterized by a significant increase in the wet kidney weight manifested by a significant elevation in kidney weight to body weight ratio after 2 weeks from the beginning of the experiment. Our results were in accordance with those reported by Shimeda *et al*. (2005), who indicated a significant increase in the kidney weight to body weight ratio in cisplatin treated male S.D. rats (5 mg/ kg). However, our results were opposing those reported by Lee *et al.* (2007) who showed that cisplatin treatment resulted in a significant decrease in kidney weight as a percentage of the total body weight. In the present work, both post- and pre- treatments of silymarin significantly prevented changes in kidney wet weight as well as kidney weight to body weight ratio values to normal after 2 weeks. Confirming our results, Gaedeke *et al*. (1996) and Shimeda *et al*. (2005) who stated that silibinin and capsaicin respectively, used as antioxidant against cisplatin-induced nephrotoxicity resulted in significant protection against cisplatin by decreasing kidney to body weight ratio. During the present work, it could be elucidated that alterations of organ-body weight ratio in cisplatin intoxicated rats could be attributed to tissue damage and altered in their functions. This is in agreement with results reported previously by Lee *et al*. (2007) and Park *et al*. (2009).

The alterations in renal structures detected in rat models correlate well with the nephrotoxic effects of cisplatin in patients treated with antitumor agent (Daugaard *et al*., 1988a, 1988b). In the present investigation, a single dose of cisplatin (5 mg/kg, i.p.), in rats resulted in the deterioration of renal corpuscle structure and increased tubular necrosis after 2 weeks. Moreover, cisplatin administration revealed that most renal corpuscles appeared hypertrophied with diminished glomeruli congested with erythrocytes and dense mesangial cells and dilated urinary space. In agreement with our results, Chirino *et al*. (2004) showed that cisplatin treatment induced mesangial cells contraction. However, our results contradicted those reported by Chirino *et al*. (2004), who suggested that the alteration in glomerular function cannot be attributed to structural damage since glomeruli exhibited normal appearance in cisplatin treated rats (7.5 mg/kg, i.p., for 3 days) .

The kidneys accumulate and retain platinum complexes to a greater extent than other organs, perhaps via mediated transport and it is the main excretory outlet for either intravenous or intraperitoneal cisplatin (Arany and Safirstein, 2003). The underlying mechanism of cisplatin-induced nephrotoxicity is still not well known but many recent *in vitro* and *in vivo* studies indicate an important role for the reactive oxygen metabolites in the pathogenesis of this effect (Matsushima *et al*., 1998). Behling *et al*. (2006) suggested that cisplatin acts mostly on the PCT of the kidney. Arany and Safirstein (2003) reported that proximal tubular epithelial cells take up this antitumor agent and this actively leads to higher concentrations than those found in the plasma; thus, cisplatin toxicity in PCT is morphologically characterized by tubular necrosis. In the present study, cisplatin caused structural alterations characteristics of acute tubular necrosis in both PCT and DCT after 2 weeks. This is in accordance with those results reported by Karimi *et al*. (2005) who stated that male Wistar rats receiving single dose of cisplatin (3 mg/kg) for 5 days showed severe tubular necrosis among kidney sections. Moreover, in the present work, PCT showed cytoplasmic debris, denudation of PCT basement membrane, swollen PCT cell with open face and pyknotic nuclei, vacuolated cytoplasm; intercellular edema and mononuclear infiltration. DCT appeared with few tubular changes. Thus, our description were in general agreement with those reported by Chirino *et al*. (2004), who observed PCT tubular necrosis, cytoplasmic vacuolization and intercellular edema in cisplatin treated male Wistar rats (at dose level 7.5 mg/kg, i.p., for 3 days). Similarly, Morigi *et al*. (2004) and Behling *et al*. (2006), reported similar description in acute cisplatin nephrotoxicity. After 2 weeks from the beginning of the present experiment, histological features of chronic nephropathy as indicated by degenerated and highly congested glomeruli were detected among kidney sections of this group. Confirming our results, El-Abd and Okda (2007), suggested that male rats receiving ribavirin (at dose level 12 mg/kg twice day) for 3 weeks, demonstrated highly congested capillaries with sever hemorrhage along with atrophied renal glomeruli. Besides, in the present study cisplatin injection resulted (after 2 weeks) in complete absence of PCT brush border, hypertrophied PCT cells with numerous cytoplasmic degenerative vacuoles, detachment of basement membrane and increased cellular infiltration. DCT exhibited similar atrophied profiles but were less severe. Similar to the present results, were those reported by Behling *et al*. (2006), who focused on the histopathological features of chronic nephropathy induced by cisplatin for 20 days in male Wistar rats including tubular atrophy and dilatation.

In the current study, glomerular and tubular atrophy was less intense in rats administrated silymarin 2 h after cisplatin (GIIb). After 2 weeks, silymarin post-treatment showed attenuation of glomerular atrophy that revealed minimal erythrocytes leakage and slightly dilated urinary space. Confirming our description, El-Abd and Okda (2007), suggested that male rats receiving i.p., injection

of silymarin (250 mg/kg for 1, 2 and 3 weeks) reduced and improved histopathological lesions induced by ribavirin (broad spectrum antiviral drug) specifically atrophied renal glomeruli. In the present study, renal tubules of this group exhibited moderate microvilli, normal shaped cells with minimal cytoplasmic degenerative vacuoles, mild to moderate tubular necrosis as well as inflammatory cell infiltration. Our results were in great accordance with those reported by Karimi *et al*. (2005), who stated that post-treatment with silymarin (50 mg/kg, i.p.) 2 h after cisplatin for 5 days, resulted in mild to moderate renal cellular injury. In agreement with Behling *et al*. (2006) who reported that gavage of administration of flavonoid quercetin (50 mg/kg) to male wistar rats receiving cisplatin (5 mg/kg) after 5 and 20 days showed reduction of acute tubular necrosis including, focal areas of broken basement membrane, swelling and flattening of PCT cells with brush border loss, diffuse interstitial edema and interstitial inflammatory cell infiltrate. In the present work, 2 weeks after silymarin treatment did not exhibit complete protection against histopathological changes induced by cisplatin. Thus, glomeruli as well as renal tubules exhibited normal cellular architecture yet very mild glomerular atrophy and tubular necrosis were rarely detected. Induction of nephrotoxicity by cisplatin is assumed to be a rapid process involving reaction with proteins in the renal tubules (Montine and Borch, 1990). Rao and Rao (1992) stated that renal damage occurs within 1 h after cisplatin administration. It is important that the protective agent is present in renal tissue before damage occurs. This might explain why complete protection did not result in our study when silymarin were given after administration of cisplatin.

In the present investigation, pre-treatment with silymarin 2 h before cisplatin administration resulted in inhibition or complete protection against cisplatin induced damage after 2 weeks; glomeruli appeared with normal dimensions, mild renal cellular injury were noted in few foci. Confirming our finding were those reported by Behling *et al*. (2006), who studied the complete protective effect of silymarin when administrated 2 h before cisplatin injection in male albino rats. However, the present work focused on the total protective and preventive effect of this treatment that significantly increased after 2 weeks, since glomerular as well as renal tubules showed normal histological features and dimensions. Our results concur with those previous reported by Gaedeke *et al*. (1996) who demonstrated that female Wistar rats receiving pre-treatment with silibinin (200 mg/kg, i.v.) 1 h prior to cisplatin administration for 11 days significantly decreased both proximal tubular and glomerular damage induced by cisplatin. Tubular defects resulting from cisplatin treatment have been ascribed largely to the generation of free radicals (Hannemann and Baumann, 1998). Cisplatin-induced damage could be increased by depleting cells of protective radical scavengers like gluthathione or superoxide dismutase (Sadzuka *et al*., 1992).

Electron microscopical examinations revealed many ultrastructural alterations in kidney preparations of cisplatin-treated animals. Since PCT were more affected than DCT, we believe that a relationship between the action of cisplatin used and the function of these tubules could be suggested. This agrees with the findings of Morigi *et al*. (2004), who studied the effect of cisplatin on the kidney of albino mice. In this study, it was also noted that cisplatin induced alterations in renal corpuscles including destructed and atrophied endothelial cells; hypertrophied podocytes with elongated, swollen and fused podocyte pedicels and widened filtration slits; thickening and highly irregular renal filtration barrier. These data are in close correlation with those reported by Kohn *et al*. (2002), who studied the nephrotoxic effect of cisplatin on kidney glomerular components in guinea pigs. Moreover, in the present study, mesangial cells (minimal in number and with slight depression in their dimensions according to morphometric measurements) appeared irregular with bizarre shaped nuclei and dense matrices. Confirming our results are those reported by L'Azou *et al*. (2002) who demonstrated that cadmium used as a nephrotoxin agent in mesangial cell culture, induced masangial glomerular cell contraction that was evident by decrease in the mesangial cells surfaces. It is conceivable that even a minor reduction in the mesangial cell area considerably affects the filtering surface of the glomeruli and could explain the decreased glomerular filtration (Rodriguez *et al*., 2000). Additionally, the present work showed serious PCT tubular lesions with loss of microvilli, pyknotic nuclei, cytopalsmic vacuolization, increased lysosomes and altered mitochondrial structure and arrangement. On the other hand, less tubular changes including minimal microvilli, pyknotic nuclei and organelles disorganization, were pronounced in DCT cells but to a lesser extent. Morigi *et al*. (2004), revealed great similarities to a certain extent with our results, where cisplatin resulted in focal and severe tubular changes in PCT and DCT cells, in male albino mice. In the present study, 2 weeks after cisplatin administration exhibited progressive and increasing glomerular cells, PCT and DCT tubular damage manifested by reduced podocytes with highly hypertrophied foot process and sharply dilated filtration slits. PCT cells with complete fragmented microvilli and increased frequency of pyknotic nuclei, cytoplasmic vacuolization, myelin figures and altered mitochondria. In agreement with our

findings, Shalaby *et al*. (2006) who studied the chronic effect of cisplatin in kidneys of male albino mice over 21 days and showed similar degenerative changes in PCT cells.

In the present study, the protective effect of silymarin was confirmed when renal tissues were observed by electron microscope. After 2 weeks, both post-treatment and pre-treatment with silymarin showed minimal changes in renal cellular structures in most kidney samples. However, some minor to moderate alteration including: slightly congested capillary loops; minimal atrophied endothelial cells; irregular mesangial and podocytes cells; PCT with reduced microvilli, moderate cytoplasmic vacuolization, irregular mitochondria; DCT cells with pyknotic nuclei and disorganized organelles. The protective effect of pretreatment of silymarin was greatly evident than those in post-treatment group, as it resulted in the complete protection and well preservation of the renal corpuscle structures as well as PCT and DCT cells structures and arrangement that did not exhibit significant differences from those in control groups.

In contrast post-treatment with silymarin reflected moderate protection with minimal alteration in renal glomerular structures and PCT cells such as partial loss of microvilli and cytoplasmic vacuolization. These findings were similar to a certain extent to those reported by Morales *et al*. (2006), who suggested that kidneys of male wistar rats receiving flavinoids i.p. quercetin (at dose level 50 mg/kg/daily for nine weeks) showed well preserved cell structures in most kidney samples including minor alteration in PCT cells such as partial loss of microvilli and isolated vacuoles could be observed.

Conclusion: The present work has shown that experimental administration of cisplatin in rats was greatly associated with many biological, histological and ultrastructual changes. Although the exact mechanism by which silymarin prevent to a great extent, cisplatin toxicity remained to be elucidated, yet our present findings suggest that silymarin protects against acute cisplatin nephrotoxicity and may be considered as a potentially useful candidate in the combination with chemotherapy by acting in the kidney as a potent scavenger of free radicals thus preventing the toxic effect of cisplatin both the histological and ultrastructural levels.

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