

# Whey protein isolate protects against cyclophosphamide-induced acute liver and kidney damage in rats

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## ARTICLE INFO

### Article history:

Received on: 23/01/2017

Accepted on: 17/03/2017

Available online: 30/06/2017

### Key words:

Cyclophosphamide; whey protein isolate; oxidative stress; inflammation; liver; kidney; rat.

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## ABSTRACT

Cyclophosphamide (CP), a common chemotherapy, interferes with the antioxidant systems of vital organs. This study investigated the protective effects of whey protein isolate (WPI) against CP-induced acute liver and kidney damage in rats. Forty adult Wistar rats were allocated into five groups. The first received the vehicles and acted as normal control. In the other groups, rats were injected with a single intraperitoneal dose of CP (200 mg/kg). The last three groups were pre-treated with oral WPI at doses of 75, 150 or 300 mg/kg/day, respectively, for 15 successive days. Forty-eight hours following CP injection, animals were investigated for serum alanine transaminase, aspartate transaminase, urea and creatinine levels, as well as hepatic and renal reduced glutathione, malondialdehyde, nitrite, interleukin 1 $\beta$ , and myeloperoxidase contents. Histopathological examination as well as immunohistochemical detection of cyclooxygenase-2 in liver and kidney were conducted. CP induced organ dysfunction, oxidative stress, inflammation, and histopathological alterations in liver and kidney. WPI significantly protected against CP-induced deterioration of liver and kidney functions showing marked dose-dependent anti-oxidant and anti-inflammatory properties demonstrated by the biochemical, immunohistochemical and histopathological results. In conclusion, the study reveals the protective effects of WPI against CP-induced acute liver and kidney damage via antioxidant and anti-inflammatory mechanisms.

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## INTRODUCTION

Cyclophosphamide (CP) is a cytotoxic alkylating agent with wide spectrum clinical uses for over 50 years and its effectiveness in the treatment of neoplastic diseases such as solid tumors and lymphomas as well as non-neoplastic diseases such as rheumatoid arthritis and systemic lupus erythematosus has been proved extensively (Lawson *et al.*, 2008). However, multi-organ toxicity mainly in the heart, testes, and urinary bladder, hindered the clinical use of CP due to its ability to damage normal tissues (Fraiser *et al.*, 1991). Though the metabolic activation of CP by hepatic microsomal cytochrome P450 mixed functional oxidase system is a prerequisite of CP therapeutic effects, toxic effects of CP like necrosis, apoptosis, and oncosis

are linked with two active metabolites, phosphoramidate and acrolein (Ludeman, 1999; Benvegnu *et al.*, 2010). Generation of reactive oxygen species (ROS) such as superoxide anion has evolved during CP metabolic activation, leads to redox imbalance and oxidative stress that result in cancer and tissue damage (Abraham *et al.*, 2011; Jnaneshwari *et al.*, 2013). Studies reported induction of haemorrhagic cystitis, hepatic and lung damage as well as cardiotoxicity and nephrotoxicity by CP treatment (Oter *et al.*, 2004; Cho *et al.*, 2010; Rehman *et al.*, 2012; Jnaneshwari *et al.*, 2014). Owing to their nutritional and biological role upon human health, whey protein products have drawn attention in recent decades (Krissansen, 2007). Whey protein belongs to a class of protein that comprises approximately 20% of the total milk proteins (Pennings *et al.*, 2011; Mitchell *et al.*, 2015). It provides high levels of essential and branched chain amino acids and possesses many beneficial bioactive properties (Hoffman and Falvo, 2004).

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Whey protein concentrate has the ability to act as an antioxidant (Bounous, 2000; Pena-Ramos and Xiong, 2001), antihypertensive, antitumor (Yoo *et al.*, 1998), hypolipidemic, antiviral (Low *et al.*, 2003), antibacterial (Ajello *et al.*, 2002), and chelating (Weinberg, 1996) agent. Dietary whey protein isolates (WPI) can stimulate immune system function, improve muscle strength and body composition, and inhibit cardiovascular diseases and osteoporosis (Watanabe *et al.*, 2000). In a previous study of our team and in many studies of other research groups, the protective effects of WPI, their enzymatic digests, and peptide fractions prepared from the enzymatic digestion against different experimental animals models of hepatotoxicity and nephrotoxicity have been reported (Gad *et al.*, 2011; Athira *et al.*, 2013; Eliwa *et al.*, 2014; Mansour *et al.*, 2015a). In addition, it has been found that WPI can be used as an adjuvant in cancer treatments by enhancing the cytotoxicity of anti-cancer drug (baicalein) (Tsai *et al.*, 2000). The current study aimed to investigate the potential protective effects of WPI against some CP-induced toxic manifestations. The study has taken in consideration that the site of metabolic degradation of CP to its toxic metabolites is the liver, and the main route of excretion of these metabolites is through the kidney, which most likely result in the susceptibility of these two organs to a greater degree of CP-induced toxicity. Therefore, this study emphasized on the study of the acute hepatotoxicity and nephrotoxicity induced by CP in rats and the role of WPI on modulating these complications in an attempt to minimize the chemotherapeutic-associated side effects on these vital organs.

## MATERIAL AND METHODS

### Animals

Forty adult male Wistar rats weighing 180-200 g were utilized in the present study. Standard food pellets and tap water were supplied *ad libitum*. Animals and food pellets were obtained from the animal house colony of the National Research Centre (NRC, Egypt). Animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals (1996, published by National Academy Press, 2101 Constitution Ave. NW, Washington, DC 20055, USA) and that their use was reviewed and approved in accordance with ethical rules for standard experimental animal studies and the Medical Research Ethics Committee (MREC) of the NRC, and all work was conducted with the formal approval of it (number: 15020).

### Drugs and chemicals

WPI was obtained from Davisco Foods International, Inc. (USA). CP (Endoxan®) was purchased from Baxter Oncology GmbH (Germany). WPI was dissolved in distilled water and freshly prepared before oral administration. CP was injected intraperitoneally in a single dose of 200 mg/kg (Mahmoud, 2014; Habibi *et al.*, 2015; Oyagbemi *et al.*, 2016). All other chemicals were of highest analytical grade available. All mandatory laboratory health and safety procedures have been complied with in the course of conducting the experimental work in this study.

### Experimental design and treatment protocol

Animals were randomly allocated into five groups (8 rats each). Rats of the 1st group received intraperitoneal injections of saline and served as normal control group. Acute CP-toxicity was induced in the remaining 4 groups by single intraperitoneal injection of CP (200 mg/kg) on the 15th day of treatment. Group 2 received only saline intraperitoneally for 15 days before CP treatment and served as CP control group. Groups 3, 4 and 5 received oral WPI (75, 150 or 300 mg/kg/day), respectively for 15 days before CP treatment on day 15. All animals were sacrificed 48 h after CP injection.

### Serum biochemical analysis

Forty-eight hours after CP injection, rats were anaesthetized with diethyl ether and blood samples were withdrawn from the retro-orbital venous plexus. Collected blood samples were allowed to stand for 10 min at room temperature then centrifuged at 4 °C using cooling centrifuge (Laborezentrifugen, 2k15, Sigma, Germany) at 4000 r.p.m for 5 min. Sera were separated for assessment of levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) using commercially available colorimetric assay kits (Biodiagnostic, Egypt) as previously described (Reitman and Frankel, 1957), in addition to serum creatinine and urea levels, using specific diagnostic kits (Biodiagnostic, Egypt).

### Hepatic and renal tissue biochemical analysis

Directly after blood sampling, rats were sacrificed by cervical dislocation under ether anesthesia. Liver and kidney tissues were collected, washed in normal saline and then divided into 3 parts. The first part was homogenized using MPW-120 homogenizer (Med instruments, Poland); the homogenate was centrifuged using a cooling centrifuge (Laborezentrifugen, 2k15, Sigma, Germany) at 3000 r.p.m for 10 min. and the supernatant was assessed for hepatic and renal levels of reduced glutathione (GSH) (Beutler *et al.*, 1963), lipid peroxides as malondialdehyde (MDA) (Uchiyama and Mihara, 1978), and nitric oxide (NO<sub>x</sub>) metabolites (Miranda *et al.*, 2001). Moreover, inflammatory markers such as myeloperoxidase (MPO), interleukin-1 $\beta$  (IL-1 $\beta$ ) were assessed using ELISA kits (Hycult Biotech, Netherlands) and (R&D Systems, USA), respectively, according to the manufacturer's instructions.

### Immunohistochemical analysis of cyclooxygenase-2 (COX-2)

For immunohistochemistry, 4  $\mu$ m thick deparaffinized liver and kidney tissue sections were used. Briefly, deparaffinized liver slices were incubated overnight with the antibodies against COX-2 diluted 1:100, Endogenous peroxidase activity was blocked by incubation in 0.075% hydrogen peroxide in PBS. For antibody detection DAKO EnVision+ System, Peroxidase/DAB kit was employed. The sections were then counterstained with hematoxylin, dehydrated using graded alcohols and xylene, and mounted with Entelan. The immunostaining intensity and cellular localization of COX-2 was analysed by light microscopy.

### Histopathological examination

The other parts of liver and kidney tissues were fixed in 10% neutral buffered formalin and embedded in paraffin wax. sections of 4  $\mu\text{m}$  thick were stained with Hematoxylin and Eosin (H&E) and examined using binocular Olympus CX31 microscope (Bancroft *et al.*, 1996).

### Statistical analysis

All values are presented as means  $\pm$  standard error of the means (SEM) of eight experiments. Comparisons between different groups were carried out using one way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test. Difference was considered significant when  $p < 0.05$ . GraphPad prism<sup>®</sup> software version 6 for Windows (USA) was used to carry out these statistical tests.

## RESULTS AND DISCUSSION

Administration of single intraperitoneal dose of CP (200 mg/kg) resulted in a significant elevation of liver and kidney function biomarkers. Liver function enzymes, ALT and AST, were elevated significantly in CP-treated rats by 51 and 85%, respectively, compared to their normal counterparts. Meanwhile, CP increased the levels of serum creatinine and urea significantly by 77 and 85%, respectively, compared to the normal group.

Pre-treatment of rats with oral WPI at 75, 150 or 300 mg/kg/day, significantly improved both liver and kidney functions in a dose-dependent manner as compared to CP-treated rats, to report normal levels of AST, creatinine, and urea in rats pre-treated with 300 mg/kg of WPI (**Table 1**). CP (200 mg/kg, i.p.) resulted in liver damage in rats as evidenced by the marked decrease in hepatic GSH, with significant increase in MDA, NOx, MPO, and IL-1 $\beta$  contents as compared to the normal control group. Pre-treatment of rats with WPI (75, 150 or 300 mg/kg) significantly elevated the decreased liver GSH in a dose-dependent manner compared to the CP-control group. On the other hand, oral WPI at 75, 150 and 300 mg/kg, for 15 days prior CP injection significantly reduced the elevation in hepatic MDA, NOx, MPO and IL-1 $\beta$  compared to the CP-control group. Of note, WPI at 300 mg/kg recorded significant decrease of MDA, NO, MPO and IL-1 $\beta$  compared to the other dose levels of WPI (**Table 2**).

Single intraperitoneal injection of CP, 200 mg/kg, resulted in severe kidney damage in rats as evidenced by significant decrease in renal GSH by 42%, along with significant dramatic increase in renal MDA, NOx, MPO and IL-1 $\beta$  content by 1.7, 1.9, 12, and 6-fold, respectively, as compared to the normal control group. Pre-treatment of rats with WPI, at 75, 150 and 300 mg/kg, significantly elevated renal GSH while decreased renal MDA, NOx, MPO and IL-1 $\beta$  in a dose dependent manner compared to the CP-control group (**Table 3**).

**Table 1:** Effect of WPI on liver and kidney functions of CP-treated rats.

Parameters	ALT (IU/L)	AST (IU/L)	Creatinine (mg/dl)	Urea (mg/dl)
<b>Groups</b>				
Normal control	123.83 <sup>b</sup> $\pm$ 3.20	137.58 <sup>b</sup> $\pm$ 2.15	4.69 <sup>b</sup> $\pm$ 0.33	26.48 <sup>b</sup> $\pm$ 0.74
CP (200 mg/kg)	187.12 <sup>a</sup> $\pm$ 1.98	254.82 <sup>a</sup> $\pm$ 7.30	8.30 <sup>a</sup> $\pm$ 0.19	48.89 <sup>a</sup> $\pm$ 2.20
WPI (75 mg/kg)+CP	167.59 <sup>ab</sup> $\pm$ 2.03	234.45 <sup>ab</sup> $\pm$ 3.90	6.00 <sup>ab</sup> $\pm$ 0.18	41.62 <sup>ab</sup> $\pm$ 1.19
WPI (150 mg/kg)+CP	151.01 <sup>abc</sup> $\pm$ 2.04	176.01 <sup>abc</sup> $\pm$ 3.20	5.24 <sup>b</sup> $\pm$ 0.22	38.91 <sup>ab</sup> $\pm$ 0.16
WPI (300 mg/kg)+CP	145.94 <sup>abcd</sup> $\pm$ 4.92	153.66 <sup>abcd</sup> $\pm$ 5.80	4.68 <sup>bc</sup> $\pm$ 0.09	31.43 <sup>abcd</sup> $\pm$ 0.41

CP, cyclophosphamide; WPI, whey protein isolate. Each value represents the mean  $\pm$  S.E.M (n=8). Statistical analysis was carried out using one-way ANOVA test followed by Tukey post hoc test. <sup>a</sup> Significantly different from saline control group at  $p < 0.05$ . <sup>b</sup> Significantly different from CP group at  $p < 0.05$ . <sup>c</sup> Significantly different from WPI (75mg/kg) group at  $p < 0.05$ . <sup>d</sup> Significantly different from WPI (150mg/kg) group at  $p < 0.05$ .

**Table 2:** Effects of WPI on hepatic contents of GSH, MDA, NOx, MPO, and IL-1 $\beta$  in CP-treated rats.

Parameters	GSH ( $\mu\text{mol/g}$ )	MDA (nmol/g)	NOx (nmol/g)	MPO (U/g)	IL-1 $\beta$ (pg/g)
<b>Groups</b>					
Normal Control	396.57 <sup>b</sup> $\pm$ 9.21	56.50 <sup>b</sup> $\pm$ 0.43	396.40 <sup>b</sup> $\pm$ 10.30	1.14 <sup>b</sup> $\pm$ 0.03	6.55 <sup>b</sup> $\pm$ 0.16
CP (200 mg/kg)	293.86 <sup>a</sup> $\pm$ 6.00	72.5 <sup>a</sup> $\pm$ 1.04	727.02 <sup>a</sup> $\pm$ 21.40	10.70 <sup>a</sup> $\pm$ 0.23	45.85 <sup>a</sup> $\pm$ 1.43
WPI (75 mg/kg) +CP	306.35 <sup>ab</sup> $\pm$ 8.63	52.12 <sup>b</sup> $\pm$ 1.64	630.64 <sup>ab</sup> $\pm$ 15.03	6.60 <sup>ab</sup> $\pm$ 0.23	27.30 <sup>ab</sup> $\pm$ 0.40
WPI (150 mg/kg)+CP	335.72 <sup>abc</sup> $\pm$ 5.78	58.40 <sup>bc</sup> $\pm$ 1.60	623.40 <sup>ab</sup> $\pm$ 10.80	4.20 <sup>abc</sup> $\pm$ 0.07	18.50 <sup>abc</sup> $\pm$ 0.34
WPI (300 mg/kg)+CP	361.36 <sup>abcd</sup> $\pm$ 4.46	50.64 <sup>bd</sup> $\pm$ 1.20	610.43 <sup>ab</sup> $\pm$ 11.08	2.33 <sup>abcd</sup> $\pm$ 0.08	11.84 <sup>abcd</sup> $\pm$ 0.24

CP, cyclophosphamide; WPI, whey protein isolate; GSH, reduced glutathione; MDA, malondialdehyde; NOx, nitric oxide metabolites; MPO, myeloperoxidase; IL-1 $\beta$ , interleukin-1beta. Each value represents the mean  $\pm$  S.E.M (n=8). Statistical analysis was carried out using one-way ANOVA test followed by Tukey post hoc test. <sup>a</sup> Significantly different from saline control group at  $p < 0.05$ . <sup>b</sup> Significantly different from CP group at  $p < 0.05$ . <sup>c</sup> Significantly different from WPI(75mg/kg) group at  $p < 0.05$ . <sup>d</sup> Significantly different from WPI (150mg/kg) group at  $p < 0.05$ .

**Table 3:** Effects of WPI on renal contents of GSH, MDA, NOx, MPO, and IL-1 $\beta$  in CP-treated rats.

Parameters	GSH ( $\mu\text{mol/g}$ )	MDA (nmol/g)	NO ( $\mu\text{mol/g}$ )	MPO (U/g)	IL-1 $\beta$ (pg/g)
<b>Groups</b>					
Normal control	225.70 <sup>b</sup> $\pm$ 2.32	71.00 <sup>b</sup> $\pm$ 1.15	299.15 <sup>b</sup> $\pm$ 2.27	0.66 <sup>b</sup> $\pm$ 0.02	4.45 <sup>b</sup> $\pm$ 0.16
CP (200 mg/kg)	130.57 <sup>a</sup> $\pm$ 1.71	121.87 <sup>a</sup> $\pm$ 3.10	580.85 <sup>a</sup> $\pm$ 6.50	8.52 <sup>a</sup> $\pm$ 0.25	29.31 <sup>a</sup> $\pm$ 0.60
WPI (75 mg/kg) +CP	197.93 <sup>ab</sup> $\pm$ 1.74	90.64 <sup>b</sup> $\pm$ 2.03	444.70 <sup>ab</sup> $\pm$ 8.90	4.45 <sup>ab</sup> $\pm$ 0.20	20.20 <sup>ab</sup> $\pm$ 0.36
WPI (150 mg/kg)+CP	199.80 <sup>ab</sup> $\pm$ 10.58	87.87 <sup>ab</sup> $\pm$ 2.09	404.90 <sup>abc</sup> $\pm$ 7.21	2.31 <sup>abc</sup> $\pm$ 0.08	13.67 <sup>abc</sup> $\pm$ 0.35
WPI (300mg/kg)+CP	216.14 <sup>b</sup> $\pm$ 4.23	82.36 <sup>abc</sup> $\pm$ 0.80	308.72 <sup>bcd</sup> $\pm$ 6.90	1.71 <sup>abc</sup> $\pm$ 0.05	9.73 <sup>abcd</sup> $\pm$ 0.20

CP, cyclophosphamide; WPI, whey protein isolate; GSH, reduced glutathione; MDA, malondialdehyde; NOx, nitric oxide metabolites; MPO, myeloperoxidase; IL-1 $\beta$ , interleukin-1beta. Each value represents the mean  $\pm$  S.E.M (n=8). Statistical analysis was carried out using one-way ANOVA test followed by Tukey post hoc test. <sup>a</sup> Significantly different from saline control group at  $p < 0.05$ . <sup>b</sup> Significantly different from CP group at  $p < 0.05$ . <sup>c</sup> Significantly different from WPI (75mg/kg) group at  $p < 0.05$ . <sup>d</sup> Significantly different from WPI (150mg/kg) group at  $p < 0.05$ .

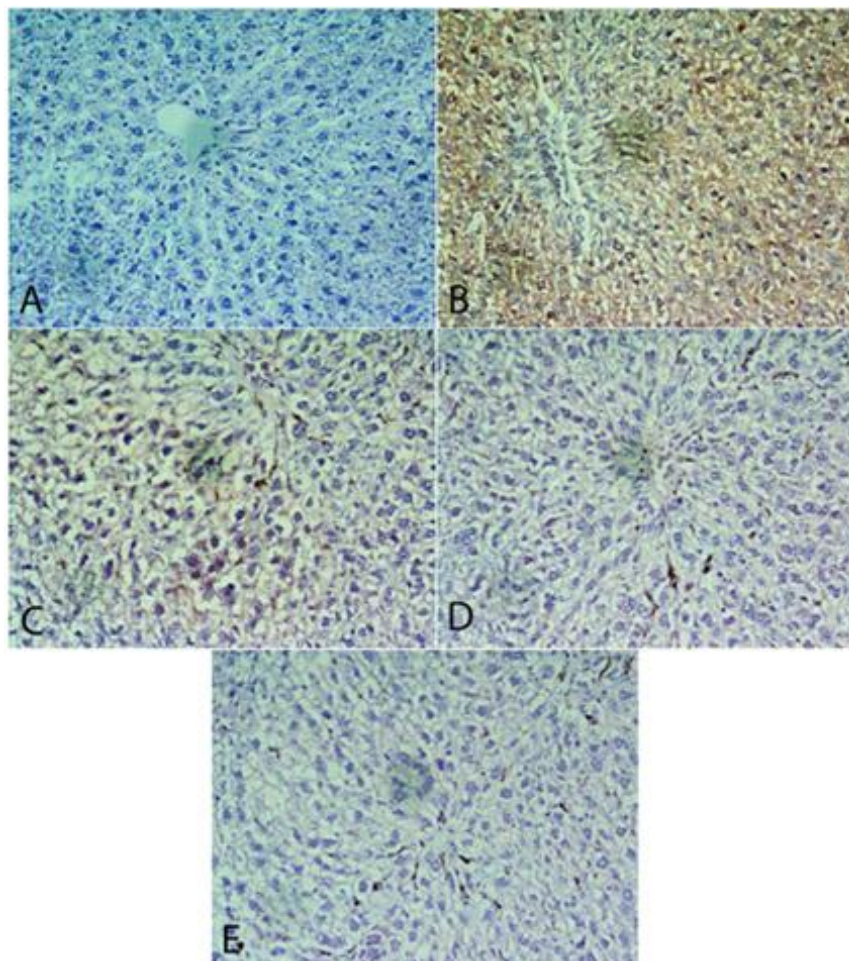
Regarding immunohistochemical examination of COX-2 in liver and kidney tissues; immunohistochemical analysis revealed non-detectable level of COX-2 expression in the livers from normal control group (**Fig. 1A**). CP injection caused marked increases in the immunoreactivity of COX-2 in the cytoplasm of hepatocytes as compared to the control group (**Fig. 1B**). WPI-pre-treated groups revealed weak expression of COX-2 at WPI (75 mg/kg) dose level (**Fig. 1C**). While, WPI at 150 and 300mg/kg, attenuated the formation of COX-2 in the livers, in a dose-dependent manner (**Fig. 1D and E**).

Renal immunohistochemical examination showed negative immunoreactivity of COX-2 expression in the glomeruli and tubules of kidney from normal control group (**Fig. 2A**). Rats treated with CP showed elevated immunoreactivity of COX-2 in the glomeruli and tubules of kidney (**Fig. 2B**). However, WPI-treated rats showed reduction in the expression COX-2 in the glomeruli and tubules of kidney in a dose-dependent manner (**Fig. 2C, D, and E**).

Concerning histopathological examination of liver and kidney tissues; liver sections of normal control rat exhibited normal hepatic cells each with well-defined cytoplasm, prominent

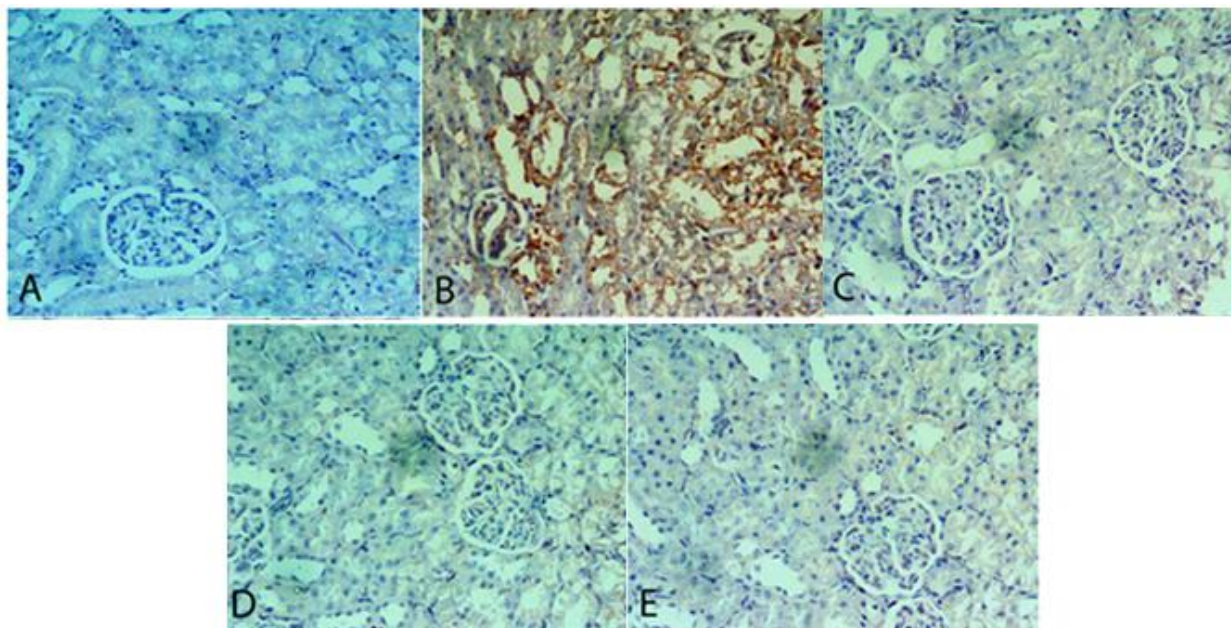
nucleus, nucleolus, central vein and blood sinusoids (**Fig. 3A**). Forty-eight hours after CP intoxication, liver tissues showed loss of hepatic architecture with centrilobular hepatic necrosis, degeneration of hepatocytes, vacuolization, and congestion of sinusoids and infiltration of mononuclear cells especially near the portal veins. Also, nuclei of most cells revealed clear signs of pyknosis and apoptosis with activation of kupffer cell (**Fig.3B**).

Treatment with WPI (75 mg/kg. p.o) for 15 days prior to CP exerted moderate improvement. Hepatic cords were slightly distorted, necrosis some hepatocytes, congestion and dilation of sinusoids. In addition, nuclei of some cells were pyknosis, apoptosis with activation of kupffer cell (**Fig.3C**). However, treatment with WPI (150 mg/kg. p.o) showed improvement in histological structure of liver sections of rats, pronounced in normalized appearance of liver in the formation of normal hepatic cords. Pyknotic nuclei and activation of kupffer cell were also observed (**Fig.3D**). Meanwhile, WPI (300 mg/kg. p.o)-treated group showed improvement in hepatic architecture and central vein slightly dilated, with persistent few pyknotic nuclei, activation of kupffer cell with mild congestion in some of the sinusoids (**Fig. 3E**).



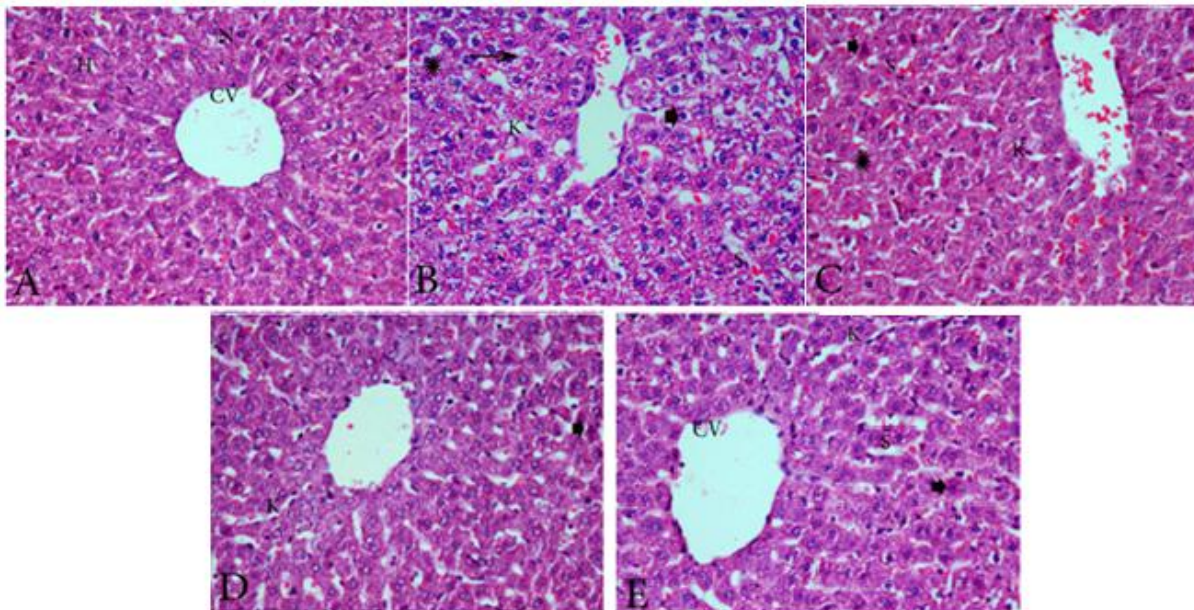
**Fig. 1:** Effect of WPI on immunohistochemical staining of COX-2 of CP-treated liver tissue in rats.

CP, cyclophosphamide; WPI, whey protein isolate; COX-2, cyclooxygenase-2. **A.** Control group showing no expression of COX-2. **B.** Cyclophosphamide group showing marked increase of COX-2 immunoreactivity. **C.** WPI (75 mg/kg. p.o) showing mild immunoreactivity of COX-2. **D.** WPI (150 mg/kg. p.o) showing weak immunoreactivity of COX-2. **E.** WPI (300 mg/kg. p.o) showing weak immunoreactivity of COX-2 (**COX-2 and hematoxylin counterstain X 400**).



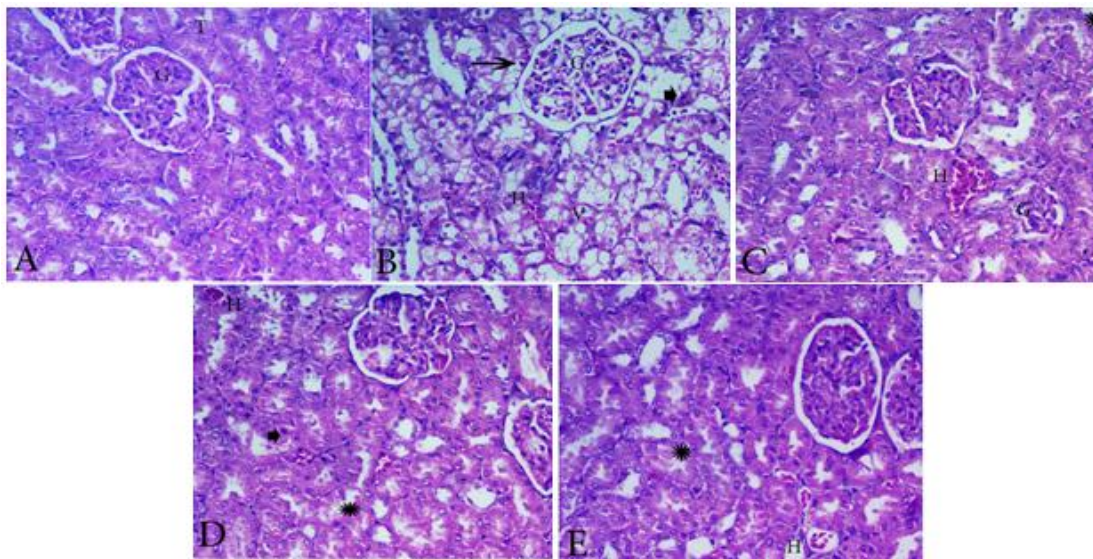
**Fig. 2:** Effect of WPI on immunohistochemical staining of COX-2 of CP-treated kidney tissue in rats.

CP, cyclophosphamide; WPI, whey protein isolate; COX-2, cyclooxygenase-2. **A.** Control group showing no expression of COX-2(**A**). **B.** Cyclophosphamide group showing increase of COX-2. **C.** WPI (75 mg/kg. p.o) showing mild immunoreactivity of COX-2. **D.** WPI (150 mg/kg. p.o) showing of weak immunoreactivity of COX-2. **E.** WPI (300 mg/kg. p.o) showing of weak immunoreactivity of COX-2. (**COX-2 and hematoxylin counterstain X 400**).



**Fig. 3:** Effect of WPI on hepatic architecture of CP-treated liver tissue in rats.

CP, cyclophosphamide; WPI, whey protein isolate. **A.** Normal liver section showing central vein (CV) normal hepatocytes (H), nucleus (N) and blood sinusoids (S). **B.** Cyclophosphamide treated liver showing loss of hepatic architecture with centrilobular hepatic necrosis (star), degeneration of hepatocytes (thin arrow), and congestion of sinusoids (S). Nuclei of most cells revealed clear signs of pyknosis (head arrow) with activation of kupffer cells (K). **C.** Whey protein isolate (75 mg/kg. p.o) showing moderate improvement, hepatic cords were slightly distorted, necrosis of some hepatocytes (star), congestion and dilation of sinusoids (S). Nuclei of some cells showed pyknosis (arrow head) with activation of kupffer cell (K). **D.** Whey protein isolate (150 mg/kg. p.o) showing improvement in histological structure of liver sections of rats and normal hepatic pyknotic nuclei (arrow head) and activation of kupffer cell (K) were also observed. **E.** Whey protein isolate (300 mg/kg. p.o) showing improvement in hepatic architecture and central vein slightly dilated. Few pyknotic nuclei (arrow head), activation of kupffer cell (K) mild congestion in some of the sinusoids (S) were also noticed. (**H&E X 400**).



**Fig. 4:** Effect of WPI on renal architecture of CP-treated liver tissue in rats.

CP, cyclophosphamide; WPI, whey protein isolate. **A.** Normal kidney section showing normal histological features of glomeruli and tubules. **B.** Cyclophosphamide treated kidney showing glomerular degeneration (G), and hypercellularity. Most of the cortical tubules showed morphologic changes, some of them being tubular necrosis, degeneration, vacuolation (V) and desquamation of tubular epithelium with interstitial hemorrhage (H). Also, nuclei of most cells revealed clear signs of pyknosis (arrow head) and apoptosis (thin arrow). **C.** Whey protein isolate (75 mg/kg. p.o) showing mild improvement, however some glomeruli were degenerated (G). Also, some tubules still necrosis (star) with interstitial hemorrhage (H). **D.** Whey protein isolate (150 mg/kg. p.o) showing more remarkable improvement and the glomeruli were normal. The kidney tubules showed necrosis, pyknotic nuclei (arrow head) with interstitial hemorrhage (H). **E.** Whey protein isolate (300 mg/kg. p.o) showing more remarkable improvement and the glomeruli were normal. The kidney tubules showed necrosis (star) with interstitial hemorrhage (H). (**H&E X 400**).

Kidney sections of normal control rat showed apparently normal histological features of glomeruli and tubules (**Fig. 4A**). Histopathological examination revealed that CP induced severe lesions in rat kidneys, evidenced by glomerular degeneration, hypercellularity and shrinkage. Most of the cortical tubules showed morphologic changes, some of them being tubular necrosis, degeneration, vacuolation and desquamation of tubular epithelium with interstitial haemorrhage. Also, nuclei of most cells revealed clear signs of pyknosis and apoptosis (**Fig. 4B**). Oral treatment with WPI (75 mg/kg. p.o) showed mild improvement, however some glomeruli were degenerated and some tubular necrosis with interstitial haemorrhage were evident (**Fig. 4C**). Treatment with WPI (150 and 300 mg/kg. p.o) showed a more remarkable improvement and the glomeruli were normal, while renal tubules showed mild necrosis with interstitial haemorrhage (**Fig. 4D and E**).

The present study demonstrated induction of acute hepatic and renal damage in rats following 48-hours of single intraperitoneal dose of CP (200 mg/kg). Our data revealed increased serum activities of ALT and AST in those rats along with high serum levels of creatinine and urea reflecting both hepatotoxicity and renal toxicity. Similar hepatotoxic effect and alteration of liver functions biochemical parameters by CP have been documented by other research groups (Zarei and Shivanandappa, 2013; Germoush and Mahmoud, 2014; Zhu *et al.*, 2015). In addition, CP-induced renal toxicity with elevation in serum creatinine and urea has been recognized (Sayed-Ahmed, 2010; Rehman *et al.*, 2012; Alhumaidha *et al.*, 2015). More importantly, CP has been found to cause clinically self-limiting

uroepithelial and renal tubular damage due to accumulation of acrolein, the toxic metabolite, in the urine at high concentrations (Hamsa and Kuttan, 2011).

A reversal of CP-induced increased serum ALT, AST, creatinine, and urea in rats was observed with WPI supplementation in a dose-dependent fashion. This indicated protective properties of WPI that resulted in a prevention of the leakage of intracellular enzymes from hepatocytes and restoration of normal renal functions. The hepatoprotective properties of WPI have been proved in several animal models of hepatotoxicity (Gad *et al.*, 2011; Mansour *et al.*, 2013; Salama *et al.*, 2015). On the other hand, debate has raised about the effect of using whey protein in the kidney functions. Consumption of high-whey-protein diets was found to cause alterations in renal health status and some metabolic parameters in rats (Aparicio *et al.*, 2011). However, at the centre of this controversy is the concern that the potential induction of renal problems arises from habitual consumption of dietary whey protein in excess of recommended amounts. Interestingly, evidences suggest that protein-induced changes in renal function are likely a normal adaptive mechanism within the functional limits of a healthy kidney (Martin *et al.*, 2005). However, the hepato- and renoprotective effects of WPI were confirmed in this study by the improvement of CP-induced oxidative, inflammatory, and histopathological alterations in both liver and kidneys. In addition to its pro-oxidant nature and its ability to elicit oxidative injury (Selvakumar *et al.*, 2005), CP and its metabolites induced depletion of GSH that may be attributed to direct conjugation of CP and its metabolites with free or protein bound -SH group (Kehrer and Biswal, 2000). In agreement, CP in

our study resulted in a significant decrease of both hepatic and renal GSH along with an increase in hepatic and renal MDA as an indicator of involvement of free radical-induced lipid peroxidation in both organs (Collins *et al.*, 1994). Herein, depletion of GSH seems to be a prime factor that permits lipid peroxidation in the CP-treated group. Moreover, our data revealed increased levels of NOx in liver and kidneys of CP-treated rats; this could be attributed to acrolein ability to activate both ROS and NO production with further production of superoxide anion, hydroxyl radical and hydrogen peroxide during CP oxidative metabolism leading to depressed antioxidant defence mechanism in different tissues (Senthilkumar *et al.*, 2006; Abraham *et al.*, 2011). Moreover, superoxide radical may react with NO producing peroxynitrite, which may impair various mitochondrial enzymes leading to more sustained intracellular ROS generation, resulting in an augmentation of oxidative/nitrosative stress (Lipton *et al.*, 1993).

The present study reported significant modulation of CP-prompted oxidative/nitrosative stress in liver and kidney by WPI supplementation in a dose dependent-manner. Noteworthy, WPI (300 mg/kg) normalized hepatic MDA as well as renal GSH and NOx contents. Several reports indicated that WPI has potential antioxidant activity due to its ability to elevate cellular GSH levels (Tseng *et al.*, 2006; Peng *et al.*, 2009). Most whey proteins are cysteine rich, including  $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin, and bovine serum albumin (Morr and Ha, 1993). Cysteine is known as an amino acid that regulates *in vivo* concentrations of GSH. The supplementation of diet with whey protein high in cysteine may promote GSH biosynthesis. The latter has been reported to be an antioxidant and anticarcinogenic tripeptide, thus improving protection against oxidant-induced cell damage (Bounous *et al.*, 1989). The elevated levels of GSH reported in the present study after treatment with WPI along with decreased levels of MDA and NOx supported previous findings and recent reports of our team that stated potent antioxidant activity of WPI at different dose levels that replenished the antioxidant pool, enzymatic and non-enzymatic, while abolished lipid peroxidation and nitrosative stress in different models of hepatotoxicity (Mansour *et al.*, 2013; Mansour *et al.*, 2015a; Salama *et al.*, 2015). Further, major components of whey protein have been investigated for their protective mechanisms against liver and brain damage (Mansour *et al.*, 2015b) as well as liver fibrosis incidence by thioacetamide (Nada *et al.*, 2015).

In addition to oxidative and nitrosative stress insult, the present study reported that CP-exacerbated liver and kidney injury was further mediated via an inflammatory process including dramatic rise in levels of hepatic and renal MPO, a marker of neutrophil infiltration (Linares-Fernandez and Alfieri, 2007; Abraham *et al.*, 2011), together with high level of IL-1 $\beta$ , a pro-inflammatory cytokine, and COX-2 expression in rat liver and kidney. In the same context, previous studies of our team and others reported inflammatory changes induced by CP in different organs through elevation of IL-1 $\beta$ , inducible NO synthase, and COX-2 activities, and revealed a cross talk between NO and COX-

1/COX-2 isoforms (Linares-Fernandez and Alfieri, 2007; Saleh and Mansour, 2016). Consistently, the current data showed that CP-induced increase in the levels of inflammatory mediators and COX-2 expression in hepatic and renal tissues was accompanied by an increase in NOx level. Similarly, an established relation between IL-1 $\beta$  and COX-2 in CP-induced cystitis has been reported (Girard *et al.*, 2008) through several lines of evidence that further suggest that IL-1 $\beta$  may cause hyperalgesia by releasing COX products through stimulation of COX-2 (Geng *et al.*, 1995; Samad *et al.*, 2001) and phospholipase A2 (Girard *et al.*, 2008) or by inducing arachidonic acid release (Dinarello, 1997).

Treatment of rats with WPI hampered CP-induced inflammation as pronounced by the decrease in MPO activity, IL-1 $\beta$  level, and COX-2 expression in hepatic and renal tissues. As stated before, WPI has a potent GSH-boosting and antioxidant activity that may correlate to its anti-inflammatory effect observed above, depending on the fact that the synthesis of many cytokines is affected by the oxidant/antioxidant balance (Kent *et al.*, 2003). The anti-inflammatory properties of WPI together with its major components,  $\alpha$ -lactalbumin and  $\beta$ -lactoglobulin have been reported before (Mansour *et al.*, 2015a). Moreover, lactoferrin, the minor component of WPI, was found to possess anti-inflammatory activities through inhibition of cytokine production such as IL-1 $\beta$ , TNF- $\alpha$  and IL-6 from lipopolysaccharide-sensitized kupffer cells (Yamaguchi *et al.*, 2001). Interestingly, recent studies reported hepato- and renoprotective effects of lactoferrin in rats through inhibition of inflammation and reduction of inflammatory markers such as TNF- $\alpha$ , IL-18, and IL-4 (Hessin *et al.*, 2015; Hegazy *et al.*, 2016).

The histopathological findings in the present study confirmed the biochemical and immunohistochemical data. Investigations of hepatic and renal tissues of animals treated with CP exposed a marked distortion in the architecture of the liver and kidney. In the liver, the cellular targets of CP in rat liver were found to be related to CP metabolites that cause death of sinusoidal endothelial cells that are more susceptible to CP-toxicity than hepatocytes with profound depletion of GSH before the onset of toxicity (DeLeve, 1996). Meanwhile, numerous data confirmed that CP-induced hepatotoxicity was associated with, at least in part, the level of GSH, which played an important role in eliminating active metabolites and defending the oxidative stress *in vivo* (Lu, 2009).

This explanation may provide further confirmation of the potential mechanism through which WPI improved the histopathologic picture of CP-treated rat liver in a dose-dependent fashion in the present study through its GSH-boosting and antioxidant properties.

Acute renal toxicity by CP was also confirmed by histopathological changes in kidney including severe tubular necrosis, oedema, haemorrhage, degeneration, tubular dilatation, and glomerular degeneration. Acute tubular necrosis, azotemia, glomerular mesangiolytic and sclerosis are reported in animals receiving as little as one dose of CP (Lavin and Koss, 1971; Raymond, 1984). Similarly, severe histopathological changes in

kidney were reported 48 hours after CP secondary to oxidative stress and inflammation (Hamsa and Kuttan, 2011).

On the other hand, WPI supplementation significantly improved renal pathologic picture to show normal glomeruli with WPI (300 mg/kg)-treatment. Several literature supported the notion that potent antioxidants through boosting cellular antioxidant armoury can manage and attenuate the renal damage induced by CP (Ghosh *et al.*, 1999; Rehman *et al.*, 2012); having GSH-boosting capacity (Xu *et al.*, 2011), WPI played the same role.

## CONCLUSION

The present findings demonstrated that WPI offers ameliorative effects against CP-induced hepatorenal damage in rats via antioxidant and anti-inflammatory properties.

**Financial support and sponsorship:** Nil.

**Conflict of Interests:** There are no conflicts of interest.

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**How to cite this article:**

Mansour DF, Salama AA, Hegazy RR, Omara EA, Nada SA. Whey protein isolate protects against cyclophosphamide-induced acute liver and kidney damage in rats. *J App Pharm Sci*, 2017; 7 (06): 111-120.