Research Article CODEN: IJPRNK IMPACT FACTOR: 1.862 ISSN: 2277-8713
Hesham Eliwa, IJPRBS, 2014; Volume 3(2): 295-314 IJPRBS

INTERNATIONAL JOURNAL OF PHARMACEUTICAL RESEARCH AND BIO-SCIENCE

EVALUATION OF THE THERAPEUTIC EFFECT OF WHEY PROTEINS ON THE HEPATOTOXICITY INDUCED BY PARACETAMOL AND ALCOHOL CO-ADMINISTRATION IN RATS.

HESHAM .A.ELIWA[1], EZZEDIN S. EL-DENSHARY[2], SOMAIA A.NADA[3], MOHAMED F. ELYAMANY[5], ENAYAT A. OMARA[4], NAGLAA ASAAF[1]

1. Pharmacology & Toxicology Department Faculty of Pharmacy, MISR University for Science &Technology.
2. Pharmacology & Toxicology Department Faculty of Pharmacy, Cairo University .
3. Pharmacology Department - National Research Centre ;
4. Pathology Dept., National Research Centre, Cairo, Egypt;
5. Pharmacology Department, Faculty of Pharmacy, Cairo University

Accepted Date: 16/04/2014; Published Date: 27/04/2014

Abstract: Hepatotoxicity induced by oral co-administration of paracetamol (APAP) (500 mg / kg) and alcohol (5 ml/ kg) to each rat for two and four weeks .On the other hand, oral administration of whey protein isolate (WPI), alpha-lactalbumin (α-LA) and beta-lactoglobulin (β-LG) are used as therapeutic agents, in two dose levels (100 and 200 mg/kg) after induction of hepatotoxicity in both two and four weeks ,then liver tissue and blood sample collected. Oral administrated of APAP (500 mg / kg) and alcohol (5 ml/ kg) for two and four weeks increase in serum levels of alanine aminotransferase (ALT), and aspartate aminotransferase (AST). While, oral administration of WPI, α-LA and β-LG after APAP and alcohol resulted in reduction in serum AST and ALT activities , serum tumor necrosis factor-alpha (TNF-α) and hepatic lipid peroxidation marker malondialdehyde (MDA),while increase oxidative stress biomarkers (hepatic reduced glutathione (GSH) and superoxide dismutase (SOD). Histopathological examination showed disruption of normal architecture, mononuclear cellular infiltration. After treatment with WPI, α-LA and β-LG, remarkable improvement in histological structure of liver tissues was observed. **Conclusion:** Induction of hepatotoxicity by APAP and alcohol improved by oral administration of whey protein (WP), α-Lactalbumin (α-LA) and β-Lactoglobulin (β-LG) after two and four weeks in rats.

Keywords: whey protein isolate, alpha-lactalbumin, beta-Lactoglobulin, paracetamol, alcohol, liver, oxidative stress.

PAPER-QR CODE

Corresponding Author: DR. HESHAM A.ELIWA

Access Online On:

www.ijprbs.com

How to Cite This Article:

Hesham Eliwa, IJPRBS, 2014; Volume 3(2): 295-314

INTRODUCTION

Ethanol is a substantial source of energy, with 7.1 kcal (29.7 kJ) per gram, a value that exceeds the energy content of carbohydrates or proteins. On average, ethanol accounts for half an alcoholic's caloric intake. It therefore displaces normal nutrients causing malnutrition, including deficiencies of folate, thiamine, and other vitamins. Secondary malnutrition also occurs through malabsorption due to gastrointestinal complications, such as pancreatic insufficiency and impaired hepatic metabolism of nutrients. In addition, alcohol promotes the degradation of nutrients, as exemplified by its effects on vitamin A *(Lieber, 1992)*. Progress in the understanding of the pathogenesis of alcoholic liver disease was achieved when it was discovered that alcohol affects the liver through not only nutritional disturbances but also its direct toxicity because of its predominant metabolism in the liver associated with redox changes and oxidative stress. Redox changes are mediated by alcohol dehydrogenase (ADH), and oxidative stress is generated mainly by the activity of the microsomal ethanol oxidizing system (MEOS) and its key enzyme cytochrome P450 2E1 (CYP2E1), which releases free radicals, (*Lieber, 2004).* In the original studies of the mechanisms of toxicity, paracetamol was found to cause liver damage through conversion by hepatic cytochrome P450 enzymes to a minor but toxic intermediate metabolite and this was subsequently identified as N-acetyl-p-benzoquinone-imine (NAPQ) *(Corcoran et al., 1980).* The

The oxidative stress caused by CYP2E1 induction and mitochondrial injury results in lipid peroxidation and membrane damage. In addition, the acetaldehyde produced by the oxidation of ethanol has toxic effects, inhibiting the repair of alkylated nucleoproteins, decreasing the activity of key enzymes, and markedly reducing oxygen use in mitochondria damaged by long-term ethanol consumption (*Lieber et al., 1989*). Moreover, acetaldehyde promotes cell death by depleting the concentration of reduced glutathione (GSH), inducing lipid peroxidation, and increasing the toxic effect of free radicals.

Oxidative stress promotes inflammation, which is aggravated by an increase of the proinflammatory cytokine tumor necrosis factor-alpha (TNF-α) in the Kupffer cells. Kupffer cells are a major source of cytokines. They also harbor CYP2E1, and its increase after chronic alcohol consumption may act as a major stimulator. Indeed, in both acute and chronic liver diseases, Kupffer cells become activated to produce cytokines and reactive oxygen radicals *(Batey et al., 1998).*

Acetaminophen (APAP; N-acetyl-para-aminophenol) is a widely used analgesic and antipyretic known to be effective and safe when consumed at therapeutic doses (1–4 g/day) *(Rumack, 2004).* However, severe liver injury resulting in liver failure can occur in some cases following an acute or cumulative overdose (10–15 g) *(Kaplowitz, 2001).* APAP overdose accounts for more

than 56,000 emergency room visits, 2600 hospitalizations and an estimated 458 deaths due to acute liver failure each year within the United States *(Lee, 2004).* Overdose of APAP is also known to cause liver injury in laboratory animals with similar characteristics as those found in patients. The murine model of APAP-induced hepatotoxicity represents the most widely used model to study the pathogenesis of drug-induced liver injury (DILI). The initiation of APAP-induced liver injury (AILI) results from the metabolism of APAP into a reactive metabolite, N-acetyl-p-benzoquinone-imine (NAPQI). At the recommended doses, APAP is considered a safe drug. However, ingestion of large quantities of this agent can result in massive hepatic necrosis in man and in experimental animals *(Prescott et al., 1971; Hinson, 1983; Webster et al., 1996).* Nephrotoxicity and extrahepatic lesions in male rodents have also been reported *(Trumper et al., 1996).* At therapeutic doses, APAP is primarily metabolized in the liver by the phase II routes, sulfation and glucuronidation, with a small fraction metabolized by hepatic cytochrome P450 to a highly reactive free radical intermediate, N-acetyl-p-benzoquinone imine (NAPQI), which binds to reduced glutathione (GSH) and therefore displays no significant toxicity.

It has been demonstrated that there is a risk of hepatic alteration when paracetamol and alcohol are ingested within short intervals of time because alcohol consumption induces CYP2E1 increasing its concentration. Alcohol is the principal substrate for this enzyme which inhibits other substrates such as paracetamol.

Milk serum proteins are defined as substances that remain soluble in milk serum *(Luhovyy et al.,2007).*These proteins are naturally formed during the production of cheese and account for 20% of the all protein in milk,such as β-lactoglobulins, α-lactalbumin, immunoglobulin's, lactoferrin, Lactoperoxidase, Glycomacropeptide, bovine serum albumin , and other proteins *(Pal et al.,2010; Hulmi et al.,2010).*Whey protein, have high nutritional value because it contains all essential amino acids in higher concentrations than vegetable protein sources *(Haraguchi et al.,2009).*

MATERIALS AND METHODS

Chemical

1- Paracetamol (APAP) (Misr Co.for pharm. IND S.A.E).

2- Alcohol (Sigma- Aldrich chemicals)

3- Alanine aminotransferase(ALT) colorimetric kit from QCA, Spain

3- Aspartate aminotransferase(AST) colorimetric kit from QCA, Spain

4- Bovine serum albumin from Oxford, England

5- Ellman's reagent (DTNB) from Sigma, USA

6- Total antioxidant capacity (TAC) colorimetric kit from Biodiagnostic, Egypt . Diagnostic kits for determination of: SOD, GSH and MDA.

(Gamma Trade 14-El Fath Street from Shehab- Giza).

7- Rat TNF-alpha ELISA Kit from RayBio®, USA

Experimental animals:

Adult male Sprague–Dawley rats weighing 100-150 gm were obtained from the animal house at the National Research Center (Giza, Egypt), and fed a standard laboratory diet and tap water ad libitum. Experimental animals were housed in an air conditioned room at 22–25 ^{0}C with a 12-h light/dark cycle. All animals received humane care and the study protocols were in compliance with institutional guidelines for the use of laboratory animals.

Collection of blood samples:

Blood samples were withdrawn from the retro-orbital vein of each animal,

under light anesthesia by diethyl ether, according to the method described by Cocchetto and Bjornsson (1983).

Preparation of liver samples

Immediately after blood sampling, animals were sacrificed by cervical dislocation and the liver tissues were rapidly removed, washed in ice-cooled saline, plotted dry and weighed. The left lobe of each liver was dissected and placed in 10% formalin in saline, to be used for histopathological examination and immunohistochemical study.

A weighed part of each liver was homogenized, using a homogenizer (Medical instruments, MPW-120, Poland), with ice-cooled saline to prepare 20% w/v homogenate. The homogenate was then centrifuged at 4000 rpm for 5 min. at 4° C using a cooling centrifuge to remove cell debris (Laborzentrifugen, 2k15, Sigma, Germany). The aliquot was divided into three parts; the 1st part was used for the assessment of reduced glutathione, the 2nd part was used for the assessment of TBARS as malondialdehyde (MDA). A second intact part of each liver, homogenized with ice-cooled phosphate buffered saline (PBS) to prepare 20% w/v homogenate, was used for the estimation of superoxide dismutase (SOD) activity.

Measurement of serum liver function enzymes:

Hepatic dysfunction was assessed by measuring the rise in serum levels of ALT and AST using commercially available kits. The results were expressed as IU/L.

Determination of hepatic reduced glutathione:

Reduced glutathione (GSH) concentration in hepatic homogenate was determined according to the method described by Beutler et al. (1963). The GSH concentration was determined using a standard curve constructed with different concentrations of an authentic sample. The results were expressed as µg/g wet tissue weight.

Determination of hepatic lipid peroxidation:

Lipid peroxidation, as an indicator of oxidative stress, was estimated by measuring thiobarbituric acid reactive substance (TBARS) that sometimes referred to as malondialdehyde (MDA) in hepatic homogenates as previously described (Ruiz-Larrea et al., 1994). The amount of MDA was expressed as nmol/g wet hepatic tissue using 1,1,3,3-tetramethoxypropane as a standard.

Histopathological examinations:

The specimens from the liver were taken and fixed immediately in 10% neutral buffered formalin, processed for light microscopy to get (5µm) paraffin sections and stained with: Hematoxylin & Eosin to verify histological details and Masson's trichrome staining to demonstrate the collagen fibers as described by Bancroft and Gamble (2002).

Statistical analysis:

The degree in variability of results was expressed as means ± standard error of means (SEM). Data were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. The level of significance was accepted at $P < 0.05$.

RESULTS:

Determination of liver enzymes alanine aminotransferase and aspartate aminotransferase activity.

Oral administration of APAP (500 mg / kg) and alcohol (5 ml/ kg) for two and four weeks produced a significant increase in serum ALT and AST activity as compared to control group. Oral administration of WP isolate, α- lactalbumin and β-lactoglobulin are able to decrease serum ALT and AST activity as compared to APAP and alcohol group.

Table (1) :Effect of oral administration of whey protein isolate , alpha-lactalbumin and beta-Lactoglobulin 100mg/kg and 200mg/kg on hepatic ALT and AST activity in rats treated with APAP (500 mg / kg) and alcohol (5 ml/ kg) after two and four weeks.

Groups	Parameters*			
	ALT (IU/L)		AST (IU/L)	
	two weeks	four weeks	two weeks	four weeks
Control	13.07 ±0.65[a]	11.17 ±0.75[a]	49.08 ±0.59[a]	30.63 ±0.39[a]
APAP & alcohol	35.32 ±1.00[b]	40.13 ±1.34[b]	87.31 ±0.43[b]	82.83 ±1.12[b]
APAP & alcohol and WP100	25.64 ±0.88[c]	15.17 ±0.61[c]	70.61 ±0.41[c]	63.24 ±1.11[c]
APAP & alcohol and WP 200	20.03 ±0.97[d]	16.02 ±1.11[c]	60.32 ±0.35[d]	57.18 ±0.99[d]
APAP & alcohol and α-LA100	30.22 ±0.74[c]	15.92 ±0.84[c]	71.24 ±0.86[c]	40.83 ±0.64[c]
APAP & alcohol and α-LA 200	28.25 ±0.72[c]	13.00 ±0.79[ac]	68.75 ±0.93[d]	34.63 ±0.42[d]
APAP & alcohol and β-LG 100	26.24 ±0.679[c]	18.7 ±0.79[c]	66.20 ±0.61[c]	33.62 ±0.42[c]
APAP & alcohol and β-LG 200	20.88 ±0.701[d]	14.78 ±0.90[d]	62.65 ±0.63[d]	34.08 ±0.42[c]

ALT, alanine aminotransferase; AST, aspartate aminotransferase.*within each column, means with different superscript letters are significantly different (P<0.05).

Determination of tumor necrosis factor-alpha in serum

Oral administration of APAP (500 mg / kg) and alcohol (5 ml/ kg) for two and four weeks produced a significant increase in serum TNF-α as compared to control group .Oral administration of WPI ,α-LA and β-LG after two and four weeks showed significant reduction in serum level of TNF-α compared to APAP and alcohol group. While oral administration of WPI (200 mg/kg) after four weeks marked decrease in serum TNF-α as compared to α-LA and β-LG group.

Table (2) :Effect of oral administration of whey protein isolate , alpha-lactalbumin and beta-Lactoglobulin 100mg/kg and 200mg/kg on serum (TNF-α) in rats treated with APAP (500 mg / kg) and alcohol (5 ml/ kg) after two and four weeks.

Groups	Parameters*	
	TNF-α (pg/ml)	
	two weeks	four weeks
Control	128.8 ±2.355[a]	120.7 ±2.721[a]
APAP & alcohol	297.3 ±3.864[b]	411.1 ±5.100[b]
APAP & alcohol and WP100	202.9 ±2.752[c]	101.8 ±2.521[c]
APAP & alcohol and WP 200	209.3 ±2.753[d]	98.7 ±2.301[c]
APAP & alcohol and α-LA100	250.9 ±3.555[c]	201.8 ±3.22[c]
APAP & alcohol and α-LA 200	230.8 ±3.531[d]	190.3 ±3.10[c]
APAP & alcohol and β-LG 100	168.6 ±4.885[c]	125.6 ±2.135[c]
APAP & alcohol and β-LG 200	170.3 ±4.889[c]	115.8±2.133[d]

TNF-α tumor necrosis factor-alpha *within each column, means with different superscript letters are significantly different (P<0.05).

Determination of hepatic GSH, SOD and MDA

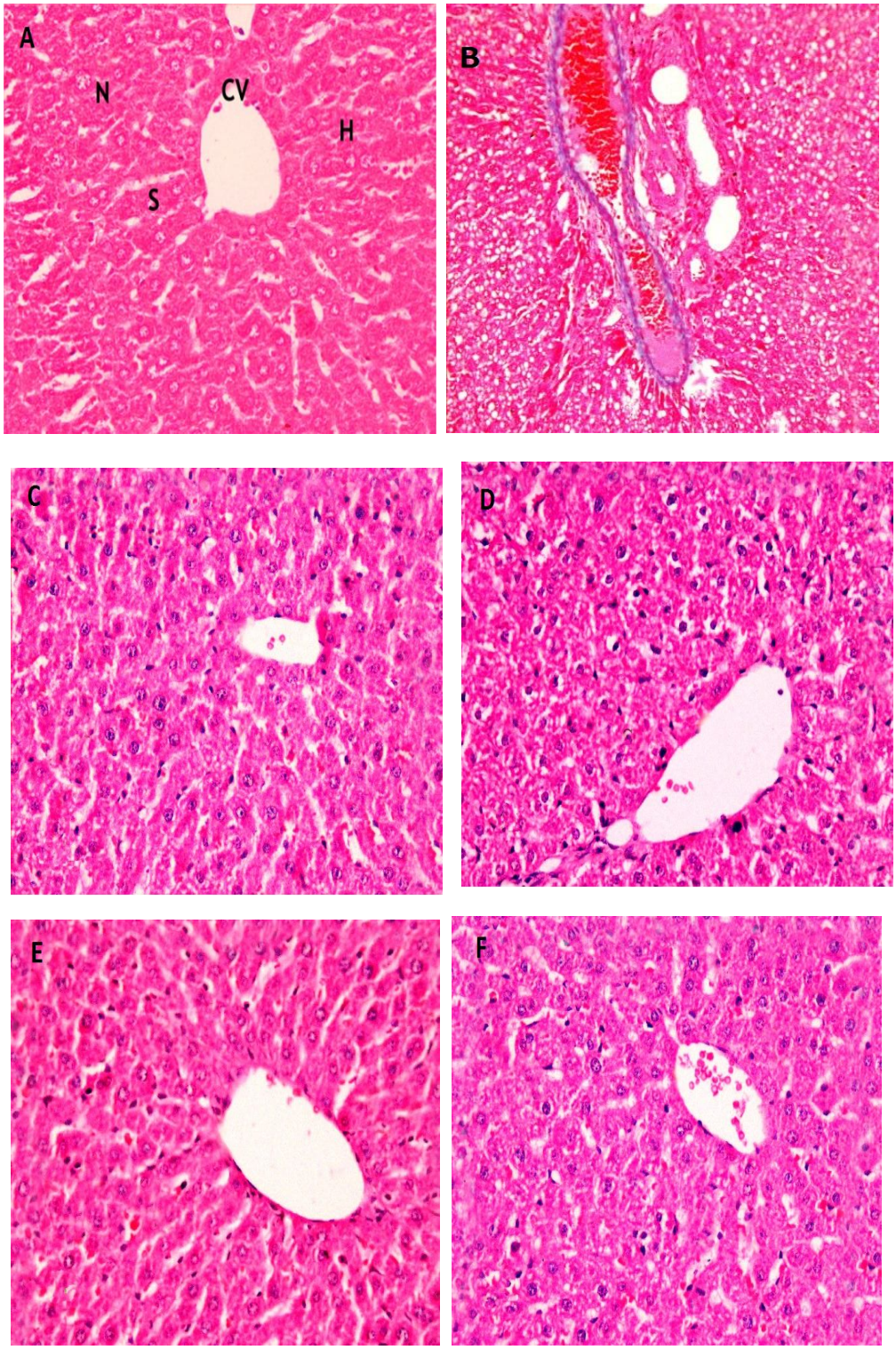
Oral administration of WPI, α-LA and β-LG decreased hepatic MDA level , while increase oxidative stress biomarkers (GSH and SOD) Treatment with α-lactalbumin significantly increased hepatic SOD activity than WPI and β-LG.

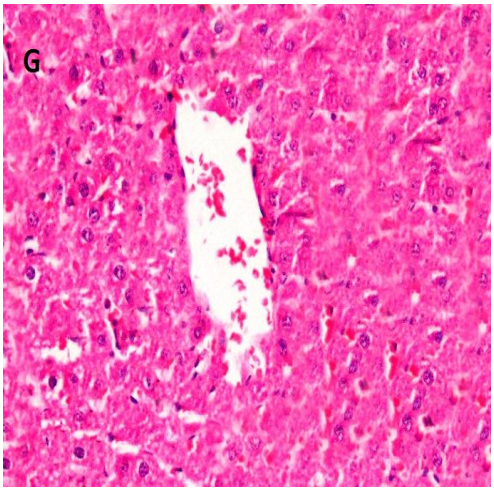
Table (3) :Effect of oral administration of whey protein isolate , alpha-lactalbumin and beta-lactoglobulin 100mg/kg and 200mg/kg on hepatic MDA , SOD and GSH in rats treated with APAP (500 mg / kg) and alcohol (5 ml/ kg) after two and four weeks.

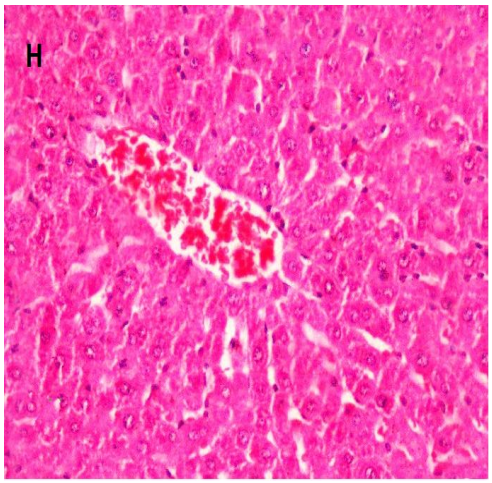
Groups	Parameters*					
	MDA		SOD		GSH	
	two weeks	four weeks	two weeks	four weeks	two weeks	four weeks
Control	43.95±0.742^{a}	36.77± 1.387^{a}	27.08±0.38^{a}	29.18±0.14^{a}	78.88±0.555^{a}	78.57± 1.023^{a}
APAP & alcohol	53.87±0.843^{b}	60.25±0.987^{b}	10.68±0.78^{b}	8.75±1.02^{b}	52.23±0.501^{b}	51.56± 0.498^{b}
APAP & alcohol and WP100	40.76±0.710^{a}	38.62±1.402^{a}	29.89±1.02^{ac}	31.88±0.99^{a}	81.45±0.623^{c}	84.1±0.621^{c}
APAP & alcohol and WP 200	35.89±0.721^{c}	26.12±1.021^{c}	32.76±0.99^{c}	36.26±1.01^{c}	85.02±0.775^{d}	92.66±0.723^{d}
APAP & alcohol and α-LA100	37.01± 0.624	33.46±1.289^{ac}	35.53±0.43^{c}	38.42±0.76^{c}	83.75±1.056^{c}	85.13± 1.223^{c}
APAP & alcohol and α-LA 200	35.32±0.603^{d}	25.77±1.312^{d}	39.23±0.41^{d}	44.2±0.54^{d}	89.23±1.278^{d}	95.23±1.376^{d}
APAP & alcohol and β-LG 100	36.31±0.697^{c}	34.87±1.342ac	30.26±0.39^{c}	38.77±0.15^{c}	85.56±0.673^{c}	89.21±1.234^{c}
APAP & alcohol and β-LG 200	32.77±0.682^{c}	30.13±1.289^{c}	35.86±0.41^{d}	40.53±0.18^{d}	88.49±0.688^{d}	96.78±1.340^{d}

GSH, reduced glutathione; MDA, Malondialdehyde; APAP paracetamol ; WPI, whey protein isolate; α-LA, alphalactalbumin; β-LG, beta-lactoglobulin.* Within each column, means with different superscript letters are significantly different (P<0.05).

Histopathological studies:







(Fig.1.A). A photomicrograph of the control liver of rat with central vein (CV) and surrounding hepatocytes (H), sinusoids (S) and nucleus (N). **(B)** Rat administration APAP and alcohol showing disarrangement of normal hepatic cells disruption of normal architecture of hepatic lobules and deposited collagen fiber in the form of very thick fibrous septa containing numerous fibroblasts in between hepatic lobules, around central veins as well as portal tracts (arrow head). Central veins were congested (long arrow), degenerated hepatocytes (D) with, and deeply stained nuclei. (**C)** Rat administration (WPI) at dose (100 mg/kg) and APAP and alcohol showing noticeable improvement, dilatation sinusoid (S) and activated of Kuppfer cells (K). Pyknotic cells (PK) and mitotic figure (M) were observed. (**D)** Rat administration (WPI) at dose (200 mg/kg) and APAP and alcohol showing mild improvement with signs of degeneration of hepatocytes is still present with pyknotic cells (PK). (**E)** Rat administration (α-LA) at dose (100 mg/kg) and APAP and alcohol showing improvement in the liver tissue with few pyknotic cells. (**F)** Rat administration (α-LA) at dose (200 mg/kg) and APAP and alcohol showing mild improvement in the liver tissue with some of degeneration of hepatocytes is still present with pyknotic cells (PK). Dilatation and heamorraghe in blood sinusoids was observed. (**G):** Rat administration (β-LG) -at dose (100 mg/kg) and APAP and alcohol showing mild improvement in the liver tissue with dilatation and heamorraghe in blood sinusoids, and activated of Kuppfer cells (K) Note binucleated hepatocytes (BN) and pyknotic cells (PK). (**H):** Rat administration (β-LG) -at dose (200 mg/kg) and APAP and alcohol showing normalization of liver tissue is observed, although there is degeneration of hepatocytes (long arrow) and activated of Kuppfer cells (K). Note binucleated hepatocytes (BN) and pyknotic cells (PK). (**H & E X 400)**).

DISCUSSION

The liver plays a central role in transforming and clearing chemicals and is susceptible to the toxicity from these agents. Certain medicinal agents when taken in overdoses and sometimes even when introduced within therapeutic ranges may induce hepatotoxicity *(Gomez-Moreno et*

al, 2008). Most instances of hepatotoxicity due to paracetamol are the result of the use of large overdoses of the drug. It was reported that paracetamol overdoses in chronic alcoholics indicate that these individuals are more susceptible to paracetamol-induced hepatotoxicity *(Cheung et al,1994).*Serious hepatotoxicity had involved the use of the therapeutic doses of paracetamol in association with chronic alcohol consumption *(Wootton et al, 1990; Tanaka et al, 2000).* Yet the effects of alcohol on paracetamol metabolism are complex. In contrast to chronic alcohol abuse, acute alcohol intoxication tends to inhibit hepatic microsomal drug metabolism. Thus acute alcohol intoxication might be expected to reduce the formation of toxic paracetamol metabolites. Animal studies indicate that acute ethanol administration tends to be protective the hepatotoxicity of large paracetamol doses. Finally, the bioavailability of paracetamol may be lower in alcoholic as compared to non-alcoholic because first pass metabolism is increased. This may partly explain the tendency of alcoholic to take large quantities of paracetamol for therapeutic purposes *(Seifert et al., 1993).*

The cytochrome P_{450} 2E1 (CYP2E1) is the major isoform which is responsible for generation of the toxic metabolites due to its ability to metabolize and activate numerous hepatotoxic substrates in the liver such as ethanol, carbon tetrachloride, acetaminophen, and N-nitroso dimethylamine, to more toxic products. Induction of CYP_{450} 2E1 by ethanol appears to be one of the central pathways by which ethanol generate a state of oxidative stress. In addition, oxidation of alcohol by CYP_{450} 2E1 produces acetaldehyde, a highly reactive compound that may contribute to the toxic effect of ethanol *(Neuman et al., 2001).*Alcohol and paracetamol ingestion leads to reduction of glutathione which is required to detoxify the reactive metabolites) in both rodents and humans as a consequence of depletion of NADPH in the cytosol *(Riordan and Williams, 2006).* At therapeutic doses paracetamol metabolized primarily in the liver to nontoxic metabolites via Phase II metabolism (conjugation) with glucuronide and sulphate, or cysteine *(Ward and Alexander-Williams, 1999)***.** A small amount of drug undergoes Phase I CYP_{450}-mediated N-hydroxylation to form N-acetyl- p-amino-benzoquinone immine (NAPQI), a toxic metabolite *(Bessems and Vermeulen, 2001).* The formation of reactive metabolites such as NAPQI is an important initiating factor for inducing liver injury. It can directly interact with macromolecules in the cell causing protein dysfunction, lipid peroxidation, damage of DNA, and oxidative stress *(Antoine et al., 2008).* Dysfunction of mitochondria may also result thereby in interrupting energy production and disrupting ionic gradients and intracellular calcium stores to result in cell death and liver damage *(Larson, 2007).* Consequently, it is important to find a method for ameliorating the toxic effects of this metabolite on the liver since consumption of alcohol and paracetamol is expected to induce hepatotoxicity. Food supplements are most likely to be effective in this respect.

Despite current advances in medical management, no definite therapy for liver failure exists, and this abnormality remains a major public health concern. Therefore, prevention of the disease and supporting the body against such disorders should strongly be taken into consideration. Some aspects of the hepatoprotective effects of whey protein on chronic liver injuries have previously been reported *(Kume et al., 2006)*; however, the mechanism of action of whey protein concentrate in ameliorating or preventing hepatocytes from injury and enhancing tissue regeneration is unclear.

In this study, whey protein products were assessed for their ability to improve the biochemical and histopathological features of hepatotoxicity induced by co-administration of alcohol and paracetamol in rats. Therefore, the current study was undertaken to evaluate the therapeutic effect of oral administration of whey protein isolate and its major protein fractions α-lactalbumin, β-lactoglobulin against hepatotoxicity induced by co-administration of paracetamol and alcohol for two and four weeks in rats.

The aminotransferases, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are enzymes that are located in the liver cells and leak out and make their way into the general circulation when the liver cells are injured. Therefore, their elevation may reflect liver injury *(Johnston, 1999).* The relevant results showed that serum liver enzymes (ALT and AST activity) were significantly elevated after oral co-administration of paracetamol (500mg/kg) and alcohol (5ml/kg) for two and four weeks. This indicates that a combination of paracetamol and alcohol induce liver injury. Our results are in accordance with Mladenovic et al (2013)**,** which showed that serum aminotransferase activity was significantly elevated after concomitant administration of paracetamol and alcohol.

It is well known that oxidative stress is a major contributor of ethanol –induced liver injury. Lipid peroxidation, as an important consequence of oxidative stress, was detected in various models of acute and chronic ethanol intoxication (*Elibol-Can et al., 2011).* Ethanol, when acutely administered, has synergistic effects with paracetamol related to lipid peroxidation and this can be one potential mechanism of aggravation of paracetamol-induced liver injury by ethanol Mladenovic et al (2013).

Since reactive oxygen species (ROS) are postulated to mediate hepatotoxic effects of ethanol and paracetamol, serum and liver MDA concentrations were significantly higher in all groups treated with paracetamol and ethanol in comparison with those of the control group. Based on these data, it could be suggested that lipid peroxidation may be an important consequence of oxidative stress in a combination of paracetamol and ethanol induced hepatotoxicity *(Anoush et al., 2009)*. The present results are in accordance with these data since a significant increase in

liver MDA levels were found after oral co-administration of alcohol and paracetamol for two and four weeks in rats.

Oral administration of whey protein was associated with decreased lipid peroxidation in rats. Lipid peroxidation leads to the generation of byproducts involved in the activation of inflammatory response and cellular damage, therefore this protective effect of whey protein on oxidative stress may also help to reduce inflammation *(Hamad et al.,2011).*

The present study which revealed that the hepatotoxicity induced by APAP and alcohol caused significant elevation in MDA levels more than the control and it was diminished by treatments with WP and its two components (α- lactalbumin and β-lactoglobulin) in two dose level (100 and 200 mg/kg).

Holt and Ju (2006) suggested that hepatocytes stress or death, as a result of the reactive metabolite induced damage, causes the release of signals that stimulate activation of the innate immune cells of the liver. Natural killer cells and neutrophils, are part of this response *(Liu et al., 2004)* and are recruited and activated. These cells produce pro-inflammatory cytokines and mediators such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and interferon (IFN)-γ *(Ishida et al., 2002).* Other mediators released by these immune cells are protective and anti-inflammatory such as IL-10 and IL-6 *(Masubuchi et al., 2003).* These results are coincides with our results which showed the significant increase in TNF-α after concomitant use of alcohol and paracetamol for two and four weeks.

Balbis et al.,(2009) suggested that WP is able to modulate the expression of the enzymes involved in GSH synthesis, in particular gamma glutamyl cysteine synthetase (gamma-GCS), which is believed to be the key enzyme in GSH synthesis *(Lu et al.,1999***);** some preliminary data seem to indicate that in basal conditions, the WP-fed rats showed moderately repressed expression of the heavy subunit (HS) of gamma-GCS (gGCS-HS) (−20%); this repression may be the result of the increased GSH level due to the high intake of cysteine; a new steady state equilibrium may be reached in WP-administered rats, where higher levels of cysteine and GSH are accompanied by slight reduced levels of gGCS-HS.

Our results revealed that hepatotoxicity induced by alcohol and paracetamol for two and four weeks significantly decreased SOD activity, GSH and TAC. These results are in accordance with various studies which suggested that GSH depletion plays a contributory role in paracetamol hepatotoxicity *(Bounous and Gold, 1991; Baruchel and Viau, 1996; James et al., 2003).*

Some reports investigated that whey protein had a potential hepatoprotective effects. These reports include Ahmad et al (2011) who reported that whey protein isolate prevented acute injury and improved liver function (serum AST, ALT) by inhibiting further tissue necrosis and

inflammatory cell infiltration due to dimethyl nitrosamine–induced hepatotoxicity and protected the hepatocytes from further degradation. Results of the present study are in accordance with the former reports as it demonstrated that administration of WPI, α-LA or β-LG after co-administration of alcohol and paracetamol for two and four weeks showed a remarkable improvement in liver enzymes AST and ALT activities as well as decreased serum TNF-α level. A significant improvement in both liver enzymes and inflammatory cytokine TNF-α was shown after treatment with α-LA or β-LG for four weeks as compared to APAP and alcohol and WPI.

Several activities of the antioxidants are important for inhibition of reactive oxygen species, which are generated during the oxidative stress. Thus, the usefulness of antioxidants in protecting cellular components against oxidative stress is well established *(Mohan et al., 2006).*

Previous reports indicated that WP has a potential antioxidant activity due to its ability to increase glutathione levels *(Peng et al., 2009; Bayram et al., 2008).* WP is well known to be rich in cysteine, α-lactalbumin, β-lactoglobulin, and bovine serum albumin *(Morr and Ha, 1993).* Cysteine is an amino acid regulates the in vivo concentrations of GSH and supplementation of the diet with whey protein high cysteine may promote GSH biosynthesis *(Kent et al., 2003)*. GSH was reported to be an antioxidant and anticarcinogenic tripeptide, and thereby improving protection against oxidant-induced cell damage *(Peng et al., 2009*). The increased TAC production reported herein in rats treated with WP accompanied with the decrease level of LP supported the earlier findings of Watanabe et al. (2000). The results of the current study and others suggested that cysteine content is responsible, in part, for the observed increase TAC via the increase in GSH. According to Bounous (2000), the suggested mechanism by which WPC induced its protection has been attributed to the increase in blood and tissue GSH concentration, which in turn increased the scavenger of the free radicals produced by APAP and alcohol.

Whey protein, α-Lactalbumin and β-Lactoglobulin exhibited significant anti-inflammatory and antioxidants effects by increasing hepatic SOD and GSH α-lactalbumin was more effective in oxidative stress inhibition and free radical scavenging than β-lactoglobulin and Whey protein, while β-Lactoglobulin depicted significant anti-inflammatory and antioxidants effects (*Hesham et al.,2012).*It has been reported that α-LA inhibits the formation of IL-6, which may contribute to its anti-inflammatory effects *(Yamaguchi et al., 2009).*

Results of the present study indicate that WP isolate, α- lactalbumin and β-lactoglobulin are able to decrease serum ALT and AST activity hepatic MDA and TNF(α) Level as compared to APAP and alcohol group in two dose level (100 and 200 mg/kg) after two and four weeks.

Our result revealed that WP isolate, α- lactalbumin and β-lactoglobulin exhibited significant increase hepatic GSH and SOD .

Alfa-Lactalbumin was the most effective WP product to increase hepatic SOD activity than β-lactoglobulin and WPI in a dose (200 mg/kg) after four weeks, while WPI showed a significant decrease in TNF (α) than α- lactalbumin and β-lactoglobulin in a dose (200 mg/kg) after four weeks.

Alfa-Lactalbumin and β-lactoglobulin in a dose (200 mg/kg) after four weeks depicted significant elevation in serum TAC than WPI in two dose level (100 and 200 mg/kg) after two and four weeks. However, despite the potent effects of WPI, α-LA and β-LG on APAP and alcohol-induced oxidative stress and the hepatic improvement they afford, the possibility of their clinical application still needs further investigation.

The results of the present study demonstrated that WPI, α-LA and β-LG provided possible treatment against APAP and alcohol -induced oxidative stress contributes to promote inflammatory reactions and liver injury even in the absence of endotoxin-mediated Kupffer cell activation. Thus, it appears that oxidative stress not only may exacerbate some aspects of ethanol-induced injury, but potentially by promoting an immune response, has a role in maintaining chronic inflammation that is important for the development of fibrosis.

Administration of WPI, α-LA and β-LG markedly counteracted the histological alterations induced by APAP and alcohol. This can be attributed to their antioxidant and chelating activities, which significantly reduced the oxidative threat leading to reduction of pathological changes and restoration of normal physiological functions. In two weeks treatment WPI showed relatively mild improvement in liver histology compared to α-LA or β-LG which improved hepatic architecture significantly compared to APAP and alcohol group. Whereas in the four-week experiment, hepatic tissue of APAP and alcohol group after four weeks showed disarrangement of normal hepatic cells with hepatic necrosis, vacuolated and fatty degenerated hepatocytes with increase eosinophility of hepatocytes. Further, inflammatory cell infiltrations were observed around the portal areas and in blood sinusoids, with thin fibrous tissue from the portal tract. Treatment with WPI showed moderate improvement in liver histology of toxic rats, while α-LA or β-LG showed almost normal appearance, the effect that was enhanced at high dose level.

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