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Protective Effects of Vitamin-P and Vitamin-C on Hypercholesterolemia -induced Oxidative Hepatic Damage and Lipid Profile Changes in Female Rats: A Comparative Study

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ABSTRACT

The current study aims to compare the oxidative protective effects of vitamin-P and vitamin-C on hypercholesterolemia-induced hepatic damage by high cholesterol diet (HCD) in female Wistar rats. Rats received experimental prepared HCD with or without vitamin-P or C for six consecutive weeks. In plasma, levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose (GLU), albumin (ALB), alkaline phosphatase (ALP), triglycerides (TG), total cholesterol (TC), high density lipoprotein (HCD) and low density lipoprotein (LDL) were determined. Levels of lipid peroxidation product, malondialdehyde (MDA), and endogenous antioxidant, reduced glutathione (GSH), as well as TC and TG were also estimated in liver. Finally, histopathological changes were assessed in hepatic tissue. HCD significantly elevated liver enzymes and lipid profile in plasma. Supplementation of vitamin-C significantly normalized this elevation more than vitamin-P. Moreover, liver concentrations of MDA, TC and TG were increased, while GSH levels were decreased by HCD. Vitamin-C showed greater ability to attenuate HCD-induced impairments in hepatic MDA, GSH, TC and TG concentrations than vitamin-P. Both vitamins protected liver tissues against HCD-induced hepatotoxicity as confirmed by the histopathological screening. In conclusion, although both vitamins demonstrated ameliorative effects against HCD-induced oxidative injury, vitamin-C had a greater protective value than vitamin-P.

INTRODUCTION

Fatty liver is a well known metabolic disease. It is usually associated with other diseases such as obesity, diabetes, and metabolic syndrome (Trauner *et al.*, 2010). Studies has indicated that individuals suffering from fatty liver represent 34% of the general population and over 75% of obese and extremely obese (Browning & Horton, 2004). Feeding of the experimental rodents with high cholesterol diet (HCD) is reported to cause hypercholesterolemia and deposit cholesterol in liver (Lee *et al.*, 2007). Oxidative stress and production of free radicals such as reactive oxygen species (ROS) have been implicated in the pathophysiology of various disease including; heart failure (Prasad *et al.*, 1996), ischemic heart disease (Ferrari *et al.*, 1998), hepatic injury (Jarrar *et al.*, 2000), and chronic renal damage and failure (Baker *et al.*, 1985; Galle, 2001).

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Similarly, lipid peroxidation and oxidative stress are one of the most important pathological mechanisms that explain metabolic changes and hepatic injury following HCD feeding (Kojima *et al.*, 2007). On the other hand, studies demonstrated that the endogenous antioxidant defense system can be regulated by sexual steroidal hormones (Pajovic & Saicic, 2008).

The female sex hormone, estrogen, was found to cause oxidative stress after interaction with its receptor (Mobley & Brueggemeier, 2002; Kobiela *et al.*, 2007). Interestingly, induction of hypercholesterolemia was found to be more easy in female rodents (Radzki *et al.*, 2009). In addition, metabolic changes following HCD was found to be more prominent in female than male rats (Terpstra *et al.*, 1982). Natural products are now an important source of novel agents for management of many metabolic diseases. One of the most abundant natural antioxidants in plants and the human diet are flavonoids. Numerous favorable pharmacological properties including antioxidant, antitumor, and anti-inflammation (Chen *et al.*, 2000; Chen *et al.*, 2001; Chen *et al.*, 2002) have been reported for naturally occurring flavonoids.

They can protect critical macromolecules, such as chromosomal DNA, structural proteins and enzymes and membrane lipids, from oxidative injury resulting from ROS exposure (Rice-Evans *et al.*, 1996; Dreosti, 2000). Vitamin-P, a quercetin-3-rutinosid or rutin, is one of the flavonoidal glycosides with a strong antioxidant activity against lipid peroxidation and ROS (Lopez-Revuelta *et al.*, 2006). Vitamin-P can be extracted from onions, apples, tea and red wine (Hertog *et al.*, 1993). Several pharmacological activities were associated with vitamin-P including antibacterial (Arima *et al.*, 2002), antitumor (Molnar *et al.*, 1981), anti-inflammatory (Selloum *et al.*, 2003), anti-diarrheal (Tamura *et al.*, 2007), antiulcer (La Casa *et al.*, 2000), anti-mutagenic (Ekram, 2006), vasodilator (Zhou *et al.*, 2006), immunomodulatory (Zhao *et al.*, 2007) and anti-diabetic (Kamalakkannan & Prince, 2006). Vitamin-C or ascorbic acid is a well known, powerful and water-soluble antioxidant. It is endogenously present in the cellular cytosol. Vitamin-C antioxidant activity is through donation of electron to the harmful free radicals generated during the oxidative stress process. Ascorbic acid is the most predominant form of vitamin-C in the human body. It prevents oxidative injury in several organs via quenching the injurious free radicals and ROS produced in biological processes (Heaney *et al.*, 2008; Verrax & Calderon, 2008). Several beneficial properties for the body have been associated with flavonoids use. Thus, the current study was designed to compare the protective effects of the well known antioxidants, vitamin-P and C, against hypercholesterolemia-induced hepatic oxidative stress in female Wistar albino rats.

MATERIALS AND METHODS

Animals

Thirty six 3 weeks old female Wistar albino rats weighing 80-100 grams were provided by the Experimental Animal Care Center (King Saud University, Riyadh, Saudi Arabia). Animal's environment was maintained under controlled conditions of temperature (22±1ºC), humidity (50-55%), and light (12 h light/dark cycles). This study was conducted in agreement with the Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research, National Institute of Health (NIH Publications No. 80-23; 1996) and approved by the Ethical Guidelines of the Experimental Animal Care Center (College of Pharmacy, King Saud University, Riyadh, Saudi Arabia).

Experimental design and diet preparation

Animals were randomly divided in to six groups, six rats in each. All groups, except normal diet (ND) group, were fed on experimental diet prepared for each group in pellet form respectively by adding 0.2% vitamin-P (P), 0.4% vitamin-C (C), 1% cholesterol+0.5% cholic acid (HCD), 0.2% vitamin-P+1% cholesterol+0.5% cholic acid (HCD+P) or 0.4% vitamin-C+1% cholesterol+0.5% cholic acid (HCD+C) in rat chow powder. The Experimental diets were prepared weekly and shade dried. All animals were kept on free access to food and water during the whole experimental period. At end of the 6th feeding week, rats were sacrificed and the trunk blood was collected in heparinized tubes. Blood samples were centrifugated at 4000 rpm for 15 min to collect plasma. Plasma samples were stored in freezer at -20ºC till analysis. Liver tissues were dissected, weighed and immediately dipped in liquid nitrogen and then preserved at -75ºC (Ultra-low freezer, Environmental Equipment, Cincinnati, Ohio, USA) for biochemical analysis. One liver section form ND, HCD, HCD+P and HCD+C groups were kept in formaldehyde for histopathological investigation.

Blood chemistry

Levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose (GLU), albumin (ALB), alkaline phosphatase (ALP), triglycerides (TG), total cholesterol (TC), high density lipoprotein (HCD) and low density lipoprotein (LDL) were estimated in plasma using commercially available diagnostic kits (Human, Wiesbaden, Germany).

Estimation of lipid contents in liver

TC and TG concentrations were determined in liver tissues using Folch et al., (1957) method (Folch *et al.*, 1957). In brief, liver tissues were homogenized in 0.15 mol/L of ice-cold KCl (10% w/w) and lipids were extracted with chloroform: methanol (2:1). After the extraction and evaporation, tissue lipids were re-dissolved in isopropanol and estimated by commercially available TC and TG diagnostic kits (Human, Wiesbaden, Germany).

Assessment of hepatic oxidative stress

Hepatic concentrations of MDA and GSH were considered as sensitive markers for oxidative stress. A TBARS assay kit (ZeptoMetrix Corporation, Buffalo, New York, USA) was used to measure lipid peroxidation products, malondialdehyde (MDA) equivalents. Briefly, 100 µl of hepatic 10 % homogenate was mixed with 2.5 ml reaction buffer (provided by the kit) and heated at 95 °C for 60 min. After cooling, the supernatant's absorbance was recorded at 532 nm. On the other hand, GSH levels were measured by Sedlak and Lindsay (1968) described method (Sedlak & Lindsay, 1968). Aliquots of 0.5 mL of liver homogenate was mixed with 0.2 M Tris buffer, pH 8.2 and 0.1 mL of 0.01 M Ellman's reagent, [5,5'-dithiobis-(2-nitro-benzoic acid)] (DTNB). Sample tubes were then centrifuged at 3000 RPM at room temperature for 15 min. The absorbance of the clear supernatants was measured at 412 nm.

Histopathological evaluation

The cross-sections from each liver was fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned at 3 µm, stained with Hematoxylin and Eosin (H & E) stain and placed in slides for light microscopic examination. Hepatic degree of degeneration, necrosis, inflammation, regeneration and fibrosis were evaluated by a histopathologist who was blinded to the

treatment groups to avoid any kind of bias.

Statistical analysis

All data were expressed as mean ± Standard Deviation (SD) and statistically analyzed using one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls multiple comparisons test. The differences were considered statistically significant at P<0.05. Graph Pad prism program (version 5) was used as analyzing software.

RESULTS

There was a significant increase in plasma levels of AST (24.09%, P<0.01), ALT (29.24%, P<0.01), ALB (16.28%, P<0.05) and ALP (50.76%, P<0.001) in HCD fed rats compared to ND group (**Table 1**). Both vitamins-P and C were able to reduce (13.65% and 17.74%, respectively) the elevated AST level significantly (P<0.05). Also, plasma level of ALT was reduced significantly (P<0.05) after both vitamin-P and C feeding (13.15% and 16.37% and P<0.01, respectively). ALB levels was significantly (13.73%, P<0.05) reduced by only vitamin-C administration to rats, while neither vitamin-P nor C was able to reduce ALP elevated levels (**Table 1**). Plasma levels of TG, TC and LDL were significantly (P<0.001) increased (138.95%, 98.95% and 79.26%, respectively), while HDL levels were reduced (25.81%, P<0.001) in HCD group as compared to ND fed rats (**Table 2**). Supplementations of vitamin-P or C along with HCD to rats for six weeks resulted in a significant decrease in plasma levels of TG (44.85% and 57.76%, P<0.001 and P<0.001, respectively), TC (26.78% and 34.34%, P<0.01 and P<0.01, respectively) and LDL (34.14% and 38.31%, P<0.001 and P<0.001, respectively) as compared to HCD fed animals. In contrast, HDL plasma levels were significantly increased (37.19% and 44.66%, respectively) following 6 weeks of vitamin-P or C administration to HCD fed rats (P<0.01 and P<0.001, respectively) (**Table 2**). Feeding of HCD to female animals significantly (P<0.01) elevated liver weights (27.8%) compared to animals fed on ND. Both vitamin-P or C significantly (P<0.01) attenuated liver weights elevation (13.59% and 18.41%, respectively) as compared to HCD group (**Figure 1**). TC and TG hepatic concentrations were significantly (P<0.001) increased in HCD group (237.3% and 59.89%, respectively) as compared to ND group. Feeding of animals with vitamin-P or C significantly (P<0.001) inhibited the elevated levels of hepatic TC (43.04% and 47.79%, respectively) and TG (35.01% and 37.01%, respectively) (**Figure 2**). HCD supplementation significantly (P<0.001) caused an increase in liver MDA levels (17.45%) and a decrease GSH levels (19.4%) as compared to ND group. Vitamin-P or C supplementation with HCD significantly (P<0.001) attenuated MDA levels increase in liver tissues (10.62% and 13.38%, respectively). However, only vitamin-C was able to significantly (P<0.05) increase the HCD-induced decrease in GSH hepatic levels (10.65%) (**Figure 3**). Histopathological investigation results are presented in **figure 4 and table 3**. Sections from ND group revealed normal looking hepatocytes, while liver sections from HCD group showed scattered foci of steatohepatosis of hepatocytes with swollen epithelial cells associated with scattered foci of periportal to lobular inflammatory cell infiltrates. Liver sections from both vitamins treated groups to HCD fed rats showed mild looking hepatocytes separated by congested central veins. The degree of hepatic degeneration, inflammation, regeneration and fibrosis were lower in HCD+P and HCD+C groups than HCD group.

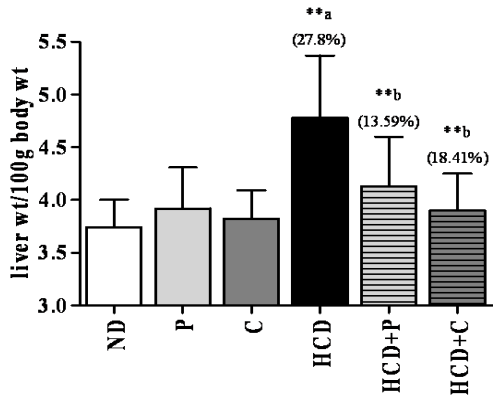

Fig. 1: Effects of vitamin P or C on liver weight per 100 gram body weight in HCD fed rats following 6 weeks of supplementation (n=6). Data were expressed as Mean±S.D and analyzed using one-way ANOVA followed by Student Newman-Keuls method as post hoc test. [(a)] ND group was compared with HCD group, [(b)] HCD group was compared with HCD+P or HCD+C. Significance was considered at [*]P<0.05, [**]P<0.01 and [***]P<0.001. Percentage of increase or decrease was indicated between brackets.

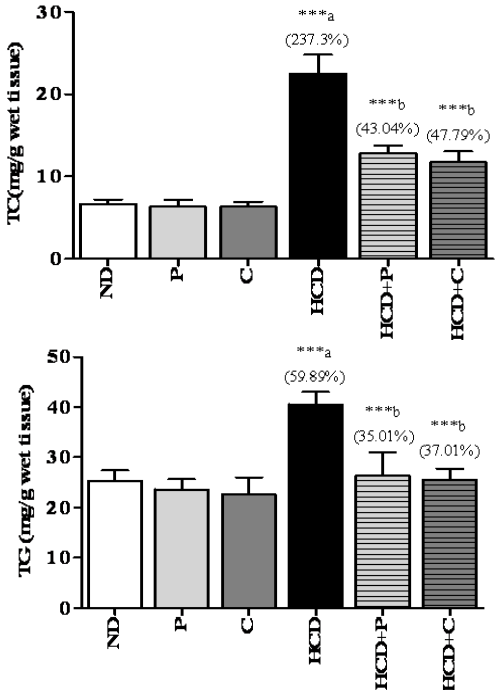

Fig. 2: Effects of vitamin P or C on hepatic TC and TG concentrations in HCD fed rats following 6 weeks of supplementation (n=6). Data were expressed as Mean±S.D and analyzed using one-way ANOVA followed by Student Newman-Keuls method as post hoc test. [(a)] ND group was compared with HCD group, [(b)] HCD group was compared with HCD+P or HCD+C. Significance was considered at [*]P<0.05, [**]P<0.01 and [***]P<0.001. Percentage of increase or decrease was indicated between brackets.

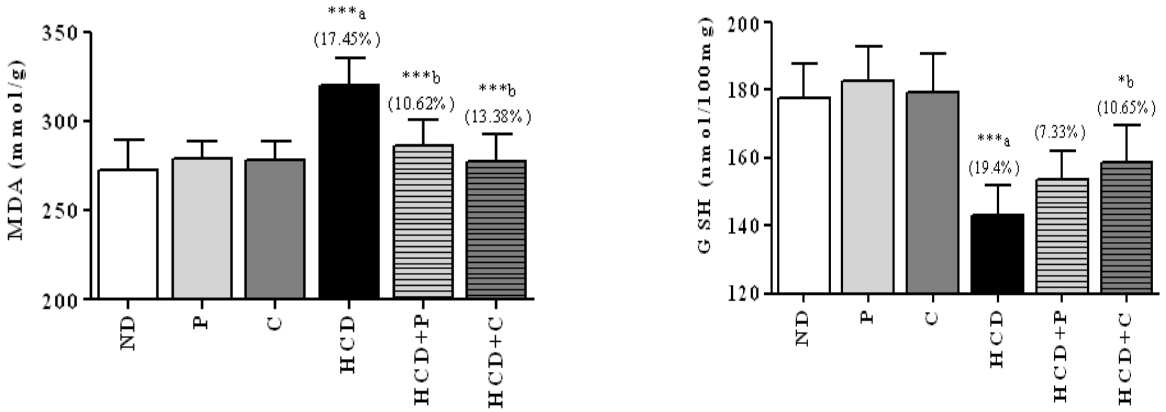

Fig. 3: Effects of vitamin P or C on hepatic concentrations of MDA and GSH in HCD fed rats following 6 weeks of supplementation (n=6). Data were expressed as Mean±S.D and analyzed using one-way ANOVA followed by Student Newman-Keuls method as post hoc test. [(a)] ND group was compared with HCD group, [(b)] HCD group was compared with HCD+P or HCD+C. Significance was considered at $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. Percentage of increase or decrease was indicated between brackets.

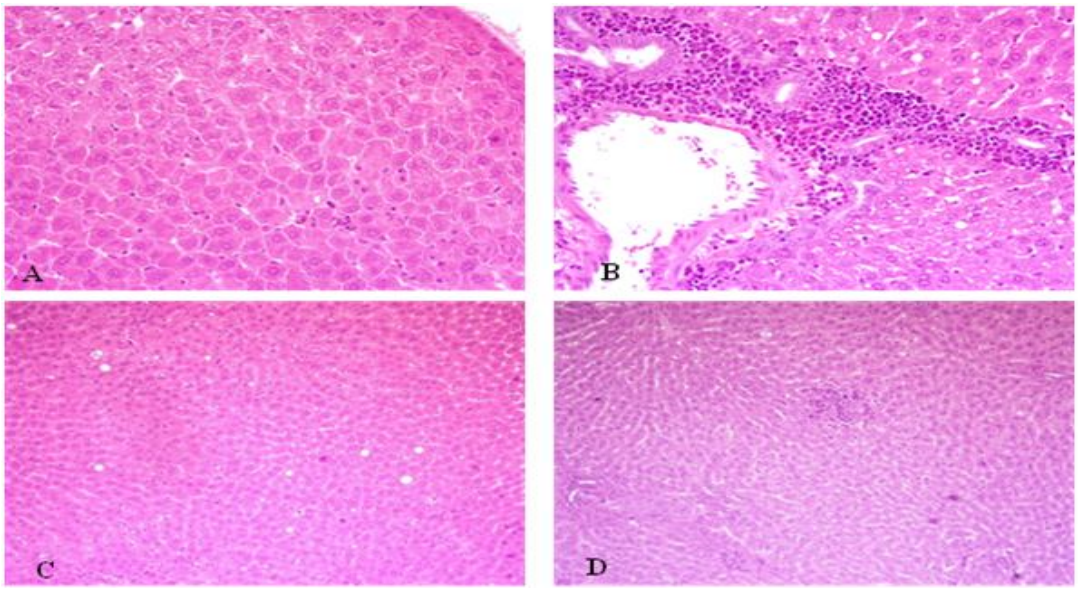

Figure 4: Histopathological sections from rats' liver showing: normal looking hepatocytes without hepatotoxicity in ND group [A]; scattered foci of steatohepatosis and swollen epithelial cells with moderate degree of hepatotoxicity associated with scattered foci of periportal to lobular inflammatory cell infiltrates in HCD group [B]; liver sections with mild degree of hepatotoxicity in HCD+P and HCD+C groups [C & D, respectively].

Table. 1: Effects of vitamin P or C on plasma level of AST, ALT, GLU, ALB and ALP in HCD fed rats following 6 weeks of supplementation (n=6).

	AST (U/L)	ALT (U/L)	GLU (mg/dl)	ALB (mg/L)	ALP (U/L)
ND	39.96±3.36	20.65±1.59	173.93±13.62	48.38±3.64	270.65±34.85
P	37.24±7.24	21.66±2.20	172.58±12.26	54.23±4.70	274.78±36.11
C	38.20±4.84	21.04±3.09	165.71±23.16	50.37±3.54	283.51±39.62
HCD	49.59±4.41^{**a}(24.09%)	26.69±3.29^{**a}(29.24%)	196.06±20.94(12.72%)	56.26±5.64^{*a}(16.28%)	408.04±48.70^{***a}(50.76%)
HCD+P	42.82±4.82^{*b}(13.65%)	23.18±2.06^{*b}(13.15%)	181.81±15.87(7.26%)	56.75±4.12(0.87%)	362.09±65.68(11.26%)
HCD+C	40.79±3.54^{*b}(17.74%)	22.32±1.31^{**b}(16.37%)	173.20±15.67(11.65%)	48.53±3.49^{*b}(13.73%)	356.11±41.35(12.72%)

Data were expressed as Mean±S.D and analyzed using one-way ANOVA followed by Student Newman-Keuls method as post hoc test. [(a)] ND group was compared with HCD group, [(b)] HCD group was compared with HCD+P or HCD+C. Significance was considered at $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. Percentage of increase or decrease was indicated between brackets.

Table. 2: Effects of vitamin P or C on plasma level of TG, TC, HDL and LDL in HCD fed rats following 6 weeks of supplementation (n=6).

	TG (mg/dl)	TC (mg/dl)	HDL(mg/dl)	LDL (mg/dl)
ND	65.62±4.86	70.06±22.08	27.43±4.95	48.32±4.24
P	69.06±5.53	74.67±9.97	28.36±2.64	46.04±5.03
C	61.58±11.58	60.48±18.93	30.67±2.75	43.59±3.55
HCD	156.80±24.59^{***a}(138.95%)	139.39±24.61^{***a}(98.95%)	20.35±1.89^{***a}(25.81%)	86.62±6.88^{***a}(79.26%)
HCD+P	86.46±12.87^{***b}(44.85%)	102.06±12.28^{**b}(26.78%)	27.92±2.88^{**b}(37.19%)	57.04±4.90^{***b}(34.14%)
HCD+C	66.23±7.78^{***b}(57.76%)	91.52±29.45^{**b}(34.34%)	29.44±3.70^{***b}(44.66%)	53.43±6.41^{***b}(38.31%)

Data were expressed as Mean±S.D and analyzed using one-way ANOVA followed by Student Newman-Keuls method as post hoc test. [(a)] ND group was compared with HCD group, [(b)] HCD group was compared with HCD+P or HCD+C. Significance was considered at $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$. Percentage of increase or decrease was indicated between brackets.

Table. 3: Histopathological scoring of degeneration, necrosis, inflammation, regeneration and fibrosis as degree of hepatotoxicity after HCD, HCD+P and HCD+C treatments to female Wistar rats.

	Degeneration	Necrosis	Inflammation	Regeneration	Fibrosis
ND	-	-	-	-	-
HCD	++	+	++	+	++
HCD+P	+	+	+	-	+
HCD+C	+	+	+	-	+

DISCUSSION

Studies indicated that the antioxidant defense system may be regulated by sex hormones (Azevedo *et al.*, 2001; Tam *et al.*, 2003). Female rodents were reported to be more susceptible to hypercholesterolemia and hepatic oxidative damage than males (Radzki *et al.*, 2009). Furthermore, estrogen level was found to be induced by high dietary fats intake making female more prone to HCD induced oxidative injury as estrogen itself was reported interact with its receptor in an oxidative stress-dependant manner (Mobley & Brueggemeier, 2002; Kobiela *et al.*, 2007; Amin *et al.*, 2011). Limited number of studies have examined the biochemical and oxidative changes following HCD on liver of female animals. Therefore, this study was designed to compare the protective effects of vitamin-P and C on hypercholesterolemia-induced hepatic oxidative injury in female Wistar albino rats. Feeding of the animals with HCD for six consecutive weeks resulted in hypercholesterolemia, fatty liver and oxidative stress. Supplementation of both antioxidant vitamins to HCD fed rats significantly ameliorated HCD-induced hepatotoxicity as confirmed by the histopathological investigation and the decrease in liver enzymes. Moreover, vitamin-P and C significantly inhibited hepatic lipids accumulation and oxidative stress.

HCD was reported in several studies to cause hepatotoxicity and fatty liver (Park *et al.*, 2002; Hirako *et al.*, 2011; Wang *et al.*, 2011). Similarly in the current study, HCD significantly induced elevation in plasma liver enzymes (ALT and AST) as well as ALB and ALP. These parameters are known to be markers for hepatotoxicity. Moreover, histopathological screening in the current study revealed several impairments in liver sections from HCD supplemented rats by showing moderate degree of fat accumulation, degeneration, fibrosis and inflammatory infiltrates. Vitamin-P has been demonstrated to exhibit numerous pharmacological activities including anti-inflammatory, vasoactive and membrane lipid peroxidation inhibitory properties (Lindahl & Tagesson, 1997; Park *et al.*, 2002; Lopez-Revuelta *et al.*, 2006). Also, vitamin-C (ascorbic acid) is well recognized to prevent various diseases including allergic rhinitis (Thornhill & Kelly, 2000), diabetes (Anderson *et al.*, 2006), heart disease (Ling *et al.*, 2002) and cancer (Enwonwu & Meeks, 1995). In the present study, supplementation of vitamin-P and C to HCD fed rats significantly prevented induction of hepatotoxicity and liver injury with vitamin-C showing more protection as it decreased the elevated levels of ALT and AST with a higher extent than vitamin-P. Histopathological screening also revealed that the degree of HCD-induced hepatic degeneration, inflammation, regeneration and fibrosis were equally lower in both vitamins treated groups compared to HCD group. The reported hepatoprotective effects of both vitamins are in accordance with other investigations in different animal models (Janbaz *et al.*, 2002; Rana *et al.*, 2010; Shenbagam & Nalini, 2011; Abhilash *et al.*, 2012).

In the present work, HCD supplementation for six weeks significantly elevated liver/body weights ratio as compared to control animals, which are in accordance with the results of other investigations (Hahn-Obercyger *et al.*, 2009; Yiu *et al.*, 2011). One possible explanation of this increment is the accumulation of hepatic lipids after HCD supplementation, which was also reported in the current study. Similarly, HCD was shown to increase lipid profile in both liver and plasma (Balkan *et al.*, 2002). These findings are in accordance with our results where hepatic cells concentrations of TG and TC as well as plasma level of TG, TC and LDL were elevated, while HDL level was decreased following six weeks of HCD. Several studies suggested multiple pharmacological actions for both vitamin-P and C including lipid lowering properties (Ziaee *et al.*, 2009; Devbhuti *et al.*, 2011). These properties were also reported for both vitamins in our work. However, vitamin-C demonstrated a stronger ability to reduce animals lipid profile than vitamin-P. The HCD-induced elevation in liver TG and TC as well as plasma TG, TC and LDL level and decreased in HDL were all significantly ameliorated by both vitamin-P and C, which explain the ability of both vitamins to inhibit the elevation in liver/body weight ration following HCD for six weeks.

Endogenous non-enzymatic defense system against oxidative stress is including the sulphadryl containing peptide namely GSH. It is widely distributed in all biological tissues. GSH inhibits ROS oxidative injuries directly via its sulfhydryl group and indirectly as a cofactor or a coenzyme in ROS enzymatic detoxification process (Morise *et al.*, 2004). Furthermore, ROS can harmfully impair lipid contents in cell membrane leading to lipid peroxidation process. Measurements of the endogenous antioxidants as well as lipid peroxidation products, as in case of the current study, have been widely used to assess the degree cellular oxidative damage. Feeding of experimental animals with HCD was reported to case oxidative stress and to induce ROS generation in different biological tissues including liver, brain, kidney and erythrocytes (Park *et al.*, 2002; Montilla *et al.*, 2006). Furthermore, studies showed HCD to alter cellular membranes lipids making the extracellular matrix to be more prone to free radical induced damage (Scheuer *et al.*, 2000). Our study showed similar results, where HCD feeding to female rats for six weeks significantly increased lipid peroxidation products (MDA) and decrease the endogenous antioxidant (GSH) in liver tissues. Both vitamin-P and C are well known for their strong ability to prevent oxidative damage and to scavenge free radicals such as ROS effectively produced through biological processes in many extracellular and intracellular reactions (Hanasaki *et al.*, 1994; Mahmoud, 2011; Ozkaya *et al.*, 2011). In the present study, HCD-induced hepatic oxidative injury was significantly ameliorated by combining vitamin-P or C with HCD. As indicated in our study, both vitamin-P and C can significantly reduce the elevated hepatic

levels of lipid peroxidation biomarker, MDA. However, only vitamin-C was capable of increasing the reduced GSH levels in liver. Although, vitamin-P is a well known antioxidant, this property may not be through induction of endogenous antioxidants in HCD animals models like in case of vitamin-C. This may be because of the widely recognized cytoprotective effects of vitamin-C (Negre-Salvayre *et al.*, 1995; Passoni & Coelho, 2008) and its ability to decrease free radicals production (Taha *et al.*, 2004). Vitamin-P is one of the naturally occurring phenolic flavonoids, which are characterized by their cytoprotective ability (Negre-Salvayre *et al.*, 1995). However, the cytoprotective effects of hydrophobic molecules like vitamin-P were suggested to be through interacting more powerfully with lipids in membranes resulting in more ability to protect cell membranes (Zhang *et al.*, 2006).

CONCLUSION

In conclusion, co-administration of vitamin-C with HCD showed more protective effects than vitamin-P against hypercholesterolemia-induced hepatic toxicity in female rats. These protective effects are suggested to be mainly through (1) the ability of both vitamins to inhibit lipid peroxidation process and (2) induction of endogenous antioxidants, in case of vitamin-C.

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