Ameliorative effect of vitamin C against 5-fluorouracil-induced hepatotoxicity in mice: A light and electron microscope study

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Abstract 5-Fluorouracil is one of the most widely used chemotherapeutic agents in case of hepatic neoplasms. The object of this study was to determine the effectiveness of vitamin C in alleviating 5-fluorouracil-induced hepatotoxicity in male mice. Thirty male albino mice were divided equally into 3 groups, each of 10 animals; group 1, mice received normal saline solution (control group); group 2, mice received 5-fluorouracil 80 mg/kg b.wt./day intraperitoneally for 4 weeks (5-fluorouracil group); group 3, mice received 5-fluorouracil 80 mg/kg b.wt./day for 4 weeks with daily injection of vitamin C (12 mg/kg b.wt./day) for 4 weeks. Animals of all groups were sacrificed and tissue samples from the liver were taken and processed for both light and electron microscopical examination. Light microscopic observations revealed that administration of 5-fluorouracil causes variable signs of hepatotoxicity which are represented by focal areas of liver cell necrosis with distortion of normal hepatic architecture; the hepatocytes showed vacuolated cytoplasm and pyknotic nuclei together with inflammatory cell infiltration. Dilated, congested hepatic sinusoids with active kupffer cells were also seen. Ultrastructure examination confirmed the light microscopic findings and demonstrated vacuolated hepatocytes cytoplasm, dilated endoplasmic reticulum, increased lysosomes, electron-dense mitochondria and pyknotic nuclei. Treatment with vitamin C with 5-fluorouracil attenuated 5-fluorouracil-induced hepatotoxicity and reverted the abnormal structural changes to near normal. In conclusion, these results suggest that vitamin C has a protective potential in ameliorating 5-fluorouracil-induced hepatotoxicity.

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Introduction

Chemotherapy has been one of the major therapeutic modalities commonly used for the treatment of a variety of cancer patients (Borek, 2004). However, while they generate acceptable outcome in the treatment of some cancer, they also induces severe toxicity and undesirable side effect (Minami et al., 2010). 5-FU is an antimetabolite that acts during S phase of the cell cycle. 5-FU is activated by thymidine phosphorylase into fluorodeoxyuridine that inhibits thymidylate synthase, thus preventing DNA synthesis; that leads to imbalanced cell growth and ultimately cell death (Shoemaker et al., 2004). 5-FU is also converted to 5-fluorouridine monophosphate (5-FUMP) and can be incorporated into RNA and interfered with RNA processing and function. 5-FU is eliminated by liver metabolism, and only a small percentage undergoes renal excretion. Dihydropyrimidine dehydrogenase (DPD) is the initial and rate-limiting enzyme in 5-FU catabolism. DPD is found in the liver, therefore it is hypothesised that no reduction in 5-FU dose needs to be made for patients with hepatic or renal dysfunction (Tateishi et al., 1999). The common clinical side effects of 5-FU include myelosuppression, diarrhea, vomiting and mucositis. Extensive investigations have been conducted on the hepatotoxicity of this anticancer drug. There is very limited information concerned with the effects of 5-FU on the histology and ultrastructure of the liver cells. Further, a variety of agents including antioxidants have been shown to attenuate the hepatotoxicity of 5-FU. Like other chemotherapeutics, 5-FU causes excessive reactive oxygen species (ROS) generation and also induces a decrease in plasma antioxidant levels, which may reflect a failure of antioxidant defence mechanism against oxidative damage. Much attention has been given to the possible role of antioxidants in protecting liver against chemotherapy-induced toxicity (Behling et al., 2006). The aim of the present work is to evaluate the protective role of vitamin C against toxicity induced by 5-FU in the liver of mice using histological and ultrastructural methods.

Material and methods

Chemicals

5-FU was denoted from Ebewe Pharma Ges.m.b.H.NFg. KG, A-4866 Unterach, Austria.

Vitamin C was purchased from Memphis Co. For Pharm. & Chemical Ind., Cairo, Egypt.

Experimental animals

Thirty adult male albino mice, each weighing 25 g were obtained from Teodor Bilharz Research Institute Imbaba, Giza. The animals were housed in cages and fed ad libitum with a standard diet and provided with free access to water, being kept under suitable laboratory conditions during the whole period for experimentation. The animals were allocated into 3 groups, each of 10 animals.
The hepatocytes are polyhedral in shape with acidophilic granulated cytoplasm and rounded centrally located nuclei. Blood sinusoids are lined with endothelial and Kupffer cells (Figs. 1 and 2).

H&E-stained sections of the liver of mouse treated with 5-FU (80 mg/kg b.wt.) daily for four weeks (group 2) showed loss of normal hepatocytes architecture, congested branches of portal vein, destruction of the bile ductules epithelium with inflammatory cell infiltration in the portal areas, some dilated portal veins showed eroded epithelial lining cells (Figs. 6 and 8). The hepatic sinusoids appeared congested, dilated with hyperplasia of Kupffer cells (Figs. 4–7). Some hepatocytes showed swollen cytoplasm with feathery degeneration, while others showed marked vacuolation and fatty degeneration (microvesicular steatosis) (Figs. 3–5 and 8). The nuclear injury observed in the present work was characterised by the presence of pleomorphic nuclei, where some nuclei appeared swollen and large, other nuclei appeared small (Figs. 4 and 7). In addition, centric nucleoli, triozemy and mitotic figure were evident among some hepatocytes (Fig. 5). However, some hepatocytes revealed necrotic changes including pyknotic nuclei (Figs. 3, 4 and 8), other hepatocytes revealed karyolytic nuclei (Fig. 6).

H&E stained sections of the 5-FU + vitamin C group (group 3) revealed manifestations of mild hepatic damage (Figs. 10–13). Preserved radially arranged hepatocytes around central vein were noticed (Fig. 9). The hepatocytes cytoplasm revealed regression in fatty changes with minimal vacuoles (Figs. 10–13). Scattered apoptotic cells were noticed (Figs. 12 and 13). Focal areas of necrosis and infiltration of mononuclear inflammatory cells were also seen (Figs. 11–13). Slight congestion and dilatation of the hepatic sinusoids with numerous Kupffer cells were also observed (Figs. 11–13).

Electron microscopic structure of the liver

Transmission electron microscopic examination of the liver of the control mice revealed that each hepatocyte exhibits a rounded large central nucleus surrounded by an envelope of 2 membranes interrupted by nuclear pores; the nucleus possessed light euchromatin and dark heterochromatin. The
The cytoplasm of the hepatocytes contains numerous mitochondria, lysosomes, rough endoplasmic reticulum and glycogen granules (Fig. 14).

Examination of the hepatocytes of animals of group 2 showed ultrastructural alterations. The nuclei of some hepatocytes showed pyknosis (Figs. 15, 17 and 20), other nuclei were round or ovoid with margined heterochromatin (Figs. 15–20). Moreover, hepatocytes cytoplasm manifested fragmented and vesiculated rough endoplasmic reticulum, proliferated smooth endoplasmic reticulum, lipid droplets and electron-dense mitochondria; some mitochondria appeared swollen with degenerated cristae (Figs. 15–20). Dilated hepatic sinusoids with active Kupffer cells and loss or destruction of hepatocytes microvilli projecting into space of Disse were also seen (Figs. 15, 16, 18 and 19). Besides, dilated bile canaliculi with loss of hepatocytes microvilli were also noted (Fig. 20).

Moreover, transmission electron microscopic examination of liver of group 3 revealed partial improvements. The hepatocytes almost restored their normal appearance and structure, the nuclei appeared oval or vesicular with normal chromatin distribution and prominent nucleoli (Figs. 21–23). The hepatocytes cytoplasm contained numerous mitochondria; some mitochondria still showed electron-dense matrix with variable shapes and sizes, rough endoplasmic reticulum and glycogen almost retained their normal appearance (Figs. 21–24). The hepatocyte cytoplasm exhibited few vacuoles as well as disappearance of the fat droplets (Figs. 21–24). In addition, some hepatic sinusoids seemed to be normal (Figs. 22 and 23).

Discussion

5-Fluorouracil (5-FU), a widely used chemotherapeutic agent, has proven efficacy in a variety of human malignancies. However, the clinical usefulness of 5-FU has been precluded because of its hepatotoxic and nephrotoxic side effects. (Skretkowicz et al., 1996; El-Sayyad et al., 2009).
In the present study, marked histological and ultrastructural alterations were observed in the liver of mice treated with 5-FU. The histological changes included marked disruption of hepatic cords, cytoplasmic lipid droplets (microvesicular steatosis), feathery degeneration, vacuolated hepatocytes cytoplasm and inflammatory cells infiltrating the hepatic tissues. Many hepatocytes’ nuclei showed features of deterioration including pleomorphic nuclei; hypertrophic nuclei, pyknotic nuclei and karyolytic nuclei. The present results agree with those obtained by Cetin et al. (2011) who reported severe degeneration and necrosis in hepatocytes, dilatation and congestion of sinusoids, increased number of Kupffer cells and inflammatory cell infiltration in portal area in rats treated with cisplatin. Similar observations have been reported in the liver of rats treated with 5-FU, cisplatin and doxorubicin El-Sayyad et al. (2009). Similar findings were reported by Zaky et al. (2006) who revealed pleomorphic nuclei in the hepatocytes of rats treated with methotrexate. The presence of fatty infiltrations represented patterns of hepatic injury as a result of exposure to 5-FU. Lipid accumulation in the hepatocytes is well known as a pathological change that occurs in the cells under a variety of deleterious conditions and could be due to an alteration in the metabolism of the hepatic cells (Wheater et al., 1990). The increase in fat was also explained by Mori (1983), who stated that increased lipid in the hepatocytes after exposure to drugs or toxins could be due to impaired synthesis of lipoproteins or due to the abnormal transport of lipoproteins via Golgi apparatus and its secretory vacuoles. These findings were in accordance with the experimental studies conducted by others (Abdelmeguid et al., 2010; El-Sayyad et al., 2009). These changes are mostly due to inflammation. Fatty changes were the landmark change in this work, this finding supported the previously reported findings by others (Koc et al., 2005; Ramachandran and Kakar, 2009; Abdelmeguid et al., 2010) where they described that following single toxic dose of cisplatin, the cytoplasm of most hepatocytes was light and filled with numerous vacuoles and lipid droplets.
and Wang (1984) suggested that, the cytoplasmic vacuolation is mainly a consequence to considerable disturbance in lipid and fat metabolism.

The intrahepatic veins, central and portal and the hepatic sinusoids were noticed in the present work to undergo marked injury indicated by dilatation of their lumina and aggregation of blood cells in some of them. The above mentioned observation is in agreement with that obtained by Sakr et al. (2011) who stated that adriamycin administration to rats resulted in dilatation and congestion in the hepatic sinusoids and in the intrahepatic veins. The present results also agree with those obtained by Klatskin and Ocean (1993) who suggested that dilatation of the blood sinusoids would be due to the direct toxic effect of the toxin on the blood sinusoids leading to their dilatation.
A marked hyperplasia of Kupffer cells and endothelial cells was observed at the light microscope level in the present work. This was in agreement with the result of Zaky et al. (2006) who reported dilatation and congestion of hepatic sinusoids with increased number of Kupffer cells in rat liver treated with methotrexate. Klatskin and Ocean (1993) reported that under a variety of conditions, the cell lining the sinusoids mostly Kupffer cells, proliferate. The proliferating Kupffer cells are almost enlarged and are often pigmented; this is due to phagocytosis of lipofuscin released from adjacent necrotic hepatocyte.

According to the present study, the hepatic tissues showed the presence of dense focal inflammatory cells or necrotic tissues. Similar findings were reported by Kallyana and Sangavai (2009) who reported inflammatory cell infiltration in the liver of rat administrated doxorubicin or 5-FU.

In the present work, ultrastructural findings in 5-FU-treated mice revealed polymorphic, atrophied, electron-dense mitochondria with ill-differentiated cristae, proliferation of smooth endoplasmic reticulum, vesiculation and fragmentation of the rough endoplasmic reticulum, increased lipid droplets, cytoplasmic vacuoles and loss or destruction of hepatocyte microvilli. The above mentioned observations were in agreement with those obtained by El-Sayyad et al. (2009) who reported that the cytoplasm of 5-FU treated rats contained vesiculated rough endoplasmic reticulum and atrophied mitochondria. In this concern, Kendry and Laszlo (1975) concluded that the alteration of the rough endoplasmic

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**Figure 17** An electron micrograph of liver section of mice treated with (80 mg/kg b.wt.) 5-flourouracil for 4 weeks showing hepatocytes and bile canaliculus (BC), the hepatocytes cytoplasm contain electron-dense, small size mitochondria, some mitochondria appeared swollen with degenerated cristae and loss of matrix (M1), other mitochondria appeared electron-dense (M), fragmented and vesiculated rough endoplasmic reticulum (RER), lipid droplets (LP), proliferated smooth endoplasmic reticulum (SER). One hepatocyte shows pyknotic nucleus (PN), other nucleus shows necrosis with peripheral nucleolus (NU). The bile canaliculus shows dilatation and loss of hepatocytes microvilli project into its lumen (BC) (×6000).

**Figure 18** An electron micrograph of liver section of mice treated with (80 mg/kg b.wt.) 5-flourouracil for 4 weeks showing hepatocytes and hepatic sinusoid, the hepatocyte cytoplasm contains electron-dense mitochondria (M) and dilated smooth endoplasmic reticulum (SER). The hepatic sinusoid showed dilitation with active Kupffer cell (KC) and destruction of hepatocyte microvilli projecting into space of Disse (arrow) (×4000).

**Figure 19** An electron micrograph of liver section of mice treated with (80 mg/kg b.wt.) 5-flourouracil for 4 weeks showing pyknotic hepatocyte nucleus (PN) with ill-defined nuclear membrane, other nucleus shows necrosis (N), hepatocyte cytoplasm shows proliferated smooth endoplasmic reticulum (SER), fragmented rough endoplasmic reticulum (RER), polymorphic electron-dense mitochondria (M), some mitochondria are swollen with loss of cristae and matrix (M1) (×4000).
The rough endoplasmic reticulum constitutes the main adverse effect of drug and toxins affecting the liver due to its important role in protein synthesis. On the other side, our results showed that glycogen rosettes were obscured within the cytoplasm of most hepatocytes and were completely absent in few cells.

**Figure 20** An electron micrograph of liver section of mice treated with (80 mg/kg b.wt.) 5-flourouracil for 4 weeks with daily injection of vitamin C (12 mg/kg b.wt./daily) for four weeks showing binucleated hepatocyte, the nucleus (N) appeared oval with nearly normal chromatin distribution and large peripheral nucleolus (Nu), numerous rough endoplasmic reticulum profiles (RER) encircling mitochondria (M) exhibited regular orientation around the nucleus, glycogen rosettes (G) and lysosomes (LY) were also noticed (×1500).

**Figure 21** An electron micrograph of liver section of mice treated with (80 mg/kg b.wt.) 5-flourouracil for 4 weeks with daily injection of vitamin C (12 mg/kg b.wt./daily) for 4 weeks showing binucleated hepatocyte, the nucleus (N) appeared oval with nearly normal chromatin distribution and large peripheral nucleolus (Nu), numerous rough endoplasmic reticulum profiles (RER) encircling mitochondria (M) exhibited regular orientation around the nucleus, glycogen rosettes (G) and lysosomes (LY) were also noticed (×1500).

**Figure 22** An electron micrograph of liver section of mice treated with (80 mg/kg b.wt.) 5-flourouracil for 4 weeks with daily injection of vitamin C (12 mg/kg b.wt./daily) for 4 weeks showing hepatocytes and hepatic sinusoids, hepatocytes (H) attained organised outlines with round nuclei. Moreover, cytoplasmic organelles become more organised. Hepatic sinusoids (HS) contain inflammatory cells (IC) (×3000).

**Figure 23** An electron micrograph of liver section of mice treated with (80 mg/kg b.wt.) 5-flourouracil for 4 weeks with daily injection of vitamin C (12 mg/kg b.wt./daily) for 4 weeks showing hepatocyte cytoplasm with few vacuoles (V), RER, few lipid droplets (LP) and glycogen (G), large centric euchromatic nucleus (N) with peripheral nucleolus (Nu) was also noticed (×6000).

Reticulum constitutes the main adverse effect of drug and toxins affecting the liver due to its important role in protein synthesis. On the other side, our results showed that glycogen rosettes were obscured within the cytoplasm of most hepatocytes and were completely absent in few cells. Similar finding
possibility of vitamin C, through its antioxidant property, to study, the proposed plan aimed to assess and examine the induced renal failure (Ferretti et al., 2008). In the current mediated lipid peroxidation (Cuddihy et al., 2008), other antioxidants that can ameliorate cisplatin-induced acute renal failure in mice were detected by Farghaly (2006) who found decrease in glycogen content in the cytoplasm of hepatocytes treated with thioacetamide. The decrease in glycogen content could be attributed to the destruction of the cell membrane including its microvilli. This might be due to the effect of free radicals on the endoplasmic reticulum which causes damage to membranes and enzymes necessary for glycogen synthesis.

According to the present study, the bile canaliculi have manifested some changes marked by slight dilatation of their lumina and destruction and loss of hepatocytes microvilli; such changes have probably occurred as a result of excretion of part of 5-FU in bile causing devastation of the bile canalicus as believed by some authors in other cases of drug treatments (Paulus and Ormgool, 1987; Al-Thani, 1993). The histological and ultrastructural alterations observed in the present work were in agreement with those obtained by El-Hoseany (2012) who reported that 5-FU induced increase in the renal and liver function injury.

Intake of antioxidant vitamins which are widely distributed in fruits could be beneficial in protection against hepatotoxicity (William, 1995). Vitamin C is a well-known antioxidant, which can protect the body from damage caused by free radicals that can be generated during normal metabolism as well as through exposure to toxins and carcinogens (Banerjee et al., 2009). In the literature, we found no reports on the protective effect of vitamin C against 5-FU induced-hepatotoxicity. However, many studies reported that vitamin C supplementation could ameliorate cisplatin-induced acute renal failure in mice (Ajith et al., 2007) and protected cells against radical mediated lipid peroxidation (Cuddihy et al., 2008), other articles suggested that vitamin C reduced the oxidative stress induced renal failure (Ferretti et al., 2008). In the current study, the proposed plan aimed to assess and examine the possibility of vitamin C, through its antioxidant property, to protect against and reduce the histopathological changes induced by 5-FU on normal liver tissues of male albino mice, which were used as biological test animals.

The present results showed that treating mice with 5-FU and vitamin C improved the histopathological and ultrastructural changes induced in the liver by 5-FU. This indicated the effectiveness of vitamin C in prevention of 5-FU hepatotoxicity and this run in agreement with Atasayar et al. (2009) who demonstrated that combined treatment of vitamin C and E with single acute dose (toxic dose) of cisplatin (7.5 mg/kg) was able to normalise the histopathological alteration induced by cisplatin on kidney when compared with the cisplatin treated group. Similar findings were reported by Ahmad and Al-Jawary (2012) who reported regression in cisplatin hepatotoxicity in the liver of rat administrated vitamin C with cisplatin and Shona et al. (2012) who reported that vitamin E reduces cisplatin hepatotoxicity in rat. The present results agree with those obtained by Chinoy et al. (2004) who revealed that ascorbic acid (vitamin C) and vitamin E are capable of completely, or almost completely, mitigating liver toxicity in mice induced by fluoride and aluminium.

The mechanism by which vitamin C decreases the hepatotoxicity induced by 5-FU, is embodied in the fact that vitamin C might ameliorate the oxidative damage by decreasing lipid peroxidation and altering the antioxidant defence system (El-Gendy et al., 2010) or by denoting electrons to free radicals and quenching their reactivity (Bendich, 1990). In addition, ascorbate prevents hepatic glutathione depletion in chemical-induced hepatotoxicity in mice, in which glutathione acted as intracellular free-radical scavengers and protected cells against radical mediated lipid peroxidation (Cuddihy et al., 2008).

In conclusion, the findings of the present study clearly indicate that 5-FU induced histopathological and ultrastructural changes in the hepatocytes of mice and that vitamin C has a partial protective role in 5-FU-induced hepatic injury. So it may be useful to use vitamin C as an adjunctive supplementation to minimise the toxic side-effects of 5-FU.

References

Figure 24 An electron micrograph of liver section of mice treated with (80 mg/kg b.wt.) 5-flourouracil for 4 weeks with daily injection of vitamin C (12 mg/kg b.wt./daily) for 4 weeks showing pyknotic nucleus of hepatocyte (N), electron-dense mitochondria (M), rough endoplasmic reticulum (RER), and dilated smooth endoplasmic reticulum (SER) (×8000).


