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Hepatic Histological and Histochemical Alterations Induced by Rosuvastatin Therapeutic Doses

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Abstract.- The aim of the present study is to determine the potential toxicity of the therapeutic doses of rosuvastatin in Wistar albino rats. A total of 80 adult Wistar male albino rats were divided randomly into 4 groups. The control group (group 1) received oral gavage administration of normal saline (1 ml /kg/day for 90 consecutive days) while group II and group III received rosuvastatin (1.25 and 2.5 mg/kg/day respectively for 90 consecutive days). Group IV received only the vehicle (carboxymethyl cellulose, 0.25% mg/kg/day) for 90 consecutive days. Liver biopsy was taken form each rat under study for histological and histochemical examination. In comparison with respective control rats, ROSU-treated animals exhibited nuclear abnormalities, dilatation of blood sinusoids and central veins, inflammatory cell infiltration, necrosis, apoptosis, congestion and hydropic degeneration. Also, ROSU exposure increased the activity of glucose-6-phosphate dehydrogenase with no change in the activity of alkaline phosphatase together with deletion in protein and glycogen hepatocytes content. The histological and histochemical alterations produced by ROSU might indicate hepatocytes insulation due to metabolic and structural disturbances caused by this drug. More histomorphological and ultrastructural investigations are needed for clear demonstration of rosuvastatin potential risk.

Key words: Rosuvastatin, statins, hepatic tissue, apoptosis, TUNEL assay, hydropic degeneration

INTRODUCTION

Statins are a class of drugs used widely for the treatment of hyperlipidemia as well as for prevention of atherosclerosis and cardiovascular events with therapy duration of 30–120 days (Wainwright, 2005; Bjornsson *et al*., 2012). Statins reduce the level of circulating atherogenic lipoproteins by inhibition of hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (McTaggart, 2003; Guthrie and Martin, 2007). Pre-marketing biochemical clinical trials and initial toxicological studies in animals suggested that statins may cause hepatotoxicity, primarily elevations in serum aminotransferases levels induced with a need of liver enzymes monitoring (Veillard and Mach, 2002; Famularo *et al*., 2007). Although the therapeutic doses of lovastatin did not cause significant liver injury, they caused hepatocellular necrosis in rabbits when given in very high doses (MacDonald *et al*., 1988). Similarly, high doses of simvastatin caused hepatocellular necrosis in guinea pigs (Horsmans *et al*., 1990). Statin-related drug-induced liver injury induced by atorvastatin, simvastatin, fluvastatin and others was reported in 1.2/100,000 users (Bjornsson *et al*., 2012; Russo *et al*., 2009).

Statins cause transaminitis with possible induction of acute liver failure, hepatitis and cholestasis (Vasudevan *et al*., 2005). One study found from the reports of the World Health Organization for deaths resulting from serious liver injury that most patients experienced liver injury after 3–4 months of statins therapy (Perger *et al*., 2003). However, the use of statins has been shown to improve liver abnormalities in patients with non-alcoholic fatty liver disease (Bjornsson *et al*., 2012; Hyogo *et al*., 2008). Some studies indicated that rosuvastatin ameliorated hepatic injury, inflammation, lipid perixodation, increased antioxidant enzymes activity and modulated immune response independent of lipid lowering effect (Awad and Kamel, 2010; Awad and El-Sharif, 2010).

Rosuvastatin is a relatively new statin as

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cholesterol-lowering drug, with liver is the main target organ (Nezasa *et al*., 2002; Famularo *et al*., 2007). Rosuvastatin is taken up by hepatocytes more selectively and more efficiently than other statins which has generated considerable controversy regarding its safety specially its probable potential hepatotoxicity (Famularo *et al*., 2007; Guthrie and Martin, 2007; Khan and Ibrahim, 2009).

Although statins hepatotoxicity is well recognized (Kaplowitz, 2004), rosuvastatin induced hepatotoxicity has been put into question (Bader, 2010). Pre-marketing studies have suggested that rosuvastatin may have lesser potential to cause liver toxicity as compared with other statins (Davidson, 2007). On the otherhand, some studies and case reports showed that treatment with rosuvastatin might cause hepatotoxicity even with low doses or short time of treatment. With this limited data exist on rosuvastatin -induced liver injury, the present study was conducted to investigate the hepatic histological and histochemical alterations induced by therapeutic doses of this drug.

MATERIALS AND METHODS

A total of 80 health adult male Wistar albino rats (*Rattus norvegicus*) of the same age (8-10 weeks old) weighing 220-250 g of King Saud University colony were used. All animals were divided randomly into 4 groups (20 animals each), kept in the laboratory conditions for a period of 7 days for acclimatization and were fed with commercial rat pellets and drinking water *ad libitum*. The daily equivalent dose (mg/kg) of rosuvastatin was calculated on the basis of the surface area ratio according to standard methods (Reagan-Shaw *et al*., 2008). Accordingly, 1.25 and 2.5 mg/kg rosuvastatin (Crestor®, AstraZeneca Pharmaceuticals LP, Wilmington) dissolved in normal saline containing sodium carboxymethyl cellulose (0.25%) were used as a daily equivalent therapeutic doses and administered orally for 90 consecutive days as follows:

Group I (Control group): The members of this group were not subjected to rosuvastatin but to one ml normal saline/kg/day.

Group II: The members of this group received rosuvastatin (1.25 mg/kg/day).

Group III: The members of this group received rosuvastatin (2.5 mg/kg/day).

Group IV: The members of this group were exposed to the vehicle (0.25% carboxymethyl cellulose dissolved in one ml normal saline)/ kg/day.

Fresh portions of the liver from each rat were cut rapidly, fixed in neutral buffered formalin (10%), then dehydrated, with grades of ethanol (70%, 80%, 90%, 95% and 100%). Dehydration was then followed by clearing the samples in two changes of xylene. Samples were then impregnated with two changes of molten paraffin wax, then embedded and blocked out. Sections (4–5 μm) were stained according to Bancroft and Stevens (1999), with the following conventional histological and histochemical stains: hematoxylin and eosin (H&E), periodic acid-Schiff (PAS) reagent, Best's carmine stain, Mallory trichrome and mercuric bromophenol blue. Histochemical reactions for alkaline phosphatase and glugose-6-phosphate dehydrogenase (G6PDH) were performed on fresh unfixed frozen sections. The demonstration of alkaline phosphatase was based on the Naphthol AS-BI method (c.f. Van Noorden and Fredriks, 1992). The specificity of the reaction for this enzyme was controlled by incubating a parallel set of sections in the incubating medium without substrate. Glugose-6-phosphate dehydrogenase detection was based on the lead method of Wachstein and Miesel (Bancroft and Stevens, 1999), and by using control consisted of parallel sections incubation in media lacking the substrate of the specified enzyme. The incubating medium consisted of NBT tetrazolium, disodium glucose-6-phosphate, nicotininamide adenine dinucleotide (NADP), sodium azide and phenazine methosulphate.

Apoptotic cells were detected by using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) detection kit (GenScript, USA), which is a sort of an immunohistochemical technique. The incubating medium consisted of proteinase K solution in PBS buffer, labeled Biotin/Streptavidin-HRP/DAB and terminal deoxynucleotidyl transferase. For negative controls, parallel sections were incubated in medium

lacking terminal deoxynucleotidyl transferase.

All experiments were conducted in accordance with the guidelines approved by King Saud University Local Animal Care and Use Committee.

RESULTS

In comparison with the control group (Fig.1a), the liver of ROSU-treated rats had lost some hepatic architecture characteristics and demonstrated several histological and histochemical alterations.

Histological changes

Occasional portal inflammatory cells infiltration mainly lymphocytes was seen in the periportal spaces of ROSU-treated rats (Fig. 1b). This infiltration was more prominent in rats received 2.5 mg than those received 1.25 mg of the drug.

Marked parenchymal necrosis was noticed in hepatocytes of ROSU-treated rats with eosinophilic cytoplasm (Fig. 1c). Apoptotic bodies in the form of intercellular rounded condensed eosinophilic bodies surrounded by clear holes were also seen (Fig. 1d).

Dilatation and congestion of hepatic sinusoids became evident in the in the liver of ROSU-treated rats (Fig. 1e). This alteration was more prominent in rats received 2.5 mg of the drug than those received 1.25 mg and was associated with hepatocytes necrosis.

The hepatic tissue of the ROSU-treated rats showed dilated central vein in all drug treated members (Fig. 1f).

Occasional perivascular edema was seen in the hepatic triads of rats exposed to ROSU administration (Fig. 2a). This change appeared in all treated groups. This alteration appeared in some hepatocytes of rats received 2.5 mg ROSU but rarely seen in those received 1.25 mg ROSU (Fig. 2b). The insulted hepatocytes exhibited poorly delineated nuclei.

Variable nuclear abnormality was mainly seen in the hepatocytes of rats received 2.5 mg ROSU and to a lesser extent in the those received 1.25 mg ROSU. These abnormalities included marked binucleation, anisokaryosis, karyolysis, karyorrhexis and karyopyknosis. Some pyknotic hepatocytes of rats exhibited clumping and condensation chromatin condensation in the periphery of the nuclei together with irregularity nuclear membranes. Hepatocytes showed nuclei disappearance while others exhibited fragmentation or dissolution of their nuclei (Figs. 2c-d).

Occasional autolytic changes and cytoplasm eosinophilia were observed in some hepatocytes especially in those with indistinct cell membranes (Fig. 2e).

Hepatocytes cytoplasm swelling and vacoulation were seen specially at high dose ROSU treated rats (Fig. 2f).

Histochemical changes

Compared with liver of control group, ROSU administration has produced significant reduction in liver glycogen specially in rats received 2.5 mg ROSU (Figs. 3a-c). Hepatocytes in the perivenous zones were more affected the those surround the periportal spaces.

Compared with liver of control group, ROSU administration has produced considerable reduction in hepatocytes protein in both group of rats received 1.25 mg and 2.5 mg ROSU respectively (Fig. 3d-f).

A marked increase in the activity of G6PDH was seen in the liver of ROSU treated rats. The activity of this enzyme was more pronounced in rats received 2.5 mg ROSU than those received 1.25 mg of the drug (Figs. 3g-i). In the control livers, this enzyme was mainly localized in the bile canalicular membranes of hepatocytes the liver. The activity of the enzyme was demonstrated clearly in the adventitia coat of blood vessels and was not affected by ROSU treatment.

None of the above histological and histochemical alterations were detected in the liver of the control rats or those received carboxymethyl cellulose**.**

Apoptosis detection

Compared with liver of control group, apoptotic cells were observed in some hepatocytes of the liver of ROSU-treated groups as seen by Tunel assay (Figs. 4a,b).

No portal fibrosis or cirrhosis were detected due to chronic therapeutic doses of rosuvastatin in

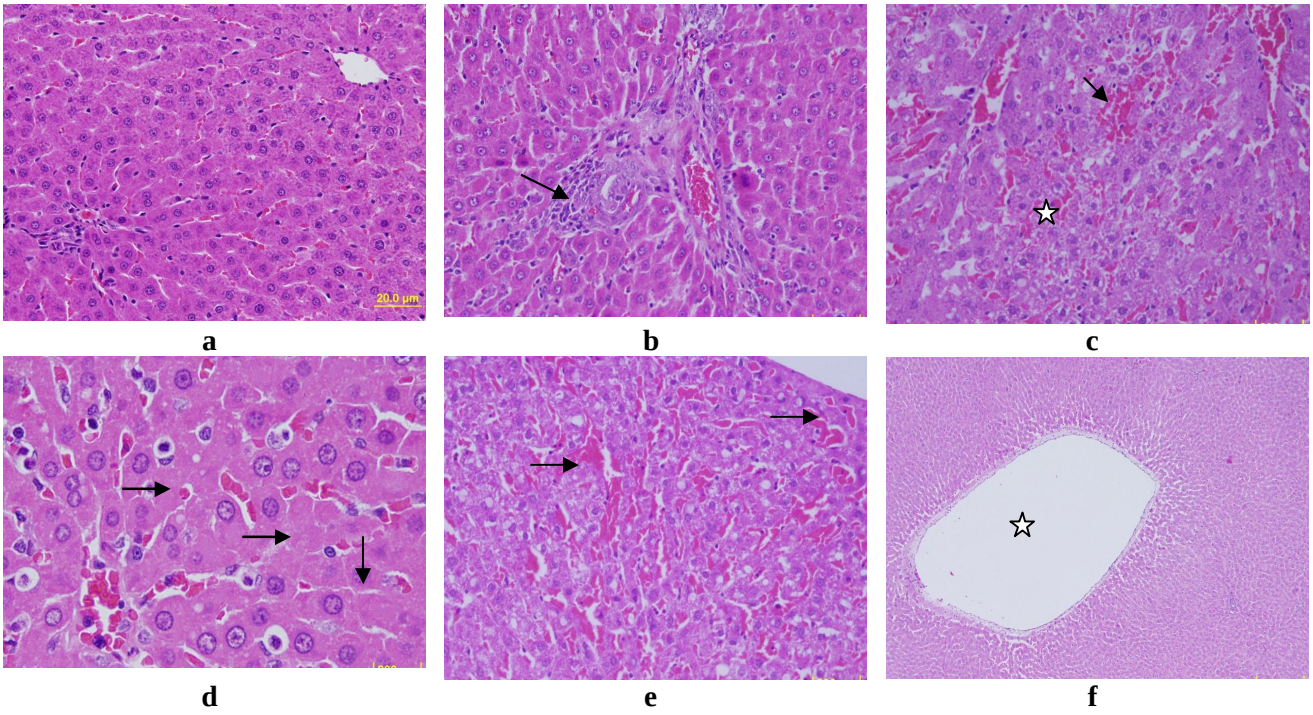

Fig. 1. Histological sections of liver of control and ROSU treated rats; a, 1ml of the vehicle/Kg/day for 90 days demonstrating normal histological architecture; b, 1.25mg/kg/day for 90 days demonstrating lymphocytes infiltration in the hepatic portal space (arrow); c, 2.5mg/kg/day for 90 days demonstrating marked necrosis (star) and scattered hemorrhages (arrow); d, 2.5mg/kg/day for 90 days demonstrating appearance of some apoptotic bodies (arrows); e, 2.5mg/kg/day for 90 days demonstrating dilation and congestion of blood sinusoids (arrows); f, 1.25mg/kg/day for 90 days demonstrating marked dilation of central vein (star).
Stain: H&E; Magnification a, b, c : 280×; d, 700×; e, 1000×; f, 240×.

the liver of any member of the treated groups over the entire period of the study.

DISCUSSION

Statins are metabolized mainly by the liver and increase aminotransferases levels with hepatic potential toxicity that might be attributed to alteration of the hepatocyte cellular membrane rather than direct liver injury (Veillard and Mach, 2002; Clarke and Mills, 2006).

The results of the present work showed inflammatory cells infiltration in the hepatic tissue due to ROSU chronic exposure. This may suggest that ROSU could interact with proteins and enzymes of the hepatic interstitial tissue by interfering with the antioxidant defense mechanism and leading to reactive oxygen species (ROS) generation which in turn may imitate an inflammatory response (Johar *et al.*, 2004).

Cell necrosis induced by ROSU chronic exposure as shown by the present work was described previously by other studies which investigated other members of statins (MacDonald *et al.*, 1988; Corsini *et al.*, 1996). Necrosis is produced as a result of cell degeneration accompanied by organelles swelling and amorphous eosinophilic cytoplasm followed by shrinking and dissolution of nuclei (Campos-Pereira *et al.*, 2012). The seen necrosis induced by ROSU may be due to statins effect on permeability of hepatocytes cell membrane that lead to depletion of cholesterol. Also, this might indicate swelling of some organelles such as mitochondria or by oxidative stress on these cells by glutathione depletion.

Apoptosis is a sort of programmed cell suicide that is highly regulated and executed via activation of specific signaling pathways. This

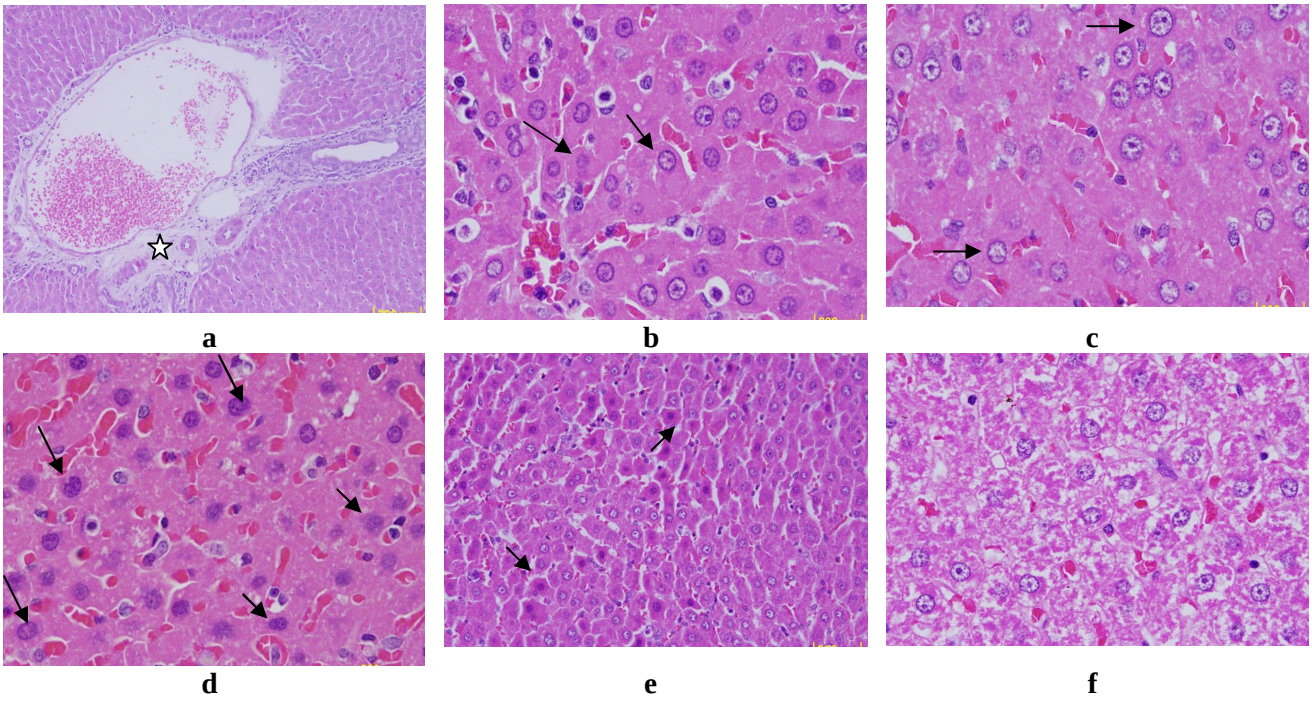

Fig. 2. Histological sections in the liver of ROSU-treated rats: a, 2.5mg/kg/day for 90 days demonstrating dilation of congested portal vein with edema around the blood vessel and lymphocytes infiltration in the portal triad (star); b, 2.5mg/kg/day for 90 days demonstrating swelling of hepatocytes (arrows); c, 1.25mg/kg/day for 90 days demonstrating anisokaryosis of hepatocytes (arrows); d, 2.5mg/kg/day for 90 days demonstrating binucleation, karyolysis and karyorrhexis of hepatocytes (arrows); e, 1.25mg/kg/day for 90 days demonstrating cytoplasmic eosinophilia in some hepatocytes (arrows); f, 1.25mg/kg/day for 90 days demonstrating hydropic degeneration (Ballooning) with cloud swelling in the cytoplasm of some hepatocytes.
Magnification: a, 240×; b, c, d, 700×; e, f, 400×. Stain: H&E.

abnormality is usually accompanied by particular morphological alterations such as DNA fragmentation, nuclear condensation, and formation of apoptotic bodies which are then engulfed by macrophages or neighboring cells without initiating an inflammatory response, death or disruption to the surrounding tissue (Pollack and Leeuwenburgh, 2001; Pollack *et al.*, 2002; Kluck *et al*., 1997). The mitochondrion plays a central role in regulating apoptosis by cytochrome *c* release into the cytosol, which then forms an "apoptosome". Some reports indicated that statins induce apoptosis by an increase caspase-9 and caspase-3 activty together with pyknosis, chromatin marginalization, and formation of dense bodies (Campos-Pereira *et al*., 2012). Cell apoptosis induced by ROSU as shown by the present work is in agreement with other findings (Guijarro *et al*., 1998; Rabkin and Kong, 2003; Erl, 2005; Kaufmann *et al*., 2006; Westwood *et al*., 2006). All tested lipophilic statins induced apoptosis of skeletal muscle cells while hydrophilic ones induced hepatic cells (Kaufmann *et al*., 2006).

The seen nuclear abnormalities induced by chronic exposure to ROSU such as marked binucleation, karyorrhexis, karyolysis and apoptosis might indicate hepatocytes cytotoxicity (Tolbert *et al*., 1992; Celik *et al*., 2003; Unal *et al*., 2005). Nuclear degeneration starts with pyknosis, followed by karyorrhexis and karyolysis lead to cell necrosis (Zamzami and Kroemer, 1999; Biradar *et al*., 2012). The results of the present work showed marked and more frequent hepatocytes binucleation in the animal exposed to ROSU than the control ones. This alteration may represents a consequence of cell injury usually seen in regenerating cells and might be due to increased cellular activity and nuclear interruption in the mechanism of ROSU detoxification (Gerlyng1 *et al*., 2008). These nuclear

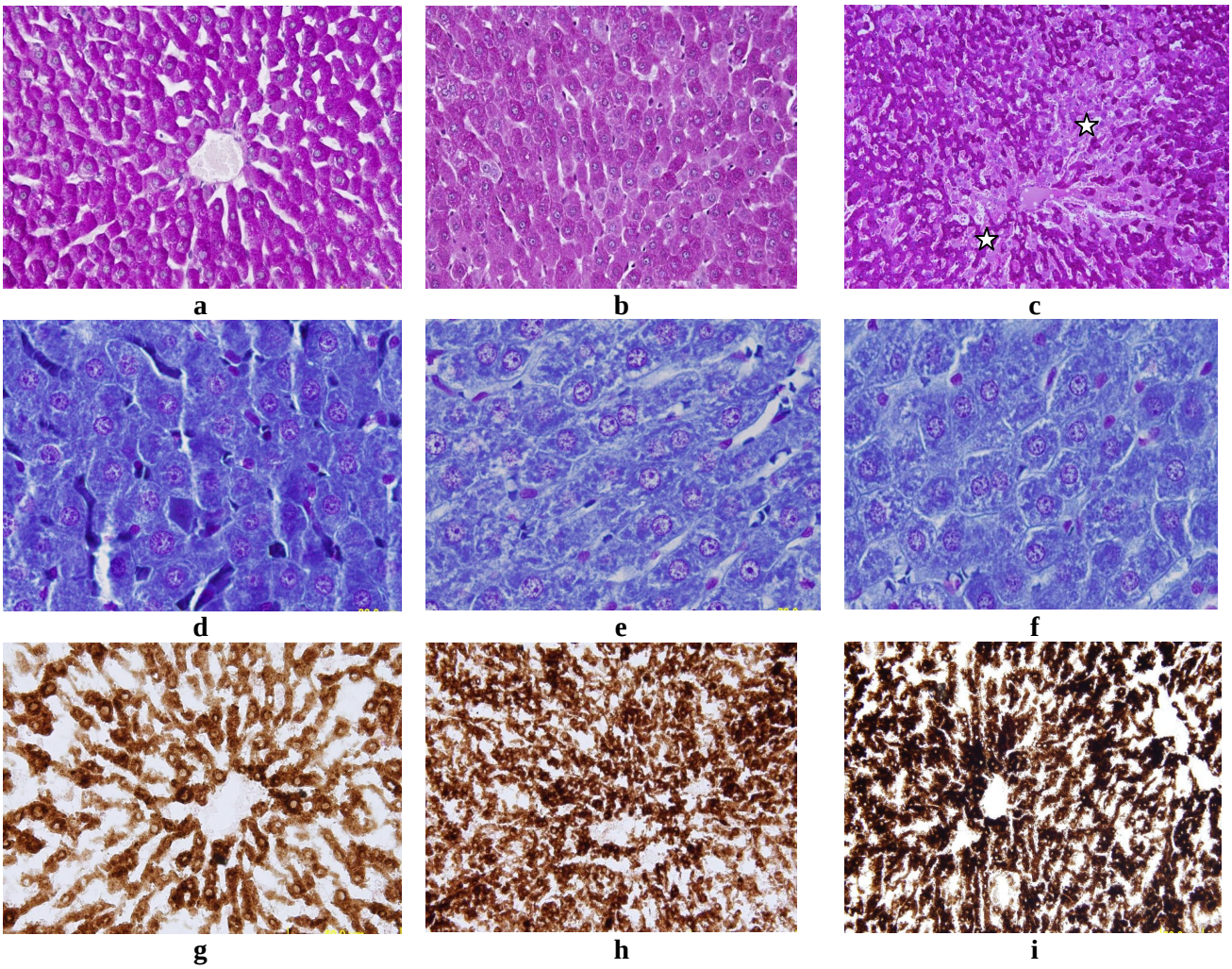

Fig. 3. Histological sections in the liver of control (a, d, g) and ROSU treated rats (d, c, e, f, h, i): a, 1 ml normal saline/kg/day for 90 days demonstrating normal content and distribution of glycogen in the hepatocytes of control group. PAS stain. 280×; b,. 1.25mg/kg/day for 90 days demonstrating significant reduction of glycogen. PAS. 280×; c, 2.5mg/kg/day for 90 days demonstrating prominent reduction of glycogen in the perivenous zones (stars). PAS. 280×; d, 1ml normal saline/Kg/day for 90 days demonstrating normal content and distribution of protein in the hepatocytes; e, 1.25 mg/kg/day for 90 days demonstrating significant depletion in protein content of the hepatocytes in comparison with the control (Fig.3d). Mercuric bromophenol blue stain; f, 2.5 mg/kg/day for 90 days demonstrating significant depletion in protein content of the hepatocytes in comparison with the control (Fig. 3d), with no significant reduction from rats received 1.25 mg/kg/day (Fig. 3e). Mercuric bromophenol blue stain; g, 1ml normal saline/Kg/day for 90 days demonstrating normal activity and distribution of G6PDH enzyme in the hepatocytes; h, 1.25 mg/kg/day for 90 days demonstrating significant increasing in the activity of G6PDH in comparison with the control (Fig. 3g); i, 2.5 mg/kg/day for 90 days demonstrating more activity of G6PDH than rats received 1.25 mg/kg/day (Fig. 3g).
Magnification: a, b, c, g, 280×; d, e, f, 700×; h, i, 240×.
Staining: a, b, c, g, h, I, PAS; d, e, f, mercuric bromophenol blue stain.

changes were dose dependent where nuclear damage was more prominent in the liver of rats received 2.5 mg ROSU than the ones received 1.25 mg. Hepatocytes nuclear abnormalities were reported by other studies used high dose of lovastatin (MacDonald and Halleck, 2004).

The distortion and swelling of hepatocytes together with the central vein dilatation might indicate that ROSU may affect the cell membrane permeability of hepatocytes and blood vessels

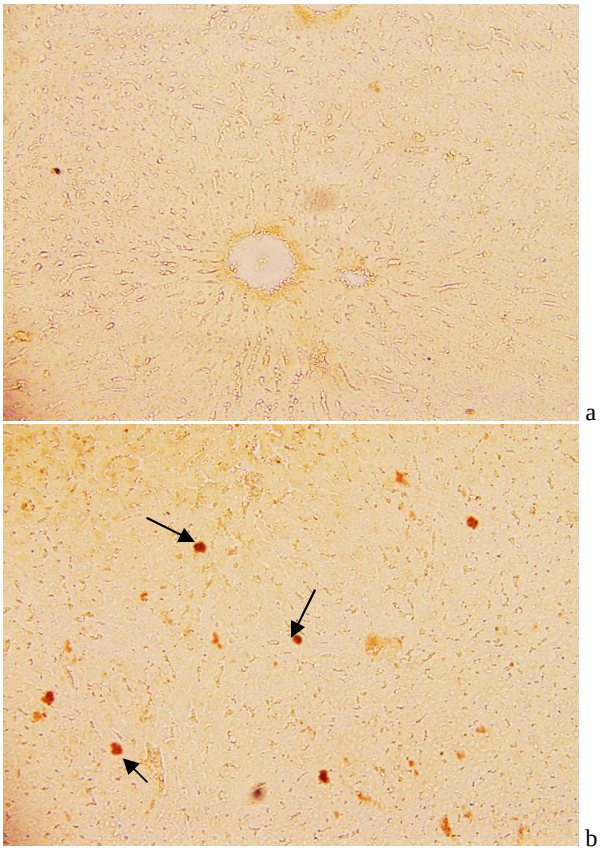

Fig. 4. Histological structure of liver of rats receiving (a) 1ml normal saline/kg/day for 90 days demonstrating no apoptosis; (b), 2.5mg/kg/day for 90 days demonstrating apoptotic cells (arrows). TUNEL assay. Magnification: a, 240×; b, 280×.

endothelial lining. Hepatocytes swelling due to ROSU exposure as seen in the present study might lead to cellular transporters adaptation (Johnson, 1995). Ischaemic or pharmacologic disruption of cellular transporters can cause swelling of parenchyma of the liver cells leading to hepatotoxicity (Ajibade *et al.*, 2012). ROSU may have acted as toxins to the hepatocytes, affecting their cellular integrity and causing defect in membrane permeability and cell volume homeostasis. These together with the hypertrophy of hepatocytes as observed in the treated rats may indicate support the cytotoxic effect of ROSU.

The present study showed that ROSU induced reduction in hepatocytes protein content. This might be due to effect of rosuvastatin on enzymes involved on protein synthesis and to the effect on the cytosol Ca^{2+} that might mediate variety of deleterious effects on the hepatocytes ribosomes and the rough endoplasmic reticulum (Kumar *et al.*, 2005; El-Daly, 2011)**.** Rosuvastatin has been noted to produce transient proteinuria while its high doses have been associated with cases of renal failure accompanied by severe proteinuria (Guthrie and Martin, 2007; Kostapanos *et al.*, 2010).

Administration of ROSU caused depletion of glycogen stores in the hepatocytes of ROSU treated rats. This alteration might be due to the effect of ROSU on glucose absorption or on the enzymes involved in the process of glycogenesis or/and glycolysis (Kumar *et al.*, 2005). Hepatocytes in the perivenous zones were more affected the those surround the periportal spaces. This might indicate that glycolysis was more affected than glycogenesis by rosuvastatin.

The results obtained in the present investigation reveal that the therapeutic doses of rosuvastatin induced considerable histological and histochemical alterations in the liver of rats. Further studies are recommended to be carried out to corroborate these findings.

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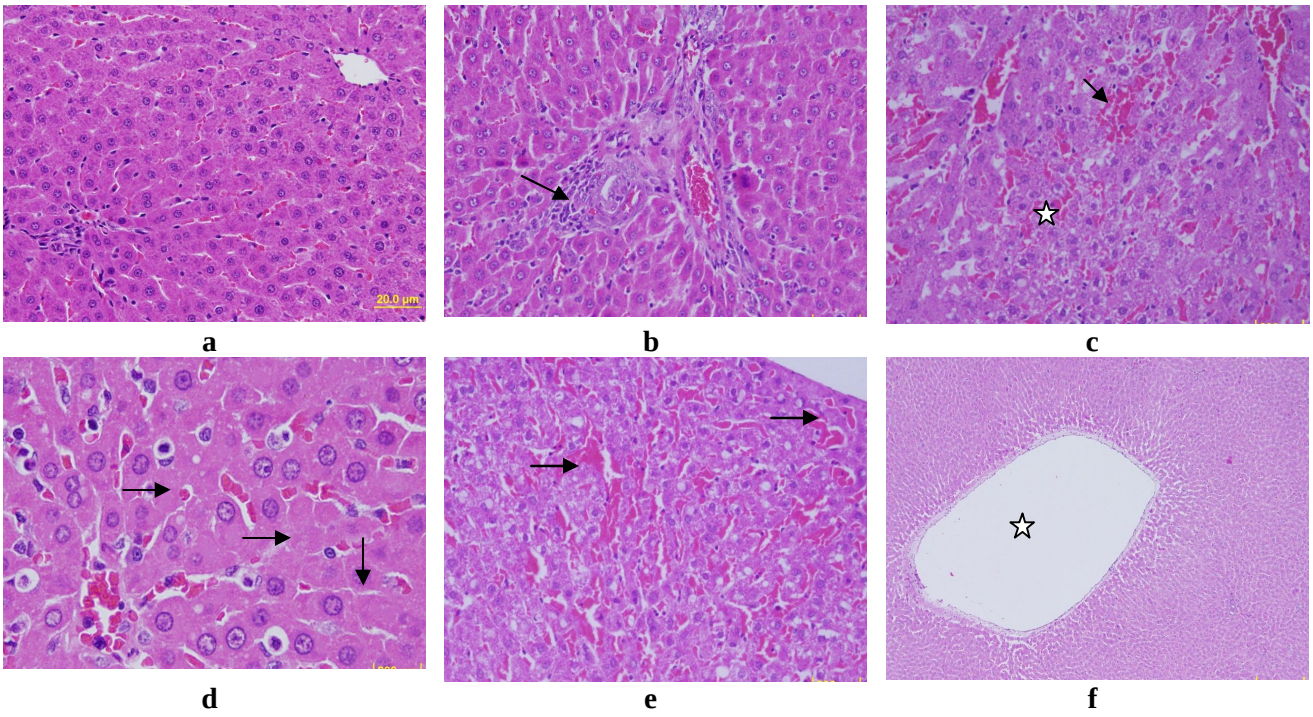

Fig. 1(a-f). Histological sections in the liver of control and ROSU treated rats received:
a, 1ml of the vehicle/Kg/day for 90 days demonstrating normal histological architecture. H&E. 280×.
b, 1.25mg/kg/day for 90 days demonstrating lymphocytes infiltration in the hepatic portal space (arrow). H&E. 280×.
c, 2.5mg/kg/day for 90 days demonstrating marked necrosis (star) and scattered hemorrhages (arrow). H&E. 280×.
d, 2.5mg/kg/day for 90 days demonstrating appearance of some apoptotic bodies (arrows). H&E. 700×.
e, 2.5mg/kg/day for 90 days demonstrating dilation and congestion of blood sinusoids (arrows). H&E 1000×.
f, 1.25mg/kg/day for 90 days demonstrating marked dilation of central vein (star). H&E. 240×.

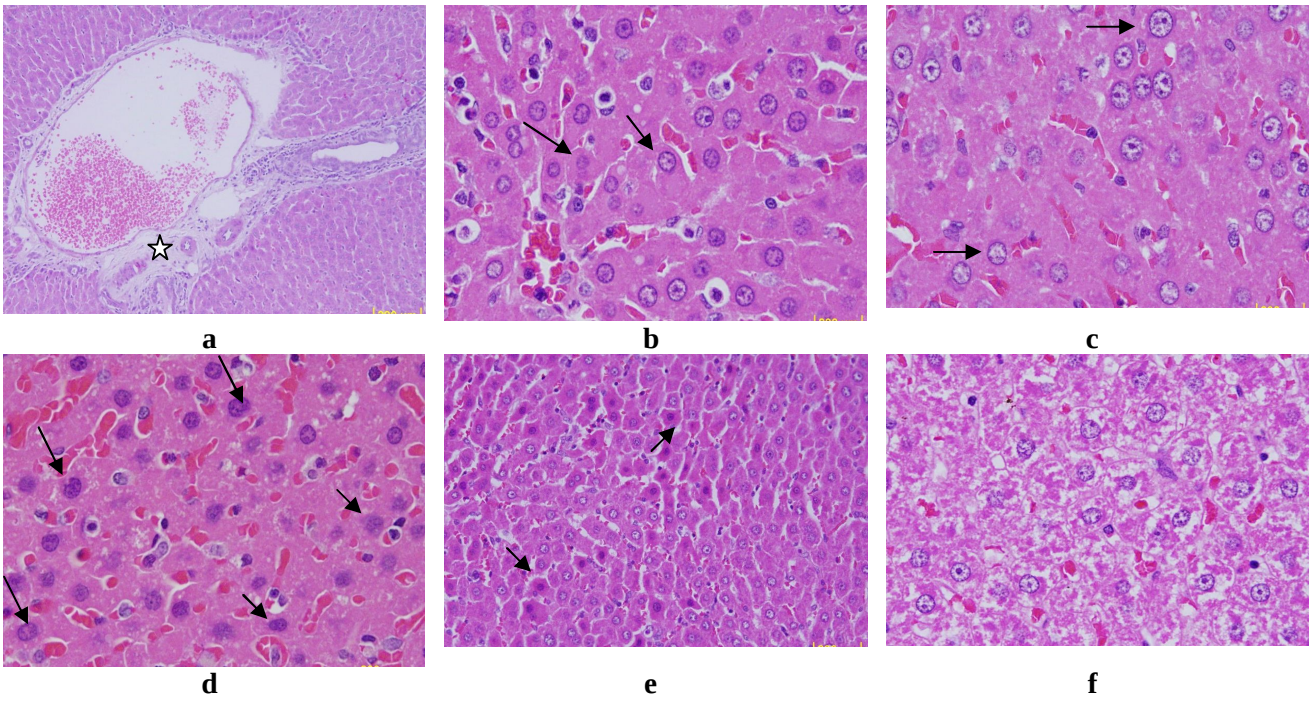

Fig. 2(a-f). Histological sections in the liver of ROSU-treated rats received:
a,. 2.5mg/kg/day for 90 days demonstrating dilation of congested portal vein with edema around the blood vessel and lymphocytes infiltration in the portal triad (star). H&E. 240×.
b, 2.5mg/kg/day for 90 days demonstrating swelling of hepatocytes (arrows)). H&E. 700×.
c,. 1.25mg/kg/day for 90 days demonstrating anisokaryosis of hepatocytes (arrows). H&E. 700×.
d,. 2.5mg/kg/day for 90 days demonstrating binucleation, karyolysis and karyorrhexis of hepatocytes (arrows). H&E. 700×.
e,. 1.25mg/kg/day for 90 days demonstrating cytoplasmic eosinophilia in some hepatocytes (arrows). H&E. 400×.
f,. 1.25mg/kg/day for 90 days demonstrating hydropic degeneration (Ballooning) with cloud swelling in the cytoplasm of some hepatocytes. H&E. 400×.

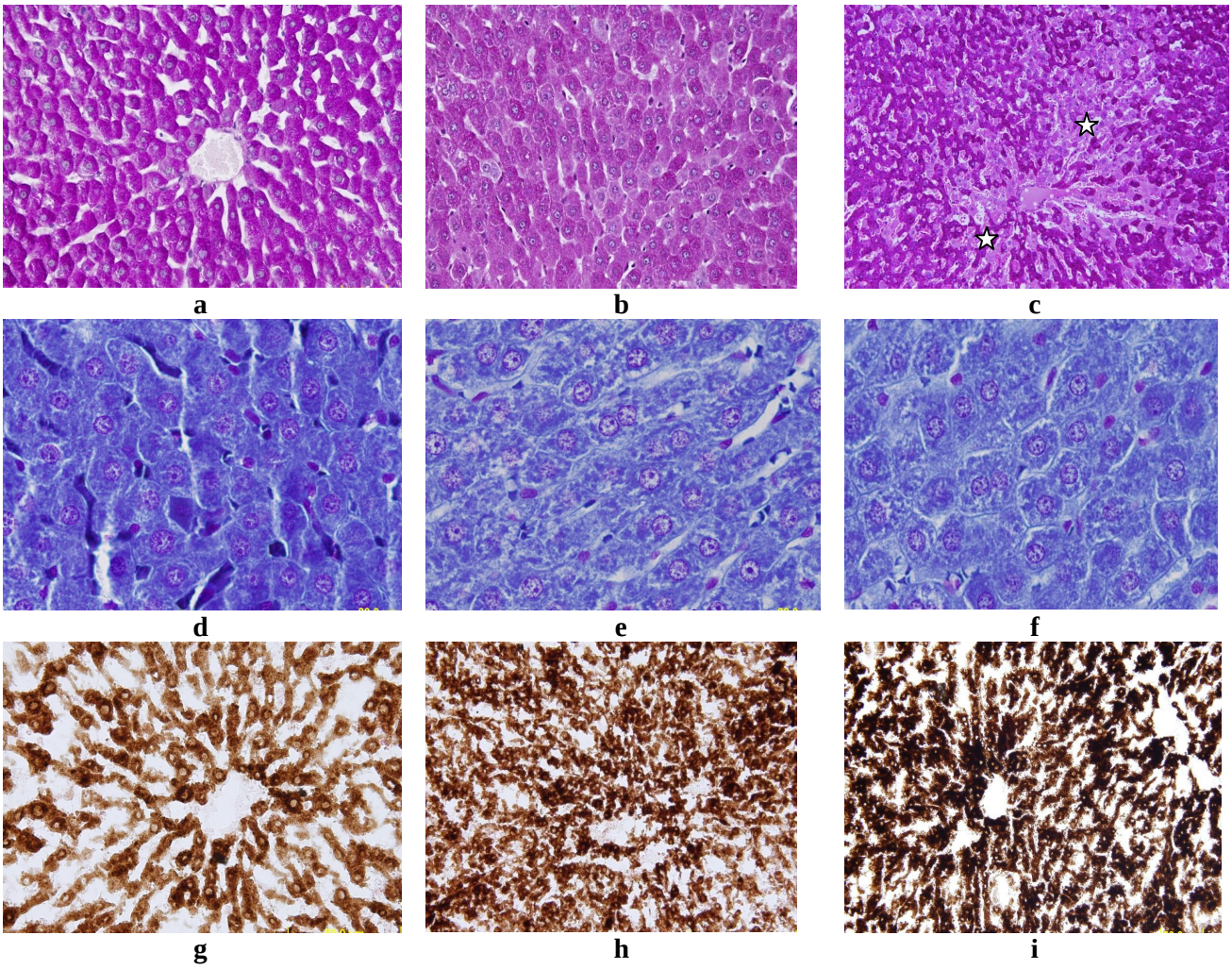

Fig. 3. Histological sections in the liver of control (a, d, g) and ROSU treated rats (d,c,e,f, h,i) received:
a, 1 ml normal saline/kg/day for 90 days demonstrating normal content and distribution of glycogen in the hepatocytes of control group. PAS stain. 280×.
b,. 1.25mg/kg/day for 90 days demonstrating significant reduction of glycogen. PAS. 280×.
c, 2.5mg/kg/day for 90 days demonstrating prominent reduction of glycogen in the perivenous zones (stars). PAS. 280×.
d, 1ml normal saline/Kg/day for 90 days demonstrating normal content and distribution of protein in the hepatocytes. Mercuric bromophenol blue stain. 700×.
e, 1.25 mg/kg/day for 90 days demonstrating significant depletion in protein content of the hepatocytes in comparison with the control (Fig.3d). Mercuric bromophenol blue stain. 700×.
f, 2.5 mg/kg/day for 90 days demonstrating significant depletion in protein content of the hepatocytes in comparison with the control (Fig. 3d), with no significant reduction from rats received 1.25 mg/kg/day (Fig. 3e). Mercuric bromophenol blue stain. 700×.
g, 1ml normal saline/Kg/day for 90 days demonstrating normal activity and distribution of G6PDH enzyme in the hepatocytes. 280×.
h, 1.25 mg/kg/day for 90 days demonstrating significant increasing in the activity of G6PDH in comparison with the control (Fig. 3g). 240×.
i, 2.5 mg/kg/day for 90 days demonstrating more activity of G6PDH than rats received 1.25 mg/kg/day (Fig. 3g). 240×.