OXIDATIVE STRESS MEDIATED HEPATOTOXICITY PRODUCED BY SIMVASTATIN

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Summary

The main objective of present study was to *in vivo* evaluation of Simvastatin hepatotoxicity. Hepatotoxicity in rat was induced by Simvastatin (20mg/kg/p.o. for 30 days) and evaluation was carried out by estimating oxidative stress markers like lipid peroxidation, reduced glutathione, super oxide dismutase and catalase along with marker enzymes for liver function like aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and γ glutamic transpeptidase (GTP) and lactate dehydrogenase (LDH), protein profile likes total bilirubin (TB), direct bilirubin (DB), total albumin (TA) and total protein (TP) and histopathological study to confirm hepatotoxicity. Simvastatin significantly ($P<0.001$) increased lipid peroxidation compared to control. Tissue levels of reduced glutathione, superoxide dismutase and catalase were significantly ($P<0.001$) decreased after treatment with Simvastatin compared to control. Serum levels of ALT, AST, γGTP, ALP and LDH were significantly ($P<0.001$) increased after treatment with Simvastatin compared to control. Simvastatin caused significant ($P<0.001$) increase in serum total bilirubin and indirect bilirubin and significant ($P<0.01$) decrease in direct bilirubin and total protein compared to control. Simvastatin hepatotoxicity was characterized by significant increase in oxidative stress markers along with marker enzymes of liver function and depletion of proteins indicated oxidative stress mediated hepatotoxicity produced by Simvastatin.

Keywords: Hepatotoxicity; Simvastatin; Oxidative stress

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Introduction

Drug-induced Hepatotoxicity (DIH) account for 9.5% of all suspected adverse drug reaction, and are the most common reason for withdrawal of drug from the market (1). Injury may be a direct toxic effect or immunological reaction to either of the drug or an active metabolite formed by bio activation (2). Although, with the exception of rare cases, DIH subsides after cessation of treatment with the drug, this represents an important diagnostic and therapeutic challenge for physicians. The present work provides an overview of the mechanisms involved in drug-induced liver disease, together with the risk factors and disease characteristics associated with DIH.

Simvastatin competitively inhibit HMG-CoA to mevalonate. Mevalonate is also a precursor of Coenzyme Q10 (CoQ10). Thus treatment with statins could lower its levels too. CoQ10 acts as an antioxidant, has membrane stabilising effects and is important for cellular mitochondrial respiration, essential for energy production in organs (3, 4).

Susceptibility to drug-induced hepatotoxicity is also influenced by genetic and environmental risk factors. Unpredictable, low frequency, idiosyncratic reactions often occur on a background of a higher rate of mild asymptomatic liver injury and, although difficult to predict, they may be detected by monitoring serum alanine aminotransferase levels. Recent and future advances in toxicogenomics and proteomics should improve the identification of risk factors and the understanding of idiosyncratic hepatotoxicity (5).

Present study was designed to induce hepatotoxicity in rat by Simvastatin, which are having clinical application. Estimating tissue oxidative stress markers along with liver function test, protein profile and histopathological study to confirm liver damage carried out in-vivo evaluation of hepatotoxicity. The main objective of present study was to assess Simvastatin hepatotoxicity.

Methods

Animals

All experiments and protocols described in present study were approved by the Institutional Animal Ethics Committee (IAEC) of M. S. University, Baroda and with permission from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

Healthy adult male Wistar rats weighing 200-250g were used. Rats were housed in polypropylene cages, maintained under standardized condition (12-h light/dark cycle, 24°C, 35 to 60% humidity) and provided free access to palleted CHAKKAN diet (Nav Maharashtra Oil Mills Pvt. Ltd., Pune) and purified drinking water ad libitium.

Experimental protocol

Animals were divided into two groups, each having 6 rats and treated accordingly. Group: 1 - rats received normal standard diet for 24 days, Group: 2 - rats received simvastatin (SMT) 20mg/kg/p.o. for 30 days (6).

Collection of serum

Blood samples were withdrawn from retro-orbital plexus under light ether anesthesia without any anticoagulant and allowed for 10 minutes to clot at room temperature. It was centrifuged at 2500 rpm for 20 minutes.

The serum obtained was kept at 4ºC until used. All the animals were euthanasiously sacrificed after blood collection with spinal dislocation method and liver removed for study of oxidative stress markers and histopathological study.

Liver homogenate preparation

Liver kept in cold conditions (precooled in inverted petridish on ice). It was cross chopped with surgical scalpel into fine slices in chilled 0.25 M sucrose, quickly blotted on a filter paper. The tissue was minced and homogenized in 10 mM Tris-HCl buffer, pH 7.4 (10%w/v) with 25 strokes of tight teflon pestle of glass homogenizer at a speed of 2500 rpm. The prolonged homogenizer under hypotonic condition was designed to disrupt as far as possible the ventricular structure of the cells so as to release soluble protein and leave only membrane and nonvascular matter in a sediment form. The clear supernatant was used for oxidative stress markers assays.

Estimatation of Lipid Peroxidation (7)

The method estimates Malondialdehyde (MDA), a product of lipid per oxidation process. One molecule of MDA reacts with two molecules of thiobarbituric acid (TBA) under mildly acidic conditions to form a pink coloured chromogen, whose intensity was measured colorimetrically at 535 nm.

Estimatation of Reduced glutathione (GSH) (8)

Glutathione present in RBC consist of sulfhydryl groups. 5,5 dithiobis 2- nitro benzoic acid (DTNB), A disulphide compound, gets readily attacked by these sulfhydryl groups and forms a yellow coloured anion which measured colorimerically at 412 nm.

Estimatation of Super oxide dismutase (SOD) (9)

Rate of auto oxidation of epinephrine & the sensitivity of this auto oxidation to inhibition by SOD were augmented as pH was raised from 7.8 – 10.2, O2, generated by xanthine oxidase reaction, caused by the oxidation of epinephrine to adrenochrome & the yield of adrenochrome produced per O2 introduced. The auto oxidation of epinephrine proceeds by least two distinct pathways only one of which is free radical chain reaction involving O2 & hence inhabitable by SOD.

Estimatation of Catalase (CAT) (10)

In the ultra-violet range H2O2 shows a continuous increase in absorption with decreasing wavelength. The décor position of H2O2 can be followed directly by the decrease in absorbance at 240 nm. The difference in the absorbance per unit time is a measure of the catalase activity.

Estimation of liver function

Estimation of marker enzymes for liver function like Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and Alkaline phosphatase (ALP) were done by using kit, Span Diagnostic Ltd, India and γ glutamic transpeptidase (γGTP) was done by using kit, Dade Behring Ltd., UK. Estimation of Lactate dehydrogenase (LDH) was done by using kit, Enzopak-Reckon diagnostics. Protein profile likes Total bilirubin, direct bilirubin; Total albumin and Total protein were done by using kit, Span Diagnostic Ltd, India.

Histopathological study

Liver was collected after the rats were sacrificed. After blotting free of blood and tissue fluids, it was kept in 5% formalin. 5-15μm thick sections was serially cut on a leitz microtome in horizontal planeand mounted on glass slide with the help of egg albumin in glycerine solution (50% v/v).

They were then stained with 10% hematoxylin for 3-5 minutes and placing in running water intensified the staining. The hematoxylin-stained sections were stained with 10% eosin for 2 minutes. The sections were observed and desired areas were photographed in an Olympus photomicroscope. The sections were viewed under 40X magnifications.

Statistical analysis

All the values are expressed as mean ± S.E.M. Statistical significance between more than two groups was tested using one-way ANOVA followed by the Bonferroni multiple comparisons test or unpaired two-tailed student's t-test as appropriate using computer based fitting program (Prism, Graphpad 3.). Differences were considered to be statistically significant when $p < 0.05$.

Results

Effect of Simvastatin on Oxidative stress markers

Simvastatin significantly (P<0.001) increased lipid peroxidation compared to control (Fig. 1). Tissue Levels GSH was significantly (P<0.001) decreased after treatment with Simvastatin compared to control (Fig. 2). Treatment with Simvastatin showed significant (P<0.001) increased in SOD compared to control (Fig. 3). Simvastatin administration caused significant (P<0.001) decreased in Catalase compared to control (Fig. 4).

Fig. 1 Effect of Simvastatin on lipid peroxidation (n = 6), *** p<0.001, * compared with control

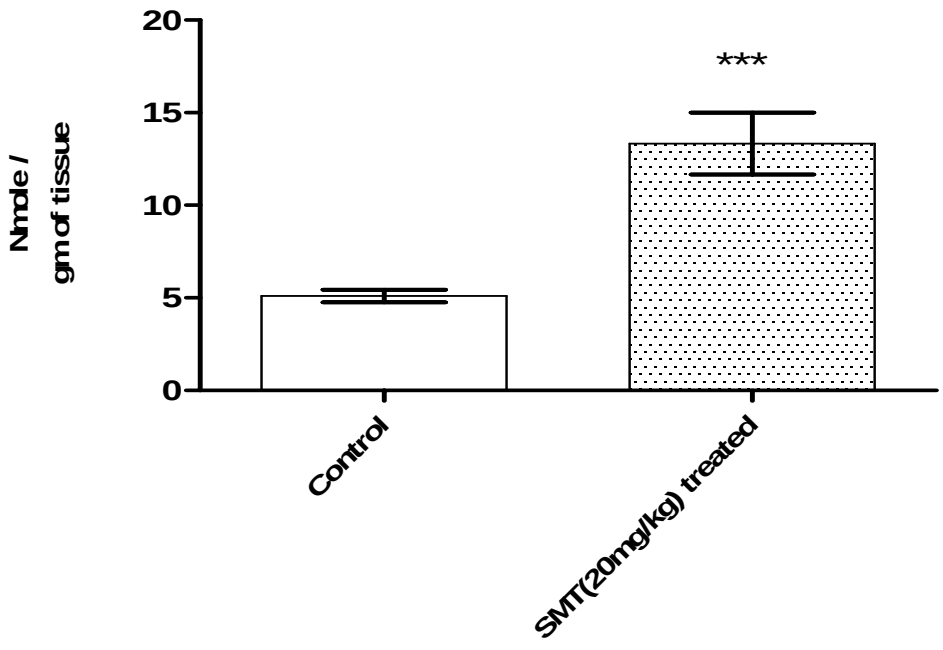


Fig. 2 Effect of Simvastatin on GSH (n = 6), *** p<0.001, * compared with control

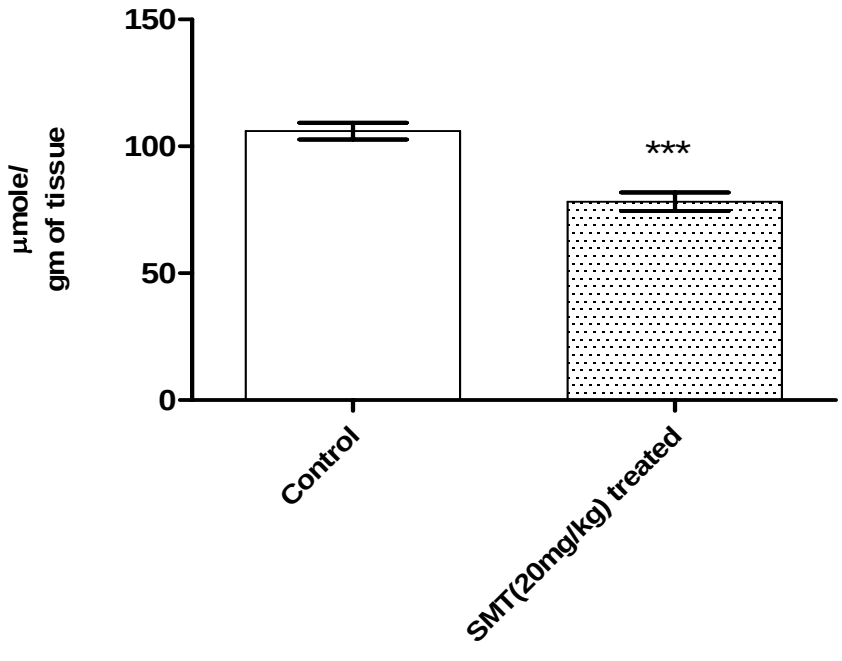


Fig. 3 Effect of Simvastatin on SOD (n = 6), *** p<0.001, * compared with control

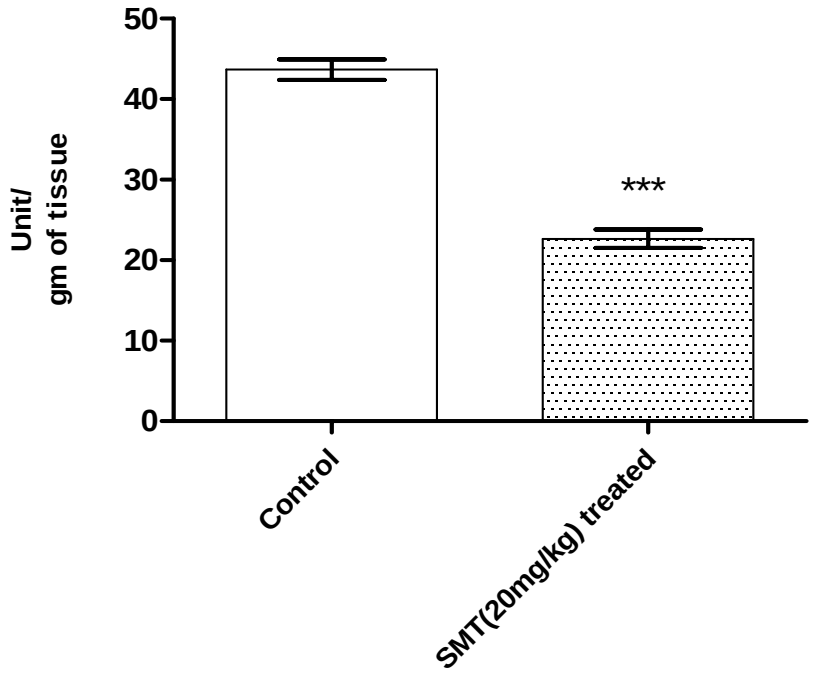
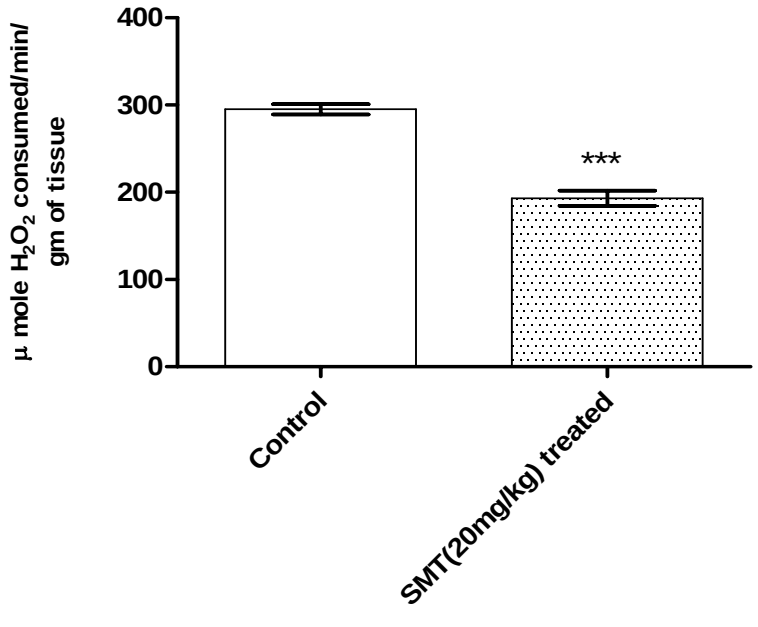


Fig. 4 Effect of Simvastatin on Catalase (n = 6), *** p<0.001, * compared with control



Effect of Simvastatin on Liver Function

Effect of Simvastatin on marker enzymes of liver Function

Serum levels of ALT and AST were significantly (P<0.001) increased after treatment with Simvastatin compared to control. Correspondingly, there was significantly (P<0.001) increased in the ratio of ALT/ AST after Simvastatin. Treatment with Simvastatin showed significant (P<0.001) increased in serum γGTP, ALP and LDH compared control *(Table: 1)*.

Groups	ALT (IU/L)	AST (IU/L)	ALT/AST	γGTP (IU/L)	ALP (IU/L)	LDH (IU/L)
Control	35.17 ± 2.613	40.00 ± 1.414	0.8800 ± 0.028	40.83 ± 1.014	152.5 ± 1.478	345.3 ± 4.702
Simvastatin treated	91.17 ± 2.786^{+++}	72.50 ± 0.991^{+++}	1.257 ± 0.051^{+++}	72.67 ±1.229^{+++}	248.0 ± 2.098^{+++}	508.3 ± 3.095^{+++}

Table: 1 Effect of SMT on Marker enzymes of liver function (n = 6), +++ p<0.001, + Compared with control

Effect of Simvastatin on Protein Profile

Simvastatin administration caused significant (P<0.001) increase in serum Total Bilirubin and Indirect Bilirubin and significant (P<0.01) decrease in Direct Bilirubin compared to control. Simvastatin produced significant (P<0.001) decrease in serum Total Protein compared to control. There ware no significant change in serum Globulin and Albumin after treatment with Simvastatin. Correspondingly the changes were observed in the ratio of Albumin/ Globulin.

Groups	Total Bilirubin (mg/dl)	Direct Bilirubin (mg/dl)	Indirect Bilirubin (mg/dl)	Total Protein (mg/dl)	Albumin (mg/dl)	A/G
Control	0.7033 ± 0.008	0.3017 ± 0.006	0.4050 ± 0.014	7.497 ± 0.116	4.995 ± 0.058	2.037 ± 0.128
Simvastatin treated	1.407 ± 0.046^{+++}	0.5000 ± 0.083^{++}	0.9067 ± 0.084^{+++}	6.483 ± 0.135^{+++}	4.660 ± 0.083	2.655 ± 0.253

Table: 2 Effect of SMT on Protein Profile (n = 6), +++ $p<0.001$, ++ $p<0.01$, + Compared with control

Effect of Simvastatin on Histopathological changes

Liver section of control rats revealed the normal hepatic hexagonal lobules and normal morphology. Liver tissue of Simvastatin treated rats showed degeneration of hepatocyte vacuolation, mild inflammation and piecemeal necrosis.

(a) **(b)**

Fig. 5 Hemayoxyline and Eosin stained section of rat liver tissue (magnification 40 X)
(a) Normal rat liver (b) Simvastatin (20mg/kg) treated rat liver

Discussion

Present study was designed to induce hepatotoxicity in rat by simvastatin that is having clinical application and *in vivo* assessment of oxidative stress mediated hepatotoxicity produced by simvastatin.

In present study increased in lipid peroxidation and depletion of antioxidant enzymes such as reduced glutathion, super oxide dismutase and catalase in Simvastatin treated animals compared to control animals indicated generation of oxidative stress. Simvastatin may cause oxidative stress mediated hepatotoxicity by reduction in mitochondrial CoQ10. The decrease in CoQ10 levels with simvastatin treatment could be the main factor responsible for activating the process of apoptosis, as seen in the increased cell death with higher simvastatin concentrations. By directly scavenging free radicals or by regenerating α- tocopherol, CoQ10 has protective effects on DNA from oxidative damage (11, 12).

CoQ10 is a carrier for both hydrogen ions and electrons in mitochondrial electron transport. The process of electron and hydrogen ion movement creates a proton electrochemical gradient across the mitochondrial inner membrane, driving ATP synthesis by the enzyme ATP synthase (13). Simvastatin reduced ATP by 50% and 80% respectively (13).

This again strengthens our hypothesis. Simvastatin is an HMG CoA reductase inhibitor. It results in depletion of mevalonate and thus leads to a reduction in cholesterol synthesis. However, depletion of mevalonate also results in a reduction in synthesis of isopentenyl pyrophosphate, geranyl and farnesyl pyrophosphate, and dolichol and ubiquinone. Isopentenyl pyrophosphate reduction may affect biosynthetic activity, while a reduction of geranyl and farnesyl pyrophosphate may potentially affect signal transduction. The effects of biosynthetic activity and signal transduction may in turn affect optimal cellular regulation, function and repair activities, potentially leading to cellular damage. It has been shown that replacement of mevalonate in rat skeletal muscle cells prevented the cytopathic effects caused by treatment with statins (14).

ALT and AST are enzymes produced within the cells of the liver, as the cells are damaged, leaks into the bloodstream leading to a rise in the serum levels. ALP is an enzyme, which is associated with the biliary tract, and is elevated; biliary tract damage and inflammation should be considered. It is used often times to confirm that the alkaline phosphatase is of the hepatic etiology by γGTP. Mild to moderate elevation of ALT, AST, ALP (1-3 times) is usually seen in drug toxicity (15, 16, 17). Elevated serum ALT, AST, ALT/AST, γGTP, ALP, LDH and CPK levels in simvastatin treated animals compared to control animals is attributed as damage to the structural integrity of liver (18), and presumptive markers of drug induced necrotic lesions in the hepatocyte.

Total bilirubin may rise in irritation of liver. Direct bilirubin fraction is that portion of bilirubin that has undergone metabolism by the liver, if the direct bilirubin is low, while the total bilirubin is high; this reflects liver cell damage or bile duct damage within the liver itself (16). The liver synthesizes albumin; it represents a major synthetic protein and is a marker for the ability of the liver to synthesize proteins. Low level indicates that the synthetic function of the liver has been markedly diminished (19).

Simvastatin treated animals significantly increased Total Bilirubin and decreased in Direct Bilirubin, Total Protein and Albumin reflects liver cell damage or bile duct damage and synthetic function of the liver has been markedly diminished, indicated drug induced hepatotoxicity. Simvastatin treated animal did not show that significant effect. No change in Albumin level in simvastatin treatment indicated simvastatin had no effect on synthetic function of liver (16).

Conclusion

Simvastatin hepatotoxicity was characterized by significant increase in oxidative stress along with marker enzymes of liver function and depletion of proteins. It can be concluded that Simvastatin caused oxidative stress mediated hepatotoxicity.

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