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Research Article

Effect of Liv-52 on Atorvastatin Induced Hepatotoxicity in Rats: A Biochemical and Histopathologic Study

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Abstract

Background and Objective: The toxic effect of atorvastatin on the liver has been associated with oxidative stress. The Liv-52 is a polyherbal formulation with known hepatoprotective properties. In this study, the preventive effect of Liv-52 against the potential hepatotoxicity of atorvastatin was investigated. **Materials and Methods:** Eighteen rats were categorized into three groups of six rats each: Healthy group (HG), atorvastatin-treated group (ATC) and Liv-52+atorvastatin-treated group (LAT). The Liv-52 was orally administered at 50 mg kg^{-1} and atorvastatin was orally given at 20 mg kg^{-1} after 1 hr of Liv-52 administration. The Liv-52 and atorvastatin were administered once daily for two months. At the end of two months, blood samples were collected from the rats. Subsequently, rats were sacrificed under high-dose (50 mg kg^{-1}) thiopental anesthesia and liver tissues were extracted. Biochemical analysis of extracted liver tissues and serum was performed. Liver tissues were additionally analyzed for histopathology. **Results:** The Liv-52+atorvastatin administration inhibited the atorvastatin-induced increase in malondialdehyde and decreased total glutathione and superoxide dismutase activities in the liver tissues. In addition, the rats receiving Liv-52 had lower levels of serum alanine aminotransferase, aspartate aminotransferase and lactate dehydrogenase compared to the atorvastatin group. Histopathologic analysis demonstrated that Liv-52 protected the liver tissue against atorvastatin-induced injury. **Conclusion:** The Liv-52 therapy may be useful in preventing atorvastatin-induced liver injury.

Key words: Liv-52, atorvastatin, hepatotoxicity, hepatotonic, oxidative stress

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Atorvastatin is a drug belonging to the statin group that exerts antihyperlipidaemic effect through inhibition of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase[1]. It is often used to treat peripheral vascular disease and stroke in addition to cardiovascular disease[2,3]. Although atorvastatin has a reasonable safe profile at therapeutic doses, it may cause various side effects such as myopathy and rhabdomyolysis[4,5]. Atorvastatin use has been reported to cause an increase in Alanine Aminotransferase (ALT) and Aspartate Transaminase (AST) activities and acute liver failure in rare cases[4,6]. Some studies have reported that the hepatotoxic effect of atorvastatin is associated with oxidative stress[7,8]. Hassan *et al.*[9] reported that atorvastatin caused oxidative liver damage by increasing malondialdehyde (MDA) in the rat livers and decreasing antioxidants including Glutathione (GSH) and catalase (CAT). Several natural compounds with antioxidant properties have drawn the attention of researchers for their potential effects against hepatotoxicity[10]. In the present study, Liv-52 was investigated, a polyherbal formulation, for its potential effect against atorvastatin-induced liver injury. The Liv-52 has been approved as an Ayurvedic medicine by the Indian Ministry of Health[11]. Treatment with Liv-52 has been observed to improve liver function by lowering elevated ALT and AST levels back to normal in cirrhotic patients[12]. In addition, Liv-52 was found to suppress liver damage by preventing increased lipid peroxidation (LPO) and depletion of superoxide dismutase (SOD) and GSH following ethanol administration[13]. The Liv-52 was thought to be useful in atorvastatin-induced hepatotoxicity, but no studies were found in this regard in the literature review. Therefore, this study was designed to investigate the effect of Liv-52 on the possible hepatic toxicity of atorvastatin in rats.

MATERIALS AND METHODS

Study area: This study was carried at the Atatürk University Medical Experiment Application and Research Centre between September and November, 2020.

Animals: Eighteen albino Wistar male rats, weighing 235-248 g, were purchased from Medical Experimental Application and Research Centre of Atatürk University (Turkey). The rats were divided into three groups and housed and fed in a laboratory (22±2°C). There was an automatic 12 hrs light-dark cycle in the environment of the rats. The study design was confirmed by the Local Animal Experiments Ethics Committee (Date: 26.12.2019 No: 16/240).

Chemicals: Atorvastatin was obtained from Pfizer (Istanbul, Turkey). Thiopental sodium was purchased by IE Ulagay (Istanbul, Turkey). The Liv-52 was purchased from Himalaya Drug (Maharashtra, India). Each Liv-52 tablet contains 65 mg *Capparis spinosa*, 65 mg *Cichorium intybus*, 33 mg *Mandur bhasma*, 32 mg *Solanum nigrum*, 32 mg *Terminalia arjuna*, 16 mg *Cassia occidentalis*, 16 mg *Achillea millefolium* and 16 mg *Tamarix gallica*.

Animal groups: The rats were categorized into three groups (per group 6 rats): Healthy group (HG), atorvastatin treated group (ATC) and Liv-52+atorvastatin-treated group (LAT).

Experimental procedure: For experiment, Liv-52 was orally administered at 50 mg kg^{-1} via gavage in the LAT group. The same volume of pure water was given orally to rats in the HG and ATC groups. One hour after having received Liv-52 and pure water, the LAT and ATC groups were given atorvastatin orally at 20 mg kg^{-1}. This protocol continued once a day for two months. Two months later, blood sample were collected from all the animals under thiopental-induced anesthesia. Subsequently, rats were sacrificed with 50 mg kg^{-1} thiopental anesthesia and liver tissues were extracted. Blood samples were analyzed for ALT, AST and Lactate Dehydrogenase (LDH) activity. Two tissue samples were taken from rats for biochemical and histopathological analysis. The MDA and tGSH levels and SOD activities were measured biochemically.

Biochemical analysis

MDA analysis of liver tissue: The MDA levels were determined by spectrophotometric measurement of the pink compound formed by the reaction of thiobarbituric acid with MDA, as described by Ohkawa *et al.*[14].

tGSH analysis of liver tissue: The tGSH levels were defined by the method described by Sedlak and Lindsay[15]. The 5,5'-dithiobis (2-nitrobenzoic acid) is reduced by reaction with sulfhydryl groups. In this method the measurement of the absorbance of the yellow colour which is the result of the reduction process[15].

SOD analysis of liver tissue: The SOD analysis was performed using the method of Sun *et al.*[16]. This assay method for determining SOD activity is based on the inhibition of nitroblue tetrazolium reduction by xanthine-xanthine oxidase resulting in the production of a superoxide. The resulting purple colour is measured spectrophotometrically.

Blood serum ALT analysis: The pyridoxal-5'-phosphate method recognised by the International Federation of Clinical Chemistry (IFCC) was used for ALT analysis. The ALT catalyses the reaction between alanine and 2-oxoglutarate. The pyruvate formed as a result of this reaction undergoes NADH-mediated reduction. There is a parallelism between NADH oxidised in this reaction and ALT activity. The detection of absorbance decrease at 340 nm was used to measure the enzyme activity (cobas 8000 autoanalyzer, Roche Diagnostic)[17].

Blood serum AST analysis: The AST analysis was conducted using the pyridoxal-5'-phosphate method according to the IFCC. The AST catalyses the reaction "L-Aspartate+2-oxoglutarate→oxaloacetate+L-glutamate". The oxaloacetate formed is reduced by NADH. The NADH oxidation is directly proportional to AST activity. The AST activity was evaluated by detecting the decrease in absorbance at 340 nm (cobas 8000 autoanalyzer, Roche Diagnostic)[18].

Blood serum LDH analysis: The LDH activity was determined using a method optimised by German Society for Clinical Chemistry. The LDH catalyses the reaction "Pyruvate+NADH+H^+→L-lactate+NAD^+". Initial NADH oxidation rate is directly proportional to LDH activity. The LDH activity was determined by detecting the decrease in absorbance at 340 nm (cobas 8000 autoanalyzer, Roche Diagnostic)[19].

Histopathological analysis: Liver tissues were kept in 10% formaldehyde solution. The tissues were washed and dehydrated by passing through (70-100%) alcohol. The liver tissues were cleared in xylol and paraffin blocks were formed. The obtained 4-5 µm sections were stained with haematoxylin-eosin and examined (Olympus DP2-SAL firmware Olympus® Inc., Tokyo, Japan). Tissues were scored between 0-3 in terms of damage (0: Normal, 1: Mild, 2: Moderate and 3: Severe).

Statistical analysis: Statistical procedures were run on the IBM SPSS Statistics Version 22.0 program. Numerical data were analysed by One-way ANOVA test. Then, *post hoc* Tukey's was applied. Numerical data were presented in "Mean Value±Standard Deviation". Kruskal-Wallis test was preferred for original data. Then, Dunn's test was used. Data were presented as median (quartile 1-3). Statistical significance was accepted at $p<0.05$.

RESULTS

Biochemical findings

MDA analysis results of liver tissue: The MDA levels were higher in the liver tissue of atorvastatin-treated rats than in HG ($p<0.001$). However, Liv-52 at 50 mg kg^{-1} inhibited the atorvastatin-induced increase in MDA ($p<0.001$) (Fig. 1a, Table 1).

tGSH analysis results of liver tissue: The tGSH level decreased in the livers of atorvastatin-treated rats according to the HG ($p<0.001$). The Liv-52 inhibited the atorvastatin-induced decrease of tGSH in liver tissues ($p<0.001$) (Fig. 1b, Table 1).

SOD analysis results of liver tissue: The SOD activity was found to be lower in the livers of ATC group compared to the healthy group ($p<0.001$). The SOD activity was higher in the Liv-52 treatment group than in the ATC ($p<0.001$) (Fig. 1c, Table 1).

ALT, AST and LDH analysis results of liver tissue: As presented in Fig. 2a-c and Table 1, ALT, AST and LDH activities in the ATC group were higher than in the healthy group ($p<0.001$). The Liv-52 inhibited the atorvastatin-induced increase in ALT, AST and LDH ($p<0.001$).

Table 1: Analysis of biochemical data obtained from experimental group

	Mean±Standard Deviation			p-values		
Parameter	HG	ATC	LAT	HG vs ATC	HG vs LAT	ATC vs LAT
MDA	3.27±0.27	6.50±0.34	3.63±0.32	<0.001	0.139	<0.001
tGSH	5.60±0.39	2.45±0.30	5.15±0.30	<0.001	0.80	<0.001
SOD	23.67±1.37	12.50±1.05	21.33±1.03	<0.001	0.09	<0.001
ALT	30.67±3.01	159.50±7.18	36.67±2.34	<0.001	0.101	<0.001
AST	39.17±2.86	212.17±7.57	43.83±3.60	<0.001	0.284	<0.001
LDH	128.33±14.42	299.50±7.58	131.33±18.22	<0.001	0.928	<0.001

MDA: Malondialdehyde, tGSH: Total glutathione, SOD: Superoxide dismutase, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, LDH: Lactate dehydrogenase, HG: Healthy group, ATC: Atorvastatin treated group, LAT: Liv-52+atorvastatin-treated group, statistical analysis was performed with One-way ANOVA-Tukey's HSD and $p<0.05$ was considered significant

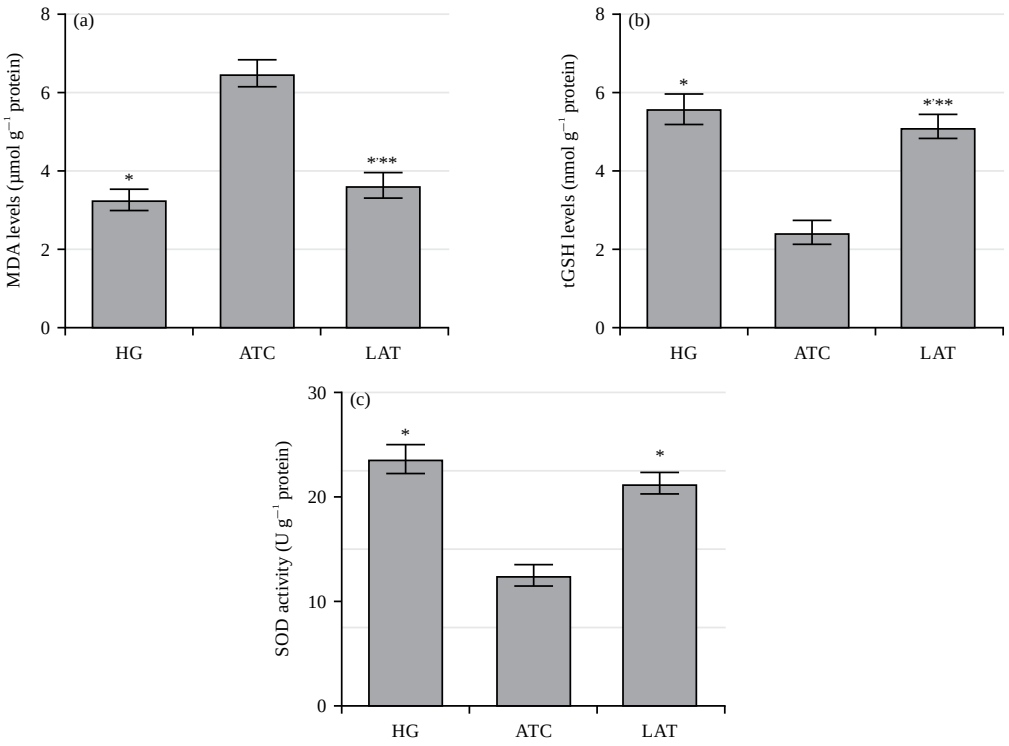

Fig. 1(a-c): Analysis of (a) MDA, (b) tGSH levels and (c) SOD activity in liver tissues

Bars show Mean±Standard Deviation, n = 6, *p<0.001 vs ATC, **p>0.05 vs HG, MDA: Malondialdehyde, tGSH: Total Glutathione, SOD: Superoxide dismutase, HG: Healthy group, ATC: Atorvastatin treated group and LAT: Liv-52+atorvastatin-treated group

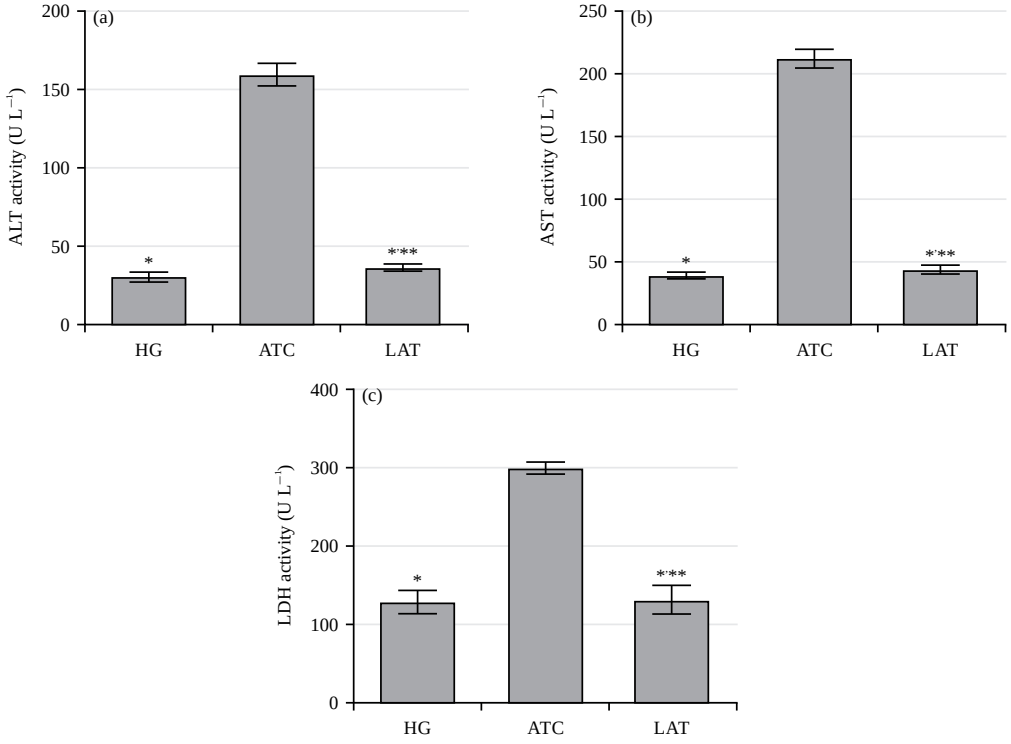

Fig. 2(a-c): Analysis of (a) ALT, (b) AST and (c) LDH activities in serum

Bars show Mean±Standard Deviation, n = 6, *p<0.001 vs ATC, **p>0.05 vs HG, ALT: Alanine Aminotransferase, AST: Aspartate Aminotransferase, LDH: Lactate dehydrogenase, HG: Healthy group, ATC: Atorvastatin treated group and LAT: Liv-52+atorvastatin-treated group

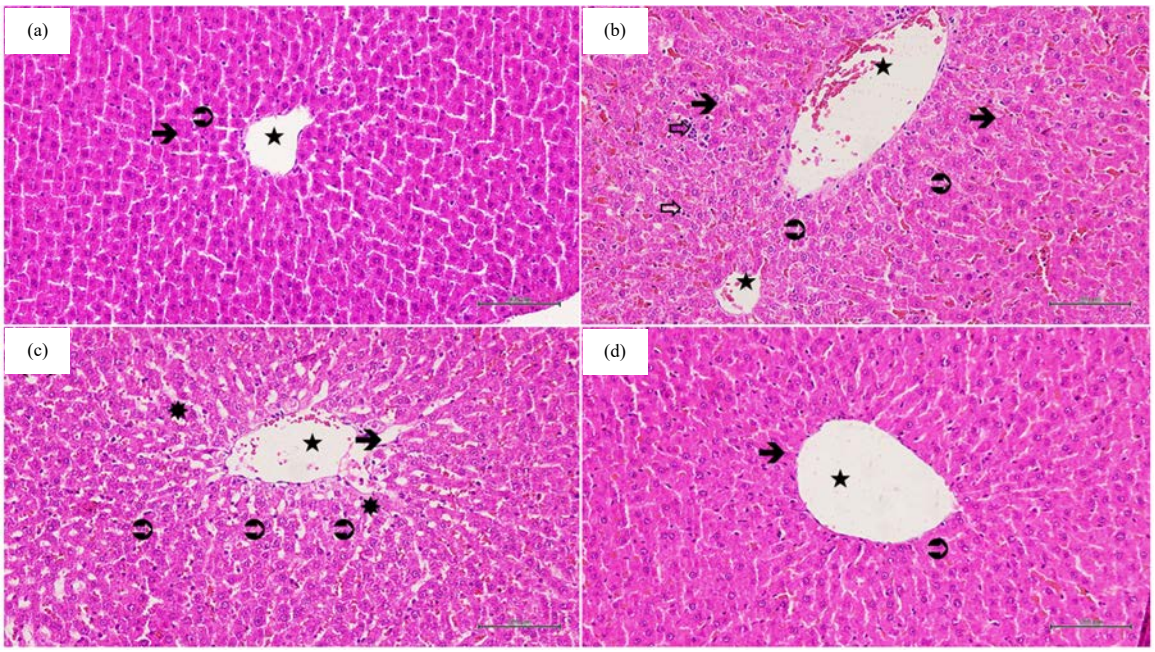

Fig. 3(a-d): Microscopic appearance of liver tissue prepared from (a) HG group, (b) ATC group, (c) ATC group and (d) LAT group
(a) ➜: Hepatocyte, ⊃: Kupffer cell, ★: Blood vessel (H&E ×200), (b) ➜: Degenerated swollen hepatocyte, ⊃: Increased Kupffer cells, ⇨: Dense polymorphonuclear cell infiltration, ★: Dilated and congested blood vessel (H&E ×200), (c) ➜: Degenerated swollen hepatocyte, ⊃: Increased Kupffer cells, ⇨: Dense polymorphonuclear cell infiltration, ✱: Oedema, ★: Congested blood vessel (H&E ×200) and (d) ➜: Hepatocyte, ⊃: Kupffer cell, ★: Blood vessel (H&E ×200), HG: Healthy group, ATC: Atorvastatin treated group and LAT: Liv-52+atorvastatin-treated group

Table 2: Analysis of hiatopathological data obtained from experimental group

	Median (quartile 1-3)			p-values		
Parameter	HG	ATC	LAT	HG vs ATC	HG vs LAT	ATC vs LAT
Degeneration	0 (0-0)	3 (2-3)	0.5 (0-1)	<0.001	0.026	<0.001
Kupffer cell activation	0 (0-0)	2 (2-3)	0 (0-0)	<0.001	0.661	<0.001
Congestion	0 (0-0)	2 (1-2)	0 (0-1)	<0.001	0.076	<0.001
Polymorphonuclear cell infiltration	0 (0-0)	3 (2-3)	0 (0-0)	<0.001	1.000	<0.001
Edema	0 (0-0)	3 (2-3)	0 (0-0)	<0.001	0.660	<0.001

HG: Healthy group, ATC: Atorvastatin treated group, LAT: Liv-52+atorvastatin-treated group, statistical analysis was performed with Kruskal Wallis test, Dunn's test and $p<0.05$ was considered significant

Histopathological findings: Analysis of the liver tissue from the healthy group revealed cords of hepatocytes, Kupffer cells and vessels were in line with normal tissue organization (Fig. 3a, Table 2). Liver sections from the group that received only atorvastatin exhibited swollen and degenerated hepatocytes, increased population of Kupffer cells compared to the control group and polymorphonuclear cell groups intensely infiltrated into the tissue. There was congestion and dilatation in the central arteries and other vessels (Fig. 3b, Table 2). Specimens from this group also exhibited intense tissue edema (Fig. 3c, Table 2). The LAT group had normal hepatocytes, Kupffer cell density relatively close to that of the healthy group and normal vascular structures (Fig. 3d, Table 2).

DISCUSSION

The present study involved a biochemical and histopathological investigation of the effect of Liv-52 on atorvastatin-induced liver toxicity in rats. The results of the biochemical assays revealed atorvastatin treatment led to a increase in the MDA amount in the liver tissue and a significant decrease in the endogenous antioxidants tGSH and SOD. The MDA, the last toxic product of LPO, increased in the liver tissue in response to atorvastatin treatment[20]. This product causes an imbalance in ion exchange across cell membranes, leading to cross-linking of membrane compounds and insufficient antioxidant enzyme activity[21]. Reports explaining the mechanism of oxidative stress in

atorvastatin-induced liver injury are scarce and controversial[7,22]. Hamzeh *et al.*[22] reported that the increase in MDA amount in cyclophosphamide-induced liver damage in experimental animals was significantly suppressed by atorvastatin treatment. Interestingly, Qin *et al.*[7] demonstrated that MDA levels increased with the increase in oxidative load in rats administered atorvastatin and damage occurred in the histological structures of liver tissues. Similarly, current study found that atorvastatin significantly increased the MDA level. Drug therapies may have varying effects on the liver tissue depending on the dosage regimen and duration of treatment[23]. Current results were in line with a report by Hassan *et al.*[9], showing atorvastatin administration over a long term caused toxic effects in the liver.

Oxidative liver damage caused by ROS has been studied in the context of changes in MDA and in antioxidant levels[24-26]. Thus, the present study measured the levels of tGSH, known as endogenous antioxidants. The GSH is a low molecular-weight tripeptide found at high concentrations in several tissues, particularly in the liver[27]. The GSH protects cells against oxidative damage through detoxification of hydroxyperoxide, peroxynitrite and lipid peroxides and scavenging of molecules such as superoxide anion, hydroxyl radicals and nitric oxide[28]. Studies have reported that GSH has an effect on oxidative stress and a possible therapeutic role in various liver diseases in which redox balance is altered[29,30]. Low hepatic GSH levels have been reported to exacerbate existing liver injury[30]. A previous study has reported that high-dose atorvastatin administration led to a decrease in GSH levels in serum samples and liver homogenates of rats[9].

In the present study, liver tissues from the group treated with atorvastatin alone exhibited a decrease in the activity of enzymatic antioxidant SOD. The SOD is one of the most essential antioxidant defense enzymes that catalyze the oxidation of superoxide radical to O_2 molecule and the reduction of another superoxide radical to hydrogen peroxide, a less reactive molecule[31]. This enzyme works together with GSH to limit the harmful effects of ROS and a decrease in its activity disrupts the structure of hepatocytes and leads to liver injury[32,33]. This study shows that atorvastatin administration caused a decrease in SOD activities in the livers of rats. Current study results seemed to be supported by Heeba and Abd-Elghany[34], who reported decreased SOD activity in hepatotoxicity cases caused by 20 mg kg^{-1} atorvastatin in rats and by Zeng and Liu[35], who reported decreased SOD activity in atorvastatin-induced oxidative liver damage in diabetic rats.

Liver function tests diagnose liver disease and assess response to treatment. Among these, serum activities of ALT, AST and LDH are considered to be the most useful biomarkers in assessing liver injury[36,37]. The serum levels of these enzymes increase when they are released into the bloodstream as a result of damage to the structural integrity of hepatocytes[36,38]. Studies have reported increased activities of ALT and AST after atorvastatin-induced liver injury[7,9,34,35]. This study showed that rats treated with atorvastatin had elevated serum ALT, AST and LDH activities, which were due to liver damage. The increase in ALT, AST and LDH activities may have resulted from the release of these enzymes into circulation due to damaged cell membrane integrity in connection with atorvastatin-induced oxidative stress.

The Liv-52, investigated for its potential effect on atorvastatin-induced liver injury in this study, significantly reduced the atorvastatin-induced increase in MDA. To our knowledge, there has been no previous study on the hepatoprotective effect of Liv-52 against atorvastatin-induced oxidative damage. However, preclinical and clinical studies have reported that Liv-52, a herbal formulation rich in phenolic compounds, reduced liver damage caused by chemical toxins[12,13]. In addition, Cimen *et al.*[39] reported that Liv-52 produced a hepatoprotective effect by significantly suppressing the increase in MDA levels caused by ischemia-reperfusion injury in rat livers. The results from the present experiment and previous studies lead to the conclusion that Liv-52 exerts its hepatoprotective effect through the inhibition of ROS-related LPO due to its antioxidant properties. Current study results also demonstrate that Liv-52 significantly suppressed the atorvastatin-induced reduction in antioxidant levels in liver tissue. These results overlapped with previous studies reporting that Liv-52 protects liver tissue from oxidative stress by inhibiting the doxorubicin-induced decrease of tGSH and SOD levels in rats[40]. Previous reports and the present study suggest that Liv-52 suppresses the production of oxidant molecules and the consumption of antioxidants, resulting in a hepatoprotective effect. Based on liver function tests, the present study also demonstrated the hepatoprotective effect of Liv-52 in atorvastatin-induced liver injury. The Liv-52 administration in rats was found to suppress atorvastatin-induced increases in ALT, AST and LDH activities, which then returned to near-normal values. These results were supported by some studies reporting that Liv-52 inhibits the increase in serum activities of ALT, AST and LDH and reduces liver injury[39,40].

There have been reports of fatal cases of hepatotoxicity due to atorvastatin[41]. Atorvastatin, which increases oxidant levels, also caused degeneration of hepatocytes, polymorphonuclear cell infiltration, congestion, dilatation and edema. The Liv-52 inhibited the amiodarone-induced increase in oxidants and decrease in antioxidants, attenuating histopathologic damage to the liver tissue. This shows that biochemical results in the present study relate with histopathologic findings. Atorvastatin may lead to several pathologic changes in hepatocytes such as degeneration, edema and inflammatory cell infiltration[42]. Atorvastatin treatment of rats caused histopathological changes such as serum and tissue MDA increase, vascular congestion with antioxidant depletion, deformed hepatocyte nuclei, inflammatory mononuclear cell infiltration[9]. Notably, atorvastatin-treated diabetic rats even developed hepatocellular necrosis[35].

The fact that Liv-52, a polyherbal formulation, has been approved as an ayurvedic medicine and has been used for many years is encouraging for clinical studies on atorvastatin-induced hepatotoxicity. Previous clinical studies have shown that Liv-52 has a protective effect on the liver without any side effects in alcohol-induced hepatic damage and hepatitis B virus infection[12]. It has also been tested in patients with cirrhosis, the end stage of liver damage and was observed to improve liver function after 6 months[12].

The limitation of this study was that the measurement of proinflammatory and anti-inflammatory cytokine levels in the liver tissues of animals treated with atorvastatin and Liv-52 may help further elucidate the mechanisms of action of atorvastatin and Liv-52.

CONCLUSION

Atorvastatin induced oxidative damage to the liver by disrupting the oxidant/antioxidant balance in the liver tissue in favor of oxidants. Histopathologic results of the present study demonstrated atorvastatin-induced oxidative and inflammatory damage to the liver tissue. However, Liv-52 significantly inhibited the deterioration of oxidant/antioxidant balance in favor of oxidants in the liver tissue. In addition, Liv-52 caused liver enzyme levels to reach normal values, which improved liver function. The Liv-52 thus, attenuated atorvastatin-induced histopathologic liver injury. The antioxidant and hepatoprotective activity of Liv-52 is mostly linked to flavonoids and phenolic acids, also known as natural antioxidants. The results of the present study suggested that Liv-52 may be useful in the treatment of liver injury during atorvastatin therapy.

SIGNIFICANCE STATEMENT

Statins, including atorvastatin, are widely used in the treatment of hyperlipidaemia. Muscle tissue and the liver are the most commonly affected by toxic effects. Given the frequency with which atorvastatin is used, studies to prevent toxic effects are even more important. This study has shown that the use of Liv-52, which is considered to be hepatoprotective, with atorvastatin significantly protects the liver. It is believed that current study will contribute to clinical studies in this field.

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