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Original Article

Esculetin Alleviates Acute Liver Failure following Lipopolysaccharide/D-Galactosamine in Male C57BL/6 Mice

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Abstract

Background: Acute liver failure (ALF) is a fatal clinical situation that rapidly leads to the loss of normal liver function. Esculetin is a natural herbal compound used for the management of various diseases such as cardiovascular and renal disorders. In this study, we evaluated the protective effects of esculetin in a mouse model of ALF.

Methods: This article is a report on an experimental study that was conducted at Iran University of Medical Sciences in 2019. Forty-eight male C57BL/6 mice were randomly divided into control, LPS/D-Gal, and LPS/D-Gal+Esculetin (40 mg/kg) groups (n=16 per group). ALF was induced with an intraperitoneal injection of lipopolysaccharide (LPS)/D-galactosamine (D-Gal).The LPS/D-Gal group received a mixture of LPS (50 µg/kg) and D-Gal (400 mg/kg). The LPS/D-Gal+Esculetin group received esculetin by gavage 24 hours and one hour before receiving LPS/D-Gal. Six hours after LPS/D-Gal injection, the mice were sacrificed. Liver injury markers, including alanine aminotransferase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP), were measured in the serum. Oxidative stress indices and inflammatory markers such as interleukin-1 beta (IL-1β), IL-6, and tumor necrosis factor-alpha (TNF-α) were measured in hepatic tissue. The histopathology of liver tissue was also assessed. The data were analyzed using one-way ANOVA, followed by the *post hoc* Tukey test.

Results: Esculetin lowered oxidative stress and myeloperoxidase activity ($P<0.001$); reduced the serum levels of ALT ($P=0.037$), AST ($P=0.032$), and ALP ($P=0.004$); and decreased the hepatic levels of IL-1β ($P=0.002$), IL-6 ($P=0.004$), toll-like receptor 4 ($P<0.001$), TNF-α ($P=0.003$), and nuclear factor-kappa B ($P<0.001$) as compared with LPS/D-Gal. Additionally, esculetin ameliorated hepatic tissue injury following LPS/D-Gal challenge.

Conclusion: Esculetin can reduce liver injury through the mitigation of oxidative burden, inflammation, and neutrophil infiltration and also exerts hepatoprotective effects against ALF.

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Keywords ● Liver failure, Acute ● Lipopolysaccharides ● Inflammation ● Oxidative stress

What's Known

- Acute liver failure is a fatal clinical situation that rapidly leads to the loss of normal liver function.
- Previous studies reported the anti-inflammatory and anti-oxidative stress effects of esculetin.

What's New

- This is the first study on the effects of esculetin on lipopolysaccharide/D-galactosamine–induced acute liver failure.
- Esculetin could protect the liver by reducing inflammation, oxidative stress, and neutrophil infiltration.

Introduction

Acute liver failure (ALF), also known as acute liver injury, is a dangerous clinical syndrome that causes high morbidity and

mortality.[1] Various factors including toxins, drugs, and viruses are allied to ALF.[1] ALF is associated with such severe complications as hepatic encephalopathy, lung injury, and cerebral edema.[1] It can lead to the rapid loss of liver function even in the absence of a history of liver disease as indicated by an increase in liver enzymes, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP).[2] Lipopolysaccharide (LPS) is a toxic compound that is found on the outer surface of Gram-negative bacteria and can trigger intense inflammatory responses.[3] The co-administration of LPS and D-galactosamine (D-Gal) to mice can lead to acute hepatitis, which is a useful experimental model in the evaluation of the pathogenesis of acute liver inflammation in humans.[4] LPS binds to the toll-like receptors (TLR) on Kupffer cells and stimulates the secretion of inflammatory cytokines such as interleukin-1 beta (IL-1β), IL-6, and tumor necrosis factor-alpha (TNF-α), which have significant roles in the development of liver damage.[5] TNF-α binds to its receptor and activates the nuclear factor-kappa B (NF-κB) pathway, stimulating the expression of inflammatory and anti-apoptotic genes.[6] ALF in humans and its animal models is associated with enhanced neutrophil infiltration, accompanied by higher activity of myeloperoxidase (MPO) as its specific biomarker. Moreover, D-Gal inhibits protein production by depleting uridine triphosphate pools in hepatocytes. This phenomenon causes the formation of reactive oxygen species (ROS) and finally leads to apoptosis.[4] Acting together, LPS and D-Gal can cause extensive damage to the liver.[4]

Esculetin is a natural herbal compound, which is chemically assigned to the category of simple coumarins.[7] Esculetin has been used in the treatment of various diseases such as cardiovascular disorders and neurological disorders and has also presented protective effects.[8] Esculetin reduces the rate of the inflammation of liver cells, neutrophil infiltration, and liver cell necrosis, in a model of hepatic injury induced by tert-Butyl hydroperoxide by reducing oxidative stress.[9] Studies showed that esculetin can inhibit various signaling pathways associated with oxidative stress such as nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2) and NF-κB in various diseases.[10, 11] Esculetin can protect cells against oxidative stress by activating the Nrf2/quinone oxidoreductase 1 pathway through the activation of the extracellular-signal-regulated kinase pathway. The excessive production of inflammatory mediators such as TNF-α, NF-κB, IL-1β, and IL-6 play important roles in the development of acute liver damage.[12] LPS can also cause extensive damage to liver tissue by triggering oxidative stress and apoptosis.[12] It has also been observed that esculetin can reduce carbon tetrachloride-induced apoptosis in the rats' liver, possibly by scavenging free radicals and inhibiting mitochondrial-dependent apoptosis pathways.[13] Various studies showed anti-inflammatory, anti-oxidative, and anti-apoptotic effects for esculetin.[8, 9] Hence, we hypothesized that esculetin could reduce LPS/D-Gal–induced liver injury in C57BL/6 mice by reducing oxidative stress and inflammation. In the light of the aforementioned research, we conducted this study to test whether esculetin could ameliorate ALF induced by LPS/D-Gal.

Materials and Methods

Animal Grouping and Applied Treatments

This article is a report on an experimental study that was conducted at Iran University of Medical Sciences in 2019. Forty-eight male C57BL/6 mice (20–25 g) were purchased from the Animal House of the Baqiyatallah University of Medical Sciences (Tehran, Iran). The animals were kept for 14 days before the experiments in a standard situation, including 22±2 °C, 50% humidity, and 12 hours of dark/light cycles, for adaptation. They had access to standard chow and water *ad libitum*. All the experiments on the animals were conducted in accordance with the ethics guidelines of Iran University of Medical Sciences (Ethics Committee's approval code: IR.IUMS.FMD.REC.1397.300) and keeping with the *National Institutes of Health* principles for the care and use of experimental animals.[14] The mice were randomly divided into three groups using the random number table randomization method: the control group; the LPS/D-Gal group, which received a mixture of LPS (50 μg/kg) and D-Gal (400 mg/kg); and the LPS/D-Gal+Esculetin group, which received esculetin at a dose of 40 mg/kg by gavage.[11] The dose of esculetin (i.e., 40 mg/kg) was selected from previous studies on the strength of its ability to alleviate the progression of asthma in a mouse model of allergic asthma[15] and its ameliorative effects in LPS-induced models of acute lung injury in mice.[11] Each group of mice was divided into two subgroups containing 10 (for all the measurements except the MPO assay) and six (for MPO measurements) animals.[16] The mice in the LPS/D-Gal group received a combination of LPS from *Escherichia coli* (50 μg/kg, Sigma-Aldrich, St. Louis, M.O., U.S.A.; 0111:B4) and D-Gal (400 mg/kg, Sigma-Aldrich, St. Louis, M.O., U.S.A.; G0500) intraperitoneally.[17] Esculetin (Sigma-Aldrich, St. Louis, M.O.,

U.S.A.; Y0001611) was given 24 hours and one hour before the administration of LPS/D-Gal orally. Six hours after the LPS/D-Gal challenge, the animals were sacrificed. Then, the blood samples and the liver tissue samples were collected for further evaluation. In our pilot study, no significant change was observed in hepatic aminotransferase enzymes in the tested control group at this dose of esculetin. Hence, such a control group was not included.

Blood Biochemical Tests

Blood samples were taken from the left ventricle of the mice and centrifuged at 3000 rpm for 10 minutes. The serum was then separated and stored at −70°C until the next assessment. The serum indices of liver function, including ALT, AST, and ALP, were evaluated using commercial kits from Pars Azmoon Company (Tehran, Iran).

Evaluation of Biomarkers Associated with Oxidative Stress in Liver Tissue

Median lobes of the liver were homogenized in 150-mM Tris-HCl buffer (pH 7.4; Sigma Aldrich, USA). After centrifuge, the supernatant was aliquoted and stored at −70°C for the following assays.

The malondialdehyde (MDA) content of the supernatant was determined according to previous studies.[18] For the determination of the MDA concentration as thiobarbituric acid reactive substances (TBARS), the supernatant was mixed with a combination of trichloroacetic acid (Sigma-Aldrich, U.S.A.) and TBARS (Sigma-Aldrich, U.S.A.) in boiling water for 90 minutes. After cooling, the samples were centrifuged at 1000 *g* for 10 minutes, and absorbance was obtained at 532 nm with the final results calculated from the tetraethoxypropane standard curve.[19]

For the measurement of ROS, a 2, 7-dichlorofluorescein diacetate fluorescent probe (DCFH-DA, Molecular Probes, Eugene, O.R, U.S.A.) was used. The interaction between intracellular ROS and DCFH-DA results in fluorescent dichlorofluorescein.[20] Briefly, 10-µL DCFH-DA (10 µM) was mixed with 150-µL tissue homogenates and incubated at 37°C for 40 minutes. The quantification of the Dichlorofluorescein (DCF) fluorescence intensity at an excitation of 488 nm and an emission of 525 nm was obtained using a fluorescence microplate reader (BioTek, Winooski, Vermont, U.S.A.).

Nitrite was estimated colorimetrically with the Griess reagent (Sigma-Aldrich, U.S.A.) in the liver homogenate as previously described.[21] Briefly, equal volumes of the liver homogenate and the Griess reagent (sulfanilamide 1% w/v, naphthyl ethylenediamine dihydrochloride 0.1% w/v, and orthophosphoric acid 2.5% v/v) were mixed and incubated at room temperature for 10 minutes with the absorbance determined at a 540 nm wavelength; the volumes were then compared with those of the known concentrations of sodium nitrite. The amount of nitrite formed was normalized to the protein content.

Catalase activity was evaluated using the Claiborne protocol.[22] For this purpose, H_2O_2 was added to the mixture of the supernatant and 50-mM potassium phosphate buffer (pH 7.0), and its decomposition was evaluated at 240 nm.

The superoxide dismutase (SOD) activity assay was measured according to previous reports.[23, 24] Briefly, the supernatant was incubated with xanthine (Sigma-Aldrich, U.S.A.) and xanthine oxidase (Sigma-Aldrich, U.S.A.) in potassium phosphate buffer (pH 7.8, 37°C; Sigma Aldrich, USA) for 40 minutes. Then, nitro blue tetrazolium (Sigma-Aldrich, U.S.A.) was added. Thereafter, blue formazan formation was monitored at 550 nm. The amount of protein that inhibited nitro blue tetrazolium reduction to 50% maximum was regarded as one nitrite unit of SOD activity.

Ultimately, the Bradford method was used to measure the amount of protein with bovine serum albumin as its standard.[25] Briefly, in this assay, Coomassie Brilliant Blue G-250 (Sigma Aldrich, USA) was dissolved in 95% ethanol and 85% phosphoric acid, and the obtained reagent was used after filtering. In this respect, 100-µL supernatant was added to the 5-mL Bradford reagent and after five minutes, its absorbance was read at 595 nm.

Determination of Hepatic TNF-α, IL-6, IL-1β, NF-κB, TLR4, and Nrf2

The level of these biomarkers in hepatic tissue was measured via the enzyme-linked immunosorbent assay according to the manufacturer's instructions (for Nrf2 and TLR4 from Cloud Clone Corp. [Houston, Texas, U.S.A.], for TNF-α from Sigma-Aldrich [St. Louis, M.O., U.S.A.], and NF-kB, IL-1β, and IL-6 from Abcam [Cambridge, M.A., U.S.A.]). The absorbance of the samples was read using a Synergy HT microplate reader (BioTek, Winooski, Vermont, U.S.A.), and final values were obtained in accordance with plotted standard curves.

Evaluation of MPO Activity

In this study, the measurement of MPO activity was used as an indicator of neutrophil infiltration and determined according to former study.[24] In brief, liver tissue homogenates were prepared

in cetyltrimethylammonium bromide buffer (SigmaAldrich, USA) and centrifuged at 15000*g* for 20 minutes. Thereafter, the samples were combined with H_2O_2 and 3,3',5,5'-tetramethylbenzidine at 37°C for five minutes. After the addition of H_2SO_4, the absorbance was determined at 450 nm.

Histopathologic Analysis of the Liver

Liver tissue samples were obtained six hours after LPS/D-Gal injection. The samples of liver tissue were fixed in 10% formalin solution, processed, embedded in paraffin blocks, sectioned at a thickness of 5 µm, and stained with hematoxylin and eosin (H&E) stain. Histological changes were randomly analyzed in selected fields at a magnification of 200X. The severity of the changes was assessed in accordance with an earlier study.[26] In this respect, scores of 1 to 3 were assigned to the specimens of minimal damage, scores of 4 to 8 to the specimens of mild damage, scores of 9 to 12 to the specimens of moderate damage, and scores of 13 to 18 to the specimens of intense damage.

Statistical Analysis

The data were reported as mean±SEM. The Kolmogorov–Smirnov test was used to ensure the normal distribution of the data. Afterward, the one-way ANOVA test, followed by a *post hoc* Tukey test, was applied using SPSS software, version 22.0. Then, the data were analyzed and the charts weredrawn in GraphPad Prism, version 8. In this study, a P value of less than 0.05 was considered a significant level.

Results

Esculetin Effects on Liver Damage Biomarkers

ALT (figure 1A), AST (figure 1B), and ALP (figure 1C), which are markers of liver damage, were significantly elevated (P<0.001) in the blood of the study mice following the LPS/G-Gal challenge. However, after the treatment with esculetin, the serum levels of ALT (P=0.037), AST (P=0.032), and ALP (P=0.004) significantly decreased in comparison with those in the LPS/D-Gal group. Nonetheless, the levels of these factors were still higher than those of the control group.

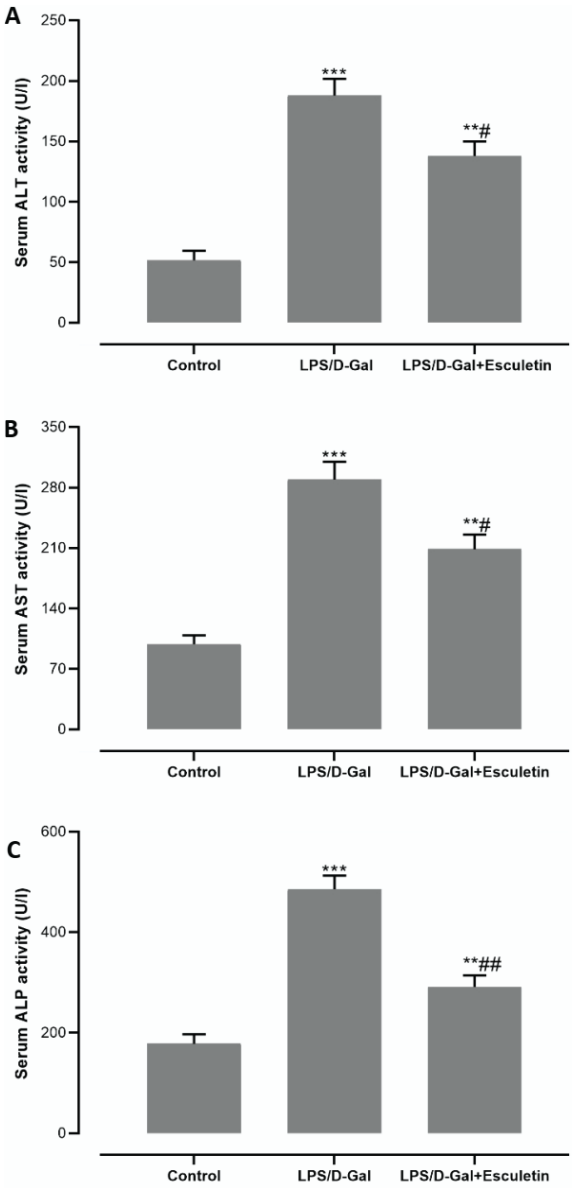

Figure 1: LPS/D-Gal challenge increased the serum biomarkers of liver function, including ALT (A), AST (B), and ALP (C). LPS/D-Gal challenge significantly increased all of these factors of liver injury compared with those of the control group (***P<0.001). Treatment with esculetin was able to significantly reduce these indices by comparison with treatment with LPS/D-Gal (for ALT #P=0.037, for AST P=0.032, and for ALP ##P=0.004); however, it failed to reduce them to the level of the control group and there was still a significant difference with the latter group (**P<0.001). The data are presented as mean±SEM (n=8). ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase; LPS: lipopolysaccharide; D-Gal: D-galactosamine; U/l: unit/liter

Esculetin Effects on Oxidative Stress Factors

Following ALF induction, oxidative stress was increased due to the excessive production of ROS, and the weakening of the antioxidant system in liver tissue, which can lead to extensive liver damage. Therefore, the effect of esculetin on the level of oxidative stress markers was explored. The mice, which received LPS/D-Gal had high levels of MDA (P=0.006, figure 2A, nitrite (P=0.007, figure 2B), ROS (P<0.001, figure 2C), and Nrf2 (P=0.059, figure 2F,) compared to the control group. The findings also showed a significant decrease in the activity of catalase (P=0.009, figure 2D) and SOD (P<0.001, figure 2E) in comparison with that of the control group. Treatment with esculetin not only reduced the

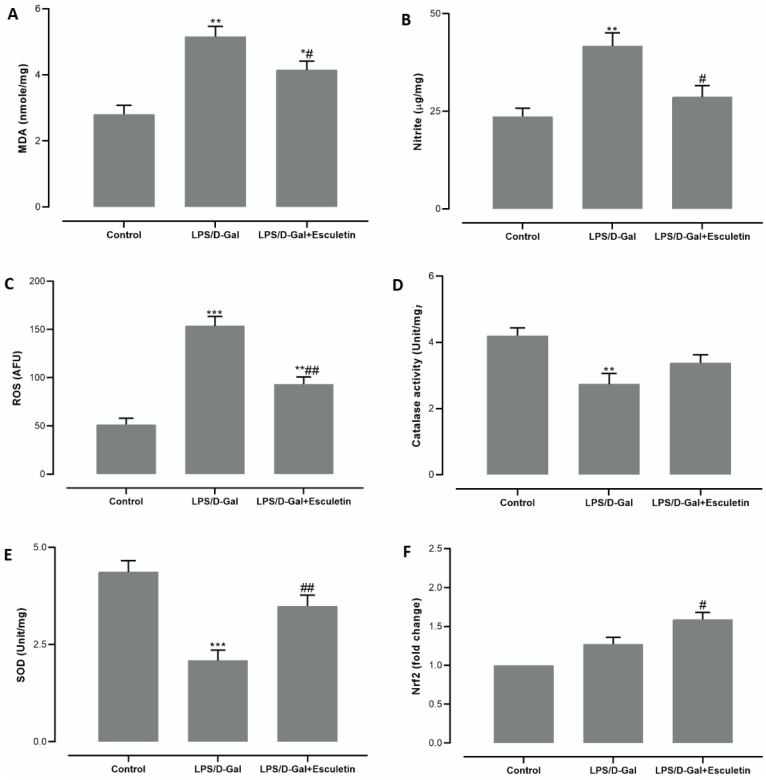

Figure 2: This figure represents the hepatic levels of MDA (A), nitrite (B), ROS (C), catalase activity (D), SOD (E), and Nrf2 (reported as fold change) (F) in the different groups. LPS/D-Gal challenge significantly increased the hepatic levels of MDA (**P=0.006), nitrite (**P=0.007), and ROS (***P<0.001) in comparison with those of the control group. Treatment with esculetin was able to significantly reduce these indices in comparison with treatment with LPS/D-Gal (MDA $^{\#}$P=0.041, nitrite $^{\#}$P=0.025, and ROS $^{\#\#}$P=0.004). LPS/D-Gal challenge decreased the hepatic levels of catalase and SOD compared with those of the control group (**P=0.009 and ***P<0.001, respectively), and treatment with esculetin increased the SOD level by comparison with treatment with LPS/D-Gal. Treatment with esculetin also increased the hepatic level of Nrf2 in comparison with the level in the LPS/D-Gal group ($^{\#}$P=0.043). The data are presented as mean±SEM (n=10). MDA: malondialdehyde; ROS: reactive oxygen species; SOD: superoxide dismutase; Nrf2: nuclear factor erythroid 2 (NFE2)-related factor 2; LPS: lipopolysaccharide; D-Gal: D-galactosamine; AFU: arbitrary fluorescence unit

levels of MDA (P=0.041), nitrite (P=0.025), and ROS (P=0.004) but also improved SOD (P=0.008) and Nrf2 (P=0.043) compared with the levels in the LPS/D-Gal group.

Effects of Esculetin on Liver Inflammatory Factors

Our experiments showed that the hepatic levels of IL-1β (P=0.002), IL-6 (P=0.004), TLR4 (P<0.001), TNF-α (P=0.003), and NF-κB (P<0.001) were significantly elevated in the LPS/D-Gal group compared to the control group (figure 3). In addition, our findings showed that esculetin administration to the LPS/D-Gal group decreased the liver levels of all of the mentioned variables significantly in comparison with the levels in the LPS/D-Gal group (P=0.029 for TNF-α, P=0.035 for NF-κB, P=0.007 for TLR4, P= 0.008 for IL-1β, and P=0.005 for IL-6). However, the levels of these factors were still higher than those in the control group.

Effects of Esculetin on Liver MPO Activity

The hepatic levels of MPO (figure 4), as an index of neutrophil infiltration, were also evaluated. The administration of LPS/D-Gal to the mice led to a remarkable increase in MPO (P<0.001) compared with the MPO level of the control group. Treatment with esculetin significantly reduced MPO (P<0.001) compared with treatment with LPS/D-Gal.

Effects of Esculetin on Liver Histology

The effects of esculetin on ALF were evaluated. The administration of LPS/D-Gal resulted in damage to liver tissue. Nevertheless, the administration of esculetin greatly reduced ALF. The protective effect of esculetin on LPS/D-Gal–induced ALF (injury) was assessed by H&E staining. Our results showed that the liver tissue of the LPS/D-Gal–challenged group indicated significant pathologic changes, including derangement of the hepatic architecture, severe hemorrhagic patches, and immense infiltration of inflammatory cells. These pathologic alterations were markedly lower following esculetin pretreatment (figure 5). In addition, the scoring of liver damage (figure 6) according to

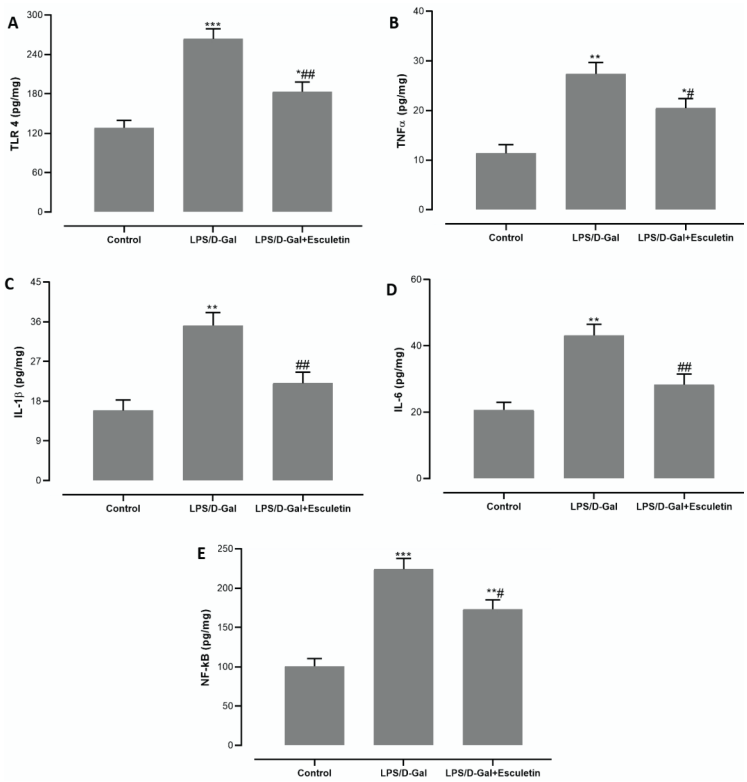

Figure 3: Hepatic levels of TLR4 (A), TNF-α (B), IL-1β (C), IL-6 (D), and NF-κB (E) are illustrated in liver tissue. LPS/D-Gal challenge significantly increased TLR4 (***P<0.001), TNF-α (**P=0.003), IL-1β (**P=0.002), IL-6 (**P=0.004), and NF-κB (***P<0.001) in comparison with those of the control group. Treatment with esculetin decreased the hepatic levels of these factors in comparison with those of the LPS/D-Gal group (TNF-α #P=0.029, NF-κB #P=0.035, TLR4, ##P=0.007, IL-1β ##P=0.008, and IL-6 ##P=0.005). The data are presented as mean±SEM (n=8). IL-1β: interleukin 1 beta; IL-6: interleukin 6; TLR4: toll-like receptor 4; TNF-α: tumor necrosis factor-alpha; NF-κB: nuclear factor kappa B; LPS: lipopolysaccharide; D-Gal: D-galactosamine

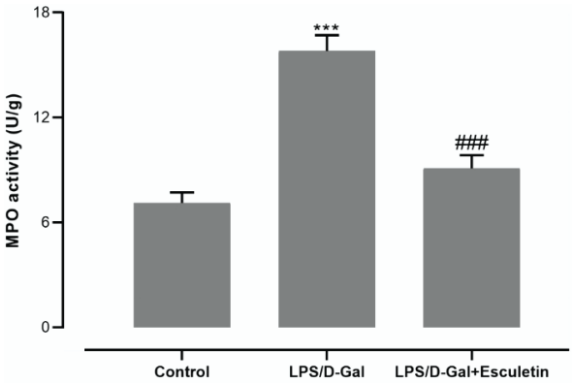

Figure 4: MPO activity, as a marker of neutrophil infiltration, is indicated in the different groups. LPS/D-Gal challenge significantly increased MPO activity in the LPS/D-Gal group (***P<0.001). In contrast, treatment with esculetin significantly decreased MPO activity in comparison with treatment with LPS/D-Gal (###P<0.001). The data are presented as mean±SEM (n=8). MPO: myeloperoxidase; LPS: lipopolysaccharide; D-Gal: D-galactosamine

its severity showed a significantly higher score for the LPS/D-Gal group than the control group (P<0.001), and this was less significant in the LPS/D-Gal group under treatment with esculetin (P=0.035). Additionally, the score of damage for the latter group was significantly lower than that of the LPS/D-Gal group (P=0.007).

Discussion

Our major finding was that esculetin prevented the development of hepatic injury through the reduction of oxidative stress and inflammation-associated factors.

LPS/D- Gal–induced ALF has been widely used to investigate both the pathologic mechanisms of this disease and the therapeutic effects of various medications.[1] In this study, following the development of the ALF model, liver injury markers, including ALT, AST, and ALP, increased in the serum. There was also a rise in oxidative stress and inflammatory indices as a result of ALF. Nonetheless, our findings showed that the use of esculetin significantly reduced liver damage caused by LPS/D-Gal. LPS, known as endotoxin, is a bacterial compound found on the outer wall of Gram-negative bacteria.[27] In the context of liver damage associated with LPS, in the first step, LPS should be attached to TLR4 so as to activate it. This receptor then activates the NF-κB pathway through specific intermediate proteins. The activated NF-κB enters the nucleus and causes several inflammatory cytokine expressions such as TNF-α, IL-1β, and IL-6.[28] Among the cytokines produced

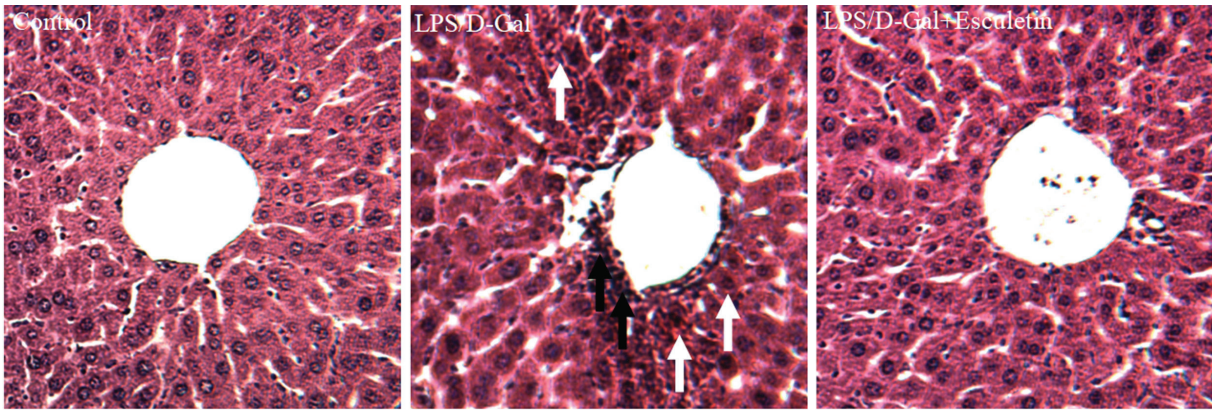

Figure 5: Histopathological changes in liver tissue (H&E, X200) are indicated in the control group, the LPS/D-Gal group, and the LPS/D-Gal group treated with esculetin at a dose of 40 mg/kg. Injection of LPS/D-Gal induced damage to liver tissue, as shown by derangement of the hepatic architecture, severe hemorrhagic patches, and immense infiltration of inflammatory cells, and the administration of esculetin greatly reduced liver injury. The solid white arrows show areas of extensive hemorrhage, and the solid black arrows show areas of inflammatory cell infiltration.

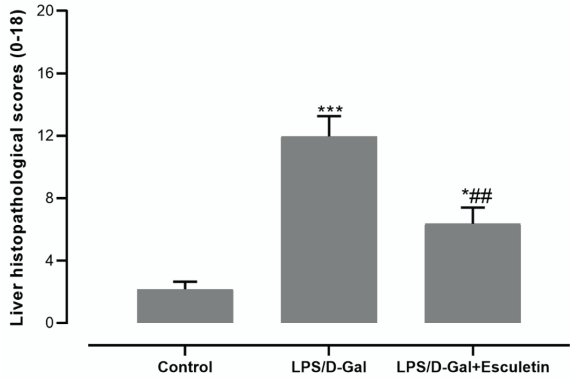

Figure 6: Scoring of liver damage according to its severity showed a significantly higher score for the LPS/D-Gal group than the control group (***P<0.001), and this was less significant in the LPS/D-Gal group under treatment with esculetin (*P=0.035). Additionally, the score of damage of the latter group was significantly lower than that of the LPS/D-Gal group (##P=0.007). The data are presented as mean±SEM. LPS: lipopolysaccharide; D-Gal: D-galactosamine

following the LPS/D-Gal challenge, TNF-α plays an important role in the development of liver damage.[29] Previous studies showed the key pathogenic role of TNF-α in liver damage caused by LPS/D-Gal, and its elevation in serum and tissue has been documented in LPS/D-Gal–induced liver damage.[30, 31] In the liver, TNF-α is primarily secreted by activated Kupffer cells, and its mechanism of action is through the induction of an imbalance in the secretion of pro- and anti-inflammatory cytokines, as well as the induction of liver cell apoptosis.[5] Moreover, IL-1β produced by immune cells in the liver leads to inflammation.[32] IL-1β, through its synergistic action with TLR4 signaling, exerts its pro-inflammatory effects,[33] which increases the production of cytokines induced by LPS from the TLR4 signaling pathway. Furthermore, in the pathologic conditions of the liver, IL-1β causes the recruitment of inflammatory cells, especially neutrophils, to liver tissue.[34] Other studies indicated that TNF-α and IL-1β together can promote cell death in hepatocytes.[2, 35] We found that the use of esculetin, as a therapeutic agent, could reduce the expression of inflammatory cytokines such as TNF-α, IL-1β, and IL-6. Therefore, it seems that esculetin can lower the severity of liver damage by modulating inflammatory cytokines and processes.

D-Gal inhibits protein production in hepatocytes, causing ROS formation and ultimately apoptosis.[36] Based on previous research, oxidative stress promotes TNF-α–induced apoptosis and necrosis in hepatocytes.[36] The focus of recent research, therefore, besides inflammation, has been on how to prevent oxidative stress. Recently, Gong and colleagues showed that pretreatment with tamoxifen prevented ALF induced by LPS/D-Gal.[37] Another study showed that the use of esculetin in the treatment of LPS/D-Gal–induced ALF diminished the degree of damage through anti-inflammatory and anti-oxidative activities.[10] In the present study, in the same line with other studies that used the same liver injury model, we found that following the LPS/D-Gal challenge, equilibrium moved toward oxidative stress factors, including MDA, ROS, and nitrite. It has been well-established that under oxidative stress conditions, the concentration of free radicals increases, causing tissue damage and cell death.

ROS causes membrane lipid peroxidation, cell and mitochondrial membrane degradation, endoplasmic reticulum dysfunction, and intracellular macromolecule damage. In the normal state, antioxidant defense systems such as SOD, catalase, and glutathione peroxidase enzymes eliminate free radicals. In our study, esculetin induced antioxidant effects by inducing antioxidant enzymes, which is marked by a significant increase in SOD and a marked decrease

in MDA. However, for the other antioxidant marker, catalase, esculetin administration at a dose of 40 mg/kg was not able to significantly increase catalase activity after the induction of ALF in our C57BL/6 mice. This insignificant effect may be ascribed to the possibly insufficient dose of esculetin and/or its short period of administration. The ALF process is associated with increased levels of liver injury biomarkers and higher levels of nitrite and nitrate.[38] Conversely, esculetin administration was capable of diminishing the liver levels of nitrite in our study, indirectly indicating its suppression of nitrosative stress.

One of the main activities of Nrf2 having been studied in the past decade is its role in resistance to oxidative stress.[39] In the cell, Nrf2 is blocked through connection to Keap1. Nrf2 is activated by oxidants and then induces the expression of a wide range of enzymes and signaling proteins to regulate drug metabolism and antioxidant defense.[39] Nrf2 also has anti-inflammatory and anti-apoptotic effects.[39] Esculetin has ROS scavenging activity inside the cell through the activation of antioxidant enzymes.[39] Han and colleagues reported that esculetin could increase the amount of Nrf2 phosphorylation and simultaneously decrease the amount of its inhibitor Keap1 (an increase in the Nrf2/Keap1 ratio).[10] In the present study, following the use of esculetin in the treatment of ALF, the amount of Nrf2 significantly increased and oxidative stress indices decreased. Consequently, it seems that esculetin plays an important role in reducing oxidative stress and protecting the liver from damage.

ALF development is followed by an increase in the infiltration of neutrophils into liver tissue, which could increase liver damage by the production of ROS and inflammatory mediators.[36] Accordingly, we measured the level of MPO, an index of neutrophil infiltration. Still, we observed that esculetin for the treatment of ALF reduced the activity of MPO, indicating decreased neutrophil filtration.

The liver tissue of our LPS/D-Gal–challenged group had notable pathologic changes, including derangement of the hepatic architecture, severe hemorrhagic patches, and immense infiltration of inflammatory cells.

The strength of this study lies in the fact that it is the first report of esculetin effects on LPS/D-Gal–induced ALF. To the best of our knowledge, this is the first report on the beneficial efficacy of esculetin in ameliorating the damage caused by ALF by reducing oxidative stress and inflammatory processes. One of the limitations of our study was the lack of other molecular and histochemical methods such as Western blotting and gene expression assays, which precluded us from a precise depiction of the beneficial effects of esculetin in our model of liver injury.

Conclusion

The present study demonstrated that the use of esculetin reduced oxidative stress, inflammation, and neutrophil infiltration in the liver tissue of mice challenged with LPS/D-Gal. Therefore, esculetin may be considered as a protective agent for acute liver injury.

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