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Research Article
Modulating Effects of Vanillic Acid on Sepsis-induced Oxidative Liver Injury in Rat Model

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Abstract

Background and Objective: Sepsis is a condition that causes multiple organ failure leading to mortality. Oxidative stress has a pivotal role in the development of sepsis causing organ failure. So, the aim of this study was to analyze the effect of vanillic acid on sepsis-induced liver damage in rats. **Materials and Methods:** Male Sprague Dawley rats were segregated into four groups; sham group, sepsis control group, vanillic acid control group and vanillic acid treated sepsis group. Sepsis was induced by cecal ligation and puncture (CLP). Rats were intraperitoneally (i.p.) injected with 100 mg kg^{-1} b.wt. vanillic acid in 0.5 mL kg^{-1} b.wt. saline upon sepsis induction and sacrificed after 24 h of treatment for biochemical and histopathological analysis. **Results:** The levels of reduced glutathione (GSH) and antioxidant enzymes (glutathione peroxidase, superoxide dismutase, catalase) were markedly reduced ($p<0.05$) in the sepsis control group. Malondialdehyde (MDA) levels, liver damage markers (AST, ALT, ALP) and pro-inflammatory cytokines (TNF-α, IL-6) in the liver tissues and plasma of rats were elevated ($p<0.05$) in sepsis control group. Treatment with vanillic acid significantly reversed ($p<0.05$) the effects of sepsis on oxidative stress markers and pro-inflammatory cytokine levels compared to sepsis control group. Histopathological changes in liver were observed in vanillic acid treated rats as compared to sepsis control group. **Conclusion:** So, it was concluded that vanillic acid is able to prevent the progression of sepsis and protect against oxidative liver injury. Further research is needed to ascertain the mechanism of vanillic acid in preventing sepsis-induced organ damage.

Key words: Liver damage, sepsis, vanillic acid, multiple organ failure, pro-inflammatory cytokines

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Competing Interest: The authors have declared that no competing interest exists.

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

INTRODUCTION

Sepsis is a critical condition that causes multiple organ failure in intensive care units leading to mortality of patients. Sepsis could be triggered by unwanted trauma during operations, infections, toxins, weak immune systems and few other factors[1]. This serious condition needs attention in the medical sector and researchers and physicians are still opting for treatment methods to decrease the mortality rate incurred by sepsis[2]. Although, it is time consuming, there is a need for a practical treatment method to solve this problem in emergency and intensive care units. The imbalance between inflammatory responses tends to increase the severity of sepsis[3]. Moreover, Reactive Oxygen Species (ROS) are responsible for the development of sepsis in vital organs such as; liver, kidney, heart and colon[4]. A state of oxidative stress under excessive production of ROS creates a critical response in the internal environment leading to organ failure characterized by the inflammatory reaction by pro-inflammatory cytokines, endothelial damage caused by lipid peroxidation, mitochondrial and DNA impairment[5]. Liver is a large organ that plays multiple roles including immunological and metabolic homeostasis and is vulnerable to the early dysfunction due to sepsis[6]. It has been reported that sepsis induced liver dysfunction could happen within 1.5 h upon Cecal Ligation and Puncture (CLP)[1]. Lack of antioxidant defense is a major factor that causes the progression of sepsis in the liver due to uncontrolled levels of ROS.

Naturally occurring antioxidants are commonly used for scavenging ROS and free radicals due to their ability to donate electrons. Numerous antioxidant compounds from plants have been studied for their liver protective potentials[6,7]. Vanillic acid (4-hydroxy-3-methoxybenzoic acid) is a common phenolic compound found in honey and other natural products including the root of *Angelica sinensis* which is a herb used in traditional Chinese medicine[8]. This secondary metabolite is the oxidized form of vanillin and is normally used as a food flavoring agent. Vanillic acid has several pharmacological values such as; liver protective, anti-diabetic, anti-inflammatory, anti-asthmatic and anti-hypertensive[9-11]. Therefore, vanillic acid was selected for this study to ascertain its modulating effects against sepsis-induced liver injury in rats. This model of study has not been conducted previously by using vanillic acid, hence the effects on sepsis-induced oxidative stress, inflammatory markers and histopathological alterations were evaluated in this research work.

MATERIALS AND METHODS

Chemicals: Vanillic acid was purchased from Sigma Aldrich, USA. Commercial kits for biochemical analysis, ELISA assay and histopathological analysis were obtained from Pure One Biotechnology Ltd. (Shanghai, China). Chemicals and reagents used for this study were purest in grade from Sigma Aldrich, USA.

Methods

Animals: Male Sprague Dawley rats aged 4-6 weeks, weighing 200-250 g were obtained from the animal housing facility of medical college district of Cangzhou People's Hospital, China. The animals were placed in polypropylene cages under controlled temperature (25±2°C) and 50% humidity under 12 h light/dark cycle. The animals were given free access to food and water, but were fasted overnight with free access to water alone on the day before experiment.

Cecal ligation and puncture (CLP) model: This study was conducted for 8 months beginning in May, 2018 at the laboratory of Medical College district of Cangzhou People's Hospital, China. The experiment was performed with the approval from animal ethics committee of Cangzhou People's Hospital (Ethical Number: CZPH201904A). The experimental procedure for CLP was done by following the method of Bacanli *et al.*[12] where rats were anesthetized with an intraperitoneal (i.p.) injection of ketamine hydrochloride (90 mg kg^{-1} b.wt.) under aseptic conditions and a midline laparotomy was performed under minimal dissection. The cecum was ligated slightly below ileocecal valve with 3-0 silk ligatures to maintain intestinal continuity and was punctured at the antimesenteric surface at two locations (1 cm apart) and small amount of feces was gently squeezed out. All animals were resuscitated with intraperitoneal (i.p.) injection of 0.5 mL kg^{-1} b.wt. saline and were maintained under same condition.

Experimental design: About 32 male Sprague Dawley rats were separated into 4 groups containing 8 animals (n = 8) in each group. The groups are described as:

- **Sham group:** The animals underwent laparotomy and resuscitated with 0.5 mL kg^{-1} b.wt. saline (i.p.) alone
- **Sepsis control group:** The animals underwent CLP and resuscitated with 0.5 mL kg^{-1} b.wt. saline (i.p.) alone
- **Vanillic acid+sepsis group:** The animals underwent CLP and immediately treated with 100 mg kg^{-1} b.wt. vanillic acid in 0.5 mL kg^{-1} b.wt. saline (i.p.)

- **Vanillic acid control group:** Animals underwent laparotomy and immediately treated with 100 mg kg^{-1} b.wt. vanillic acid in 0.5 mL kg^{-1} b.wt. saline (i.p.)

Laparotomy and CLP were performed in aseptic conditions under anesthesia. The dose of vanillic acid was selected based on previous literature[13]. All animals were sacrificed through decapitation under anesthesia after 24 h of treatment to collect the liver organ and blood serum. Liver tissues were cleaned and rinsed with PBS and separated for biochemical and histopathological experimentations. The samples were stored at -80°C until further analysis.

Determination of the oxidative stress markers: Oxidative markers such as; reduced glutathione (GSH), antioxidant enzymes (GPx, SOD and CAT) and lipid peroxidation (MDA) levels were determined through biochemical analysis by following the protocols of Aydin *et al.*[5] and Bacanli *et al.*[12]. The liver tissues were homogenized in phosphate buffer saline (pH 7.4) in 5 folds volume. The homogenate (10% tissue) were centrifuged at 10,000 ×g for 15 min in 4°C. A portion of the supernatant obtained was de-proteinized using 4% sulfosalicylic acid (Sigma Aldrich, USA) for GSH analysis. The remaining supernatant was used for analysis of antioxidant enzymes (GPx, CAT, SOD) and lipid peroxidation levels (MDA formation). All the biochemical analysis were performed using commercially available assay kits (Pure One Biotechnology Ltd., Shanghai, China) following the protocol provided by the manufacturer. Briefly, the GSH levels were read spectrophotometrically at 412 nm by measuring the formation of 5-thiol-2-nitrobenzoic acid. The lipid peroxidation levels were read spectrophotometrically at 535 nm by measuring the formation thiobarbituric acid conjugate with MDA. The units for GSH and lipid peroxidation are given as nmol mg^{-1} tissue. Activity of antioxidant enzyme GPx was read spectrophotometrically at 340 nm by measuring the rate of oxidation of NADPH to $NADH^{+}$. The unit is given as µg GSH consumed/min/mg tissue. Activity of SOD was read spectrophotometrically at 470 nm by measuring the inhibition rate of pyrogallol auto-oxidation. The unit is given as units/mg tissue. Activity of CAT was read spectrophotometrically at 240 nm by measuring the decomposition rate of H_2O_2. The unit is given as µmol H_2O_2 consumed/min/mg tissue.

Determination of serum liver damage markers: Liver damage marker enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were determined in serum biochemical analysis following the protocols of Mihailovic *et al.*[14]. Blood collected from the animals were left at room temperature for 30 min to clot and then centrifuged at 2000 ×g for 10 min in 4°C. The serum obtained was used for analysis of AST, ALT, ALP and pro-inflammatory cytokines. Commercial diagnostic kits were used for determination of serum AST, ALT and ALP levels, following the protocol provided by the manufacturer. The units for AST, ALT and ALP levels are given as units/liter of serum.

ELISA assay for determination of pro-inflammatory cytokines: The levels of pro-inflammatory cytokines TNF-α and IL-6 were determined in the serum of rats through ELISA assay by using commercially available kits (Pure One Biotechnology Ltd., Shanghai, China) following the protocol provided by the manufacturer. The analysis was performed according to the method mentioned by Santhanam *et al.*[15].

Histopathological analysis: Liver tissue sections were fixed in 10% buffered formaldehyde for 48 h, embedded in paraffin and sectioned to 5 µm thickness using microtome. The sections were stained with hematoxylin and eosin, viewed under light microscope with photographic facility (Olympus BX61, USA) and photographed at 100× magnification to study the histopathological alterations caused by sepsis and the protective measure of vanillic acid.

Statistical test: All experimental results were expressed as mean±standard error of mean of 8 animals (n = 8). The statistical test for all experiments was performed with windows statistical package for social sciences version 19.0 (SPSS Inc., USA) using one-way analysis of variance (ANOVA) and Tukey's multiple group comparison *post-hoc* analysis. Significance level for the tests were fixed at 95% interval, where p-values less than 0.05 ($p<0.05$) were significant.

RESULTS

Levels of GSH, MDA and antioxidant enzymes (GPx, CAT, SOD): The levels of tissue GSH and MDA of all animal groups were shown in Table 1. The level of GSH was significantly

Table 1: Vanillic acid modulates the levels of reduced glutathione (GSH) and malondialdehyde (MDA) on sepsis-induced liver injury in rats

Groups	GSH (nmol mg^{-1} tissue)	MDA (nmol mg^{-1} tissue)
Sham group	3.15±0.35	18.39±0.89
Sepsis control	1.17±0.28#	46.27±4.16#
Vanillic acid+sepsis	2.49±0.47**	25.03±1.27**
Vanillic acid control	3.32±0.38**	16.88±1.08**

Data presented as mean and standard error of mean of 8 animals (n = 8), #p<0.05 compared to untreated sham group, **p<0.05 compared to sepsis control group

Table 2: Vanillic acid modulates the levels of antioxidant enzymes on sepsis-induced liver injury in rats

Groups	SOD (Units/mg tissue)	CAT (μmol H_2O_2 consumed/min/mg tissue)	Gpx (μg GSH consumed/min/mg tissue)
Sham group	148.58±12.31	15.26±0.82	88.66±5.81
Sepsis control	106.56±13.32#	9.32±0.68#	35.18±3.94#
Vanillic acid+sepsis	128.30±10.72**	12.14±0.93**	72.38±6.17**
Vanillic acid control	145.56±11.43**	15.18±0.75**	85.40±2.86**

Data presented as mean and standard error of mean of 8 animals (n = 8), #p<0.05 compared to untreated sham group, **p<0.05 compared to sepsis control group, SOD: Superoxide dismutase, CAT: Catalase, GPx: Glutathione peroxidase

Table 3: Vanillic acid modulates the levels of serum liver damage markers on sepsis-induced liver injury in rats

Groups	AST (U L^{-1})	ALT (U L^{-1})	ALP (U L^{-1})
Sham group	127.86±16.12	85.48±9.92	532.54±18.21
Sepsis control	819.72±19.17#	282.38±14.21#	823.12±17.38#
Vanillic acid+sepsis	428.48±15.87**	131.70±12.62**	632.24±15.30**
Vanillic acid control	136.64±18.26**	80.56±8.57**	517.82±17.54**

Data presented as mean and standard error of mean of 8 animals (n = 8), #p<0.05 compared to untreated sham group, **p<0.05 compared to sepsis control group, AST: Aspartate Aminotransferase, ALT: Alanine Aminotransferase and ALP: Alkaline Phosphatase ALP

Table 4: Vanillic acid modulates the levels of pro-inflammatory cytokines in plasma of sepsis-induced liver injury in rats

Groups	TNF-α (pg mL^{-1})	IL-6 (pg mL^{-1})
Sham Group	117.80±16.88	96.30±8.61
Sepsis control	281.66±17.23#	227.86±5.68#
Vanillic acid+sepsis	152.48±18.72**	148.38±7.71**
Vanillic acid control	108.24±12.44**	102.27±5.89**

Data presented as mean and standard error of mean of 8 animals (n = 8), #p<0.05 compared to untreated sham group, **p<0.05 compared to sepsis control group, TNF-α: Tumor necrosis factor-alpha, IL-6: Interleukin-6

(p<0.05) reduced in sepsis control group as opposed to the sham group, but vanillic acid significantly increased (p<0.05) the GSH level in vanillic acid treated sepsis group as opposed to sepsis control group. Hepatic MDA formation significantly elevated (p<0.05) in sepsis control group as opposed to the sham group, but vanillic acid significantly (p<0.05) reduced the levels of MDA in vanillic acid treated sepsis group as opposed to sepsis control group. The activities of antioxidant enzymes GPx, SOD and CAT of all animal groups were given in Table 2. The activities of GPx, SOD and CAT were significantly reduced (p<0.05) in sepsis control group as opposed to the sham group, but vanillic acid significantly elevated (p<0.05) the activities of GPx, SOD and CAT in vanillic acid treated sepsis group as opposed to sepsis control group. Vanillic acid control group showed almost similar results to sham group.

Serum AST, ALT and ALP levels: The levels of serum liver damage marker enzymes ALT, AST and ALP in all animal groups were displayed in Table 3. The levels of serum biomarkers ALT, AST and ALP were significantly increased (p<0.05) in sepsis control group as opposed to the sham group. Vanillic acid significantly (p<0.05) decreased the levels of ALT, AST and ALP in vanillic acid treated sepsis group as opposed to sepsis control group. Vanillic acid control group showed no significant difference to sham group.

Pro-inflammatory cytokine (TNF-α and IL-6) levels: Levels of pro-inflammatory cytokines TNF-α and IL-6 for all animal groups determined through ELISA assay were shown in Table 4. The manifestation of TNF-α and IL-6 were significantly high (p<0.05) in sepsis control group as opposed to the sham group. Vanillic acid significantly reduced (p<0.05) the expressions of the pro-inflammatory cytokines in vanillic acid treated sepsis group as opposed to sepsis control group. Vanillic acid control group results did not differ significantly from sham group.

Histopathological alterations due to sepsis: Histopathological changes of liver sections in all animal groups were shown in Fig. 1(a-d). The sham group showed normal hepatocytes with distinct central vein and nuclei. In contrast, the sepsis control group showed heavy distortion in the hepatocyte arrangements. There were signs of lobular inflammation, centrilobular necrosis, deranged central vein and fatty changes in the sepsis control group. Vanillic acid treated sepsis group exhibited mild inflammation and slightly dilated sinusoidal spaces. Overall hepatocyte arrangements were almost normal in the vanillic acid treated sepsis group. The vanillic acid control group had healthy hepatic arrangement with distinct central vein similar to the sham group.

DISCUSSION

Initiation of sepsis can be triggered by several factors such as; infections, trauma and toxic exposure but oxidative

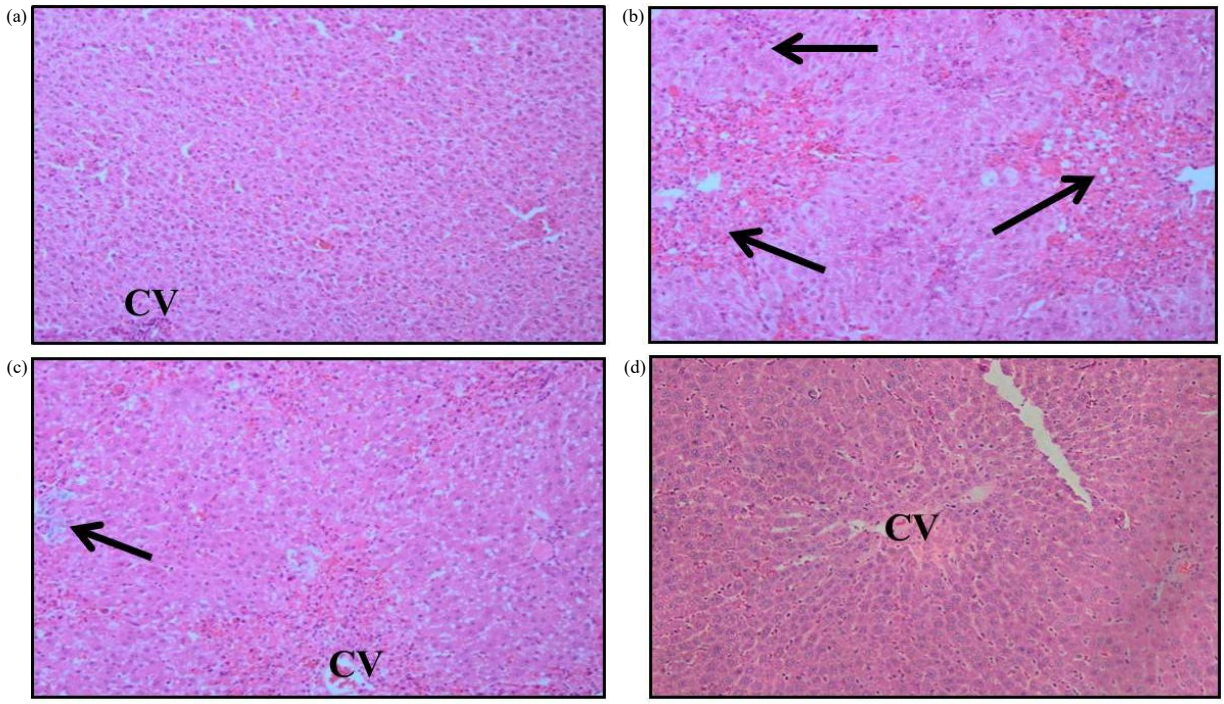

Fig. 1(a-d): Histopathological alterations observed in the liver tissue of all groups under hematoxylin and eosin staining, 100× magnification, (a) Sham group showing normal hepatocytes with distinct central vein and nuclei, (b) Sepsis control group: Hepatic cells showing necrotic cells, lobular inflammation and deranged central vein, black arrows indicated signs of lobular inflammation and necrosis, (c) Vanillic acid treated sepsis group: Hepatocytes showing signs of recovery with reduced inflammation and slightly dilated sinusoidal spaces, black arrow indicates signs of dilated sinusoidal and (d) Vanillic acid control group showing healthy arrangement of hepatocytes with distinct central vein
CV: Central vein

stress and inflammatory response are responsible in the progression of sepsis in organs, leading to organ failure[16,17]. From the results of this study, it was observed that the levels of hepatic GSH and the activities of GPx, CAT and SOD were reduced in the sepsis control group compared to sham group. This indicated the liver was under oxidative stress, causing depletion of GSH, which is an antioxidant and reduced activities of antioxidant enzymes that are supposed to control ROS formation. Moreover, the levels of MDA were elevated in the sepsis control group as opposed to the sham group. High MDA levels showed lipid peroxidation was taking place in the hepatic cells at an uncontrolled condition. Therefore, sepsis initiated through CLP had progressed to the liver organ through oxidative stress causing damage to the hepatic cells. Vanillic acid treatment in the CLP performed sepsis group significantly prevented the progression of sepsis in liver by restoring the levels of GSH, GPx, SOD and CAT. The levels of MDA were reduced significantly by vanillic acid treatment, which showed lipid peroxidation of hepatic cells was prevented. The ameliorative potential of vanillic acid on the oxidative stress markers is similar to the results of Bacanli *et al.*[12] and Taner *et al.*[18].

Serum biomarker enzymes ALT, AST and ALP were commonly measured in experimental models involving liver organ to identify the extent of damage that has occurred in the organ. These biomarkers are found in high concentrations in the blood serum once the hepatic cells are severely damaged. The results of serum biomarkers showed elevated levels of AST, ALT and ALP in sepsis control group compared to the sham group. This proved liver injury was prominent due to sepsis, causing the leakage of these marker enzymes into the blood serum. Treatment with vanillic acid significantly reduced the levels of serum ALT, AST and ALP in sepsis induced group. Vanillic acid significantly prevented the sepsis induced hepatic damage. These results are comparable to the findings of Zhong *et al.*[19]. Inflammatory response is a cause for sepsis progression leading to organ damage and failure[20]. The levels of

pro-inflammatory cytokines TNF-α and IL-6 estimated through ELISA assay exhibited prominent increase in the sepsis control group in contrast to the sham group. This indicated inflammation was developed in the hepatic cells due to sepsis. Vanillic acid treated sepsis group showed remarkably reduced expression of TNF-α and IL-6 in disparity to the sepsis control group. Therefore, vanillic acid prevented inflammatory response and ultimately protected the liver from damage due to sepsis. These findings are in agreement with the results of Maurya *et al.*[4] and Zhong *et al.*[19].

Histopathological alterations due to sepsis induced oxidative liver injury was evidenced in the results of sepsis control group. The extent of liver damage was observed in the tissue sections of sepsis control group with enormous lobular inflammation and centrilobular necrosis. These sightings can be related to the biochemical results of sepsis control group indicating oxidative stress and inflammatory responses. Vanillic acid clearly prevented most of the damage caused by sepsis as evidenced in the histopathological results. The normal hepatic cell arrangements and mild inflammation related the prevention of oxidative stress and inflammation by vanillic acid. The architecture of damaged liver tissue and a recovered liver tissue by vanillic acid treatment can be related to the findings of Alomar and Al-Attar[21]. The histopathological results of vanillic acid control group showed normal hepatic arrangements with distinct central vein similar to the sham group. This indicated vanillic acid did not cause any damage to the liver.

Overall, this study provided evidence that of vanillic acid was able to prevent the progression of sepsis in liver from the CLP. Expressions of oxidative stress markers and inflammatory mediators were significantly reversed in the liver by the i.p. injection of vanillic acid, therefore it can be said that vanillic acid worked from the extracellular environment by preventing sepsis from initiating oxidative injury in liver organ. The histopathological findings supported that vanillic acid treated liver tissues were not affected by sepsis induced by CLP.

CONCLUSION

This study concluded that vanillic acid had significant protective effects against sepsis-induced oxidative liver injury. Vanillic acid was able to prevent oxidative stress and pro-inflammatory cytokines, hence inhibited the progression of sepsis in liver. Therefore, vanillic acid can be recommended for treatment against sepsis. Further research is needed to ascertain the mechanism involved in prevention of sepsis in organs by vanillic acid.

SIGNIFICANCE STATEMENT

This study investigated the potential of vanillic acid, a phenolic antioxidant in prevention of sepsis progression in liver. Sepsis is a serious problem in intensive care units leading to mortality of patients due to multiple organ failure. Exploring the potential of antioxidant compounds like vanillic acid in preventing the progression of sepsis in organs will be beneficial for the future of medical industry.

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