ORIGINAL ARTICLE

A Study on the Protective Effect of Antioxidants on Damage Induced by Liver Ischemia/Reperfusion in a Rat Model

Yong Ho Ahn¹, Pu Reum Seok², Su Jin Oh², Jin Woo Choi², Jae-Ho Shin²¹Department of Clinical Laboratory Science, Dongnam Health University, Suwon, Korea²Department of Biomedical Laboratory Science, Eulji University, Sunnam, Korea

모델 랫드에 간 허혈/재관류로 유발된 손상에 대한 항산화제의 보호 효과에 관한 연구

안용호¹, 석푸름², 오수진², 최진우², 신재호²¹동남보건대학교 임상병리과, ²을지대학교 임상병리학과**ARTICLE INFO**Received June 26, 2019
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The hepatic ischemic model has recently been widely used for the epidemiological study of ischemic reperfusion injury. This study was carried out to investigate the protective effect of vanillin, which is known to have antioxidant and anti-inflammatory effects, against hepatic and renal injury using an ischemia-reperfusion rat model, and we also investigated the mechanism related to vanillins' protective effect. The test material was administered at a concentration of 100 mg/kg for 3 days, followed by ligation of the liver for 60 minutes to induce ischemia reperfusion. As control groups, there was a negative control, sham control and ischemia-reperfusion-only ischemia reperfusion control, and the controls groups were compared with the drug administration group. In the vanillin group, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were significantly inhibited compared with the AST and ALT activities of the ischemia-reperfusion group, and histopathological examination showed significant reduction of both inflammation and necrosis. The malondialdehyde (MDA) and superoxide dismutase (SOD) levels were significantly different from the ischemia-reperfusion group. In conclusion, vanillin showed a hepatocyte protective action by alleviating the cellular inflammation and cell necrosis caused by hepatic ischemia-reperfusion, and vanillin mitigated inflammatory changes in the kidney glomeruli and distal tubules. The protective effect is considered to be caused by vanillin's antioxidant function. Further studies such as on cell death and possibly vanillin's same effect on damaged tissue will be necessary for clinical applications such as organ transplantation.

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INTRODUCTION

Postoperative dysfunction and death are still serious problems, and the main cause is ischemia-reperfusion injury during surgery [1, 2]. It is reported that massive bleeding during surgical resection of hepatocellular carcinoma results in unstable cardiovascular events, and

* Corresponding author: Jae-Ho Shin
Department of Biomedical Laboratory Science, Eulji University, 553
Sanseong-daero, Sujeong-gu, Sunnam 13135, Korea
E-mail: shinjh@eulji.ac.kr
* ORCID: <https://orcid.org/0000-0002-2141-1090>

that transfusion of hepatocellular carcinoma results in deterioration of liver function after surgery, increased recurrence rate of liver cancer and decreased survival after surgery [3–6]. In addition, because hepatic artery and portal vein are blocked at the same time, damage to liver ischemic injury and intestinal congestion can't be avoided. This ischemic reperfusion injury is a major cause of mortality and morbidity in hepatectomy. Therefore, the long-term protective effects of drugs such as antioxidant substances or iron poisoning drugs in the ischemic reperfusion injury of the liver have been studied [7–10]. The exact mechanism of ischemic reperfusion injury has not been elucidated, but the production of oxygen free radicals by ischemia reperfusion has been reported to be one of the important factors [11]. Ischemic reperfusion injury is a pathophysiological process that is exacerbated by resumption of blood flow and oxygen transport after hypoxic injury of the tissue. Clinical significance of this injury is more severe in the process of solid organ transplantation, spontaneous injury, hypoxic shock, and liver resection [12]. Reperfusion injury begins with direct physical damage by reactive oxygen species (ROS), inflammatory cytokines, etc., and is exacerbated by nonspecific and specific immune responses that are followed by these injuries [13]. In addition, pulmonary injury due to ischemia reperfusion in the tissues causes many problems, and clinically it is directly or indirectly related to severe shock. Vanillin (4-hydroxy-3-methoxy-benzaldehyde) is a widely used spice in the world and inhibits mutation prevention and carcinogenesis. In addition, it has been reported that inhibition of mutation by chemical and physical mutations and inhibition of invasion and metastasis of cancer cells have been reported. In the rat model, drug-induced effects were observed in liver cancer induction and cancer development in other organs [14–16]. Vanillin has been reported to inhibit the DNA-dependent protein kinase directly by inhibiting the non-kinetic DNA end-binding, the main pathway for the repair of double-stranded DNA breakdown in human cells [17]. Furthermore, vanillin has antimicrobial and antioxidant properties and has been

used as a temporary preservative and therapeutic agent [18, 19]. Therefore, in this experiment, we examined the effect of vanillin on the damage of liver and kidney induced by ischemia reperfusion model in rats. In addition, antioxidant mechanisms and changes of each drug were observed by biochemical markers and immunohisto-pathologic methods.

MATERIALS AND METHODS

1. Experimental method

Five weeks old males Sprague-Dawley rats were divided into four groups; control group (N=6), sham group (N=6), ischemic reperfusion group (N=8), and vanillin group (N=8). The experiment was conducted in accordance with the guidelines of the Animal Screening Commission of the Eulji University were administered to the animal study group. The incubation environment was changed from 12 hours/day (9:00 am to 9:00 pm) to 12 hours/day, relative humidity is $50 \pm 10\%$, ventilation frequency is 10~12 times/hour, lighting time of illumination 150~300 Lux. The diet and drinking water were supplied for free consumption, and the daily intake was measured. After 1 week acclimation period, 100 mg/kg of vanillin (Aldrich Sigma Chemical, St. Louis, Mo., USA) was orally administered to vanillin group for 3 days. The rats were anesthetized by intraperitoneal injection of Zoletil (Virbac, Korea) and Rompun (Bayer, Korea) at a ratio of 1:2 in body weight of 0.1 mL/kg, followed by abdominal incision at 37°C. After ligation for 1 hour to induce ischemia, clips were removed and blood was collected from the tail 6 hours after reperfusion. After 24 hours from the day of induction of ischemia, rats were autopsied and samples were separated and stored at -80°C .

2. Liver function test and kidney function test

After 6 hours of ischemia reperfusion, the tail vein was collected, and after 24 hours, blood samples were collected from the abdominal aorta at the time of autopsy were centrifuged at 3,000 rpm for 15 minutes at 4°C , and then the serum were separated. Aspartate aminotrans-

ferase (ALT), alanine aminotransferase (ALT), blood urea nitrogen (BUN), and creatinine were measured in the serum.

3. Histopathological examination

Liver and kidney tissues were fixed in 10% neutral formalin, embedded in paraffin, cut into 4 μm with a microtome, and stained with hematoxylin and eosin (H&E). Immunohistochemistry staining was performed on a γ -methacryloxypropyltrimethoxysilane-coated-slide glass (Matsunami, Japan). Antigen resuspension was treated with 0.1% trypsin solution at 37°C for 30 minutes. To remove endogenous peroxidase, the slides were incubated with 0.3% H_2O_2 methanol solution at room temperature for 30 minutes. Then, the slides reacted with goat serum diluted 1:10 with PBS, and incubated with primary antibody interleukin-6 (IL-6, Abcam, USA) and tumor necrosis factor- α (TNF- α , Abcam, USA) overnight at 4°C. The biotinylated goat anti-rabbit IgG secondary antibody was reacted at room temperature for 30 minutes. Avidin-Biotin complex (Vector Laboratories, Burlingame, CA) solution the reaction was carried out at room temperature for 30 minutes, and stained with DAB (3,3'-diaminobenzidine, Sigma, USA) containing 10N HCl and 0.3% H_2O_2 for 30 minutes. After staining with hematoxylin,

the slides were observed under a microscope.

4. Determination of malondialdehyde (MDA) content

MDA was tested by Esterbauer and Cheeseman [62], and hemoglobin components were removed from liver and kidney tissues preserved by freezing (-80°C) in heparin-added PBS solution. After homogenization with butylated hydroxytoluene (BHT) solution, centrifuge at $10,000 \times g$ for 5 minutes to prepare supernatant. Samples and MDA standards were dispensed into microcentrifuge tubes, and 100 μL of SDS-solubilizing agent was added thereto, followed by reaction at room temperature for 5 minutes. After adding 250 μL of thiobarbituric acid (TBA), the reaction was carried out at 95°C for 60 minutes. The mixture was cooled on ice for 5 minutes and then centrifuged at 3,000 rpm for 15 minutes. The supernatant was dispensed into 200 μL , and the MDA content was measured at 532 nm absorbance (OxiSelect TBARS assay kit, San Diego, USA).

5. Determination of Superoxide dismutase (SOD) content

SOD activity was performed according to Arthur and Boyne's method [63]. The serum was diluted 1:5 with sample buffer, and SOD standards were prepared. 10 μL of

Figure 1. Effect of vanillin on serum AST, ALT, BUN, and creatinine 6 hours and 24 hours after ischemia-reperfusion. Values are mean $\pm$ SEM of 6 to 8 rats per group. **, *Significantly different ($P < 0.01$, $P < 0.05$) from each control group, respectively. ***, *Significantly different ($P < 0.01$, $P < 0.05$) from each sham group, respectively.

standard material and the diluted specimen into the sample well and add 200 μ L of diluted Radical Detector. 20 μ L of Xanthine Oxidase diluted in sample buffer was added to each well and shaken well at room temperature for 20 minutes. SOD content was measured at 460 nm (SOD assay kit, Cayman Chemical, Ann Arbor, MI, USA).

6. Statistics

One-way ANOVA and Tukey's multiple comparison tests in the Prism 6 for Windows Version 6.01 (GraphPad software, Inc., California, USA) program were used to indicate the mean value of the mean error (mean $\pm$ SEM). A *P*-value less than 0.05 was considered statistically significant.

RESULTS

1. Results of serum AST and ALT measurements

The results of serum AST test after 6 hours of ischemia reperfusion showed that the ischemic reperfusion group

had a significant difference ($P<0.05$) compared to negative control and sham group ($P<0.01$), and vanillin treated group tended to be lowered (Figure 1). After 24 hours serum AST showed a significant increase ($P<0.01$) in the ischemic reperfusion group compared to the negative control group and sham group vanillin treated group tended to be lowered (Figure 1). Serum ALT levels at 6 hours after ischemia reperfusion were significantly higher ($P<0.01$) in the ischemic reperfusion group compared to the negative control group and sham group. Serum ALT at 24 hours, compared to negative control group, ischemic reperfusion group was increased significantly ($P<0.05$) (Figure 1).

2. Serum blood urea nitrogen (BUN), creatinine measurement results

The results of serum BUN test after 6 hours of ischemia reperfusion was significantly increased ($P<0.01$) in the ischemic reperfusion group compared to the negative control group. Vanillin treated group showed no

Figure 2. Effect of vanillin on histopathological changes in ischemia-reperfusion induced rat liver. H&E stain and immunohistochemical stain for IL-6 and TNF- α ($\times 400$).

significant change. After 24 hours, serum BUN showed control and sham group was significantly increased ($P < 0.05$) in compared with the ischemic reperfusion group. Vanillin treated group tended to decrease (Figure 1). After 6 and 24 hours, creatinine level was tended to decrease in the vanillin group compared to the ischemic reperfusion group. But there was no significant change (Figure 1).

3. Histological examination

1) H&E stain

In the liver tissue, control and sham group maintained normal structure of hepatocytes, portal vein, and bile duct tissue. In the ischemic reperfusion group, infiltration of inflammatory cells, partial necrosis and slight evidence of bleeding were observed around the portal vein. In the vanillin group, the pathological damages were significantly decreased (Figure 2). In kidney tissue, nuclear and cytoplasm of proximal tubules and small cubic cells with well-preserved corpuscles were well preserved in kidney tissue and granular cytoplasm. However, in ischemic

reperfusion group, degeneration of glomeruli and disappearance of proximal tubule in the vanillin group, the degeneration of the glomeruli, the proximal tubule-like edge, and the area of the follicle were reduced with time (Figure 3).

2) IL-6 immunochemical staining findings

Immunohistochemical expression of IL-6 antibody was strongly detected in the necrotic area and inflammatory cells in the hepatic necrosis of liver tissue of ischemia-reperfusion group. However, antibody staining intensity decreased in vanillin group (Figure 2).

Immunohistochemical expression of IL-6 antibody was strongly observed in the glomeruli, proximal tubules and collecting tubules that were inflamed in the renal tissues of the ischemic reperfusion group, but the decrease of expression intensity was observed in the vanillin group (Figure 3).

Figure 3. Effect of vanillin on histopathological changes in ischemia-reperfusion induced rat kidney. H&E stain and immunohistochemical stain for IL-6 and TNF- α ($\times 400$).

Figure 4. Effect of vanillin on MDA liver and kidney tissue level 24 hours after ischemia-reperfusion. Effect of vanillin on serum SOD level 6 hours and 24 hours after ischemia-reperfusion. Values are mean $\pm$ SEM of 6 to 8 rats per group. ++ Significantly different ($P<0.01$) from each control group, respectively. # Significantly different ($P<0.05$) from 24 hour I/R group. *, ** Significantly different ($P<0.05$, $P<0.01$) from each I/R group, respectively.

3) TNF- α immunochemical staining

In the ischemic reperfusion group (Figure 2), the TNF- α immunoreactivity was strongly positive in the portal vein, periportal necrosis area, and inflammatory cells. In the vanillin group, the expression level of TNF- α antibody was decreased. In the renal tissues of the ischemic reperfusion group (Figure 3), TNF- α immunoreactivity was observed in proximal tubules and collecting tubules, and the expression of TNF- α immunochemical staining was decreased in the group treated with vanillin.

4) Malondialdehyde (MDA) content measurement

Twenty-four hours after ischemia reperfusion, liver tissue MDA was significantly increased ($P<0.01$) in the ischemic reperfusion group compared to the control group and the sham group, and decreased significantly ($P<0.05$) in the vanillin group compared to the ischemia reperfusion group. MDA in renal tissue 24 hours after ischemia reperfusion was significantly higher ($P<0.01$) in ischemic reperfusion group compared to control group and sham group (Figure 4).

5) Superoxide dismutase (SOD) content measurement

Six hours after ischemia reperfusion, the ischemic reperfusion group was increased compared to the control and sham group. Vanillin group was tended to be decreased compared to the ischemic reperfusion group. After 24 hours, serum SOD levels were significantly increased ($P<0.05$) in ischemic reperfusion group

compared to sham group and vanillin group was significantly decreased ($P<0.01$) compared with ischemia-reperfusion group (Figure 4).

DISCUSSION

Hepatic ischemia reperfusion injury occurs inevitably during liver transplantation and hepatic resection. When blood flow is blocked, local ischemia occurs in the tissues, and organ failure and septicemia occur. During ischemia, anaerobic glucose degradation occurs, accumulating lactic acid in the tissues, which leads to acidification of body fluids, which promotes secretion of catecholamine, leading to cardiac tachycardia and vasoconstriction. Catecholamine and acidemia due to local ischemia cause systemic inflammatory response syndrome through secretion of inflammatory mediators resulting in systemic tissue damage and dysfunction [20]. Leukocytes stimulated by mediators also stimulate the systemic inflammatory response by promoting the secretion of cytokines, proteases, oxidants, etc., such as TNF- α , IL-6 and IL-1 [21]. Increased glutathione (GSH) deficiency and lipid peroxidation due to oxidative stress induce highly reactive free radicals and oxides and promote the oxidation of sulfhydryl groups and thioethers similar to the nitration and hydroxylation of aromatic compounds [22]. This reaction not only induces cell structure damage but also induces cellular responses through the activation of NF- κ B mediated signal transduction [23]. Activation of

these transcription leads to the expression of various inflammatory mediator genes including cytokines and adhesion molecules. Cell damage can be divided histologically into cell death and cell necrosis. Cellular damage after ischemic reperfusion injury in the liver occurs most frequently in the first 3 to 6 hours after reperfusion, and cellular damage, which is expressed as cellular necrosis, is known to occur more than 3 hours after reperfusion injury [24]. In this study, morphological changes of hepatic tissue after ischemia reperfusion showed increase of inflammatory cells and partial necrosis around the portal vein, and hepatic function was significantly decreased. The ischemic reperfusion injury experienced during renal transplantation surgery is not only an acute renal failure due to acute tubular necrosis after transplantation, but also an important factor in chronic renal failure [25].

In addition to renal transplantation, ischemic reperfusion injury also causes renal dysfunction due to the same pathophysiologic process in all surgical or traumatic procedures, such as abdominal aortic aneurysm surgery, which reduces blood flow to the kidneys [26, 27]. In the present study, after the ischemia reperfusion, the proximal end of the proximal tubule was disappeared, the coronary structures such as collecting tubules disappeared, and renal function was reduced. Dame and Juul [28] were expressed weakly in the glomeruli, distal tubules and collecting tubules during the development of IL-6 in human fetal kidneys, and not expressed in proximal tubules, and expression of IL-6 in renal injury and acute renal failure of the patients [29-32]. These results were consistent with the results of this study. Vanillin is a powerful antioxidant. This destroys free oxygen and inhibits protein oxidation and lipid peroxidation. These antioxidant properties eradicate free radicals and have anti-inflammatory and anti-angiogenic activity [33].

Vanillin inhibits peroxynitrite mediation in cells and inhibits enzymatic activity of metalloproteinase-9 secreted from cancer cells, inhibiting the migration and invasion of cancer cells [34]. In addition, it has been reported that inhibition of mutation by chemical and

physical mutations and inhibition of invasion and metastasis of cancer cells have been reported [14-16]. In the rat model, drug-induced effects were observed in liver cancer induction and cancer development in other organs [35]. Furthermore, vanillin has antimicrobial and antioxidant properties and has been used as a temporary preservative and therapeutic agent [18, 19]. In the present study, liver function values were lowered in the vanillin group than in the ischemia-reperfusion group. BUN and creatinine levels were lower in the vanillin group than the ischemia-reperfusion group. Histologic findings showed similar results.

This radical is bound to oxygen and becomes lipid peroxide such as hydroperoxide, endoperoxide, and polyperoxide, and is decomposed into MDA. In the early stage of ischemic reperfusion injury, ROS was produced to over-produce free radicals, and MDA, which is the lipid peroxidation caused by destruction of unsaturated fatty acids, was increased in the cell membrane, and the endogenous It causes the reduction of the antioxidant GSH [36]. This study also showed that MDA was significantly decreased in the drug-treated group compared to the ischemia-reperfusion group. MDA in liver tissues is used as a substrate for glutathione peroxidase (GSH-Px) and glutathione sulfur transferase (GST), enzymes that protect against oxidative cell damage. Active oxygen, lipid peroxides and electrophilic substances, which is involved in the final detoxification process, and performs various functions such as removal of lipids, oxidants and foreign substances in the cells, amino acid transport and storage, and liver decryption [37].

SOD is the most powerful antioxidant enzyme produced in the human body and inhibits the harmful effects of active oxygen. Indeed, a considerable amount of SOD is present in the cells in the liver. Cataracts in the liver tissue were severely depleted by ischemia reperfusion injury, and adequate administration of antioxidants could significantly prevent such exhaustion. Biological defense systems that can protect biomaterials from ROS or free radicals are largely distinguished as enzymatic defenses by

enzymes such as SOD and nonenzymatic defense systems that terminate or terminate the chain reaction of ROS or free radicals [38], oxygen-bearing organisms have SOD, which converts superoxide anion to H_2O_2 . Catalase is a radical scavenging enzyme that degrades H_2O_2 into water and oxygen to convert it into water [39]. In this process, SOD is exhausted. In this study, the SOD of the 24-hour ischemia-reperfusion group was significantly lower than that of the control group, consistent with the results of other investigators, such as the results of MDA [36, 40, 41]. In the group treated with vanillin, injury of organs due to ischemic reperfusion promoted SOD production and also used as an antioxidant to inhibit the production of oxygen free radicals and to protect against liver and kidney toxicity. In the future, further studies such as cell death and the same effect on damaged tissue will be necessary for clinical applications such as organ transplantation. This study was conducted to investigate the protective effect of vanillin on liver and kidney damage in rat liver ischemia reperfusion injury model. As a result, the protective effect was confirmed, which is thought to be due to the antioxidative effect of each substance.

요약

허혈 재관류 손상은 기관 이식, 외과적 혈관 재개통 및 출혈 시에 발생하며 조직 및 기관 기능 장애를 유발한다. 최근 허혈 재관류 손상의 기전적 연구를 위해 간장 허혈 모델을 많이 이용하고 있다. 본 연구는 허혈 재관류 랫드 모델을 이용하여 항산화와 항염증 효과를 가진 것으로 알려진 Vanillin에 의한 간장 및 신장 손상에 대한 보호 효과를 알아보고 관련된 기전을 조사하기 위하여 실시하였다. 시험물질은 각각 100 mg/kg의 농도로 3일간 투여한 후, 60분동안 간을 결찰하여 허혈 재관류를 유도하여 관찰하였으며, 음성대조군, sham대조군 및 허혈재관류만 실시한 허혈 재관류대조군을 따로 두어, 약물투여군과 비교하였다. Vanillin 처치군에서는 AST, ALT 활성이 허혈 재관류대조군에 비해 유의하게 억제되었고, 조직병리학적 관찰에서도 염증 부분과 괴사부분이 현저하게 감소하였다. MDA와 SOD는 허혈 재관류군에 비해 유의적인 변화를 보였다. 이상의 결과를 종합하면 Vanillin은 간장 허혈 재관류에 의한 세포염증 및 세포괴사를 완화시켜 간세포 보호작용을 나타내었고, 신장의 사구체 및 원

위세뇨관에 염증 변화를 완화 시키고 있어 세포 손상을 방어하는 것으로 생각되며, 이러한 방어효과는 항산화 기능에 의한 영향으로 사료된다.

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Author's information (Position): Ahn YH¹, Professor; Seok PR², Researcher; Oh SJ², Researcher; Choi JW², Researcher; Shin JH², Professor.

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