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Protective Effects of Vanillic Acid on Arsenic-Induced Hepatotoxicity and Diabetes in Mice; the Role of PPARγ and NF-κB Signaling

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Abstract

Background: Arsenic (As), a toxic metalloid present in drinking water, is one of the environmental pollutants associated with diabetes in humans. Vanillic acid (VA), a bioactive compound derived from plants has various medicinal activities.

Methods: This study was conducted on NMRI male mice for 8 weeks. forty mice were randomly divided into control group, As group (50 ppm), VA (100 mg/kg) group, and two groups receiving As (50 ppm) and VA with doses of 50 mg/kg and 100 mg/kg. After 56 days of the study, the mice were fasted overnight and on day 57, fasting blood glucose was measured, and glucose tolerance test was performed. On day 59, mice were euthanized and serum factors, markers of oxidative stress, tumor necrosis factor-α (TNF-α), and expression nuclear factor kappa B (NF-κB) and Peroxisome Proliferator-Activated Receptor Gamma (PPARγ) proteins were measured.

Results: The As significantly increased fasting blood sugar, the activity level of liver function enzymes, thiobarbituric acid reactive substances (TBARS), nitric oxide (NO), TNF-α, and NF-κB expression. Furthermore, As decreased hepatic total thiol (TT) and activity levels of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and expression of PPARγ. VA decreased the altered liver enzymes, hyperglycemia, NO, TBARS, TNF-α and the expression of NF-κB. Furthermore, increased the hepatic activity of the CAT, SOD, and GPx, TT and the expression of PPARγ.

Conclusion: The administration of VA at doses of 50 and 100 mg/kg demonstrated significant mitigation of the toxic effects induced by As on the liver.

Keywords: Arsenic, Diabetes Mellitus, Liver Injury, Vanillic acid.

Introduction

Diabetes is classified as a chronic disease affecting approximately 25% of the global population. It is a metabolic disorder resulting from either inadequate insulin secretion by the islet cells of the pancreas or insulin resistance, which ultimately leads to elevated blood glucose levels (1). The likelihood of endangering human health has increased due to the increased use of industrial chemicals, pharmaceuticals, and environmental toxins in recent decades. Arsenic (As) is one of these contaminants, and long-term exposure to it has been linked to several health issues and illnesses in people (2-6). It has been determined that oxidant-antioxidant imbalance and pro-inflammatory cytokine increases are the basic

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mechanisms of As-induced hepatotoxicity. As a front-line immunological organ, the liver's function is closely related to the immune response excessive inflammation in the absence of infection results in hepatotoxicity and tissue destruction. The fundamental mechanisms of hepatotoxicity caused by As have been identified as oxidant-antioxidant imbalance and pro-inflammatory cytokine increases (7-9). The As has drawn attention recently as a possible risk factor for type 2 diabetes. It remains unclear how As plays a role in the development of diabetes. Human study data substantiate the link between As and diabetes in populations where drinking water As levels above 500 µg/L. Exposure to As may worsen the consequences of diabetes in those who already have it by affecting gluconeogenesis, insulin secretion, and lipid metabolism in healthy individuals (10). Since the liver is essential to the metabolism and detoxification of toxins, it is thought to be the perfect location for As -induced fatalities. Currently, oxidative stress is a well-established mechanism behind As-induced hepatotoxicity. Therefore, a workable solution to this problem and to improve the antioxidant-defense system by using specific antioxidants to reduce the As -generated oxidative stress must be employed (11). Vanillic acid (VA) is a naturally occurring compound derived from various fruits, vegetables, and plants. It is a pharmacologically significant derivative of benzoic acid, known for its potential health benefits. Historically, Angelica sinensis and vanilla beans have been recognized as primary sources of VA, contributing to its availability in natural products (12). Additionally, VA has been linked to a wide range of pharmacological actions, hepatoprotective, anti-oxidant, carcinogenesis, apoptosis, cardioprotective, and anti-inflammatory properties (13-17). The hepatoprotective effect of VA is through immune-mediated suppression of liver inflammation (17). However, most studies showed that VA can suppress the over-expression of nuclear factor-κB (NF-κB) and tumor necrosis factor (TNF-α) during body malfunction and reduce inflammation (18). Studies have shown that inflammatory cytokines (NF-κB and TNF-α) play a critical role in diabetes (19). Peroxisome Proliferator-Activated Receptor Gamma (PPAR-γ) is a nuclear receptor that regulates gene expression related to glucose and lipid metabolism, as well as the differentiation of pre-adipocytes into adipocytes (20). It plays a crucial role in fatty acid storage and enhances insulin sensitivity, making it significant for type 2 diabetes management (21). Treatment with VA led to a reduction in body weight and suppressed the expression of PPARγ (22).

However, the role of VA as an anti-diabetic and hepatoprotective agent has not yet been investigated. Therefore, the present study was designed and conducted to investigate the anti-diabetic and hepatoprotective effects of VA.

Materials and Methods

Materials

Vanillic acid, D-glucose, sodium arsenite, Bradford reagent, thiobarbituric acid (TBA), and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), along with other chemicals and reagents, were obtained from Sigma-Aldrich Chemical Company (USA). Antibodies specific for PPAR-γ, NF-κB, and GAPDH, as well as anti-rabbit immunoglobulin (IgG) conjugated with horseradish peroxidase (HRP) and anti-mouse IgG (HbL) conjugated with HRP, were sourced from Cell Signaling Technology (USA). All other chemicals utilized in the study were of analytical grade.

Animals

This study was conducted at the Toxicology Research Center, Basic Medical Sciences Research Institute, Ahvaz Jundishapur University of Medical Sciences in Ahvaz, Iran. The research adhered to the principles of animal care by the guidelines set forth by the Animal Ethics Committee and received approval from the Institutional Animal Care and Use Committee of AJUMS, following the NIH guidelines for animal care and use. Forty laboratory animals were maintained under controlled conditions, with a room temperature of 22-24 °C, humidity levels of 40-60%, and a

12-hour light-dark cycle. The animals had unrestricted access to food and tap water.

Efforts to minimize animal suffering were prioritized throughout all phases of the study.

Experimental Design

The As and VA doses were selected based on the previous studies (23, 24) and then dissolved using distilled water.

Adult male mice were randomly divided into 5 groups (8 in each):

Group I (Normal control group) received the normal saline daily.

Group II (Diabetic control) received As (50 ppm).

Group III received 100 mg/kg VA.

Groups IV and V (As + VA group): mice received 50 and 100 mg/kg VA.

All mice received water containing 50 ppm As or normal saline for 8 weeks. In the treatment groups, in addition to the daily administration of As, in the last two weeks, they received VA with doses of 50 and 100 mg/kg per day orally by gavage.

Sample preparation

Treatments were administered via gavage to the mice. Blood samples were collected 24 hours after the two weeks of last VA administration under anesthesia with ketamine/xylazine (10/100 mg/kg) for serum analysis and stored at -80 °C. Then, histopathological and other oxidative stress tests were performed on liver tissue samples.

Fasting blood sugar (FBS) and glucose tolerance test (GTT)

At the end of eight weeks, on day 57, a blood sugar (BS) meter (Accu-Chek, Switzerland) was used to perform FBS and GTT tests. All mice were fasted for eight hours before measuring glucose levels. 30 minutes after As administration, 2 g/kg of D-glucose (intra peritoneal) was given to the animals to check GTT. Blood samples were collected from the tail vein of mice at different time points (0, 15, 30, 60, 90 and 120 minutes) after D-glucose injection (25).

Biochemical analysis

Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) tests were performed using the obtained plasma for liver function tests according to the instructions of commercial kits of Pars Azmoun Iran.

Assessment of liver oxidative stress

Preparation of the tissue sample to perform the desired tests was done as previously described (25). After preparing tissue samples, oxidative stress parameters such as thiobarbituric acid reactive substances (TBARS), Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), total thoil (TT) were measured using ZellBio commercial kits and according to the manufacturer's instructions.

Effect of vanillic acid on inflammatory

The level of Nitric oxide (NO) in liver tissue was determined using commercial kits according to the manufacturer's protocol (ZellBio GmbH, Germany). The amount of NO was expressed as nmol/mg protein. Hepatic TNF-α level was measured using a ZellBio GmbH (ELISA) assay kit (Germany) according to the manufacturer's instructions, and absorbance was recorded at 450 nm using a microplate reader, then the results were prepared as pg/mg of protein.

Expression of NF-κB and PPAR-γ proteins

After the prepared tissues were lysed with RIPA buffer. Lysates were removed at 4 °C using centrifugation at 14,000 rpm for 20 minutes. Protein concentration was determined by the Bradford method. Briefly, NF-κB and PPAR-γ proteins were electrophoresed on sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE). Then Protein bands were transferred to polyvinylidene fluoride (0.2 μm Immune-Blot™) (PVDF) membranes and blocked in skim milk (5% (w/v) at 4 °C for 24 h. The blocked PVDF membranes were incubated for 120 min with

primary antibodies anti-NF-κB and anti-PPAR-γ. The membranes were then incubated with the appropriate secondary antibody. Then, the obtained protein bands were analyzed using a JS 2000 scanner (BonninTech, China). The density of bands was normalized to GAPDH as a control in Western blot.

Histopathology analysis

The pieces of liver tissues were placed in 10% formalin for histological examination. Sections of five micrometers were embedded in paraffin and stained with hematoxylin-eosin (H&E). Six slides per animal were used for histological evaluation.

Statistical analysis

Data are presented as the mean accompanied by the standard error of the mean (SEM). To assess differences between groups, we conducted one-way and two-way analyses of variance (ANOVA). Following these analyses, Tukey's post hoc test was applied to identify specific group differences. All statistical analyses were performed using GraphPad Prism software, version 9. A significance level of $p< 0.05$ was established to determine statistical relevance.

Results

Effect of VA on FBS and GTT

As shown in Fig. 1, the FBS level in the group that received only As was higher than the control group ($p< 0.001$), but VA at both doses of 50 and 100 mg/kg could significantly decrease the FBS compared to As group ($p< 0.001$). GTT in all groups peaked within 15 minutes. The results show that the BS level in the control group decreased to the baseline 120 minutes after the injection of D-glucose, in contrast, the BS level in the group exposed to the toxic substance remained higher than the baseline level, indicating impaired glucose tolerance. Both doses of VA (50 and 100 mg/kg) significantly prevented glucose tolerance compared to the As group.

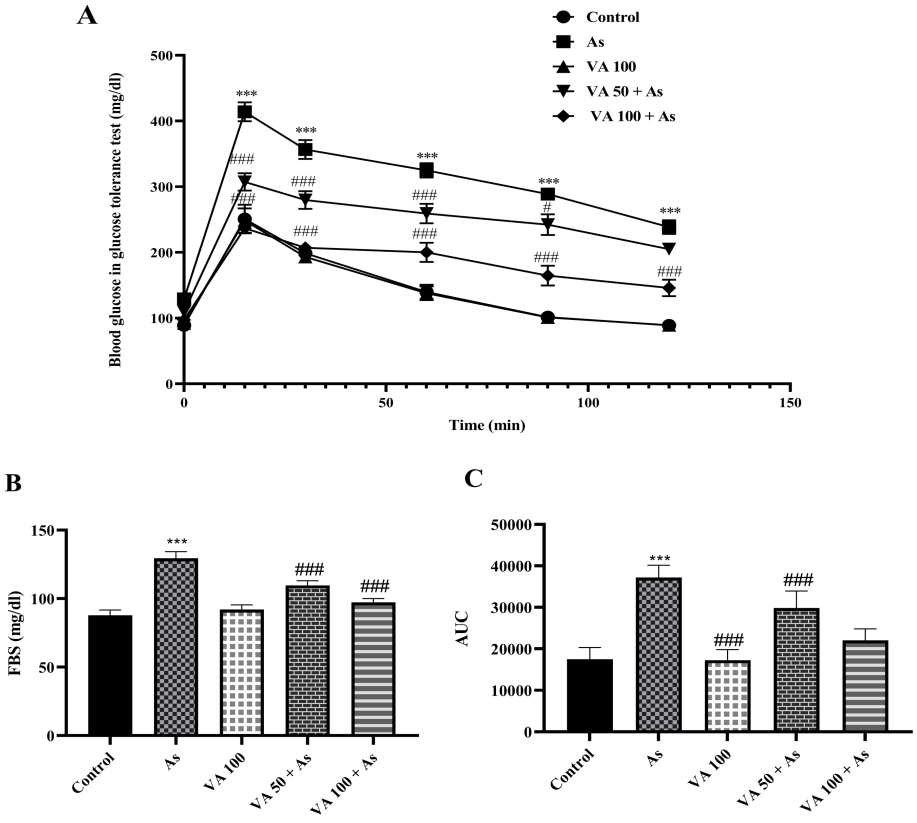


Fig. 1. The effects of vanillic acid (VA) on the glucose tolerance test (A): (GTT), fast blood sugar (B): (FBS) and Area under curve (C): (AUC) in arsenic (As) induced diabetes in mice.

Effect of vanillic acid on liver function parameters

As expected, and shown in Fig. 2, administration of As caused a significant increase in the activity of ALT, AST and ALP compared to the control group ($p< 0.001$). In the groups treated with VA at doses of 50 and 100 mg/kg, the levels of these enzymes significantly decreased compared to the As group ($p< 0.001$).

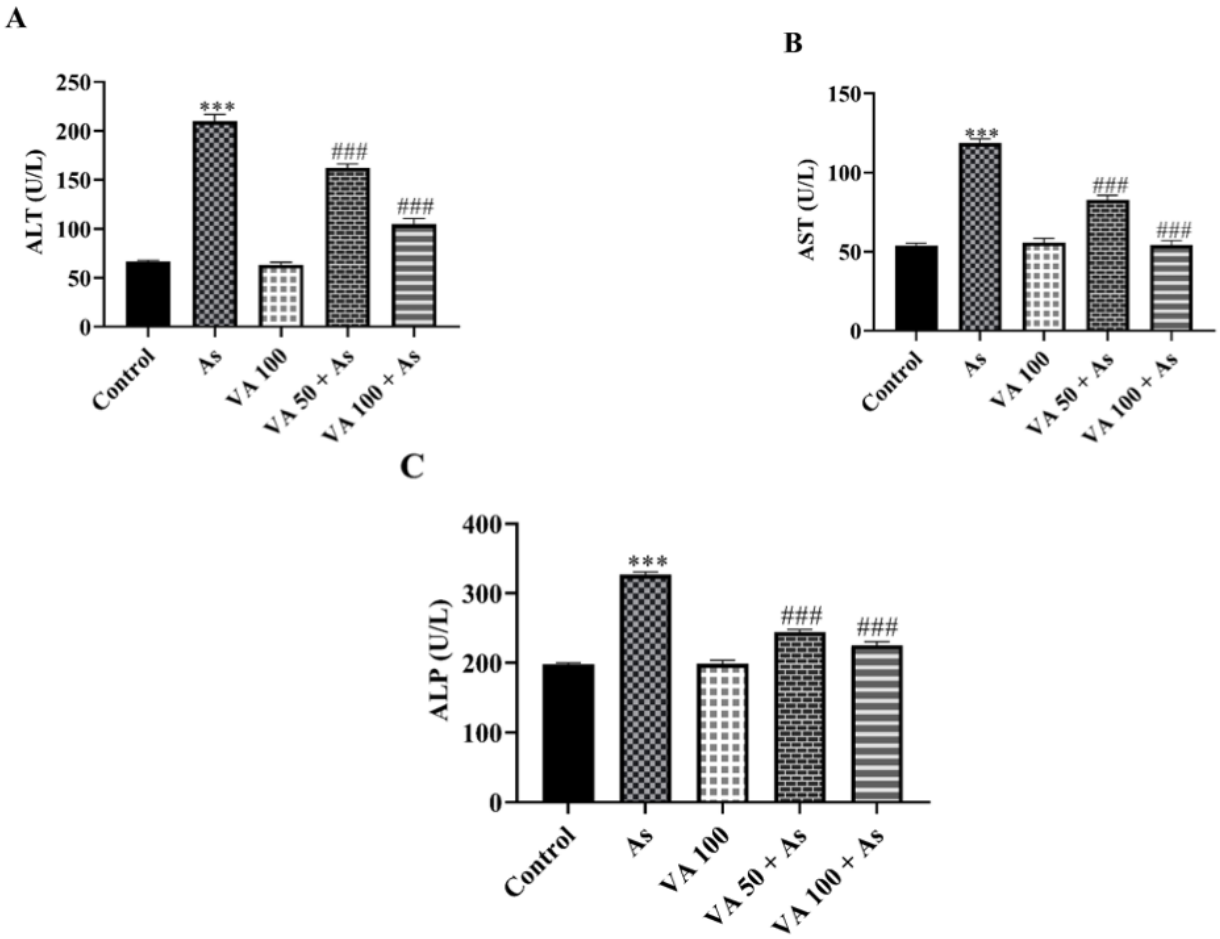


Fig. 2. The effects of vanillic acid (VA) on the activity level of (A): alanine aminotransferase (ALT), (B): aspartate aminotransferase (AST), (C): alkaline phosphatase (ALP) in arsenic (As)-induced hepatotoxicity in mice.

Effect of vanillic acid on oxidative stress hepatic biomarkers

The data in Fig. 3 shows that the activity of CAT, SOD, and GPx enzymes and the content of TT decreased significantly in the group receiving the toxic substance compared to the control group ($p< 0.001$), at the same time, the administration of As decreased the level of TBARS, compared to the control group ($p< 0.001$). Treatment with VA at doses of 50 and 100 mg/kg led to a significant increase in SOD, CAT, GPx, and TT levels and a decrease in TBARS levels compared to the As group ($p< 0.001$).

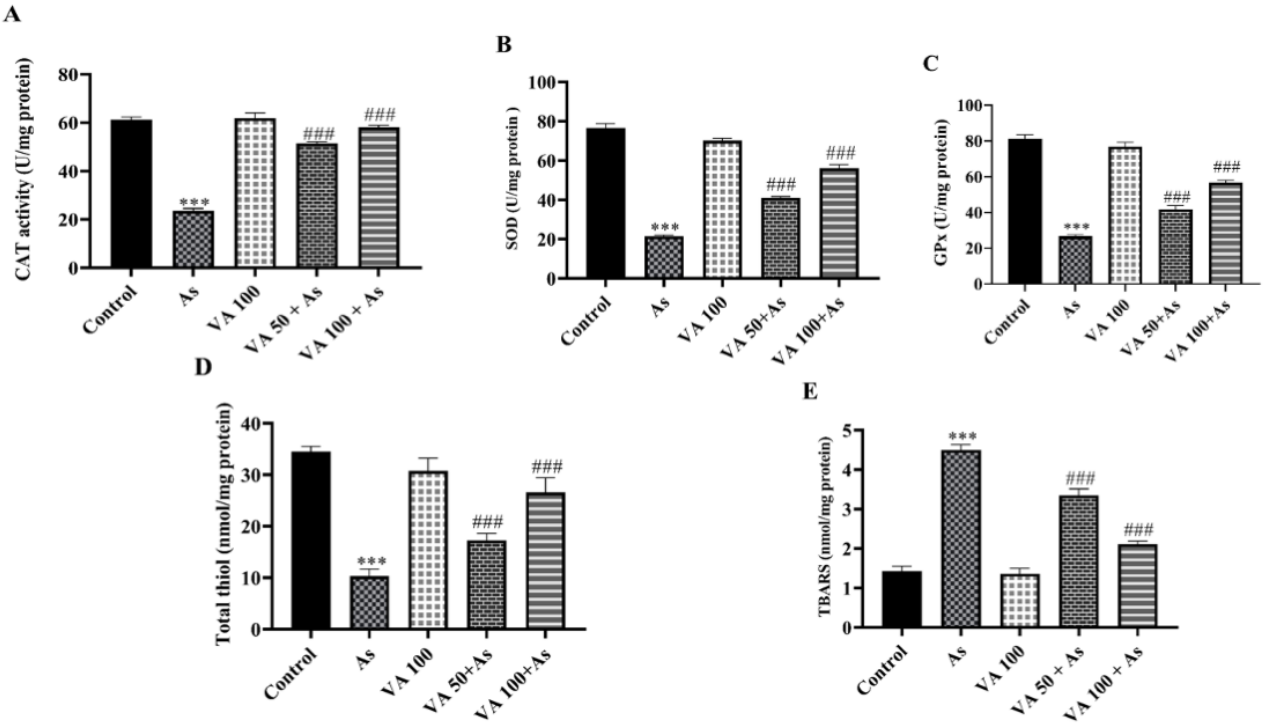


Fig. 3. The effects of vanillic acid (VA) on the level of (A): CAT (catalase), (B): SOD (superoxide dismutase), (C): GPx (glutathione peroxidase), (D): TT (total thiol), and (E): SOD (superoxide dismutase) in arsenic (As) induced hepatotoxicity in mice.

Effect of vanillic acid on inflammatory

In Fig. 4, the results show that the administration of As led to a significant increase in the levels of NO and TNF-α ($p< 0.001$). Meanwhile, the levels of these markers in the group treated with VA at doses of 50 and 100 mg/kg decreased significantly compared to the As group ($p< 0.001$).

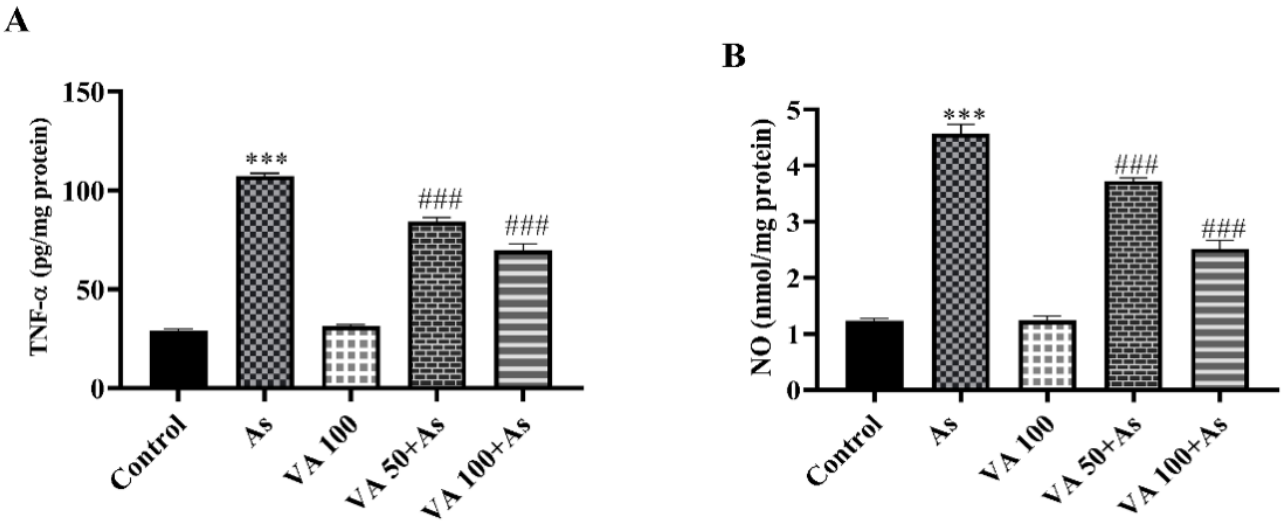


Fig. 4. The effect of vanillic acid (VA) on the level of (A): tumor necrosis factor-α (TNF-α) and (B): nitric oxide (NO) in arsenic (As)-induced hepatotoxicity in mice.

Effects of vanillic acid on the expression level of PPAR-γ and NF-κB

In Fig. 5, this suggests that VA may reduce inflammation by reducing NF-κB signaling ($p< 0.001$). Also, in the As group, PPAR-γ gene expression decreased and NF-κB increased compared to the control group ($p< 0.001$). Simultaneous treatment with VA at doses of 50 and 100 mg/ kg significantly increased PPAR-γ gene expression ($p< 0.001$) compared to the As group. Based on the western blot results, it was observed that the intensity of the PPAR-γ protein band was significantly ($p< 0.001$) higher in animals treated with VA (50 mg/kg and 100 mg/kg) compared to animals treated with As.

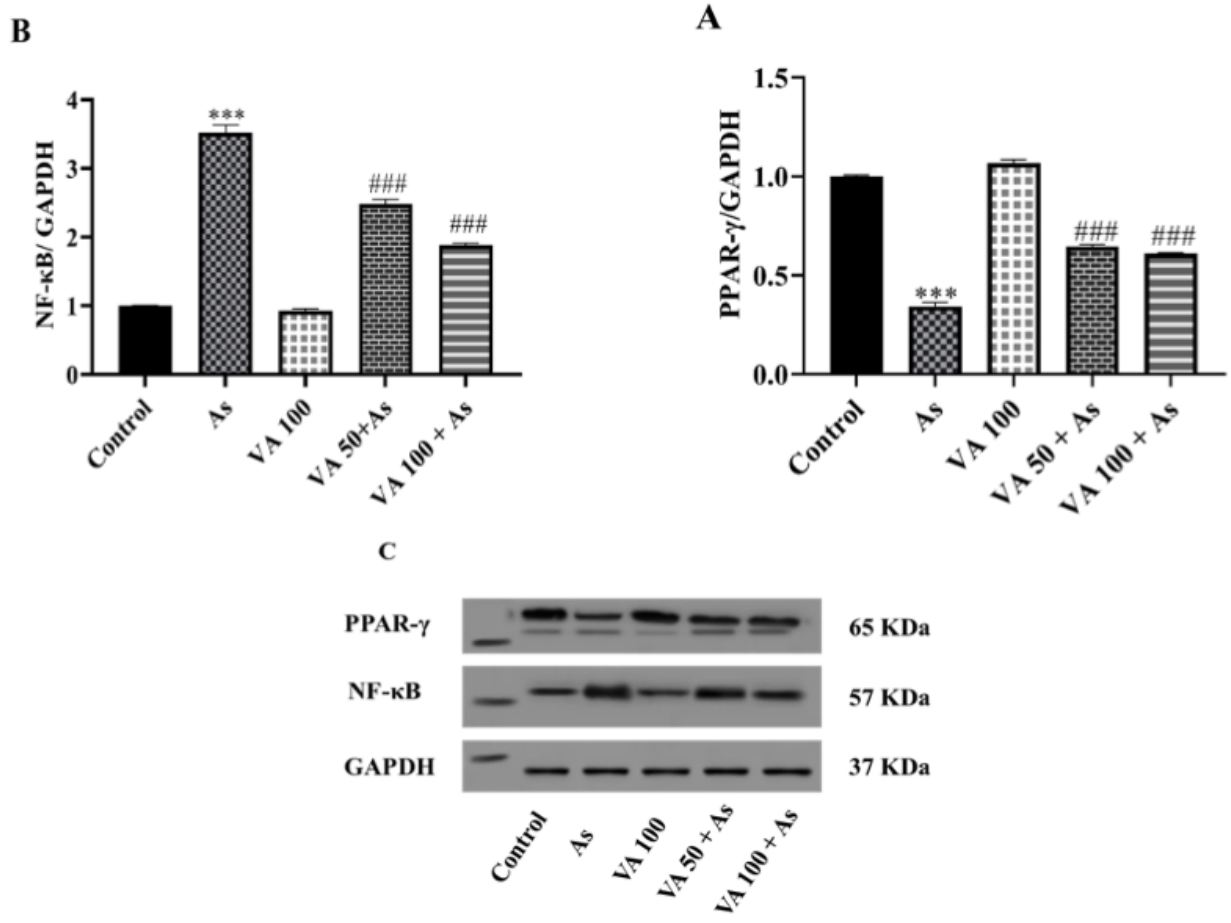


Fig. 5. The effect of vanillic acid (VA) on the expression level of (A): peroxisome proliferator-activated receptor gamma (PPAR-γ), tumor necrosis factor (NF-κB) and (C) Western blot images in arsenic (As)- induced hepatotoxicity in mice.

Effect of vanillic acid on histopathological alteration in liver

In the control group, the liver exhibited a regular lobular structure, with liver cells arranged radially around the central veins. In contrast, the animals in the As group showed a disruption of this normal architecture, characterized by inflammation and an accumulation of red blood cells. The group receiving VA at a dose of 50 mg/kg showed only slight improvement. However, the pathological changes in the liver caused by As were significantly reduced in the group treated with VA at a dose of 100 mg/kg (Fig. 6).

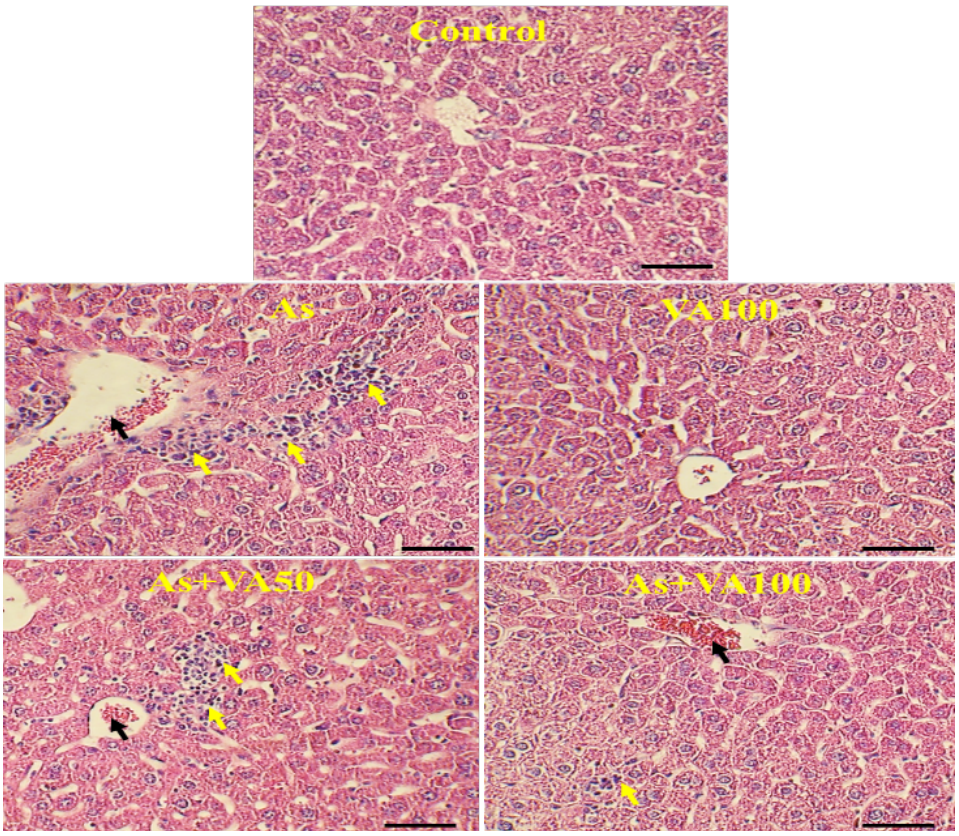


Fig. 6. Effects of vanillic acid (VA) on histopathological changes and lesions in hepatotoxicity induced by arsenic (As) in mice. The effects of vanillic acid are obvious in H&E. Yellow arrows: inflammation and black arrows: accumulation of red blood cells. (Magnificence: 250 x and scale bar: 100 micrometer).

Discussion

The As is recognized as one of the most hazardous substances globally, due to its extensive occurrence in the environment, particularly within the Earth's crust, as well as its toxic characteristics and potential carcinogenic effects (26). Individuals are commonly exposed to As through several pathways, including the consumption of contaminated food and water, inhalation of airborne particles, and direct dermal contact with contaminated materials (27). As a life-threatening factor, As can have chronic effects on the body, including diabetes (28). Because the symptoms of chronic As poisoning do not appear immediately, incorporating antioxidants into the diet can help prevent chronic damage from heavy metals, including As (29, 30). In the present study, we exposed mice to As through drinking water for 8 weeks. BS results, including FBS, GTT, and area under the curve (AUC), showed that As increased blood sugar factors such as FBS, GTT, and AUC. This suggests that the beta cells responsible for insulin secretion have been damaged, leading to decreased insulin secretion. The BS results obtained in the study by Molavinia et al. align with the findings of the present study. (9). One of the critical factors that increase in hepatotoxicity caused by diabetes is serum factors such as ALT, AST and ALP (31). This study also shows that As caused hepatotoxicity and increased serum factors. Treatment with VA at a dose of 50 and 100 mg/kg significantly reduced these damages. In the study conducted by Pourmafi et al., an increase in serum factors associated with As and diabetes was observed, which aligns with the results of the current study. Additionally, betaine was shown to reduce serum factors in Pourmafi's research. Similarly, in our study, VA, recognized as an antioxidant, also demonstrated the ability to decrease serum factors (32). Understanding the interplay between oxidative stress and

antioxidant defense mechanisms is essential for elucidating hepatotoxicity, particularly concerning diabetes and exposure to As. Oxidative stress arises when there is a disruption in the balance between the generation of reactive oxygen species (ROS) and the body's ability to neutralize or eliminate these detrimental compounds. This imbalance can lead to cellular damage and inflammation, ultimately contributing to various pathological conditions, including liver dysfunction (27, 33). In the context of diabetes, elevated levels of glucose can exacerbate oxidative stress, further compromising the liver's ability to detoxify and regenerate. Concurrently, As exposure has been shown to induce oxidative damage, which can overwhelm the liver's antioxidant defenses. The liver plays a pivotal role in metabolizing glucose and xenobiotics, making it particularly vulnerable to the combined effects of oxidative stress and toxic exposures. Therefore, a comprehensive understanding of how these factors interact is vital for developing effective therapeutic strategies to mitigate hepatotoxicity and improve health outcomes in affected individuals (34). In diabetes, this balance is often disrupted, leading to increased oxidative stress that may exacerbate hepatotoxicity. In a study, Vinothiya et al. investigated the effect of VA on diabetic rats and, it was shown that VA can restore the function of the antioxidant defense system and elevate the levels of CAT, SOD, GPx, and TT meanwhile reduced TBARS levels (35). The results of this study were consistent with our study results. It has been shown that VA can be effective in improving the function of the damage caused by diabetes.

TNF-α is an essential cytokine that plays a significant role in mediating inflammation and influencing physiological processes such as cell proliferation, differentiation, and apoptosis (36). NF-κB is a transcription factor that regulates the expression of genes involved in inflammation, and cell survival (37). The connection between TNF-α and NF-κB is crucial; when TNF-α binds to its receptor, it triggers a signaling cascade that leads to the release of NF-κB, allowing it to translocate into the nucleus and promote the transcription of pro-inflammatory genes, thus sustaining the inflammatory response. In turn, TNF-α stimulates NO production through the upregulation of inducible nitric oxide synthase (iNOS), while NO can modulate NF-κB activity, inhibiting it at low concentrations and enhancing it at higher levels. Inactivating this pathway with appropriate treatments can heal diseases by reducing inflammation (38-40). The relationship between NF-κB and PPAR-γ is crucial in diabetes and inflammation. NF-κB is an important transcription factor that regulates the expression of inflammatory cytokines and is activated by pro-inflammatory signals, which can lead to insulin resistance and β-cell dysfunction in diabetes. On the other hand, PPAR-γ is a nuclear receptor that helps manage fatty acid storage and glucose metabolism, and it has anti-inflammatory effects by inhibiting pro-inflammatory cytokines and reducing NF-κB activity. This reciprocal relationship indicates that PPAR-γ can help reduce inflammation by inhibiting NF-κB, while NF-κB can suppress PPAR-γ expression, worsening inflammation and insulin resistance (41). Previous studies showed that As exposure leads to increased levels of TNFα, NO, and NF-κB expression in the liver tissue of mice (9, 42). In the study of Daryagasht et al., PPAR-γ decreased in exposure to As (43). Also, our research results were consistent with previous studies and As increased levels of TNFα, NO, and expression of NF-kB. In the present study, VA was able to reduce the TNFα level, NO level, expression of NF-κB and increased the expression of PPAR-γ.

In the present study, according to the factors that were measured, it was shown that VA with a dose of 50 and 100 mg/kg can reduce the damage of diabetic mice caused by As. Targeting the mentioned pathways could provide therapeutic strategies for managing diabetes and its inflammatory complications. This underscores the importance of further research to clarify these mechanisms and explore potential interventions.

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Conflict of interest
The authors confirm the absence of any known conflicts of interest related to this manuscript, and no significant financial support was received for this research that could influence its results.

Ethics approval
All experimental procedures were carried out and approved by the Institutional Animal Care and Use Committee of the AJUMS (Ethics ID: IR.AJUMS.ABHC.REC.1401.058).

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