Vitamin E and *Lactobacillus* Provide Protective Effects Against Liver Injury Induced by HgCl₂: Role of CHOP, GPR87, and mTOR Proteins

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Abstract

**Background and Objective:** Mercury is one of the most harmful heavy metals and its toxicity causes severe multi-organ dysfunction. This study was designed to explore novel molecular pathways involved in the hepatoprotective effect of vitamin E (Vit-E) and *Lactobacillus plantarum* (Lac-B) against mercury toxicity.

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Method: Acute hepatotoxicity was induced by administration of high dose of mercuric chloride (HgCl₂) in male rats, Vit-E or/and Lac-B were given along with HgCl₂ for 2 weeks. The effects of those antioxidants were studied focusing on their anti-apoptotic, anti-oxidative stress and anti-inflammatory efficacies. Histopathological examinations were also conducted.

Results: The administration of HgCl₂ induced liver injury which manifested by elevation in serum ALT and AST. Liver MDA, caspase-3 and TNF-α levels were markedly increased; whereas, GSH level and SOD activity were declined. HgCl₂ significantly elevated the expressions of hepatic CHOP, GPR87, NF-κB and mTOR. Histopathological examination revealed massive hepatocyte degeneration following HgCl₂ administration. Treatment with Vit-E or/and Lac-B restored the normal levels of the previously mentioned parameters, as well as improved hepatic architecture.

Conclusion: Vit-E and Lac-B provided protective effect against HgCl₂-induced hepatotoxicity via reduction of oxidative stress and inflammation, and downregulation of CHOP, GPR87, NF-κB and mTOR proteins’ expressions.

Keywords
mercury, hepatotoxicity, lactobacillus plantarum, vitamin E, CHOP, mTOR

Introduction

Mercury is extensively spread in the environment, which leads to a continuous human exposure to even low levels of this heavy metal.⁴ People can be exposed to mercury by consumption of specific types of fish, dental amalgam or even at work.⁵ The main source of mercury is the natural degassing of the earth’s crust, including oceans and rivers. It has been documented that low level of mercury exposure caused immune system alterations and reduced mice resistance to viral infections.³ Mercury toxicity is attributed to its capability to diminish free sulfhydryl groups of GSH and other antioxidants enzymes.

Numerous studies illustrated that oxidative stress evokes various intracellular events, such as proliferation, cell-cycle arrest, and apoptosis.⁴,⁵ In addition, exposure to inorganic mercury can exert a dose-dependent cytotoxicity by producing high levels of H₂O₂, which is normally quenched by pyruvate and catalase.⁶ Mercuric chloride (HgCl₂) may damage the function of many organelles such as lysosomes that keep proton gradient through the membrane and decline renal glutathione peroxidase activity with upregulation of heme oxidase function. Several studies have found increased risk of pulmonary, renal, and CNS systems among dental workers.⁷ Mercury induces disruption of the cytochrome c oxidase system/ATP energy function,⁸,⁹ and inhibits enzymes needed to change porphyrins to ATP causing progressive porphyrinuria, leading to low energy and digestive injuries.¹⁰

Many studies indicated that oxidative stress represents a dangerous event correlated to the neurotoxic effects of HgCl₂.¹¹ The levels of various reactive species are dramatically increased upon HgCl₂ exposure.¹² Although it is broadly sulfhydryl reactive, yet signaling cascade implicated in mediating HgCl₂-induced liver injury is not fully investigated.

This initiates our interest to study a new mechanistic role of HgCl₂ hepatotoxicity at molecular level and to find a way to protect against this toxicity using a combination of vitamin E (Vit-E) and Lactobacillus plantarum (Lac-B). Vit-E is considered as a major lipid soluble element in the defense system inside the cell, and it can be obtained from diet. It has a powerful protection action against complications of various diseases due to its antioxidant role.¹³ Lac-B, a lactic acid bacterium, is used for dairy and meat fermentation, also it can be used as a probiotic with favorable actions on gut and metabolic illnesses.¹⁴

Materials and Methods

Chemicals
Vit-E and HgCl₂ were obtained from Sigma Chemical Co. (Sigma, St. Louis, MO, USA). Lac-B was obtained from local pharmacy. The primary antibodies of CHOP, GPR87, mTOR and NF-κB were obtained from Santa Cruz (Santa Cruz Biotechnology, CA, USA).

Experimental Animals
Thirty Wistar adult male albino rats weighing 150-200 g were obtained from the Animal House, Faculty of Pharmacy, King Saud University. Animals were kept at temperature of 20-22°C; they were fed with standard rat pellet chow with free access to tap water ad libitum. The Experimental protocol was approved by the Research Ethics Committee, King Saud University (KSU-SE-19-38).

Experimental Design

After 1-week acclimation, rats were allocated into 5 groups each contains 6 rats; they were treated as follows: the first group was served as the normal control group and administered distilled water; in the second group, rats were intoxicated subcutaneously with 5 mg/kg HgCl₂¹⁵ once daily; the rats in the third group were treated with Vit-E at a dose of 100 mg/kg/day, orally¹⁶; the fourth group was orally treated with 6 × 10¹⁰ CFU of Lac-B 1.8701/kg in 1 mL normal saline¹⁷; and the fifth group was treated with the combination of Vit-E and Lac-B. All treatments were given daily along with HgCl₂ for 2 weeks.

After completion of all treatments, rats were subjected to a gradual concentration of CO₂, then sacrificed by decapitation.
Blood samples were collected, and sera were separated by centrifugation at 3000 rpm for 20 min. The livers were also collected; parts of livers were homogenized in phosphate buffer to yield 20% homogenates. Then the homogenates were centrifuged for 20 min at 3000 rpm at 4°C, and the supernatants were kept at -80°C. Other parts of livers were rapidly frozen under liquid nitrogen and stored at -80°C for Western blotting. Parts of livers from each group were kept in 10% formalin for histopathological examination.

**Biochemical Analysis**

**Examination of the liver enzymes.** The activity of liver enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) was measured in the serum using kits obtained from Randox (Crumlin, UK).

**Determination of oxidative stress biomarkers.** Malondialdehyde (MDA) was estimated in the liver tissue following the method of Mihara and Uchiyama. Reduced glutathione (GSH) was determined using the method of Ellman. Superoxide dismutase (SOD) activity was evaluated following the procedure of Marklund and Marklund.

**Determination of liver inflammatory and apoptotic biomarkers.** Liver tumor necrosis factor alpha (TNF-α), cysteine–aspartic acid protease 3 (caspase-3) and interleukin 6 (IL-6) levels were measured using a highly sensitive ELISA kit (Immuno-Biological Laboratories Co., Ltd. Takasaki-Shi, Gunma, Japan).

**Western blot analysis.** Western blots were performed to determine the proteins expressions of CHOP, GPR87, mTOR, and NF-κB. Protein bands were visualized using the ECL-Plus detection system (Amersham Life Sciences, Little Chalfont, Buckinghamshire, UK) according to the manufacturer’s instructions. Positive immunoreactive bands were quantified densitometrically and compared with control.

**Statistical Analysis**

Data were expressed as mean ± SEM for quantitative measures. The statistical comparisons were performed using 1-way analysis of variance (ANOVA), followed by Tukey-Kramer multiple comparisons test. The level of significance was set at $P < 0.05$, $P < 0.01$, and $P < 0.001$. Statistical tests were conducted using GraphPad Prism 5.00 (GraphPad Prism, San Diego, California, USA).

**Results**

**Lac-B and/or Vit-E Retained Liver Functions in HgCl₂-Induced Hepatotoxicity**

The current work showed marked elevation of the activity levels of serum ALT and AST enzymes in rat exposed to HgCl₂ compared to control rats. While the administration Lac-B and/or Vit-E concurrently with HgCl₂ significantly lowered the activity of liver enzymes (Figure 1).

**Lac-B and/or Vit-E Attenuated Oxidative Stress, Inflammation, and Apoptosis Induced by HgCl₂**

Further assessment of the hepatic protective effects of Lac-B and/or Vit-E was conducted by measuring the levels of oxidative, inflammatory, and apoptotic markers. Figure 2 shows the significant increase of MDA level and decrease of GSH and SOD following HgCl₂ intoxication; accordingly, indicated high oxidative damage. In the same figure, the administration of Lac-B and/or Vit-E concurrently with HgCl₂ significantly lowered the activity of liver enzymes (Figure 1).

**Histological analysis.** Liver samples were fixed in 10% formaldehyde, and thinly sliced sections were used for histopathological examination using hematoxylin and eosin (H&E) stain.

**Western blot analysis.** Western blots were performed to determine the proteins expressions of CHOP, GPR87, mTOR, and NF-κB. Protein bands were visualized using the ECL-Plus detection system (Amersham Life Sciences, Little Chalfont, Buckinghamshire, UK) according to the manufacturer’s instructions. Positive immunoreactive bands were quantified densitometrically and compared with control.
Moreover, HgCl₂ intoxicated rats exhibited a significant elevation of the hepatic NF-κB (\( P < 0.001 \)) relative to control rats. On the other hand, rats received the antioxidants in question concurrently with HgCl₂ showed a significant reduction in the expression of NF-κB (Figure 4). In addition, HgCl₂ toxicity provoked apoptosis by causing a significant increase in capsase-3 expression in the liver of intoxicated rats (\( P < 0.001 \)). However, the use of Lac-B and/or Vit-E produced remarkable reduction in apoptosis by down-regulating capsase-3 expression (Figure 3).

**Lac-B and/or Vit-E Modulated the Expression of CHOP, GPR87, and mTOR**

The CHOP, GPR87, and mTOR were significantly overexpressed following HgCl₂ intoxication compared to control group (Figure 4). Interestingly, the administration of Lac-B or Vit-E either alone or in combination downregulated the expressions of those proteins in comparison to HgCl₂ intoxicated group (Figure 4).

**Lac-B and/or Vit-E Improved the Histopathological Changes Induced by HgCl₂ Overdose**

The hepatoprotective effects of Lac-B and/or Vit-E were confirmed by the histological examination of liver sections stained with H&E. The liver sections from the rats of the negative control group demonstrate normal hepatocytes and normal blood sinuoids (Figure 5A). The liver section from a rat administered HgCl₂ shows moderate hepatocyte with ballooning and binucleated hepatocytes, central vein congestion (Figure 5B). The liver section from a rat administered Vit-E with HgCl₂ shows a hepatic tissue with normal structure and architecture with dilated congested sinuoids, central vein conge-stion (Figure 5C). The liver section from a rat administered Lac-B concurrently with HgCl₂ shows moderate healing of the necrosis and a slight decrease of the dilated sinuoids (Figure 5D). Lastly, the liver section from a rat administered a combined therapy of Vit-E and Lac-B with HgCl₂ shows a marked improvement in hepatocyte degeneration with normal sinuoids (Figure 5E).
Discussion

Human may be exposed to HgCl₂ poisoning by multiple routes including oral, inhalation or skin exposures, since it is extensively distributed in the environment. The environmental levels of mercury are rising because of the discharge from hydroelectric, mining and paper industries. It can be found in some skin lightening products and the filling of dental amalgam. Therefore, the aims of the current study are to examine the potential hepatoprotective effects of Vit-E and/or Lac-B against HgCl₂-induced liver damage in rats, and to further investigate the mechanisms underlying those effects.
In this study, we examined the effect of \textit{HgCl}_2 intoxication on the activity of liver enzymes, and we found that \textit{HgCl}_2 cause significant elevation of serum ALT and AST activities. These enzymes are considered as critical and initial markers in the diagnosis of liver injury, as these enzymes could be released into the circulating blood directly due to hepatic cell damage.\textsuperscript{22,23} Our results were parallel to the outcomes of previous studies which revealed the increasing of the liver enzymes activities post excessive exposure of \textit{HgCl}_2.\textsuperscript{22,23}

Our study revealed the administration of \textit{HgCl}_2 caused significant increase in the levels of oxidative stress (MDA), inflammatory (IL-6, TNF-\textit{\alpha}, and NF-kB), and apoptotic (caspase-3) markers, while GSH level and SOD activity were lowered post \textit{HgCl}_2 injection. It has been approved that when \textit{HgCl}_2 is accumulated within the hepatic cells, this provokes oxidative stress and subsequent liver injury, and it is believed that the principal mechanism of hepatotoxic effect of \textit{HgCl}_2 is the liberation of free radical and production of reactive oxygen species (ROS).\textsuperscript{24} In addition, the inflammation is usually associated with the production of ROS,\textsuperscript{25} and the inflammatory cytokines such as IL-6, TNF-\textit{\alpha} and NF-kB are important transcription factors known to be sensitive to oxidative stress,\textsuperscript{26,27} see graphical abstract figure.

Additionally, our study revealed the upregulation of the protein’s expressions of CHOP, GPR87, and mTOR in the liver tissues from \textit{HgCl}_2 intoxicated rats. It is documented that in normal healthy conditions, CHOP is expressed at very low levels\textsuperscript{28}; while in diseased states the expression of CHOP is markedly increase and the apoptosis is triggered.\textsuperscript{28,29} Furthermore, Arfelt and colleagues documented the great relationship between the activation of NF-\textit{\kappa}B and GPR87-mediated cancer progression.\textsuperscript{30} Because mTOR is involved in the liver metabolism process of a drug,\textsuperscript{31} the balanced level of mTOR expression is vital for retaining hepatic cell homeostasis and prevention of liver damage and inflammation. Thus, the dysregulation of mTOR activity may result in liver injury, inflammation and even carcinogenesis.\textsuperscript{32}

The administration of Vit-E and/or Lac-B could reduce the \textit{HgCl}_2-oxidative stress and prevent the overexpression of apoptotic and inflammatory biomarkers in liver tissue; moreover, using of those antioxidants improved the liver morphology. It has been demonstrated that Vit-E and Lac-B alleviated \textit{HgCl}_2-induced testicular atrophy by reducing the testicular MDA, and serum inflammatory cytokines.\textsuperscript{5} It has been shown that Lac-B decreased the inflammation of rat’s paw edema induced by carrageenan (a food additive which can initiates inflammation), diminished the expression of inflammatory cytokines, and improved its morphological architecture.\textsuperscript{33} Moreover, in clinical studies, Lac-B exhibited antioxidant and anti-inflammatory activities in healthy individuals and diabetic patients.\textsuperscript{34,35} Likewise, Vit-E has shown the antioxidant properties against \textit{HgCl}_2-induced reproductive toxicity.\textsuperscript{36} Additionally, Vit-E possessed a protective action in human lung cell exposed to \textit{HgCl}_2, through inhibition of oxidative stress and apoptosis.\textsuperscript{37}

To sum up, this study suggested that administration of Vit-E and/or Lac-B has the potential to protect the liver from the toxicity induced by \textit{HgCl}_2 and also has the ability to attenuate and reverse \textit{HgCl}_2-induced oxidative stress, apoptosis, and inflammation in liver tissue. Additionally, the modulation of CHOP, GPR87, and mTOR may explain further mechanisms of liver injury and protection.

\textbf{Author’s Note}

AA and IH conceived of the study, participated in its design and coordination; IH, SA and WA carried out the animal experimentation and biochemical analyses; AA and IH conducted the gene and protein analyses, IHII analyzed the data; AA drafted the manuscript and all authors read and approved the final manuscript.

\textbf{Declaration of Conflicting Interests}

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