

Protective effect of selenium-enriched lactobacillus on CCl₄-induced liver injury in mice and its possible mechanisms

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Abstract

AIM: To study the protective effects and mechanisms of Se-enriched lactobacillus on liver injury caused by carbon tetrachloride (CCl₄) in mice.

METHODS: Seventy-two ICR mice were randomly divided into four groups: normal group, CCl₄-induced model group, low Se-enriched lactobacillus treatment group (L-Se group), and high Se-enriched lactobacillus treatment group (H-Se group). During a 3-wk experimental period, the common complete diet was orally provided daily for normal group and model group, and the mice in L-Se and H-Se groups were given a diet with 2 and 4 mg of organoselenium from Se-enriched lactobacillus per kg feed, respectively. From the 2nd wk of experiment, the model group, L-Se group, and H-Se group received abdominal cavity injection of olive oil solution containing 500 mL/L CCl₄ (0.07 mL/100 g body mass) to induce liver injury, and the normal group was given olive oil on every other day for over 2 wk. In the first 2 wk post injection with CCl₄, mice in each group were killed. The specimens of blood, liver tissue, and macrophages in abdominal cavity fluid were taken. Then the activities of the following liver tissue injury-associated enzymes including glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) as well as malondialdehyde (MDA) content were assayed. Changes of phagocytic rate and phagocytic index in macrophages were observed with Wright-Giemsa stain. Plasma TNF- α level was measured by radioimmunoassay. The level of intracellular free Ca²⁺ ([Ca²⁺]_i) in hepatocytes was detected under a laser scanning confocal microscope.

RESULTS: During the entire experimental period, the AST and ALT activities in liver were greatly enhanced by CCl₄

and completely blunted by both low and high doses of Se-enriched lactobacillus. The Se-enriched lactobacillus-protected liver homogenate GSH-Px and SOD activities were higher or significantly higher than those in model group and were close to those in normal group. CCl₄ significantly increased MDA content in liver homogenates, while administration of Se-enriched lactobacillus prevented MDA elevation. Phagocytic rate and phagocytic index of macrophages decreased after CCl₄ treatment compared to those in normal control, but they were dramatically rescued by Se-enriched lactobacillus, showing a greatly higher phagocytic function compared to model group. CCl₄ could significantly elevate plasma TNF- α and hepatocyte [Ca²⁺]_i level, which were also obviously prevented by Se-enriched lactobacillus.

CONCLUSION: Se-enriched lactobacillus can intervene in CCl₄-induced liver injury in mice by enhancing macrophage function activity to keep normal and beneficial effects, elevating antioxidant-enzyme activities and reducing lipid peroxidation reaction, inhibiting excessive release of TNF- α , preventing the dramatic elevation of [Ca²⁺]_i in hepatocytes.

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Key words: Se-enriched lactobacillus; Liver injury; Carbon tetrachloride; Macrophage; Ca²⁺

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INTRODUCTION

Hepatic fibrosis represents the response of the liver to diverse chronic insults such as parasitic disease, chronic viral infection (hepatitis B and C), immunologic attack (autoimmune hepatitis), hereditary metal overload, toxic damage, etc. Because of the worldwide prevalence of these insults, liver fibrosis is a common pathological process of hepatic disease, leading to the development of irreversible cirrhosis^[1,2]. If treated properly at fibrosis stage, cirrhosis could be prevented^[3]. However, there are no effective anti-fibrosis drugs till date. Selenium (Se), an essential nutrient element, can prevent the malignant transformation of normal cells and the activation of oncogenes with anticarcinogenic effects within a physiological dosage range^[4,5]. It has been found in clinical investigations that the blood selenium of hepatocarcinoma patients is

low, and the progress of liver injury and carcinomatous change are related to the degree of selenium deficiency^[6]. In addition, some studies indicate that selenium is closely related to the inhibition of hepatic fibrosis^[7,8]. However, the supplement of selenium is relevant to selenium style and dosage^[5]. Se-enriched lactobacillus is an organoselenium agent. We have demonstrated that concomitant Se-enriched lactobacillus administration to mice and rats subjected to CCl₄-induced liver injury results in a reliable hepatoprotection against hepatic damage, as well as an effective action on enhancing peripheral blood lymphocyte proliferation activity and RBC immune function^[9,10]. Therefore, the aim of this study was to further evaluate the beneficial action of Se-enriched lactobacillus on reversing a well-established liver injury and the possible mechanisms by analyzing the activities of several liver tissue injury-associated enzymes, phagocytic function activities in macrophages, plasma TNF- α level and change of hepatocyte intracellular free Ca²⁺ [Ca²⁺]_i homeostasis.

MATERIALS AND METHODS

Preparation of a complete diet with Se-enriched lactobacillus

Lactobacillus was grown to a density of 5.88×10^7 /mL in a medium containing sodium selenite to obtain Se-enriched lactobacillus. The Se-enriched lactobacillus was then processed into an organoselenium agent with 11.45 mg of selenium/g, then mixed into the common complete feed to produce the palletized complete diet with 2 and 4 mg of organoselenium/kg feed according to compound feed processing method in a feed processing plant.

Animals

Seventy-two ICR mice, equal number of males and females, weighing 27.74 ± 0.37 g, were obtained from Experimental Animal Center of Nanjing Medical University, China. The mice were housed at room temperature (20-25 °C) in a 12-h light and dark cycle. Free access to water and food was allowed during the experimental period. All mice were randomly divided into four groups: normal control group ($n = 18$), model control group ($n = 18$), low Se-enriched lactobacillus treatment group (L-Se group, $n = 18$), and high Se-enriched lactobacillus treatment group (H-Se group, $n = 18$). During a 3-wk experimental period, the common complete diet was orally provided daily for normal group and model group, and the mice in L-Se and H-Se groups were given a diet with 2 and 4 mg of organoselenium from Se-enriched lactobacillus/kg feed, respectively. In the 2nd wk of experiment, hepatic injury was induced in mice by carbon tetrachloride (CCl₄). The mice in model group, L-Se and H-Se groups were given abdominal cavity injection of olive oil solution containing 500 mL/L CCl₄ (0.07 mL/100 g body mass) every other day for over 2 wk. The mice in normal group received abdominal injection of olive oil at the same dose and frequency.

Phagocytic function detection of macrophage

The macrophage activities were observed in six mice of each group in the first 2 wk post injection with CCl₄. The mice received abdominal cavity injection of 1 mL saline suspension containing 5% chicken's erythrocytes, followed by 1 mL saline after 1 h. Abdominal cavity fluid was collected

from the killed mice and incubated for 30 min at 37 °C, then centrifuged at 1 000 r/min for 10 min to obtain the remaining deposit for smearing and staining with Wright-Giemsa stain. The phagocytic rate and phagocytic index of macrophages were counted under a light microscope.

Collection of specimens

In the first 2 wk post injection with CCl₄, six animals were chosen from each group and anesthetized with ether before they were killed to collect blood samples by enucleating eyeballs, which were put into test tubes containing anticoagulant heparin sodium (1:500). Plasma was obtained by centrifugation at 3 000 r/min for 20 min and stored for analysis. Then, the animals were killed and the liver was quickly washed *in situ* with ice-cold isotonic saline, followed by stripping the capsules. Parenchyma (0.5 g) was sheared into broken bits and put into test tubes with 4 mL physiological saline for tissue homogenates. Tubes were centrifuged at 4 000 r/min for 30 min at 4 °C to separate upper fluid, which was stored at -20 °C for analysis.

Laser scanning confocal microscopic analysis of intracellular free Ca²⁺ in hepatocytes

Hepatocyte isolation In the 1st wk post injection with CCl₄, six mice from each group were exsanguinated and liver was minced with dissecting scissors into approximately 1 mm³ pieces. The pieces of liver were placed into 3 mL D-Hank's solution containing 0.25% trypsin (1:250, Amresco Co. USA) for 1 h digestion at 37 °C, and then added into small 1640 medium (pH 7.2) containing 10% neonatal bovine serum (Sijiqing Co., Hangzhou, China). Streptomycin and penicillin (1×10^5 U/L for each) were used to terminate the digestion. The released cells were filtered through sterilized nylon membranes and washed in D-Hank's solution via two centrifugations at 1 000 r/min for 5 min after erythrocytes were dissolved with 0.85% amchlor solution, and then resuspended in 1640 medium containing 10% neonatal bovine serum.

Fluo-3/AM loading of hepatocytes Twenty microliters of suspension samples containing freshly isolated hepatocytes from each group was placed in 13 mm culture dishes. The glass bottom of the culture dishes were covered with a layer of 0.1 mg/mL ConA (Fluka Co., USA) and dried at 37 °C in order to allow cells to adhere. The cells were loaded with 15 μ L of 0.1 μ g/mL Fluo-3/AM (Calbiochem Co., USA) for 30 min at 37 °C, and washed thrice with Hank's solution (pH 7.2) to remove the extracellular Fluo-3/AM.

The fluorescence intensity of [Ca²⁺]_i in hepatocytes was determined by ACAS Ultima-312 laser scanning confocal microscopy. Total images were scanned in each experiment and the data were stored in disks for analysis.

Parameters of plasma and liver homogenates in mice with hepatic injury

The activities of glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) as well as malondialdehyde (MDA) content in liver tissue homogenates were assayed by spectrophotometry using commercial kits (Nanjing Jiancheng Biotechnology Institute, China). Plasma TNF- α

level was measured by ^{125}I -based radioimmunoassay using commercial reagents (Radio-Immunity Institute of General Hospital of Chinese Liberation Army). In general, the procedures indicated by the kits were performed strictly according to the manufacturer's protocol.

Statistical analysis

Results were expressed as mean \pm SE. Statistical analysis was performed by Student's *t*-test (STATISTICA, Statsoft Inc., Tulsa, USA) on a conventional personal computer. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of Se-enriched lactobacillus on AST and ALT activities of liver homogenates in mice with CCl_4 -induced hepatic injury

As shown in Table 1, during the entire experimental period, CCl_4 significantly increased AST activity in liver homogenates compared to normal control ($P < 0.05$). This effect was completely prevented by both low and high doses of Se-enriched lactobacillus ($P < 0.05$). Upon exposure to CCl_4 , ALT activity in liver homogenates was not affected in the 1st wk of treatment, but significantly enhanced in the 2nd wk ($P < 0.05$). This effect was also blunted by a high dose of Se-enriched lactobacillus ($P < 0.05$).

Table 1 Changes of AST and ALT activities in mice with hepatic injury after Se-enriched lactobacillus treatment (mean \pm SE)

Index	Group	Post-injection with CCl_4	
		1 wk	2 wk
AST (U/mg tissue)	Normal	20.43 \pm 0.54 ^a	17.68 \pm 1.21 ^a
	Model	28.05 \pm 1.03 ^c	26.81 \pm 0.46 ^c
	L-Se	23.60 \pm 0.93 ^a	17.86 \pm 1.75 ^a
	H-Se	18.90 \pm 0.94 ^a	18.90 \pm 2.26 ^a
ALT (U/mg tissue)	Normal	31.64 \pm 0.23	42.93 \pm 0.42 ^a
	Model	31.15 \pm 0.32	50.61 \pm 1.05 ^c
	L-Se	30.71 \pm 0.39	49.47 \pm 0.26 ^c
	H-Se	28.99 \pm 0.13 ^{ac}	43.64 \pm 1.61 ^a

^a $P < 0.05$ vs model group; ^c $P < 0.05$ vs normal group.

Effect of Se-enriched lactobacillus on GSH-Px and SOD activities of liver homogenates in mice with CCl_4 -induced hepatic injury

As demonstrated in Table 2, GSH-Px activity in the liver homogenates slightly decreased in the 1st wk, and significantly decreased in the 2nd wk after CCl_4 treatment. This effect was blunted by a high dose of Se-enriched lactobacillus ($P < 0.05$). Similarly, during the entire period of treatment, SOD activity dramatically decreased after CCl_4 treatment ($P < 0.05$), which was rescued by both low and high doses of Se-enriched lactobacillus.

Effect of Se-enriched lactobacillus on MDA content in liver homogenates of mice with CCl_4 -induced hepatic injury

Figure 1 shows that CCl_4 significantly increased MDA content in liver homogenates compared to normal control during the entire experimental period ($P < 0.05$). However, administration of Se-enriched lactobacillus prevented MDA elevation.

Table 2 Changes of GSH-Px and SOD activities in mice with hepatic injury after Se-enriched lactobacillus treatment (mean \pm SE)

Index	Group	Post-injection with CCl_4	
		1 wk	2 wk
GSH-Px (U/mg tissue)	Normal	6.43 \pm 0.67	6.40 \pm 0.38 ^a
	Model	6.24 \pm 0.36	5.21 \pm 0.24 ^c
	L-Se	7.68 \pm 0.33	5.67 \pm 0.25
	H-Se	8.10 \pm 0.21 ^a	6.45 \pm 0.31 ^a
SOD (U/mg tissue)	Normal	60.57 \pm 2.25 ^a	61.34 \pm 0.83 ^a
	Model	47.88 \pm 2.15 ^c	48.95 \pm 1.19 ^c
	L-Se	54.43 \pm 2.43	52.70 \pm 2.14 ^c
	H-Se	52.61 \pm 3.46	51.50 \pm 2.20 ^c

^a $P < 0.05$ vs model group; ^c $P < 0.05$ vs normal group.

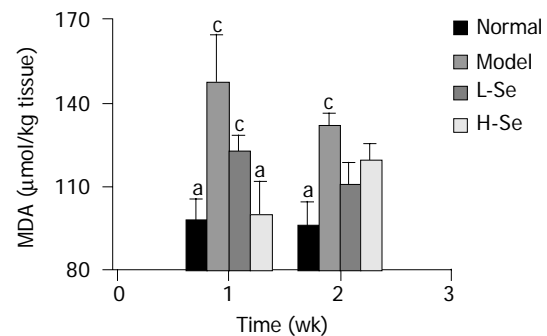


Figure 1 Change of MDA content in liver homogenates of mice with hepatic injury after Se-enriched lactobacillus treatment. ^a $P < 0.05$ vs model group; ^c $P < 0.05$ vs normal group.

Effect of Se-enriched lactobacillus on macrophage activities in mice with CCl_4 -induced liver injury

During the entire experimental period, the phagocytic rate of macrophages significantly decreased after CCl_4 treatment compared to normal control ($P < 0.05$), and was dramatically rescued by both low and high doses of Se-enriched lactobacillus, showing an obviously higher phagocytic rate than that in model group ($P < 0.05$). The phagocytic index of macrophages slightly decreased in the 1st wk, and significantly decreased in the 2nd wk after CCl_4 treatment. This effect was protected by high dose of Se-enriched lactobacillus ($P < 0.05$, Table 3).

Table 3 Changes of phagocytic function in mice with hepatic injury after Se-enriched lactobacillus treatment (mean \pm SE)

Index	Group	Post-injection with CCl_4	
		1 wk	2 wk
Phagocytic rate (%)	Normal	17.25 \pm 0.81 ^a	15.25 \pm 1.18 ^a
	Model	11.67 \pm 1.00 ^c	10.64 \pm 0.56 ^c
	L-Se	14.92 \pm 0.33 ^{ac}	12.67 \pm 0.49 ^a
	H-Se	20.42 \pm 0.86 ^{ac}	15.17 \pm 0.60 ^a
Phagocytic index (RBC/macrophage)	Normal	1.75 \pm 0.11	1.69 \pm 0.08 ^a
	Model	1.46 \pm 0.08	1.17 \pm 0.06 ^c
	L-Se	1.52 \pm 0.12	1.36 \pm 0.07
	H-Se	1.72 \pm 0.04 ^a	1.55 \pm 0.09 ^a

^a $P < 0.05$ vs model group; ^c $P < 0.05$ vs normal group.

Effect of Se-enriched lactobacillus on plasma $\text{TNF-}\alpha$ in mice with CCl_4 -induced liver injury

During the entire experimental period, CCl_4 significantly

elevated plasma TNF- α level compared to normal control ($P < 0.05$). This effect was completely prevented by both low and high doses of Se-enriched lactobacillus, showing an obviously lower value than that in model group ($P < 0.05$) and no significant difference as compared to normal group (Figure 2).

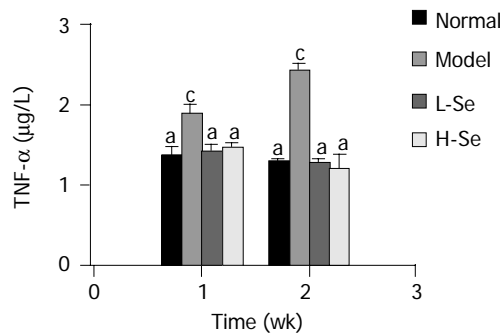


Figure 2 Change of plasma TNF- α level in mice with hepatic injury after Se-enriched lactobacillus treatment. ^a $P < 0.05$ vs model group; ^c $P < 0.05$ vs normal group.

Effect of Se-enriched lactobacillus on hepatocytes [Ca²⁺]_i in mice with CCl₄-induced liver injury

Figure 3A shows changes of hepatocyte [Ca²⁺]_i fluorescent visualizations in mice with hepatic injury after Se-enriched lactobacillus treatment. In model group, the hepatocyte [Ca²⁺]_i content significantly raised and was 5.5-fold higher than that in normal group ($P < 0.05$), but hepatocytes [Ca²⁺]_i in mice with liver injury after Se-enriched lactobacillus treatment was significantly downregulated ($P < 0.05$) and close to that in normal control (Figure 3B).

DISCUSSION

Liver fibrosis is a common pathological process of hepatic disease, leading to the development of irreversible cirrhosis in patients. In recent years, the mechanism of hepatic fibrosis has been partly disclosed^[11]. If treated properly at fibrosis stage, cirrhosis could be prevented^[3]. Several drugs, including cytokines, antioxidants, chemical drugs, soluble type II receptor for TGF- β_1 , and antibodies directed against TGF- β_1 have been used to block liver fibrosis, but their effects are not as good as expected^[12]. The present study has demonstrated that Se-enriched lactobacillus is effective in treating mice hepatic injury and fibrosis based on both lipid peroxidation reaction and functional analysis. The underlying protective mechanism may involve enhanced immunity, and downregulation of TNF- α release and equilibration of hepatocyte [Ca²⁺]_i homeostasis.

There are various kinds of liver injuries all over the world, causing great affliction to patients. Searches for effective ways to inhibit fibrogenesis and to prevent the development of cirrhosis are of great significance. Selenium can prevent the malignant transformation of normal cells and the activation of oncogenes with anticarcinogenic effects within a physiological dosage range^[4,5]. Selenium is closely related to the inhibition of hepatic fibrosis^[7,8]. Se-enriched lactobacillus is an organoselenium agent. The present study showed that

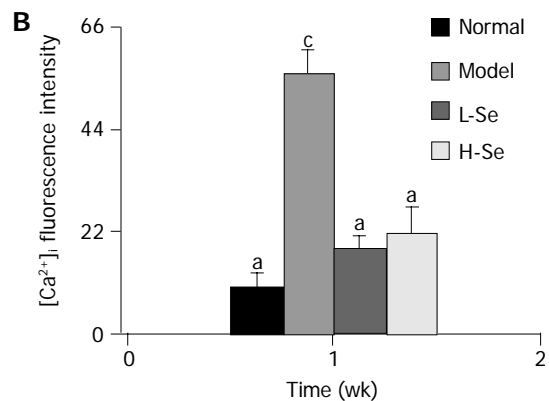
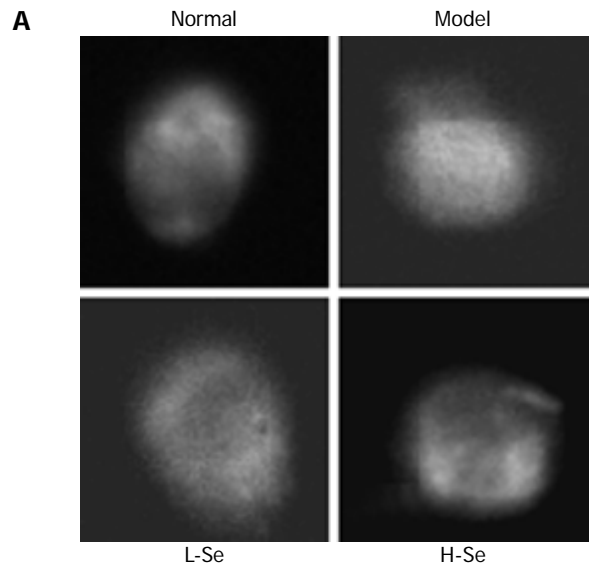


Figure 3 [Ca²⁺]_i fluorescence visualizations (A) and intensities (B) in mice with hepatic injury after Se-enriched lactobacillus treatment. ^a $P < 0.05$ vs model group; ^c $P < 0.05$ vs normal group.

ALT, AST and MDA in mice after Se-enriched lactobacillus treatment dropped significantly when compared to those in model group, indicating that Se-enriched lactobacillus reduces the violent lipid peroxidation reaction induced by CCl₄ and prevents hepatic injury.

GSH-Px, a selenium-containing enzyme, is a primary selenium-dependent, glutathione-utilizing peroxidase in the liver^[13-15]. The decreased GSH-Px activity has been implicated in animals fed with a selenium-deficient diet^[16,17]. Conversely, selenium supplement can also impact GSH-Px activity directly^[18,19]. In this study, CCl₄ caused an obvious decrease of GSH-Px activity in liver homogenates, but the enzyme activity was higher after Se-enriched lactobacillus treatment, suggesting that Se-enriched lactobacillus can ameliorate GSH-Px activity or enhance its activity by participating in GSH-Px biosynthesis in mice with CCl₄-induced liver injury. SOD is one of the potent antioxidant enzymes in cells and catalyzes the conversion of superoxide ions into oxygen and hydrogen peroxide. In our study, SOD activity was dramatically decreased by CCl₄, but the effect was rescued by both low and high doses of Se-enriched lactobacillus. GSH-Px and SOD are the important antioxidant enzymes of antioxidant

defense systems for stabilizing oxidative reactions. Decreased activity and content of these enzymes cause the descent of antioxidant ability in the body^[20]. Therefore, Se-enriched lactobacillus may prevent experimental liver injury by modulating or enhancing GSH-Px and SOD activities.

Macrophages are important immune cells of the immune system and the first line of defense to infection and inflammation in the body, and play a very important role in regulating the body immune status. Many experiments have demonstrated that the macrophage phagocytosis in mice with CCl₄-induced liver injury can be reduced^[16,21,22]. In our study, Se-enriched lactobacillus was found to be able to enhance the immunity of the body by increasing the phagocytic rate and phagocytic index in macrophages in L-Se group and H-Se group. This fact may be relevant to the protection of its biomembrane against oxidative destruction. Mayanski *et al.*^[22], showed that structural and functional integrity of the cell membrane system including macrophages in mice with CCl₄-induced liver injury is damaged, thus depressing the activity of macrophages.

To understand the mechanism, we evaluated the effect of Se-enriched lactobacillus treatment on blood TNF- α concentration in mice with liver injury. There is evidence that TNF- α is a primary and key endogenous mediator inducing acute inflammatory conditions^[23-25]. When an inflammatory reaction occurs, TNF- α is expressed by both infiltrating inflammatory cells such as macrophages in the blood and hepatocytes in liver injuries. Excessive TNF- α can indirectly induce liver damage by further enhancing the release of mass inflammatory mediators including free radicals, prostaglandin, leukotriene, thromboxane, interleukin-1, and TNF- α from macrophages, and also promote liver injury by increasing nitric oxide production from liver cells^[26,27]. The present study showed that plasma TNF- α level due to CCl₄ increased obviously, but the effect was significantly blunted by Se-enriched lactobacillus, indicating that Se-enriched lactobacillus downregulates excessive release of TNF- α .

It was reported that changes of intracellular cation homeostasis are closely related to the mechanism of hepatic cell injury^[28,29]. Elevation of [Ca²⁺]_i concentration is associated with the development of cell damage^[30,31]. In the present study, CCl₄ caused the increase of hepatocyte [Ca²⁺]_i, suggesting that the dramatic elevation of hepatocyte [Ca²⁺]_i due to CCl₄ is closely related to CCl₄-induced hepatocellular injury. However, the hepatocyte [Ca²⁺]_i level in mice with liver injury after Se-enriched lactobacillus treatment was significantly lower than that in model group, indicating that Se-enriched lactobacillus may prevent disturbance of [Ca²⁺]_i homeostasis. Other investigations showed that hepatocyte [Ca²⁺]_i elevation is caused by the decreased activities of Na⁺/K⁺- and Ca²⁺-ATPases in hepatocyte membranes^[32,33]. In addition, it is possible that CCl₄ may release Ca²⁺ by inhibiting the endoplasmic reticulum Ca²⁺ pump so that a passive leak of Ca²⁺ from the endoplasmic reticulum results in the increase of [Ca²⁺]_i and induces cell death^[30,34,35]. Thus, Se-enriched lactobacillus may play an important role in preventing liver injury. The exact mechanisms remain to be explored.

In conclusion, Se-enriched lactobacillus can significantly reduce CCl₄-induced liver injury in mice by enhancing macrophage function activity to keep normal and beneficial effects, elevating antioxidant-enzyme activities and reducing

lipid-peroxidation reaction, inhibiting excessive release of TNF- α and preventing dramatic elevation of hepatocyte [Ca²⁺]_i concentration.

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