Study on injury effect of food additive citric acid on liver tissue in mice

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Abstract To investigate the damaging effect and action mechanism of the food additive citric acid (CA) on mouse liver, 40 healthy male Kunming mice were randomly divided into control group (0.9 % saline), low CA dose (120 mg/kg), middle dose (240 mg/kg) and high dose groups (480 mg/kg). All experimental mice have received peritoneal injection of the corresponding reagent each week for 3 weeks. After 7 days since the third injection, morphological changes were observed by light microscope; activities of T-SOD, glutathione peroxidase (GSH-Px), caspase-3, and contents of hydrogen peroxide (H_2O_2) and malonyldialdehyde (MDA) in the liver were evaluated using the corresponding assay kits; DNA fragmentation was assayed using agarose gel electrophoresis. Microscopical detection showed a series of hispathological changes in mouse livers treated with CA, such as indiscriminate liver cell cord, blood clot in central veins, and lymphocyte infiltrating. Biochemical examination suggested the gradually but moderately reduced T-SOD activity and elevated H_2O_2 level with the increase of CA dose ($P > 0.05$), and the gradually reduced GSH-Px activity and increased MDA content depending on graded doses with a significant difference ($P < 0.05$) between the high dose group and the

control group. According to cell apoptosis assays, caspase-3 activity were significantly higher in all treatment groups than in the control ($P < 0.05$) in a dose-dependent manner. Contrasting to the control, characteristic DNA laddering was observed when injected with any of the three graded doses. It can be concluded that certain concentrations of CA cause oxidative damage of the liver by means of the decrease of antioxidative enzyme activities, thus resulting in MDA level elevation and DNA fragmentation inducing active caspase-3.

Keywords Citric acid · Mouse · Liver · Oxidative damage · Cell apoptosis

Introduction

Citric acid (CA), a flavoring agent and preservative, is used widely in food and daily chemical goods. As a kind of the most effective single organic acid, citric acid has broad applications in the area of poultry production and disease prevention. For instance, in the review by Heo et al. (2013), it was reported that addition of citric acid or its salts can enhance the growth and the feed to gain ratio of weaned and growing-finishing pigs. Through monitoring the effect of graded concentration of lanthanum citrate on lung cancer cells, Li and Chen (2000) observed the negative

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effect of citric acid on the proliferation of the human lung cancer cell PG at concentrations of 0.05, 0.1, 0.5 and 1.0 mmol/l.

With widespread application of citric acid in food, industry, chemicals and other fields, the general population is more and more frequently exposed to citric acid via ingestion of food and dermal contact with this compound and other products containing citric acid. So it is inevitable that some safety problems have arisen concerning its use, and its toxicity on living organism has become the focus of research nowadays. Much research has already indicated the significant damage of long-term use of overdose of citric acid on body's tissues. One reasonable explanation is that citric acid entering the organism can be absorbed by the detoxifying organs (e.g., liver) and act as an adjuvant to complex with metal ions contained in the detoxification enzymes [e.g., glutathione peroxidase (GSH-Px), superoxide dismutase (T-SOD)] and inactivate these enzymes. Furthermore, detoxifying this kind of xenobiotics leads to the generation of free radicals [e.g., hydrogen peroxide (H_2O_2)] by means of oxidation/reduction reaction, exerting a damaging impact on body's tissues. To the best of our knowledge, although many parameters have been used to indicate the toxic effects of citric acid or citrate on living organisms, there still remains limited information on its detailed effects in the livers. It was reported from clinical biochemistry examination of drink poisoning in children that baby's liver becoming enlarged was related to the addition of citric acid into drinks. In addition, by monitoring the short-term effect of single dose citric acid on mouse tissue, Aktaç et al. (2003) discovered that citric acid treatment caused injury of hepatocyte membranes, cytoplasmic vacuolization in hepatocyte, karyopyknosis and so on, suggesting the toxic effects of citric acid on mice. To reveal how this food additive exerts the damaging effect on the liver, Zhang and his coworkers performed a biochemical analysis on citric acid-treated mice and the result showed a significant decrease in the activities of antioxidative enzymes such as T-SOD and GSH-Px, and a series of pathological changes such as disorganized hepatocyte cords, blood clot in central veins, lymphocyte and neutrophil infiltrating (Zhang et al. 2011).

On the other hand, whether toxicity of citric acid to the body is associated with cell apoptosis or not also deserves study. Koca et al. (2005) reported that citric

acid could significantly increase micronucleus frequency in red blood cells of *Tinca tinca*. Türkoğlu showed that citric acid decreased the mitotic index in *Allium cepa* chromosomes (Türkoğlu 2007). Yılmaz et al. also discovered that citric acid significantly decreased mitotic index and increased chromosomal aberrations at the concentration of 100 and 200 $\mu\text{g ml}^{-1}$, which in turn induced cell apoptosis (Yılmaz et al. 2008). Also, some studies suggested that CA exerts its cytotoxic effect on glial cells by inducing apoptosis (Lan et al. 1999; Navarro-Escobar et al. 2010). However, there are still very few studies for exploring whether citric acid or citrate could induce apoptotic cell death in mouse liver. It was just in recent years that some researchers found necrotic changes caused by this xenobiotic substance, such as vacuolated cytoplasm in hepatocytes, and chromatin decrease in mouse liver (Aktaç et al. 2003). One of the approaches examining the harmful influence of citric acid on living organisms is to determine its effects on oxidative stress enzymes and its metabolites whose changes in activity and content indicate injury degree of the body. Among these enzymes and its metabolites, T-SOD, GSH-Px, H_2O_2 and malonyldialdehyde (MDA) are the most important ones. Another way is testing the extent of DNA fragmentation and caspase-3 activity that represent cell apoptosis index. Therefore, we estimated the effects of graded doses of citric acid on liver tissue in mice. In this study, we first tested the activities of oxidative stress enzymes and levels of its catalytic products, then DNA ladder and caspase-3 activity assay, and finally the histopathological changes of liver tissue.

Materials and methods

Material

Citric acid (pure) was obtained from the Chengdu Kelong Chemical Reagent Factory (ChengDu, China). The chemical properties of citric acid are as follows: chemical formula is $\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$, molecule weight is 210,14 g/mol. T-superoxide dismutase Assay Kit (No. 20111129), and Glutathione Peroxidase Assay Kit (No. 20111125) were purchased from the NanJing JianCheng Bioengineering Institute (NanJing, China). Hydrogen Peroxide Assay Kit (No. 20111201), Maleic Dialdehyde Assay Kit (No. 20111125), and Caspase-3

Assay Kit were from the Beyotime Institute of Biotechnology (JingSu, China).

Animals and treatments

Forty healthy male Kunming mice used in this experiment (weighing 20 ± 2 g), were purchased from the Animal Center of the Henan Province (ZhengZhou, China). They were randomized into four groups of 10 mice each: the control group, citric acid low, middle and high dosage groups, respectively. The animals were housed in stainless steel cages within an air-conditioned room, and allowed standard mouse chow diet and water ad libitum for the duration of experiment. After 1 week adaptation, animals from four experiment groups were treated as below:

Control Physiological saline (0.9 % NaCl, intraperitoneally)

Low dose group citric acid dissolved in physiological saline (120 mg/kg intraperitoneally)

Middle dose group citric acid dissolved in physiological saline (240 mg/kg intraperitoneally)

High dose group citric acid dissolved in physiological saline (480 mg/kg intraperitoneally)

Animals received intraperitoneal injection once a week for 3 weeks. Seven days after the third injection, the mice were sacrificed by cervical dislocation. The liver was removed, washed free from extraneous materials and kept in ice till further processing.

HE staining of liver sections

The liver lobes were cut into about $3 \text{ mm} \times 4 \text{ mm}$ blocks, and fixed in 10 % neutral formaldehyde for 48 h, followed by washing under current water for 24 h. Blocks of liver tissue were dehydrated using graded alcohol with a concentration of 50–100 %, vitrified by dimethylbenzene, dipped, embedded, sliced, and spread, followed by dewaxing with dimethylbenzene and rehydration with graded alcohol (100–50 %). Slides were placed in hematoxylin stain for 4 min, differentiated in 1 % hydrochloric-alcohol solution and stained in 0.01 % eosin for 2 s, rinsed in 95 % alcohol, dehydrated in absolute alcohol and cleared in xylenes for 15 min before coverslipping. Finally, morphological changes of liver tissue from the four experimental groups were observed under light microscope.

Determination of activities of T-SOD, GSH-Px and contents of H_2O_2 , MDA

Firstly, 10 % mouse liver homogenates were prepared. According to the instructions in the corresponding assay kits, the activities of T-SOD, GSH-Px, and contents of H_2O_2 , MDA in the liver were determined using the methods of xanthine oxidase, DTNB, Ammonium Molybdate and TBA, respectively.

Measurement of caspase-3 activity

A 10 mM diluent solution of the AMC reference standard (Beyotime Institute of Biotechnology, Shanghai, China) was firstly prepared, then this solution was used to prepare a standard curve to determine the moles of product produced in the caspase-3-containing reaction. Fresh liver tissues from the four experimental groups were collected. According to the sample quantity, 100 μl of lysates was added to per 0.003–0.01 g of liver tissue sample. Tissue lysates were well homogenized in an ice bath, centrifuged at 16,000–20,000 r/min for 10–15 min at 4 °C. The caspase-3 activity was assayed as described in the kit protocol.

DNA ladder assay

One liver tissue sample from each group was randomly selected for weighing 0.1 g of liver tissue sample and cut into small pieces. DNA isolation was performed according to the method described in the DNA Ladder Detection Kit. The extracted DNA was stained with 5 % bromophenol blue and then electrophoresed on a 1.2 % agarose gel (90 V, 35 min) containing 0.5×10^{-3} mg/ml ethidium bromide (EB) for identifying DNA fragments. The DNA marker with 7 bands (2,000, 1,600, 1,000, 750, 500, 250, 100 bp) was purchased from Beijing Dingguo Changsheng Biotech Co., Ltd, China.

Statistical analysis

Data are expressed as “mean $\pm$ SD”. Data comparisons were carried out using one way analysis of variance (one-way ANOVA) followed by LSD test and Duncan test to compare means between the different treatment groups. Difference between any

two groups with $P < 0.05$ was considered as statistical significance.

Results

The effect of citric acid on liver structure in mice

The results of light microscopic examination showed the varying degrees of necrotic changes in the liver of mice treated with different doses of citric acid (Fig. 1). There were basically complete liver tissue structure in the control group (Fig. 1a). Compared to the control, lost structure of hepatic lobules, cytoplasmic vacuolization in hepatocytes, small venule bleeding, inflammatory infiltration around the central vein (Fig. 1b), picnotic nuclei, chromatin condensation, obscure cell contour, liver cell turgescence, central venous congestion (Fig. 1c), severe injury of liver structure, hepatocyte necrosis, more obvious karyopyknosis, characteristic vacuolation and granular denaturation around liver cell nuclei, liver cell cord derangement (Fig. 1d) as degenerative changes in mouse liver tissue could be observed with the increase of the citric acid level.

The effect of citric acid on the activity of antioxidant enzymes and levels of free-radical metabolites in mouse liver

According to enzyme activity assay results, it was demonstrated that T-SOD and GSH-Px activities were significantly lower in all treated groups than that in the controlled group. The statistical analysis showed no significant difference in T-SOD activity between CA-treated groups ($P > 0.05$). With citric acid dosage increasing, however, T-SOD activity in the four experimental groups increased gradually. Any dose of citric acid significantly decreased GSH-Px activity in a dose-dependent manner ($P < 0.05$), but, the differences between the three treatment groups were not statistically significant.

Free radical metabolites assay indicated that citric acid dose-dependently induced an increase in the MDA levels in all treatment groups (Table 1). Specifically, the MDA content in the high dosage group markedly increased ($P < 0.05$, vs control group). Whereas, H_2O_2 level in all treated groups was nearly

unchanged or even moderately lower than in the control ($P > 0.05$).

The effect of citric acid on caspase-3 activity in mouse liver

A simple linear regression analysis was performed with pNA concentrations and their OD values, which thus generated a standard curve. With reference to Fig. 2, regression equation representing the relativity of pNA concentration and OD value was as follows: $y = 447.99x - 3.2675$. The correlation coefficient was 0.9985, suggesting a high correlation between both items. Caspase-3 activities per unit of protein weight between different experimental groups were statistically analyzed by the software SPSS 18.0. The results summarized in Table 2 showed that citric acid increased caspase-3 activity in all treatment groups when compared to the control. And this increase was dose-dependent. In detail, compared with the control group, there is a highly significant difference in the low and the middle dosage groups ($P < 0.01$), and a significant difference in the high dosage group ($P < 0.05$), however, there is no statistically significant difference between the three treated groups ($P > 0.05$).

The effect of citric acid on DNA damage in mouse hepatocytes

To determine whether a certain level of citric acid can contribute to an accelerated hepatocyte death, DNA laddering assay was performed to identify apoptosis. The result shown in Fig. 3 revealed that DNA laddering, which is indicative of apoptotic events, occurred in hepatic cell samples from three treated groups, whereas cell apoptosis in the control sample was not detected. In detail, DNA laddering was much more apparent in high dosage group (Lane H) than in the other two dosage groups. Analysis of the results indicated that the apoptotic effect of citric acid on hepatocytes was dose-dependent.

Discussion

The effects of CA in living organisms can be investigated in various ways. Among these methods, graded-dose toxicity tests are commonly used. In this method, many parameters can be used to test the effects

Fig. 1 HE staining showing the effects of citric acid injection on liver tissue structure in mice. *panel a*: control group; *panel b*: low dosage group (120 mg/kg); *panel c*: middle dosage group (240 mg/kg); *panel d*: high dosage group (480 mg/kg). *Left* panels represent $10\times$ liver tissues; *right* panels represent $40\times$ liver tissues

Table 1 The effect of citric acid on the activities of antioxidant enzymes and levels of free radical metabolites in the liver of mice

Groups	T-SOD activity/ (U/mg)	GSH-Px activity/ (U/mg)	MDA content/ (mmol/gprot)	H ₂ O ₂ content/ (mmol/gprot)
Control (saline)	309.76 ± 1.88 ^a	110.63 ± 1.32 ^a	4.01 ± 0.55 ^a	28.14 ± 0.33 ^a
Low dosage (120 mg/kg)	255.68 ± 2.26 ^a	98.82 ± 1.47 ^{ab}	4.35 ± 1.01 ^a	24.06 ± 0.23 ^a
Middle dosage (240 mg/kg)	257.59 ± 1.81 ^a	96.03 ± 1.77 ^{ab}	4.47 ± 0.20 ^a	24.98 ± 0.46 ^a
High dosage (480 mg/kg)	291.08 ± 2.20 ^a	76.17 ± 1.61 ^b	5.63 ± 0.51 ^b	27.81 ± 0.36 ^a

Different small letters within the same column mean significant difference ($P < 0.05$); the same letters represent no significant difference ($P > 0.05$)

Fig. 2 pNA standard curve for caspase-3 activity**Table 2** The effect of citric acid on Caspase-3 activity (UI/mg) in the liver of mice

Groups	Mice number (n)	Enzyme activity (UI/mg)
Control	10	$(1.46 \pm 0.28) \times 10^{6a}$
Low dosage	10	$(1.86 \pm 0.33) \times 10^{6b}$
Middle dosage	10	$(1.94 \pm 0.17) \times 10^{6b}$
High dosage	10	$(1.96 \pm 0.20) \times 10^{6b}$

Different small letters within the same column mean significant difference ($P < 0.05$); the same letters represent no significant difference ($P > 0.05$)

of CA. Some of these parameters are body weight, blood profile, histopathological, and biochemical examination. In the present study, the effects of different doses (120, 240, and 480 mg/kg) of citric acid injected intraperitoneally in mouse liver were investigated. It has been reported that body weight decreased in mouse by the effects of citric acid in chronic studies (Qin et al. 2004). Similarly, Aktaş et al. (2003) determined that injection of CA in Balb/C mice led to a significant decrease of body weights. However,

Fig. 3 Citric acid-induced hepatocyte apoptosis measured by the DNA Ladder Assay. Four samples representing the four treatment groups were loaded in the corresponding lanes. Electrophoresis was performed on 1.2 % agarose gel. C control, L low dosage group, M middle dosage group, H high dosage group

our study did not put into evidence the decrease of body weight in mice by the effect of CA, probably due to the influences of a variety of factors such as breeding condition, injection dosage of CA and so on. Additionally, we also could not find any significant change of liver weights by intraperitoneal injection of citric acid in mice (Data not shown).

Although the liver weights were not changed significantly, light microscopy examination revealed the pathological changes in mouse liver, such as cytoplasmic vacuolization in hepatocytes, picnotic nuclei, small venule bleeding and inflammatory infiltration. Again, similar findings were obtained in the mice with oral treatment of citric acid (Aktaş et al. 2003). Our results suggested the hepatotoxic effect of CA and even severe damage induced by CA exposure in mouse liver. However, the mechanism of damaging effects of this food additive on mouse liver needs to be clarified by more detailed studies as follows.

Up to date, there are few reports whether the damage of CA exposure on the liver of mice is due to the mechanism of oxidative stress. For this reason, we tested the activities of antioxidant enzymes (e.g., T-SOD, GSH-Px) and levels of free radical metabolites (e.g., H_2O_2 , MDA) in the liver of mice treated with different doses of citric acid. SOD, an important metal-containing antioxidant enzyme, is a kind of effective free radical scavenger converting naturally-occurring but harmful superoxide radicals to molecular oxygen and H_2O_2 (Kapasi et al. 2004).

Theoretically, citric acid can act as an adjuvant to remove the copper or zinc ions contained in SOD and thus markedly decrease SOD activity. Consistently, it has been reported by Zhang et al. (2011) that T-SOD activity in the liver treated with a high dose of CA (480 mg/kg) was significantly reduced ($P < 0.05$) when compared with the control. However, somewhat differently, we observed a gradual decrease in SOD activity with the increase of the dose of injected citric acid, but the difference was not statistically significant ($P > 0.05$). One possible reason may be that only a portion of CA peritoneally injected into the body can capture copper or zinc ions in SOD to form metal-citric acid complex, thus just leading to inactivity of some superoxide dismutases.

GSH-Px, one of the most important antioxidant, is a selenium-containing enzyme which plays a role in the detoxification of H_2O_2 . The mechanism of the effect of citric acid on its activity is almost identical to that mentioned above. The result of this study showed that CA decreased GSH-Px activity in a dose-dependent manner, and the GSH-Px activity in the high dose (480 mg/kg) group was significantly different from that in the control group ($P < 0.05$). The findings of this study coincided with the results of Zhang et al. (2011). The possible mechanism is the following: when a higher level of citrate is injected into the body, it can react with metal selenium to form a complex which in turn reduces GSH-Px activity.

Free radicals produced during the course of the metabolism can attack polyunsaturated fatty acids in the biomembranes to result in lipid peroxidation of cellular membranes, finally forming lipid hydroperoxide, such as MDA (García-Gil et al. 2012). So the MDA levels in body are usually used as indicator of the extent of lipid peroxidation. In our study, MDA levels showed an ascendant trend with the increase of CA dose and showed a significant increase ($P < 0.05$)

in the high dose group, indicating high doses of citric acid can do great damage to cell membranes. It is possible that the measurable reduction of antioxidant enzyme activities (i.e., T-SOD, GSH-Px) by exposure to high dose of CA may lead to a deficiency in free radical removal, furthermore speeding up the lipid peroxidation reaction followed by the generation of high levels of MDA in mouse hepatocytes. Another metabolic waste produced during metabolism is H_2O_2 which is also harmful to the body. Its breakdown is catalyzed by catalase converting H_2O_2 to water and oxygen and thereby mitigating its toxic effects, so catalase activity was negatively correlated with H_2O_2 level (Rezvani et al. 2006). The higher catalase activity is, the lower the H_2O_2 level is. But our findings did not show any obvious reduction ($P > 0.05$) in the H_2O_2 content in the liver of CA-treated mice compared with the control. This is possibly due to the nonspecificity of the enzymes degrading H_2O_2 or the incapability of CA to impact on catalase activity by complexing with metal ions because of the lack of metal ion in this enzyme (Flora et al. 2003).

As described previously, the insufficient scavenging of free radicals caused by the weaker activities of antioxidant enzymes such as T-SOD or GSH-Px accelerates caspase-3 activation and apoptosis induction. So, caspase-3 activation and nuclear DNA fragmentation, serving as the important indicators of cell apoptosis, were detected in this present study. Caspase-3 activity assay result suggested that, in contrast with the control, the activity of enzyme per milligram of total protein in the three treatment groups rose by 32, 34, and 27 %, respectively. In addition, there was a marked difference ($P < 0.05$) between the three treatment groups and the control group, indicating that a low dose of CA (120 mg/kg) could promote cell apoptosis by increasing caspase-3 activity.

In addition, in order to measure DNA fragmentation using gel electrophoresis, we treated mouse hepatocytes with graded doses of citric acid (120, 240 and 480 mg/kg). DNA laddering in mouse hepatocytes was already observed when just injected with 120 mg/kg of citric acid. Similar DNA laddering was also observed when the livers were injected with the middle and high doses of CA. Compared to the control, the significantly increased intensity in DNA “ladder” occurred after peritoneal injection with any dose of citric acid, indicating the occurrence of

apoptosis in hepatocytes. Here, it should be mentioned, summarizing the above results, that not only the stronger caspase-3 activity but also the greater DNA band intensity appeared in the high dose group when compared with the two other treatment groups, suggesting that citric acid at 480 mg/kg concentration contributes more to cell apoptosis, which is in agreement with the result of Zhang et al. (2011).

Thus, according to above-mentioned results, it can be concluded that citric acid indeed has damaging effects on organs in a dose-dependent manner, and controlling the consumption of citric acid is important for the health of the living organism.

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