ORIGINAL RESEARCH

Effect of Tea Polyphenols on Nuclear Factor Erythroid 2-related Factor 2 (NRF2) and Kelch-like ECH-associated Protein 1 (KEAP1) Gene Expression in Mice with Acute Cadmium Poisoning

Tingting Tao, MM; Xiaoshan Li, BD; Linyu Li, MM

ABSTRACT

Background • Cadmium poisoning is mainly caused by inhalation of cadmium dust or cadmium compound dust, which greatly harms people's lives. Tea polyphenols extracted from green tea have wide biological properties, including anti-cardiovascular disease, anti-tumor, anti-inflammatory, and immune regulation. The transcription factor nuclear factor erythroid 2-related factor 2 (NRF2) and Kelch-like ECH-associated protein 1 (KEAP1) are involved in the regulation of cadmium-induced oxidative damage. However, whether tea polyphenols relieve acute cadmium poisoning via regulating NRF2 and KEAP1 gene expression remains unclear.

Objective • To explore the influences of tea polyphenols on NRF2 and KEAP1 gene expression in mice with acute cadmium poisoning.

Design • This is an animal experiment that adopts hematoxylin and eosin (HE) staining and immunohistochemistry (IHC) staining.

Setting • This study was carried out in Zunyi Medical and Pharmaceutical College.

Participants • Fifty specific pathogen-free (SPF) male Kunming mice aged 9 weeks, weighing 18-22 g were divided into five groups: normal group, model group, low-dose tea polyphenols group, middle-dose tea polyphenols group, and high-dose tea polyphenols group.

Interventions • Tea polyphenols were administered intraastrically into mice with doses of 50 mg/kg, 100 mg/kg, and 200 mg/kg for 10 consecutive days, respectively.

Observation indicators • (1) liver coefficient, (2) pathological liver injury, (3) liver function, (4) oxidative damage, and (5) NRF2 and KEAP1 gene expression.

Results • The liver coefficient, pathological liver injury, serum aspartate transaminase and alanine transaminase levels of the model group were higher relative to the normal group ($P < .05$). Relative to the model group, different doses of tea polyphenols treatment significantly relieved liver coefficient, pathological liver injury, serum aspartate transaminase, and alanine transaminase levels ($P < .05$). Malondialdehyde content in liver tissues of the model group was significantly higher compared to the normal group, while glutathione together with glutathione peroxidase contents of the model group was lower ($P < .05$). Compared to the model group, malondialdehyde content in liver tissues declined while glutathione together with glutathione peroxidase contents were elevated after different doses of tea polyphenols treatment ($P < .05$). Relative to the normal group, NRF2 expression in the liver tissues of the model group was significantly lower, while KEAP1 expression was higher ($P < .05$). Relative to the model group, NRF2 expression in the liver tissues was elevated after treatment of different doses of tea polyphenols, while KEAP1 expression was declined ($P < .05$).

Conclusion • Tea polyphenols can relieve liver injury in mice with acute cadmium poisoning by regulating NRF2 and KEAP1 expression. Our study might provide a promising treatment strategy for acute cadmium poisoning. (*Altern Ther Health Med.* [E-pub ahead of print.])

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INTRODUCTION

As a kind of environmental pollutant, cadmium exists widely in the production and living environment and seriously threatens the health of animals and human beings.[1] Cadmium-related compounds are highly water-soluble and can enter plants through environmental water, air, and soil and accumulate in animals and humans through the food chain, causing acute and long-term chronic pathological effects and damaging many tissues and systems.[2] The most

common sources of cadmium exposure to humans are factories for non-ferrous metal smelting, electroplating, and cadmium compounds as raw materials or catalysts or cigarette smoke.[3] Studies have shown that trace cadmium entering the body will cause a series of damage to the liver, kidney, and reproductive organs through biological amplification and accumulation.[4] Recently, numerous studies have shown that cadmium can stimulate oxidative damage.[5] Cadmium toxicity cannot directly induce redox reactions to produce free radicals. Still, it can disrupt the oxidation balance of animals in a variety of ways or ways, such as through the interaction between transporters, depleting glutathione or displacing other metal ions in enzymes, causing oxidative stress, which results in increased levels of reactive oxygen species and reactive nitrogen species in the body and damage to DNA, proteins or lipids.[6] However, there is no good way to alleviate the stress effect of cadmium on animal tissues and reduce the accumulation of cadmium in the body. How to effectively treat the organ damage caused by cadmium poisoning and reduce the accumulation of cadmium in the body has been a difficult problem for medical workers.

The transcription factor nuclear factor erythroid 2-related factor 2 (NRF2) has been considered to be the master modulator of a cellular defense against toxic damage.[7] During an oxidative stress response, NRF2 is dissociated from its cytosolic inhibitor Kelch-like ECH-associated protein 1 (KEAP1), translocated to the nucleus, as well as combined with antioxidant-response elements on target gene promoters.[8] In a word, the NRF2-antioxidant system belongs to a type of the most cellular protection and defense systems against oxidative damage, and it is of great significance to clarify its role in cadmium-stimulated nephrotoxicity.[9]

Recently, more and more focus has been paid to the utilization of natural active ingredients with antioxidant activity to prevent heavy metal poisoning.[10] Tea polyphenols (TPs), a kind of polyphenolic compounds extracted from tea leaves, and green TPs extracted from green tea have the strongest activity and wide biological properties, including anti-cardiovascular disease, anti-tumor, anti-inflammatory, and immune regulation.[11] As reported previously, TPs can prevent the growth and metastasis of colorectal cancer by promoting the immune system and decreasing inflammatory responses.[12] TPs exert anti-inflammatory effects in cardiovascular diseases.[13] Among them, the powerful free radical scavenging antioxidant properties, inhibition of lipid peroxidation, and other functions have attracted much attention.[14] At present, the research on its application value is more and more extensive.

Herein, this study was designed to investigate the influences of TPs on NRF2 and KEAP1 gene expression in mice with acute cadmium poisoning. The novelty of our study is that we demonstrate that TPs can relieve liver injury in mice with acute cadmium poisoning by regulating NRF2 and KEAP1 expression, which might provide a novel sight for the treatment of acute cadmium poisoning.

MATERIAL AND METHODS

Animals

Fifty specific pathogen-free (SPF) male Kunming mice aged 9 weeks, weighing 18-22 g, were obtained from Chengdu Dashuo Animal Technology Co., LTD. Mice were housed in a standard environment which was characterized by 12 h light/dark cycle, 22–25 °C and 40–60% humidity with free access to water and forage. The study has received appropriate ethical approval from an animal care and use committee.

Animal grouping

After one week of adaptive feeding, 50 mice were randomly divided into 5 groups: normal group (NG), model group (MG), low-dose TPs group, middle-dose TPs group as well as high-dose TPs group, with 10 mice in each group. The NG was intraperitoneally injected with normal saline. The MG was intraperitoneally injected with cadmium chloride; the dose was 2.5 mg/(kg·d), and continued for 3 days. The intraperitoneal injection of cadmium chloride in the TPs (Purity 98.06%) treatment group was the same as the MG. After 3 days, TPs were administered intragastrically into mice with doses of 50 mg/kg, 100 mg/kg, and 200 mg/kg for 10 consecutive days, respectively based on preliminary experiments.

Weight changes in liver tissues

After the experiment, the mice were weighed, and their livers were quickly removed after death, cleaned with normal saline, and weighed. Calculation: organ coefficient = liver weight (g)/body weight (g) × 1000.

Hematoxylin and eosin (HE) staining

Briefly, 4 μm frozen liver sections after air-dried were fixed in 10% neutralized buffered formalin (NBF) for 10 min, and then rinsed in 95% ethanol for 5 min. The slides were then washed three times and stained with hematoxylin (Beyotime, Shanghai, China) for 40 s. After washing, the sliced slides were soaked in ammonia for 4 times, rinsed with tap water for 4 times, and then soaked in 95% ethanol for 20 times. Next, the sections were stained in eosin (Beyotime, Shanghai, China) for 10 s. The slides were dehydrated twice in 95% ethanol, three times in 100% ethanol, treated twice with xylene, and attached to a mounting medium.

Immunohistochemistry (IHC) staining

The specimens were taken for paraffin-embedding, dewaxing, and rehydrating. Then, the slides were washed and heated. Afterward, 1% hydrogen peroxidase was implemented to block endogenous peroxidase activity. The specimens were taken to incubate with anti-NRF2 (Abcam, ab62352, 1/100) and anti-KEAP1 (Abcam, ab227828, 1/500) overnight. Following washing, the samples were taken to incubate with rabbit anti-lgG secondary antibodies (Abcam, ab125938, 1/2000) for 2 h. Finally, DAB solution was taken for colorization and followed by counterstaining of hematoxylin. The mean microscopically positive staining of tumor cells

was calculated independently and blindly by two investigators based on clinical parameters.

Determination of serum and liver tissue indexes in mice

Mouse orbital blood was obtained with a 1.5 ml eppendorf (EP) tube, followed by centrifugation, and the supernatant was absorbed. An automatic biochemical instrument (BS-600M, Mindray, Nanjing, China) detected aspartate transaminase (AST) and alanine transaminase (ALT) to reflect the liver function of mice.

After rinsing and removing blood from mouse liver tissues with normal saline, 10% liver tissue homogenates were prepared. The prepared homogenates were centrifuged in a frozen centrifuge. After centrifugation, the homogenates were abandoned for precipitation, and the supernatant was left for the determination of malondialdehyde (MDA), glutathione (GSH) together with glutathione peroxidase (GSH-Px) using the MDA assay kit, GSH assay kit and GSH-Px assay kit (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted by homogenate. After reverse transcription using the ImProm-IITM reverse transcriptase (Promega Corporation, USA), real-time PCR was implemented to detect KEAP1 NRF2, and β-actin mRNA. The analysis software on 7500 fluorescence quantitative PCR instruments was used to analyze the experimental results using SYBR Green PCR Master Mix (Applied Biosystems, USA). The thermal cycling conditions for the RT-PCR: Predenaturation at 93-95°C for 3~5 min, and then entered the cyclic amplification stage: 94°C 30 s → 55°C 30 s → 72°C 60 s, cycling 30~35 times, and finally amplified at 72°C for 5~10 min. Relative gene expression was calculated by the standard $2^{-\Delta\Delta Ct}$ method. The primers were as below: KEAP1: forward primer: 5'-GAGAATCTACGTCCTTGGAGG-3', reverse primer: 5'-CAGGTGTCTGTATCTGGGTC-3', NRF2: forward primer: 5'-AAAATCATTAACCTCCCTGTTGAT-3', reverse primer: 5'-CGGCGACTTTATTCTTACCTCTC-3', β-actin: forward primer: 5'-GGAGATTACTGCCCTGGCTCCTA-3', reverse primer: 5'-GACTCATCGTACTCCTGCTTGCTG-3'.

Statistical analysis

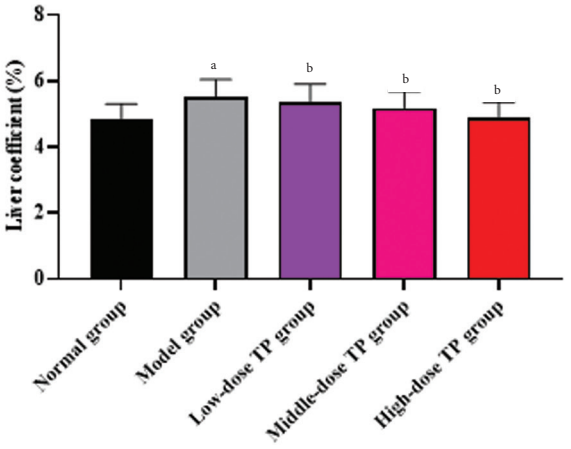
The data obtained from the experiment were expressed as mean ± standard error. SPSS 13.0 software (IBM, Armonk, New York, USA) was used to test the significance of differences between groups by one-way analysis of variance, followed by LSD-t test. $P < .05$ indicated significance when the data met the assumptions of the statistical tests used.

RESULTS

Effects of different doses of TPs on the liver coefficient

It was displayed in Figure 1 that the liver coefficient of the MG was (5.52±0.53)% higher relative to that of (4.82±0.48)% in the NG ($P < .05$). Relative to the MG, different doses of TPs treatment [(5.36±0.54)%, (5.18±0.51)% and (4.87±0.49)%] significantly lessened the liver coefficient ($P < .05$). However, no difference was discovered in the liver coefficient among different doses of TPs treatment ($P > .05$).

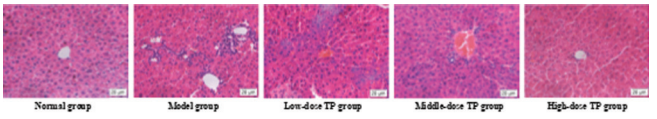
Figure 1. Effects of different doses of TPs on liver coefficient.



[a]$P < .05$, compared with the normal group.
[b]$P < .05$, compared with the model group.

Abbreviation: TP, tea polyphenol.

Figure 2. HE staining detected the effects of different doses of TPs on pathological liver injury.



Abbreviation: TP, tea polyphenol.

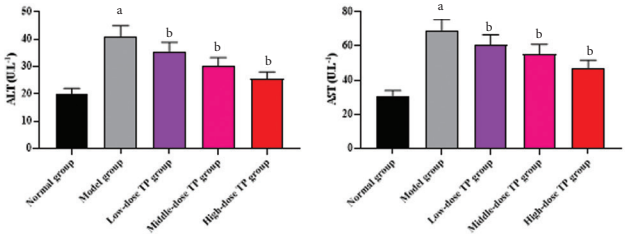
Effects of different doses of TPs on pathological liver injury

Figure 2 shows that the liver tissue of the NG was stained evenly, and the cells were arranged in an orderly way. Relative to the NG, the liver cells in the MG were swollen, with severe liver inflammation and liver cell death. The treatment of different doses of TPs significantly improved liver damage and reduced liver inflammation. However, no difference was discovered in pathological liver injury among different doses of TPs treatment ($P > .05$).

Influences of different doses of TPs on serum levels of AST and ALT

Compared with the NG, serum ALT and AST levels of mice in the MG [(40.78±4.12) $U.L^{-1}$ and (68.85±6.87) $U.L^{-1}$] were elevated ($P < .05$). Relative to the MG, serum ALT, and AST levels of mice were declined after treatment of different doses of TPs [(35.26±3.58) $U.L^{-1}$, (30.17±3.06) $U.L^{-1}$, (25.48±2.55) $U.L^{-1}$ and (60.49±6.12) $U.L^{-1}$, (55.37±5.57) $U.L^{-1}$ and (46.97±4.72) $U.L^{-1}$] ($P < .05$, Figure 3). However, no difference was discovered in serum ALT and AST levels among different doses of TPs treatment ($P > .05$). All these results suggested that TPs could reduce liver damage and promote liver repair in mice with acute cadmium poisoning.

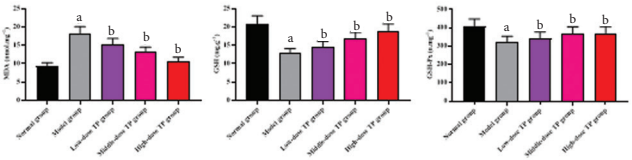
Figure 3. Influences of different doses of TPs on serum levels of AST and ALT.



[a]$P < .05$, compared with normal group.
[b]$P < .05$, compared with model group.

Abbreviations: TP, tea polyphenol; ALT, alanine transaminase; AST, aspartate transaminase.

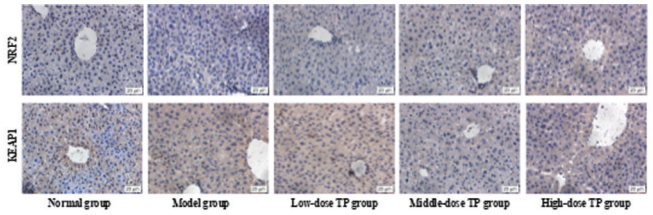
Figure 4. Influences of different doses of TPs on MDA, GSH and GSH-Px contents in liver tissues.



[a]$P < .05$, compared with normal group.
[b]$P < .05$, compared with model group.

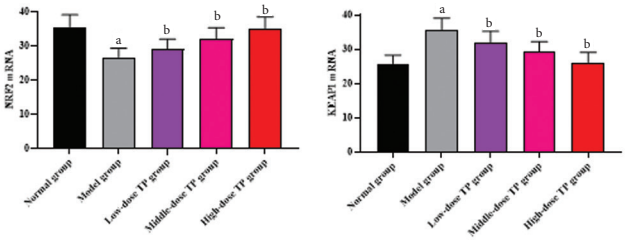
Abbreviations: TP, tea polyphenol; MDA, malondialdehyde; GSH, glutathione; GSH-Px, glutathione peroxidase.

Figure 5. Influences of different doses of TPs on KEAP1 and NRF2 expression in liver tissues.



Abbreviations: TP, tea polyphenol; NRF2, nuclear factor erythroid 2-related factor 2; KEAP1, kelch-like ECH-associated protein 1.

Figure 6. Influences of different doses of TPs on KEAP1 and NRF2 mRNA in liver tissues.



[a]$P < .05$, compared with the normal group.
[b]$P < .05$, compared with the model group.

Abbreviations: TP, tea polyphenol; NRF2, nuclear factor erythroid 2-related factor 2; KEAP1, Kelch-like ECH-associated protein 1.

Influences of different doses of TPs on MDA, GSH as well as GSH-Px contents in liver tissues

MDA content in liver tissues of the MG was (18.28±1.83) nmol.mg^{-1}, was elevated relative to that of (9.35±0.95) nmol.mg^{-1} in the NG, while GSH and GSH-Px contents of the MG were [(12.85±1.29) mg.g^{-1} and (321.56±32.24) u.mg^{-1}], declined relative to those of [(20.88±2.17) mg.g^{-1} and (407.28±40.15) u.mg^{-1}] in the NG($P<0.05$). Compared to the MG, MDA content in liver tissues were declined while GSH and GSH-Px contents were elevated after treatment of different doses of TPs [(15.34±1.56) nmol.mg^{-1}, (13.21±1.32) nmol.mg^{-1}, (10.68±1.09) nmol.mg^{-1}; (14.64±1.48) mg.g^{-1}, (16.87±1.69) mg.g^{-1}, (18.97±1.92) mg.g^{-1} and (342.67±34.25) u.mg^{-1}, (367.84±36.85) u.mg^{-1}, (368.49±36.97) u.mg^{-1}] ($P < .05$, Figure 4). However, no difference was discovered in MDA, GSH as well as GSH-Px contents among different doses of TPs treatment ($P > .05$). All these results suggested that TPs could reduce oxidative stress and improve liver function in mice with acute cadmium poisoning.

Influences of different doses of TPs on KEAP1 and NRF2 expression in liver tissues

Relative to the NG, NRF2 expression was significantly lower while KEAP1 level was higher in the liver tissues of the MG. In contrast to the MG, NRF2 level in the liver tissues were elevated while KEAP1 level was declined after different doses of TPs treatment (Figure 5). However, no difference was discovered in KEAP1 and NRF2 expression among different doses of TPs treatment ($P > .05$). All these results suggested that TPs could relieve liver injury in mice with acute cadmium poisoning by upregulating NRF2 and downregulating KEAP1 expression.

Influences of different doses of TPs on KEAP1 and NRF2 mRNA in liver tissues

Relative to the NG, the NRF2 mRNA level in the liver tissues of the MG was significantly lower (26.68±2.68), while the KEAP1 mRNA level was higher (35.53±3.56) ($P < .05$). Compared to the MG, NRF2 mRNA levels in the liver tissues were elevated [(29.03±2.96), (32.14±3.23) and (35.01±3.51)] after treatment of different doses of TP, while the KEAP1 mRNA level declined [(32.03±3.24), (29.17±3.06) and (26.16±2.92)] ($P < .05$, Figure 6). However, no difference was discovered in KEAP1 and NRF2 mRNA expression among different doses of TPs treatment ($P > .05$). All these results suggested that TPs could relieve liver injury in mice with acute cadmium poisoning by upregulating NRF2 and downregulating KEAP1 expression.

DISCUSSION

As one of the heavy metals widely used in modern times, cadmium has brought great benefits to industry, while its harm to the environment and human health is becoming more and more obvious.[15] Cadmium in the body can cause great oxidative damage to tissue cells and induce tissue to produce reactive oxygen species.[6] Oxidative cell membrane

structure generates lipid peroxidation, which reduces the capacity of organs to cope with oxidative stress.[16] Under cadmium exposure, all organs of the body will be affected to varying degrees, among which the liver is the most seriously affected.[17] Phenolic compounds are rich in -OH groups and have the properties of antioxidant and chelating metal ions.[18] Numerous studies have demonstrated that plant phenolic compounds have a strong regulatory effect on oxidative stress.[19] Liu et al. have proposed that resveratrol protects against cadmium poisoning through inhibition of mTORC1/2 pathways.[20] Grape seed procyanidins extract has a protective effect against renal oxidative damage induced by cadmium.[21] It is one of the main directions of modern toxicology to search for plant phenolic compounds that can effectively prevent and treat cadmium poisoning.[22]

Studies have confirmed that plant phenolic components such as flavonoids and anthocyanins can protect cadmium-induced body damage by inducing antioxidant enzymes and reducing lipid peroxidation levels.[23] As an excellent natural antioxidant, TPs are closely related to the antioxidant properties of TPs, which have been proved by a large number of experimental studies to have pharmacological activities such as anti-tumor, anti-aging, and regulating blood lipids.[24] Altaf S Darvesh et al. have indicated the chemopreventive and antineoplastic effects of TPs in hepatic cancer.[25] Zhang et al. have indicated that TPs can play a neuroprotective role by regulating intestinal flora.[26]

The results of our study revealed that the liver coefficient and pathological liver injury of the MG were increased relative to the NG, while different doses of TP treatment significantly relieved the liver coefficient and pathological liver injury in contrast to the MG. ALT and AST belong to the main enzymes that reflect liver parenchymal damage, and the level of enzyme activity is consistent with the degree of liver cell damage.[27] At the same time, oxidative stress was proven to play a vital role in the development of liver injury.[28] MDA is the main product of peroxidation between free radicals and lipids, and its content indirectly reflects the degree of peroxidation of body tissues.[29] GSH is a non-enzymatic antioxidant and free radical scavenger, which can combine with heavy metals and free radicals, and convert them into non-toxic substances to be discharged from the body, and alleviate the damage of toxic substances to cells.[30] GSH-Px is an important catalytic decomposition enzyme in the body, which can remove hydrogen peroxide and lipid peroxides produced in the body, so as to reduce the damage caused by high reactive oxygen species.[31] In our study, we found that the serum ALT and AST levels of mice in the MG were significantly increased compared with the NG, but the serum ALT and AST levels of mice were further declined after different doses of TP treatment. Meanwhile, MDA content in liver tissues of the MG was elevated relative to the NG, while GSH and GSH-Px contents of the MG were lower. Compared to the MG, MDA content in liver tissues were declined while GSH and GSH-Px contents were elevated after different doses of TP treatment. All above results implied that TPs could relieve the liver injury in mice with acute cadmium poisoning via reducing oxidative stress, which was similar to previous study.[32] Consistently, it has been reported that green TPs protect against acetaminophen-stimulated liver injury through modulating drug metabolizing enzymes and transporters.[33] Wang et al. have indicated that E Se tea extract ameliorates CCl_4 induced liver fibrosis via enhancing antioxidant and anti-inflammatory abilities.[34]

NRF2-KEAP1 pathway is a master modulator for the endogenous antioxidant response, and NRF2 nuclear translocation is the precondition for activating this antioxidant pathway.[35] Once across the nuclear membrane, NRF2 facilitates the transcription of downstream target genes to hinder oxidative damage.[36] Of note, NRF2-KEAP1 pathway is involved in the regulation of cadmium-induced oxidative damage.[37] Besides, it has been documented that quercetin, a natural antioxidant, exerts a protective function in cadmium-stimulated oxidative damage by promoting the NRF2-KEAP1 pathway.[38] In line with the above literatures, our study also proved that relative to the NG, NRF2 expression in the MG was lower, whereas KEAP1 expression was higher. Compared to the MG, NRF2 expression was elevated after treatment with different doses of TPs, while KEAP1 expression was declined. All these results suggested that TPs could relieve liver injury in mice with acute cadmium poisoning by upregulating NRF2 and downregulating KEAP1 expression. Similarly, Zhang et al. have indicated that E Se tea alleviates acetaminophen-induced liver injury by activating the Nrf2 signaling pathway.[39]

There are some limitations of our study. First, the interaction between TPs and NRF2 and KEAP1 expression remains to be further explored. Second, in vivo, studies were not conducted to verify the impacts of TPs on liver injury and oxidative damage. Therefore, the effect of TPs on liver injury and oxidative damage can be further evaluated by transfecting NRF2 and KEAP1 knockdown/overexpression plasmids in vitro at a later stage, and the interaction between TPs and NRF2 and KEAP1 expression will be further explored in the future, in order to explore the long-term effect and the of TPs on liver injury casused by acute cadmium poisoning.

In conclusion, TPs can relieve liver injury in mice with acute cadmium poisoning by enhancing antioxidant through upregulating NRF2 and downregulating KEAP1 expression. Our study might provide a novel sight for the treatment of acute cadmium poisoning. TPs might be as a preventive measure against cadmium toxicity, especially in populations at high risk due to environmental exposure.

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