Citronellal ameliorates doxorubicin-induced hepatotoxicity via antioxidative stress, antiapoptosis, and proangiogenesis in rats

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Abstract

Doxorubicin (DOX) is a very effective broad-spectrum anticancer drug, yet its clinical application is badly restricted due to its serious side effects. Citronellal (CT), a specialized metabolite of plants found in \textit{Cymbopogon} spp., is proved to exhibit many beneficial properties. In the current study, we intended to investigate the effect of CT on DOX-induced hepatotoxicity in rats. Rats were treated with CT (200 mg/kg b.w./day orally), and given DOX (2.5 mg/kg b.w./week, intraperitoneally) to induce hepatotoxicity for six consecutive weeks. The results showed that CT administration could attenuate the DOX-induced pathological changes of liver tissues and ameliorated the inappropriate alteration of liver function biomarkers (serum glutamic aspartate aminotransferase, glutamic pyruvic transaminase, and albumin) in serum and oxidative stress parameters (malondialdehyde, superoxide dismutase, and reduced glutathione) in the liver. Moreover, CT mitigated the Bax/Bcl\textsubscript{-}2 ratio and caspase-3 expression to inhibit cell apoptosis. Further study indicated that CT therapy could enhance the protein levels of p-PI3K, p-Akt, and CD31 in the liver. These results demonstrate that CT can ameliorate DOX-induced hepatotoxicity in rats mediated by antioxidative stress, antiapoptosis, and proangiogenesis.

KEYWORDS

angiogenesis, apoptosis, citronellal, doxorubicin, hepatotoxicity, oxidative stress, PI3K/Akt signaling pathway

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1 | INTRODUCTION

Doxorubicin (DOX), an anthracycline antibiotic, is proved to exhibit a superior curative effect on various solid and hematological malignant tumors, but the clinical application of this drug is badly restricted by its low selectivity and serious toxic effects on the nontarget organ.[1,2] Liver, the main metabolic organ of DOX, is one of the organs most vulnerable to DOX damage. Studies have also demonstrated that circa 40% of the patients who were treated with DOX experienced varying degrees of hepatic damage.[3] However, there is no clinically recognized drug that can effectively alleviate the hepatotoxicity induced by DOX.

The mechanisms concerning DOX-induced cell damage are complicated and not yet completely understood, including free radical production, mitochondrial dysfunction, lipid disorders, and excessive calcium.[4] Among these possible mechanisms, oxidative stress is considered to serve an extremely crucial role in the development of DOX-induced hepatic injury.[5] DOX with anthraquinone structure can be metabolized into unstable semiquinone intermediate, which can induce the overproduction of reactive oxygen species (ROS) through redox cycling and inactivating endogenous antioxidants such as superoxide dismutase (SOD) and reduced glutathione (GSH). As a result, the subtle balance between the generation of oxidants and antioxidant defense systems is broken, excessive ROS binding closely to organelles and cell membranes, leading to oxidative organ damage.[6,7] What is more, these DOX-derived ROS can directly or indirectly activate death receptor (extrinsic) and mitochondrial-dependent (intrinsic) pathways, both of which can promote the release of Cytochrome C and activate caspase-3, eventually lead to apoptosis that has been proved to be the main form of cell death caused by DOX.[8,9] Therefore, antioxidative stress and antiapoptosis therapy should be an effective method to treat DOX-induced hepatotoxicity.

Microvascular endothelial cells (ECs) are the crucial building blocks of the hepatic vascular net, which not only participate in the material exchange, energy metabolism, and removal of toxic substances between cells and blood, but also serve an extremely crucial role in the repair of damaged hepatic.[10,11] It is reported that DOX can destroy cardiac ECs/endothelial function and suppress angiogenesis, leading to cardiomyopathy in rats.[12] However, it is not clear whether DOX has any effect on hepatic microvascular ECs.

Natural products derived from plants are a significant source for developing hepatoprotective drugs.[13] Citronellal (CT; C10H18O; molecular mass: 154.24, CAS: 106-23-0) is a kind of acyclic monoterpenoid aldehyde, which is usually isolated via short path distillation from the oils of some Cymbopogon spp. such as Cymbopogon jwarancusa (Jones) and Cymbopogon winterianus Jowitt.[14,15] Considerable studies have stated that CT possesses a great diversity of pharmacological activities, including antimicrobial and anthelmintic activity, antioxidant, anti-inflammatory activity, and EC protective effects.[16–21] In addition, recent studies have reported that CT could also ameliorate the hepatotoxicity induced by a high-cholesterol diet.[22] Yet, the effects of CT on DOX-induced hepatotoxicity have not yet been investigated.

In this context, we investigated the potential protective effects of CT against DOX-induced hepatic damage in vivo rat models. Our findings demonstrated that CT could ameliorate the DOX-induced hepatotoxicity through antioxidative stress, antiapoptosis, and proangiogenesis.

2 | MATERIALS AND METHODS

2.1 | Reagents

CT, with a purity of 98% was obtained from Yuke (Beijing, China). DOX hydrochloride was purchased from Duly (Nanjing, China) and dissolved in normal saline an hour before administration to make a 1-mg/mL solution. The doses of CT and DOX were chosen based on previous studies.[20,23]

2.2 | Animals

Male Sprague-Dawley rats (n = 40; weight: 200-250 g; age: 7-week-old) were obtained from Henan Laboratory Animal Center (Henan, China). All rats were maintained in standard cages (22 ± 1°C temperature; 45%-65% relative humidity; 12-hour light/dark cycle). The animals were given standard rat chow and water ad libitum during the experimental period. All animal experiments in the course of this study were performed based on the guidelines of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of Xinxiang Medical University, Henan, Xinxiang (Approval ID: 2014-0005).

Forty rats were acclimatized for one week and then allocated equally in four groups (n = 10/group):

1. Control group: Treated with normal saline orally for 6 consecutive weeks.
2. CT group: Administered CT (200 mg/kg) orally once a day for 6 consecutive weeks.
3. DOX group: Treated by intraperitoneal injection with DOX (2.5 mg/kg b.w.) every 1 week for 6 consecutive weeks.
4. CT + DOX group: Treated by intraperitoneally injected with DOX (2.5 mg/kg) every 1 week while administered CT (200 mg/kg) orally once a day for 6 consecutive weeks.

After the last administration of DOX, all rats were fasted for 12 hours, weighted and anesthetized with 10% chloral hydrate, taken blood samples from the abdominal aorta and centrifuged at 3000 rpm for 15 minutes at room temperature (RT) to get their serum, which was then stocked at 4°C. The liver of each rat was quickly removed and washed, one part of which was stocked at −80°C, and the other part was fixed in 4% paraformaldehyde (PFA) solution.

2.3 | Histopathological analysis

All hepatic samples which were fixed by PFA for 16 hours, were dehydrated with ascending ethanol (70%, 80%, 90%, and 100%). After paraffin
embedding. Ultra-Thin Semiautomatic Microtome (Leica, Oskar, Germany) were employed to cut the samples into slices (5-μm), which were then stained with hematoxylin-eosin (H&E) and Masson trichrome (MT). The staining results of these slices were observed and photographed with an optical microscope (×400; Olympus, Shinjuku, Japan) and analyzed with ImageJ V1.8.0.112 (NIH, Bethesda, MD).

2.4 Evaluation of liver function

The Commercial Kits (Jiancheng, Nanjing, China) were used to detect the differences of liver function in rats of each group by assessing the serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and albumin (ALB), following the manufacturer’s protocols.

2.5 Evaluation of oxidative stress

Hepatic tissue samples, which were preserved at ~80°C, were prepared in a homogenizer and centrifuged at 4°C, 3000 rpm for 15 minutes to collect supernatant, which was then used the BCA Protein Kit (Jiancheng) to quantify the protein concentrations. After that, the Commercial Kits (Jiancheng) were adopted to detect the levels of malondialdehyde (MDA), SOD, and GSH of supernatant, following the instructions given.

2.6 Immunofluorescence analysis

For immunofluorescence, the paraffinized liver tissue sections (5-μm) were dewaxed in xylene and rehydrated in descending order of ethanol (100%, 90%, 80%, and 70%). After three washes with phosphate-buffered saline (PBS) for 15 minutes, antigen retrieval of these sections was achieved by maintaining them in sodium citrate solution (pH 6.0) at 95°C for 25 minutes and blocked with bovine calf serum (5%) for 1 hour at RT. The slices were then incubated with appropriate concentration of primary antibodies against Bcl-2 (catalog number: AF6139; dilution 1/1000; Affinity), Bax (catalog number: AF0120; dilution 1/1000; Affinity), and β-actin (catalog number: AF7018; dilution 1/10 000; Affinity, US) overnight at 4°C. After three PBS washes for 15 minutes, sections were then incubated with peroxidase-labeled anti-rabbit secondary antibody (catalog number: S0001; dilution 1/5000; Affinity) at 38°C for 1 hour, the protein bands were visualized through chemiluminescence using the ECL Kit (catalog number: KF003; dilution 1/5000; Affinity) and then measured by ChemiDoc Imaging System with Image Lab V5.1 (Bio-Rad, Hercules, CA). β-Actin was utilized as the internal control for equal loading.

2.8 Immunohistochemistry analysis

Like immuno Fluorescent, all slices were dewaxed and rehydrated, washed, and repaired antigens. Three percent H2O2 solution was then used to block the activity of endogenous peroxidase in these slices for 15 minutes at RT. The slices were then incubated with appropriate concentration of primary antibodies against caspase-3 (catalog number: AF6311; dilution 1/150; Affinity), p-PI3K (catalog number: AF3242; dilution 1/150; Affinity), p-Akt (catalog number: AF0016; dilution 1/150; Affinity), and CD31 (catalog number: GB12063; dilution 1/300; Servicebio, China) overnight at 4°C. After three PBS washes for 15 minutes, sections were then incubated with a secondary horseradish peroxidase-conjugated secondary antibodies for 30 minutes at RT and then developed by 3, 3′-diaminobenzidine (Solarbio) to visualize positive immunoreactivities. After three PBS washes for 15 minutes, sections were re-stained with hematoxylin. Finally, a light microscope (×400; Olympus) was used to observe and photograph the stain results, which were then analyzed by ImageJ V1.8.0.112 (NIH).

2.9 Statistical analyses

Results were presented as the mean ± SEM and analyzed by GraphPad Prism 6 (GraphPad, San Diego, CA), and the multiple comparisons were performed using one-way analysis of variance followed by Turkey’s test. Differences were considered statistically significant at P < .05.

3 RESULTS

3.1 Effect of CT on liver histological structure

H&E staining proved that rats in the control had a normal hepatic architecture (Figure 1A). In contrast to the control group, liver
tissues taken from the DOX group showed severely histological alterations, including microvesicular steatosis, macrovesicular steatosis, hepatocellular ballooning, and nuclear pyknosis. While in the DOX + CT groups, the degree of liver lesions was significantly reduced.

MT staining proved that rats in the control group were presented only a few collagen fibers in their liver tissues (Figure 1B). Conversely, the amounts of deposited collagen fibers surrounding hepatocytes in the DOX group were notably increased. On the other hand, the rats in the DOX + CT group had remarkably decreased the amount of collagen fibers deposited in their livers tissues. Histomorphometric results further prove this discovery (Figure 1C). In summary, all the above findings support that CT treatment can ameliorate the DOX‐induced damage of the liver histological structure.

3.2 | Effect of CT on liver function

The serum levels of ALT, AST, and ALB were measured as liver function biomarkers. As shown in Figure 2A, DOX treatment significantly reduced serum ALB, but it was markedly increased in the DOX + CT group. As presented in Figure 2B,C, the serum levels of ALT and AST in the DOX group were remarkably increased. However, these elevated scores were notably mitigated in the DOX + CT group. These findings suggest that CT can effectively protect liver function against DOX‐induced hepatotoxicity.

3.3 | The antioxidative effect of CT on hepatic tissue

The levels of MDA, SOD, and GSH in liver tissues were tested as oxidative stress parameters. As presented in Figure 3A, after the administration of DOX, the hepatic level of MDA was drastically increased. But the DOX + CT group exhibited significant mitigation in the content of MDA vs the DOX group.

Moreover, DOX treatment remarkably decreased the hepatic levels of GSH and SOD, which were significantly reversed by CT therapy (Figure 3B,C). These above data imply that CT may serve as an antioxidant to attenuate DOX‐induced hepatotoxicity.

3.4 | The antiapoptosis effect of CT on hepatic tissue

The expression of Bcl-2 (antiapoptotic factor), Bax (proapoptotic factor), and caspase-3 (apoptotic executive protein) in liver tissues...
were detected by immunofluorescence stain, Western blot, and immunohistochemistry stain to explore the effect of CT on hepatocyte apoptosis caused by DOX. As presented in Figure 4A, in contrast to the control group, the fluorescence intensity of Bcl-2 was significantly declined and the fluorescence intensity of Bax was obviously increased in the DOX group hepatic tissues. However, the administration of CT caused a drastic rise in the fluorescence intensity of Bcl-2 and significantly suppressed Bax fluorescence intensity compared to the DOX group. Western blot revealed that the ratio of Bax/Bcl-2 protein was drastically raised in the DOX group, while CT treatment reversed this increase (Figure 4B).

Moreover, immunohistochemistry analysis of caspase-3 protein expression revealed that DOX administration markedly raised the percentages of caspase-3 positive areas, which were significantly reversed by CT therapy (Figure 4C,D). These findings indicate that CT can effectively mitigate DOX-induced hepatocyte apoptosis.

3.5 | The activated effect of CT on phosphoinositide 3-kinase/protein kinase B signaling pathway in hepatic tissue

To future investigate the molecular mechanism underlying the anti-apoptosis effect of CT in rat liver, the expression of p-PI3K and p-Akt were detected by immunohistochemistry stain and displayed in Figure 5. The administration of DOX caused a significant decrease in p-PI3K and p-Akt levels, while treatment with CT restored the DOX-induced changes in p-PI3K and p-Akt levels. These findings indicate that CT can effectively attenuate DOX-induced hepatocyte apoptosis via activating phosphoinositide 3-kinase/protein kinase B (PI3k/Akt) signaling pathway.

3.6 | The proangiogenesis effect of CT on hepatic tissue

The expression of CD31 (angiogenesis marker) protein in the liver tissues was detected by immunohistochemistry stain and presented in Figure 6. DOX administration induced a significant increase in the percentages of CD31-positive areas. Interestingly, the cotreatment of CT significantly augmented the percentages of CD31-positive areas in liver tissues versus the DOX group. These findings indicate that CT treatment can effectively promote angiogenesis in the liver.

4 | DISCUSSION

Nowadays, chemotherapy has become indispensable to humans for treating cancer. DOX is a chemotherapeutic drug with a
wide-spectrum and superior curative activity. Nevertheless, DOX will inevitably cause multiple organ damage during the course of cancer treatment. In our present study, we aimed to investigate in vivo whether CT had a potentially ameliorative effect on the DOX-induced hepatotoxicity. To clarify that, we performed a histopathological examination, liver function test, oxidation parameters detection, immunofluorescence, Western blot, and immunohistochemistry analysis. Our results demonstrate that CT can ameliorate the DOX-induced hepatotoxicity by antioxidant stress, antiapoptosis, and proangiogenesis.

For evaluating the DOX-induced hepatotoxicity, we performed a histopathological examination and biochemical analysis of liver function biomarkers (ALT, AST, and ALB). The results showed that DOX administration for 6 weeks (2.5 mg/kg b.w./week) could cause severe steatosis and fibrosis in rat liver. Meanwhile, the levels of transaminases in serum were drastically increased and the serum ALB level was decreased. The outcome results were comparable to the previous report.\textsuperscript{23,24} On the other hand, CT administration for 6 weeks (200 mg/kg b.w./day) could notably alleviate DOX-induced histopathological changes. Meanwhile, it declined the elevated serum levels of ALT and AST and enhanced the suppressed serum ALB level. These findings suggest that the CT can ameliorate the DOX-induced hepatotoxicity.

**FIGURE 4** Effect of CT on DOX-induced hepatocyte apoptosis in rats. A, Immunofluorescence staining of Bcl-2 and Bax in liver tissues, Bcl-2 (green), Bax (red) in all of the groups’ liver tissues. DAPI (blue) was used to stain the nucleus. B, Western blot analysis for the expression of Bax and Bcl-2. C, Immunohistochemistry staining of caspase-3 in liver tissues. D, Quantitative analysis of the positive area of caspase-3. Data are shown as mean ± SEM (n ≥ 6). Different small letters (a-d) above bars indicate significant (P < .05) differences between all groups. Bax, Bcl-2-associated x protein; Bcl-2, B-cell lymphoma-2; caspase-3, cysteiny1 aspartate specific proteinase-3; CT, citronellal; DOX, doxorubicin
Oxidative stress burden is considered as the root and initiating mechanism of DOX-induced hepatotoxicity. Lines of evidence have confirmed that DOX therapy can reduce the content of endogenous antioxidants and increase the contents of oxygen free radicals like hydrogen peroxide and superoxide anion. These excessive and hard-to-scavenge free radicals can further lead to lipid peroxidation and cell damage. MDA is the main product of lipid peroxidation, which is considered as a specific biomarker of oxidative injury. SOD and GSH are the crucial endogenous antioxidants to protect cells from oxidative damage. The results of the current study showed that the DOX treatment could increase liver MDA level, while decrease SOD and GSH level in liver tissues, which were consistent with previous reports. In the current research, we found that DOX administration significantly elevated the ratio of Bax/Bcl-2 protein levels in liver tissues, which was consistent with the previous experimental results. On the opposite side, CT therapy significantly reduced the increment of the Bax/Bcl-2 protein ratio and caspase-3 protein level by DOX. These findings indicate that CT could inhibit DOX-induced apoptosis.

PI3K/Akt is an ROS-sensitive signaling pathway, which is involved in cell proliferation, survival and has been considered as a pivotal part in DOX-induced apoptosis. Akt (as known as protein kinase B) is the key mediator of the PI3K/Akt signaling pathway, which can be phosphorylated and activated by p-PI3K and regulating the expression and activities of downstream apoptosis-related proteins (Bax, Bcl-2, and caspase) to attenuate apoptosis. What is more, early studies have reported that the DOX-induced apoptosis was accompanied by the downregulation of Akt phosphorylation and

**FIGURE 5** Effect of CT and DOX on the hepatic protein of PI3K/Akt signaling pathway in rats. A, Immunohistochemistry staining of p-PI3K in liver tissues. B, Immunohistochemistry staining of p-Akt in liver tissues. C, Quantitative analysis of the positive area of p-PI3K. D, Quantitative analysis of the positive area of p-Akt. Data are shown as mean ± SEM (n ≥ 6). Different small letters (a-c) above bars indicate significant (P < .05) differences between all groups. Akt, protein kinase B; CT, citronellal; DOX, doxorubicin; PI3K, phosphoinositide 3-kinase.
inhibited by the activation of the PI3K/Akt pathway,
our results showed that DOX treatment could significantly decrease the levels of p-PI3K and p-Akt protein in liver tissues, which was similar to the previous experimental results. In addition, the activation of the PI3K/Akt signaling pathway plays vital roles during the processes of angiogenesis and improves cell viability/restoration. The administration of CT significantly upregulated the suppressed levels of p-PI3K and p-Akt protein by DOX. These findings indicate that CT could inhibit the DOX-induced hepatotoxicity via activating PI3K/Akt pathway.

It is well-documented that intrahepatic microvascular ECs are crucial for liver regeneration, providing oxygen, trophic support, and angiocrine signals to the growing tissues by neovascularization. CD31, a glycoprotein expressed by the newly formed vasculature, is a specific marker of vascular ECs, and its level can reflect the degree of angiogenesis. Interestingly, in the current research, we found the CD31 protein level in the liver was significantly increased after DOX (2.5 mg/kg b.w./week) treatment for 6 weeks. We suggest that this phenomenon could be an intrinsic response to hepatic damage for the regeneration of the liver. More importantly, CT treatment significantly enhanced the expression of CD31 compared to the DOX group. These results demonstrate that CT can accelerate intrahepatic angiogenesis, enhancing hepatic microvascular EC density to promote the regeneration of damaged liver induced by DOX.

5 | CONCLUSION

To our best knowledge, this is the first investigation with clear evidence that CT could be a beneficial agent on DOX-induced hepatotoxicity in rats. The ameliorative effect of CT against DOX-induced hepatotoxicity is probably through antioxidative stress, antiapoptosis, and proangiogenesis. Clinically, the combination of CT and DOX may serve as a new strategy for chemotherapy.

ACKNOWLEDGMENTS

This study was supported by the National Natural Science Foundation of China (81874312, U1804197, 81673423, and 1704168), Research Foundation of Henan Province (194200510005, 18HAS-TIT047, 2018GGJS102, and 2017GGJS108), and the Research Foundation of Xinxiang Medical University (XYBSKYZZ201626 and XYBSKYZZ505319).

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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