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Involvement of mitochondrial dysfunction in hepatotoxicity induced by *Ageratina adenophora* in mice*#

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Ageratina adenophora is a noxious plant and it is known to cause acute asthma, diarrhea, depilation, and even death in livestock (Zhu et al., 2007; Wang et al., 2017). *A. adenophora* grows near roadsides and degraded land worldwide (He et al., 2015b). In the areas where it grows, *A. adenophora* is an invasive species that inhibits the growth of local plants and causes poisoning in animals that come in contact with it (Nie et al., 2012). In China, these plants can be found in Yunnan, Sichuan, Guizhou, Chongqing, and other southwestern areas (He et al., 2015a) and they have become a dominant species in these local regions. It threatens the native biodiversity and ecosystem in the invaded areas and causes serious economic losses (Wang et al., 2017). It has been reported that *A. adenophora* can grow in the northeast direction at a speed of 20 km per year in China (Guo et al., 2009). Because of the damage caused by *A. adenophora*, it ranks among the earliest alien invasive plant species in China (Wang et al., 2017).

Previous studies have reported that *A. adenophora* causes hepatotoxicity in different species of animals. Chronic respiratory disease and exercise intolerance have been reported in horses in Australia due to ingestion of *A. adenophora* (O'Sullivan, 1985). Experimental feeding of *A. adenophora* samples to cattle from sub-Himalayan region in India has been seen to cause anorexia and photosensitization. Rats administrated with a diet mixed with purified extracts from *A. adenophora* leaves developed hepatotoxicity and cholestasis (Katoch et al., 2000; Kaushal et al., 2001).

Mitochondria have a pivotal role in the production of adenosine triphosphate (ATP), which is the energy currency of the cell (Koh et al., 2018). There are hundreds of mitochondria in each hepatocyte, and each organelle has 2 to 10 copies of mitochondrial DNA (mtDNA) (Robin and Wong, 1988). The changes of mass and copy number of mitochondria are associated with cell growth and differentiation (Shay et al., 1990), but also can be caused by a variety of noxious stimuli (Nugent et al., 2007; Tiao et al., 2007). Liver damage is closely related to mitochondrial dysfunction (Shi et al., 2018). According to our previous studies, *A. adenophora* had toxic effects on renal cells, splenocytes, and hepatocytes by activating apoptosis in Saanen goat through mitochondrial pathway (He et al., 2015a, 2015b, 2016). In addition, apoptosis is closely related with mitochondria (Song et al., 2018). These results led us to investigate the changes occurring in the mitochondria during the process of hepatotoxicity caused by *A. adenophora*.

After six weeks of treatment with *A. adenophora* extract, histopathological analysis was carried out on the liver tissues. Based on the microscopic examination,

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normal hepatic sinusoid and hepatic lobules can be found clearly in the control and the group treated with a dosage of 100 g/kg *A. adenophora* (Figs. 1a and 1b). However, we observed varying degrees of pathological changes in the liver in the 200 and 300 g/kg dosage groups. Light vacuolar degeneration was seen in the 200 g/kg dosage group (Fig. 1c). We observed many foci of hepatocytes degeneration mainly in the periportal areas in the high dosage group (Fig. 1d). Degenerated hepatocytes were swollen with hydropic cytoplasm and unclear nucleus. Inflammatory cells were also universally observed in the necrotic areas (Fig. 1d). The histological evaluation demonstrated that the livers subjected to medium and high dosages of *A. adenophora* exhibited destruction of normal hepatic structure to a certain degree.

To investigate the changes in the mitochondrial structure induced by *A. adenophora*, transmission electron microscopy (TEM) was used to measure the ultrastructure in the control and *A. adenophora*-treated groups. As shown in Fig. 2, the most characteristic change was the cristal abnormalities caused by *A. adenophora* and these were heterogeneous in sizes and shapes compared to the control group. Partially swollen mitochondria with unusual and sparse cristae were observed in *A. adenophora*-treated groups (Figs. 2b–2d).

Furthermore, we determined the quantitative degree of mitochondrial swelling using flow cytometry (FCM) with appropriate forward/side scatter (FSC/SSC) plot parameter setting. FSC is closely related to the cell size, while SSC correlates with the granularity and the refractive index. The quantitative degree of mitochondrial swelling was expressed by the ratio of FSC/SSC. The FSC/SSC ratios in *A. adenophora*-treated (100, 200, and 300 g/kg dosages) groups were 0.850±0.035, 0.941±0.014, and 1.190±0.049, respectively. All of the results obtained were higher than that in the control group (0.770±0.009, $P<0.01$; Fig. 3).

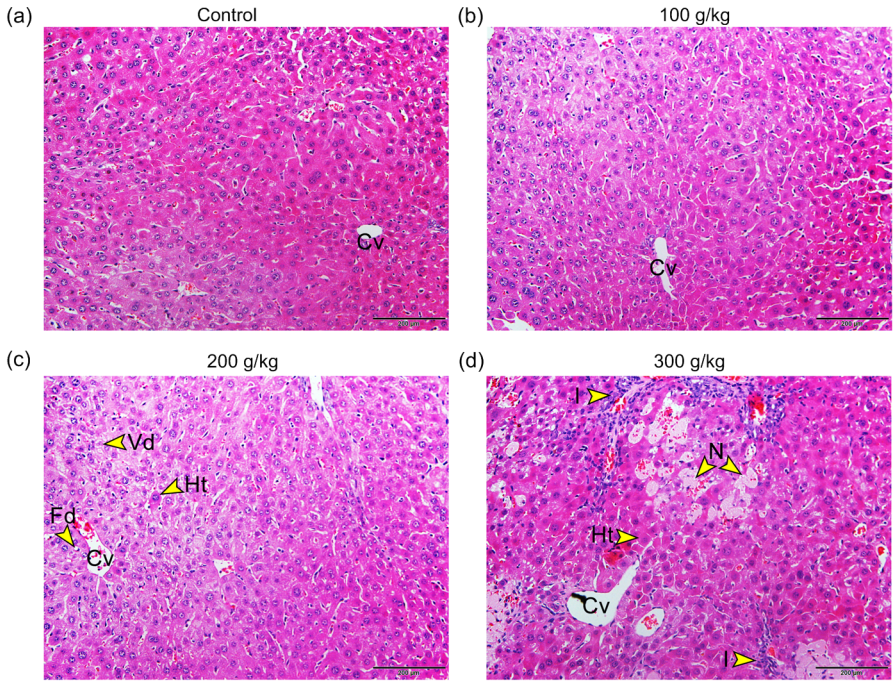

Fig. 1 Histopathological observation of in the liver sections from the control and *A. adenophora* exposure groups
Central vein (Cv) was clearly found in all groups. Normal hepatic cord arrangement and hepatic lobules were found in the control (a) and 100 g/kg dosage group (b); varying degrees of pathological changes in the liver appeared in the 200 g/kg (c) and 300 g/kg (d) dosage groups. Slight vacuolar degeneration (Vd, yellow arrowhead), fatty degeneration (Fd, yellow arrowhead), and hepatocytic hypertrophy (Ht, yellow arrowhead) were present in the 200 g/kg dosage group (c). Flake-like or focal hepatocytic necrosis (N, yellow arrowhead), inflammatory cellular infiltration (I, yellow arrowhead), and hepatocytic hypertrophy (Ht, yellow arrowhead) appeared in the 300 g/kg dosage group (d). In addition, other alterations were also seen in the livers in the 200 and 300 g/kg dosage groups, including hepatic cord disorder, hypertrophy in hepatocytes, and hepatic sinusoidal stenosis. Hematoxylin and eosin (H&E) staining, bar=200 μm (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

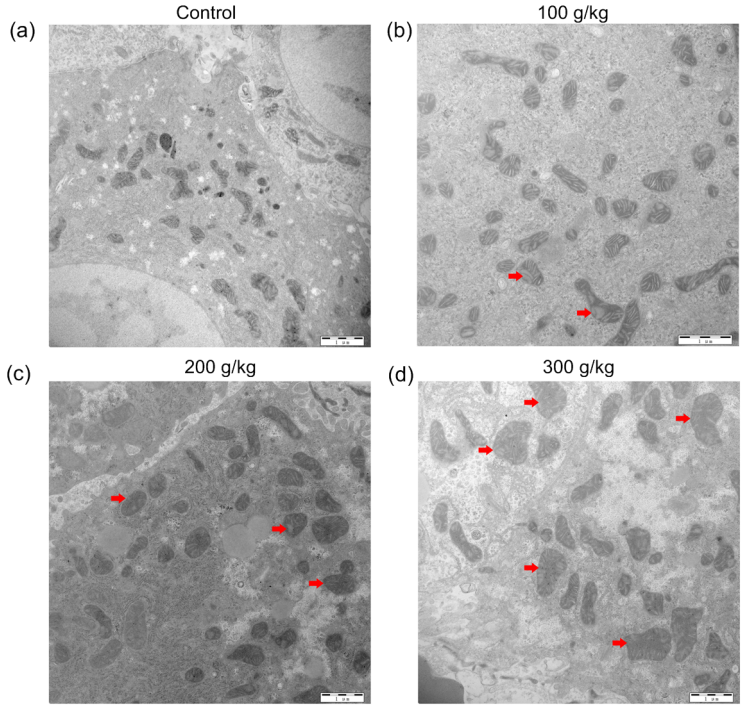

Fig. 2 Scanning of liver mitochondria by transmission electron microscopy (TEM)

(a) In control group, mitochondria of normal shape, cristae, and ultrastructure were present; (b) In the 100 g/kg dosage group, the structures and membranes of the mitochondria were disrupted, the cristae partially disappeared, and vacuolization occurred (red arrows); (c) In the 200 g/kg dosage group, pleomorphic mitochondria were observed (red arrows); (d) In the 300 g/kg dosage group, swollen mitochondria with unusual and sparse cristae were observed (red arrows). Bar=1 μm (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

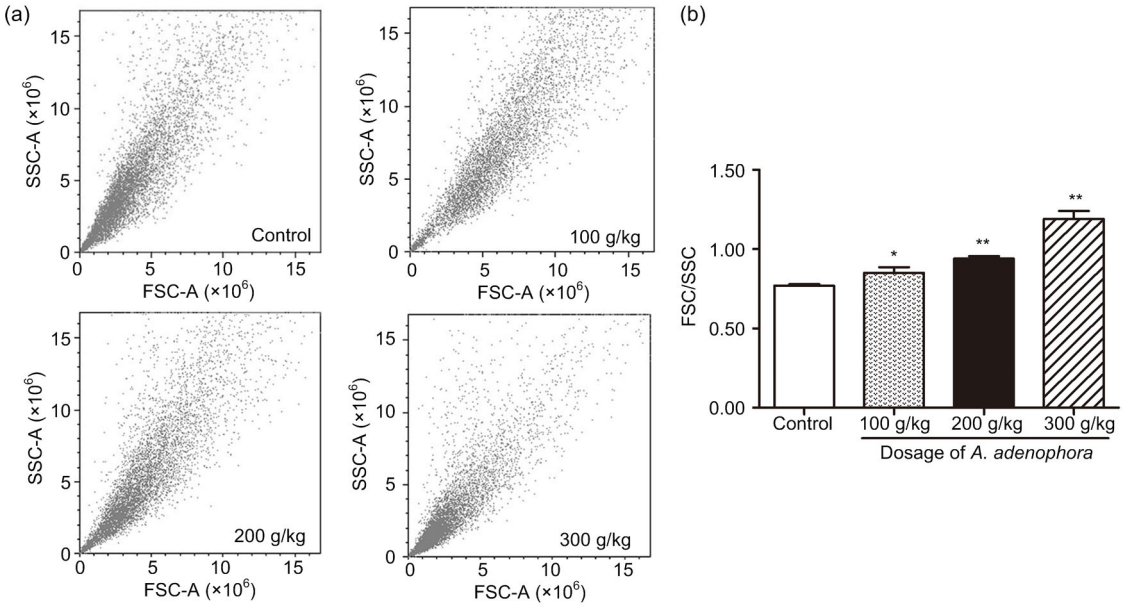

Fig. 3 Effects of *A. adenophora* on mitochondrial morphology in the liver

(a) Forward scatter area (FSC-A) and side scatter area (SSC-A) of the control and the groups treated with dosages of 100, 200, and 300 g/kg *A. adenophora*, were measured by flow cytometry (FCM). (b) Quantitative evaluation of mitochondrial swelling by FCM. The degree of mitochondrial swelling is represented by FSC/SSC ratio. The data are represented as mean±standard deviation (n=3). $^{*}P<0.05$, $^{**}P<0.01$, vs. the control group

To detect the effect of *A. adenophora* on the mitochondrial function, ATPase activity (Na^+K^+-ATPase and $Ca^{2+}Mg^{2+}$-ATPase) and mtDNA copy number were measured in the control and *A. adenophora* administration groups. The results showed that treatment with *A. adenophora* led to the decrease in the contents of Na^+K^+-ATPase and $Ca^{2+}Mg^{2+}$-ATPase in a dose-dependent manner compared with the control group (P<0.01; Fig. 4a). Cellular mtDNA is also a key indicator of mitochondrial function. In an effort to understand the changes in the levels of mtDNA, quantitative real-time polymerase chain reaction (qRT-PCR) was used to measure the copy number of mtDNA. As shown in Fig. 4b, the copy number of mtDNA showed a decreasing trend in the *A. adenophora*-treated groups compared with that in the control group (P<0.01).

A. adenophora is one of the most harmful invasive plants in China and it is toxic to animals (Yang et al., 2017). Previous studies have confirmed that hepatotoxicity could be caused by *A. adenophora* in mice, rat, and goat (Sani et al., 1992; Katoch et al., 2000; He et al., 2016). Liver, which is the target organ of *A. adenophora*, plays a vital role in the detoxification of ingested toxin. In the present study, we investigated the effects of *A. adenophora* in inducing hepatotoxicity in mice that had consumed *A. adenophora* for 42 d.

Mitochondria are the major cellular loci for ATP production (Wang et al., 2009). It is known that most liver diseases are closely associated with the depletion of mitochondrial energy production (Koh et al., 2018). ATP generated in the cell (including Na^+K^+-ATPase and $Ca^{2+}Mg^{2+}$-ATPase) is an important extracellular ligand in the autocrine signal transduction, intercellular communication and nerve transmission, and has a wide range of physiological and pathophysiological roles (Guo et al., 2018; Taruno, 2018). Cellular ATP is an indicator of mitochondrial function. Our previous study demonstrated hepatotoxicity caused by *A. adenophora* through activation of mitochondrial pathway in goat (He et al., 2016). However, there is little information about the changes in the structure or function of mitochondria. In the present study, we found that the levels of Na^+K^+-ATPase and $Ca^{2+}Mg^{2+}$-ATPase had a tendency to decrease when induced by *A. adenophora* in mice liver (Fig. 4a). This indicated that *A. adenophora* damaged hepatic function by decreasing ATP availability.

Apart from ATP, mtDNA is another indicator of mitochondrial function. Mitochondria are an important source of reactive oxygen species (ROS), but have incomplete DNA repair capacity (Shay et al., 1990). The mtDNA can be damaged oxidatively by ROS (Larosche et al., 2010). Our recent study showed that ROS was elevated by *A. adenophora* in mice liver (Sun et al., 2018). However, no studies have been reported on the effect of hepatopathy induced by *A. adenophora* in hepatic mtDNA. In this study, we quantified mtDNA copies by qRT-PCR. A decreasing trend of mtDNA copy number was found in *A. adenophora*-treated groups (Fig. 4b). This result was supported by the observation through histopathology (Fig. 1) and TEM (Fig. 2) in the present study, which suggests that decreased mtDNA copy numbers are highly associated with liver dysfunction.

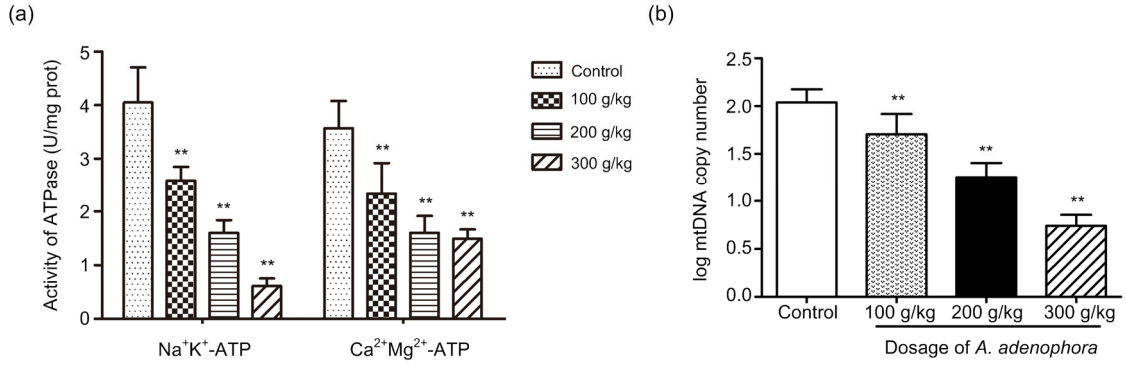

Fig. 4 Effects of *A. adenophora* on ATPase levels and mtDNA copy numbers in all groups

(a) Changes of ATPase levels, including $Ca^{2+}Mg^{2+}$-ATPase and Na^+K^+-ATPase, were measured by chemical kits. (b) Relative mtDNA copy numbers were measured by qRT-PCR with a value of *Cytb* to *β-actin*. ** P<0.01, vs. the control group. All data are represented as mean±standard deviation (n=6). prot: protein; *Cytb*: cytochrome b

In brief, our present study demonstrates that the hepatotoxicity induced by *A. adenophora* is associated with mitochondrial dysfunction in mice liver tissues, which is accompanied by the decreased levels of Na^+K^+-ATPase and $Ca^{2+}Mg^{2+}$-ATPase as well as mtDNA copy number. With the increase in dosage of *A. adenophora*, the pathological lesions in liver and mitochondria were aggravated, which is supported by the observation of hematoxylin and eosin (H&E) staining and TEM, respectively.

Materials and methods

Detailed methods are provided in the electronic supplementary materials (Data S1).

Contributors

Wei SUN performed the experimental research and data analysis, and wrote the manuscript. Chao-rong ZENG contributed to data analysis and writing the manuscript. Dong YUE contributed to the animal experiment and qRT-PCR analysis. Yan-chun HU designed this study and contributed to writing and editing the manuscript. All authors read and approved the final manuscript. Therefore, all authors have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Wei SUN, Chao-rong ZENG, Dong YUE, and Yan-chun HU declare that they have no conflict of interest.

This study was reviewed and approved by the Animal Care and Use Committee of Sichuan Agricultural University (SYXK2014-187), China. All animal operations, collection samples, and procedures were carried out in accordance with the guidelines approved.

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List of electronic supplementary materials

Data S1 Materials and methods

中文概要

题　目：紫茎泽兰引起小鼠线粒体功能障碍从而导致肝脏毒性损伤

目　的：研究紫茎泽兰造成小鼠肝脏毒性损伤后，肝脏线粒体结构和功能发生变化的情况。

创新点：紫茎泽兰可以导致肝脏细胞发生凋亡和焦亡，从而产生中毒损伤，然而尚未有报道其对线粒体超微结构和功能的改变。本研究通过多种手段解决了这一问题。

方　法：将 40 只小鼠随机分成 4 组，分别饲喂不同浓度的紫茎泽兰饲料（对照组、100、200 和 300 g/kg 紫茎泽兰添加饲料组）。采用苏木精-伊红染色法（H&E）研究肝脏损伤情况，利用透射电子显微技术研究线粒体超微结构改变。同时，结合实时荧光定量聚合酶链反应（qRT-PCR）、流式细胞术和化学分析方法对线粒体 DNA 拷贝数、肿胀度和三磷酸腺苷（ATP）酶活性的改变进行探究。

结　论：紫茎泽兰导致线粒体超微结构的改变，增大了线粒体肿胀度，降低了钠钾 ATP 酶（Na^+K^+-ATPase）和钙镁 ATP 酶（$Ca^{2+}Mg^{2+}$-ATPase）活力，同时减少了 DNA 拷贝数，从而引起肝脏损伤。

关键词：紫茎泽兰；肝毒性；线粒体功能障碍；三磷酸腺苷（ATP）酶；线粒体 DNA