Effect of autophagy-associated proteins on the arecoline-induced liver injury in mice

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Abstract. Arecoline can be used to treat diseases including glaucoma and tapeworm infection, however, long-term administration can cause severe adverse effects, including oral submucosal fibrosis, oral cancer, hepatic injury and liver cancer. Autophagy serves a role in these injuries. The present study established a mouse model of arecoline-induced hepatic injury and investigated the role of autophagy-associated proteins in this injury. The results indicated that the expression levels of the autophagy marker protein microtubule associated protein 1 light chain 3 B (MAP1LC3B) and autophagy-promoting protein beclin 1 were elevated in the injured hepatic cells, while the expression levels of a well-known negative regulator of autophagy, mammalian target of rapamycin (mTOR), were reduced. Following treatment of the hepatic injury with glutathione, the liver function improved and liver damage was reduced effectively. Compared with the control group, the expression levels of both MAP1LC3B and beclin 1 were significantly upregulated in the glutathione-treated mice, but the expression of mTOR was significantly downregulated. It may be concluded that in the process of protecting against arecoline-induced hepatic injury, glutathione cooperates with mTOR and beclin 1 to accelerate autophagy, maintaining stable cell morphology and cellular functions.

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Introduction

Areca catechu is a species of palm widely cultivated in India, Indonesia, Sri Lanka, the Philippines and other South and Southeast Asian countries (1). A total of four alkaloids are presented in the betel nut, including arecoline, arecaidine, guavacoline and guavacine (2). Arecoline is the major alkaloid, soluble in water or any concentration of ethanol (3). In Southeast Asia, South Pacific Islands and the surrounding areas, people chew the betel nut frequently for its comforting and pleasant effects (4). Betel nut is the fourth most frequently consumed recreational drug globally, after nicotine, ethanol and caffeine. As a common clinical drug, arecoline can be used in the treatment of tapeworm infection due to its insecticidal properties (5) and for protection of vascular endothelial cells to prevent atherosclerosis (6). However, due to its cytotoxicity and immunotoxicity, long-term administration of arecoline can lead to cellular DNA damage and promotion of apoptosis (7). Arecoline can cause submucosal fibrosis and oral cancer (8), and promote apoptosis and damage of liver cells, leading to hepatic cirrhosis and hepatic cancer (9-11). Several researchers have reported that arecoline-induced liver injury involves not only apoptosis but also autophagy (9,12,13), however, the exact role of autophagy remains to be elucidated.

Autophagy is a highly conserved, self-protective behavior of eukaryotic cells when subjected to adverse external stimuli, including hypoxia, sudden pressure elevation and drug stimulation (14,15). A variety of autophagy-related (ATG) proteins are involved in the autophagy process, including the ATG12-ATG5 conjugation and beclin 1 (16,17). Under different conditions, the level of autophagy is strictly regulated by various signal transduction pathways (18). Mammalian target of rapamycin (mTOR), a factor involved in the regulation of autophagy, serves a role of a ‘gatekeeper’ (19). Microtubule associated protein 1 light chain 3 (MAP1LC3), as a specific substrate of autophagy, is essential for autophagosome formation and is recognized as a marker of autophagic activity (20-22). Therefore, the present study detected cellular autophagic activity by measuring the content of MAP1LC3B.

L-glutathione is commonly used in the clinical treatment of liver disease and drug-induced hepatic injury, and serves a role in the protection and repair of hepatocytes by scavenging free radicals and superoxide ions (23). A previous study by
the authors of the present study has found that MAP1LC3B expression increased in the injured and L-glutathione treated hepatic cells (24), however, the mechanism remains to be elucidated. In the present study, the function and regulation of autophagy were further investigated by detecting the expression of mTOR, beclin 1 and MAP1LC3B, and the results may provide an insight into the protection or treatment for drug-induced liver damage.

Materials and methods

Chemicals and mice. Arecoline hydrobromide (98%) and L-glutathione were purchased from J&K Scientific Ltd. (Beijing, China). Mouse aspartate aminotransferase enzyme-linked immunosorbent assay kit (AST ELISA kit; cat. no. BPE20184) and mouse alanine aminotransferase (ALT) ELISA kit (cat. no. BPE20168) were purchased from Shanghai Lengton Bioscience Co., Ltd. (Shanghai, China). The following reagents were used for immunohistochemistry and western blotting: Rabbit anti-mTOR (cat. no. ab32028), anti-beclin 1 (cat. no. ab55878), anti-MAP1LC3B (cat. no. ab63817) polyclonal antibodies purchased from Abcam (Cambridge, UK); and anti-caspase-3 (cat. no. ab90437) polyclonal antibodies purchased from Abcam (Cambridge, UK) for western blotting; and the PV-6000 immunohistochemical detection kit (cat. no. K145213B) from OriGene Technologies, Inc. (Beijing, China). A total of 80 Male ICR mice (age, 6-8 weeks; weight, 20±2 g) were provided by the Laboratory Animal Center of Binzhou Medical University (Yantai, China). All animal experiments were performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, the animal use protocol was reviewed and approved by the Ethics Committee of Binzhou Medical University (Yantai, China).

Animal treatments. All experimental mice were housed in plastic cages in a pathogen-free environment, with controlled temperature (23-25°C) and humidity (50-70% relative humidity), with ad libitum access to food and water, and a 12-h light/dark cycle. These animals were randomly divided into four groups, with 20 mice in each group (n=20). Arecoline hydrobromide was dissolved in physiological saline (0.9% NaCl). The control group mice were given physiological saline gavage (4 ml/kg/day) and the model group mice were given arecoline hydrobromide (20 mg/kg/day). The low-dose glutathione group mice were given a mixture of L-glutathione (1 mg/kg/day) and arecoline hydrobromide (20 mg/kg/day), and high-dose glutathione group mice were given a mixture of L-glutathione (2 mg/kg/day) and arecoline hydrobromide (20 mg/kg/d). During the experiment, physiological saline, arecoline hydrobromide or a mixture of L-glutathione and arecoline hydrobromide were administered to corresponding group of mice by gavage twice daily for 15 or 30 days. The dosage of arecoline hydrobromide was determined according to a previous study (25).

Behavior and bodyweights of the experimental mice. During the experiment, the behavior of mice was observed using an open field test (26) and the mental states were detected through mechanical stimulus method (27). The bodyweights were recorded on days 15 and 30.

Serum collection and liver function measurement. Blood samples were collected from the orbit sinus after ether anesthesia and spinal cord dislocation on days 15 or 30 (10 mice/group). During ether anesthesia, the respiratory rate of mice was monitored by the biological signal analytical system (BL-420F; Chengdu Techman Software Co., Ltd. Chengdu, China) and controlled within 90-120 beats/min. Marker enzymes of liver function (ALT and AST) in the serum were assessed with the respective ELISA kits following the manufacturer's protocol in an automatic biochemical analyzer.

Liver morphology. The morphological alterations of the liver were observed. Liver samples were dissected, partly placed in liquid nitrogen immediately for western blotting, and partly in 10% formalin solution, fixed for 48 h at room temperature and progressively dehydrated prior to embedding in paraffin. The paraffin blocks were cut into 4-µm-thick sections, deparaffinized, rehydrated and stained with hematoxylin for 15 min and eosin for 5 min at room temperature. Histological examination was performed under a light microscope (magnification, x400; Olympus BX43; Olympus Corporation, Tokyo, Japan).

Immunohistochemistry. After deparaffinization and rehydration in a descending alcohol series, each section was incubated with 0.01 mol/l citrate buffer (pH 6.0) in a microwave at 98°C for 10 min for antigen retrieval. Endogenous peroxidase was blocked with 3% H2O2 for 20 min at room temperature. Each sample was pretreated with normal goat serum (OriGene Technologies, Inc.) for 30 min at room temperature to block nonspecific binding and incubated with rabbit anti-mTOR (diluted 1:800), rabbit anti-beclin 1 (diluted 1:120) or rabbit anti-MAP1LC3B (diluted 1:60) antibodies overnight at 4°C. Human colon carcinoma tissue obtained from a 50-year-old male patient (Collected in January 2017 from the Pathology Department, of Binzhou Medical University Hospital, Binzhou, China) was used as the positive control. The participant provided written informed consent. An ethical approval for the use of human samples was obtained from the Ethics Committee of the Binzhou Medical University. For the negative control, the primary antibody was replaced with phosphate-buffered saline. A biotinylated goat anti-rabbit immunoglobulin G antibody (cat. no. ZB-2305; OriGene Technologies, Inc.; 1:400) was used as the secondary antibody and incubated with each section at 37°C for 15 min. These sections were subsequently exposed to streptavidin-horseradish peroxidase conjugate (Beijing Bioneeds, Technologies Co Ltd. Beijing, China; cat. no. CJ30H; 1:500) at 37°C for 10 min. The peroxidase reactivity was visualized by the application of 3,3′-diaminobenzidine solution for 5 min at room temperature. These sections were counterstained with hematoxylin for 10 min and mounted. Two sections were selected from the right and left liver lobes separately in each mouse, and five non-repeating fields of view were observed for each section. The percentage of positive immunoreactivity cell area for mTOR, beclin 1 or MAP1LC3B and the reactive intensity were determined using a light microscope at a magnification of x400 (Olympus BX43; Olympus Corporation). The proportions of liver tissues that stained positively in the cytoplasm and/or cytomembrane were scored according the following criteria: i) 0, <1% cell staining; ii) 1, staining in 1-10% cells;
Western blotting. The proteins of liver samples were prepared with radioimmunoprecipitation assay lysis buffer and phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology, Shanghai, China). Protein concentration was detected with a BCA Protein Assay kit (Beyotime Institute of Biotechnology) with a loading mass of 20 µg/well. The samples were separated by 12% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% nonfat milk in PBST buffer (PBS +0.1% Tween 20) for 2 h at room temperature, then incubated with primary antibodies overnight at 4°C and secondary antibodies (Beijing ZhongShan Golden Bridge Biotechnology Co., Ltd Beijing, China; 1:5,000) at 37°C for 0.5 h. The following primary antibodies were used: mTOR (1:1,000), beclin1 (1:1,000), MAP1LC3B (1:1,000), caspase-3 (1:1,000) and GAPDH (cat. no. SC-32233; 1:10,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Protein bands were visualized by Enhanced Chemiluminescence kit (Beyotime Institute of Biotechnology). Integral optical density (IOD) value measured with Image-Pro Plus (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA) was used to calculate the relative expression of the target protein (ratio) using the following equation: Ratio=IOD target protein/IOD GAPDH.

Statistical analysis. All experiments were repeated three times independently. Data were analyzed using SPSS 19.0 software (IBM Corp., Armonk, NY, USA) and presented as the mean ± standard deviation. Multigroup comparisons were carried out by one-way analysis of variance with Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Alterations in bodyweights and serum markers of liver function in the experimental mice. In the model group, the mice exhibited hair loss, thinning and increased irritability. The activity of mice was decreased (data not shown), and weight losses were significantly more severe in the model mice compared with the control group (P<0.05; Fig 1). Glutathione administration groups exhibited minor alterations in behavior or bodyweight, especially in the high-dose glutathione group, when compared with control and model groups. The serum levels of ALT and AST were measured on days 15 and 30 of the experiment. Serum levels of ALT and AST in the model group significantly increased compared with the control group and high-dose glutathione group (all P<0.05). On day 15, there was no significant difference in serum levels of ALT and AST between the model group and the low-dose group, whereas the serum levels in the low-dose glutathione group were significantly lower compared with the model group on day 30 (P<0.05; Fig 1).

Liver morphology of the mice. In the control group, it was observed that the liver capsule was smooth, the sections were gray and red, and the texture was uniform and soft. Under the microscope, the structures of the hepatic lobule and portal area were clear, without obvious signs of the presence of fibrous tissue; the liver cells were aligned and the cytoplasm was eosinophilic uniformly. In the model group, the livers were clearly congested, swollen and rough. Hepatocellular edema, a large number of hepatocytes undergoing hepatic steatosis and mild necrosis could be observed on day 15 in the model group. On day 30 in model group, the livers exhibited severe edema and multifocal dotted necrosis; fibrosis and hyperplasia were visible around the portal vein, with inflammatory cell infiltration. In the low-dose glutathione group, cell morphology was similar to that of the model group, with occasional hepatic cell degeneration. In the high-dose glutathione group, the livers exhibited slight congestion, mild hepatocyte edema, slight dotted necrosis and no significant fatty degeneration, however, obvious hepatic cell degeneration was observed (Fig 2).

Expression of autophagy-associated proteins mTOR, beclin 1 and MAP1LC3B in mouse livers. High levels of mTOR expression were observed in the cytoplasm in the control group, however these levels were reduced in the model group. On day 15 of the experiment, mTOR expression was significantly lower in the glutathione treatment groups compared with the control group and model group (both P<0.05), especially in the high-dose glutathione group. On day 30, compared with the control group, mTOR expression decreased significantly in all other groups (including the model group and glutathione treatment groups; all P<0.05; Fig 3). The trend of beclin 1 expression was different compared with that of mTOR expression. On day 15 of the experiment, expression levels of beclin 1 increased significantly in the glutathione treatment groups compared with the control group and model group (both P<0.05). On day 30, beclin 1 expression was significantly higher in all groups compared with the control group (all P<0.05; Fig 4). The expression of MAP1LC3B was similar to that of beclin 1. On day 15, the expression of MAP1LC3B in all drug-administrated groups was significantly higher than the control group (P<0.05) and MAP1LC3B expression in the high-dose glutathione group was also higher than the model and low-dose glutathione group. On day 30, MAP1LC3B expression in all drug-administrated groups was higher than the control group and glutathione-administrated groups were also higher when compared with the model groups (all P<0.05; Fig 5). The results of western blotting indicated that mTOR expression decreased obviously in all groups compared with the control group. The expression of beclin1 increased in both glutathione treatment groups compared with the control group, which was consistent with the results for MAP1LC3B and caspase-3 expression (all P<0.05; Fig 6).

Discussion

Hepatic injury caused by the long-term administration of particular drugs, including acetaminophen, amoxicillin-clavulanate and antiepileptic drugs are common clinical phenomena (29,30). The degree of hepatocyte damage depends on the
Figure 1. Alterations in mouse bodyweights and serum ALT and AST levels (U/l). The bodyweight in the model group was significantly lower compared with other groups on day 15 and 30 after administration. Serum ALT and AST levels significantly increased in the model and low-dose glutathione groups compared with control and high-dose glutathione groups respectively. *P<0.05. Data are presented as the mean standard deviation. ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Figure 2. Morphology of the mouse livers. (A1) Gross observation revealed that the normal control liver capsule was smooth and the sections were grey and red, and uniformly soft. (A2) The liver of the model group was clearly congested, swollen and brittle. (A3) The liver of the low-dose glutathione group was light yellow and moderately swollen. (A4) The liver of the high-dose glutathione group was slightly congested. (B1) Histological examination of the liver revealed that hepatic cells were aligned and cytoplasm was uniformly eosinophilic in the control group. (B2) Severe edema with multifocal dotted necrosis and inflammatory cell infiltration in the model group (black arrow indicates inflammatory cell infiltration). (B3) Edema with dotted necrosis in the low-dose glutathione group (black arrow indicates dotted necrosis). (B4) Mild edema with a slight dotted necrosis and no obvious fatty degeneration in the high-dose glutathione group (black arrow indicates slight dotted necrosis). (Original magnification, x400).

Figure 3. Expression of mTOR determined by immunohistochemistry. (A) Control group. (B) Model group. (C) Low-dose glutathione group. (D) High-dose glutathione group. The positive expression of mTOR was localized to the cytoplasm (original magnification, x400; black arrows indicate positive staining). (E) On day 15 of the experiment, mTOR expression was significantly lower in the glutathione-treated groups compared with the control group (*P<0.05 and model group). On day 30, mTOR expression was significantly lower in all groups compared with the control group. *P<0.05 vs. Control group; **P<0.05 vs. model group. mTOR, mammalian target of rapamycin.
accumulation of toxicity and the protection afforded by physiological or exogenous factors (31,32). Among these cell protection mechanisms, autophagy is currently widely investigated (13).

Under normal physiological conditions, the autophagy level of cells is low, designated as 'basic autophagy', which primarily maintains the stable state of cells (15). However, autophagy is induced by pathological factors, including starvation, ischemia/hypoxia and injury (15,33). To maintain homeostasis and ensure cell survival, energy is generated by the degradation of damaged organelles and protein aggregates (15,33,34). Several studies have demonstrated that autophagy may serve dual roles in alcoholic liver disease, hepatic ischemia/reperfusion injury and bile duct ligation or carbon tetrachloride cirrhosis models (35-37). As a cellular self-protective mechanism, autophagy significantly increases in hepatic injury caused by various factors, including drug use, alcohol consumption and chronic hepatitis B and C (38,39). Furthermore, autophagy may attenuate fibrosis and reduce hepatic injury by inhibiting inflammatory reaction and decreasing oxidative stress response (40). Therefore, autophagy may be a new therapeutic target for liver injury and fibrosis (40). However, reports on autophagy in the context of arecoline-induced hepatic injury are scarce.

Arecoline is a commonly used Chinese medicine extracted from the betel nut with numerous properties, including parasite expulsion and antiatherosclerotic properties (5,6). However, due to its cytotoxicity and immunotoxicity, long-term use of arecoline can damage cellular DNA and promote cell apoptosis (7). Long-term chewing of the betel nut can lead to oral submucosal fibrosis and oral cancer (4). Several studies
have shown that the long-term administration of arecoline accelerates apoptosis, degeneration and necrosis of hepatic cells, leading to liver cirrhosis and liver cancer (9-11). The betel nut has been identified as a first-class carcinogen by the International Agency for Research on Cancer (41,42). In the present study, intragastric administration of arecoline resulted in alterations in mice behavior and bodyweight, increased levels of AST and ALT, and induction of histological hepatocyte edema and spotty necrosis, and these alterations aggravated with the progress of administration. The above results demonstrated that the excessive use of arecoline may damage liver cells. Thangjam and Kondaiah (43) reported that arecoline-induced cytotoxicity could promote the generation of reactive oxygen species (ROS) and activate inflammatory cells in stress. Furthermore, in the presence of ROS, arecoline may inhibit adenosine monophosphate-activated protein kinase (AMPK) phosphorylation and induce autophagy (44).

However, the exact associations between ROS, AMPK and autophagy in arecoline-induced hepatic injury remain unclear, and further investigation is required.

In clinical practice, L-glutathione is commonly used to protect undamaged liver cells and repair damaged cells by scavenging free radicals and superoxide ions (23). In the present study, L-glutathione was used to treat the hepatic injury induced by arecoline. Compared with the model group, the behavior and serum levels of AST and ALT altered only marginally in the low-dose glutathione group. In the high dose glutathione group, the serum levels of AST and ALT were restored to normal, and histological examinations indicated cell regeneration with minimal necrosis, suggesting that glutathione could protect liver function and promote the regeneration of hepatocytes.

MAP1LC3 is required for autophagosome formation and the level of MAP1LC3 is proportional to the number of autophagic vacuoles present in cells, and, therefore, the autophagic activity can be assessed by measuring the expression of MAP1LC3 (20-22). The phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB)/mTOR signaling pathway is involved in the inhibition of autophagy (45). Under nutrient-rich conditions, the mTOR signal is activated and autophagy is inhibited (19). In the case of nutrient deficiency, mTOR is suppressed, and autophagy is activated by the negative feedback of the PI3K/PKB/mTOR signaling pathway (46). The PI3K-VPS34/beclin 1 pathway is involved in the positive regulation of autophagy (47). Beclin 1, as a ‘platform’ for molecular reactions, promotes the localization of autophagy-associated proteins to the phagosome and regulates the formation and maturation of autophagosome by binding with VPS34 (48). Cursio et al (49) investigated the role of autophagy in hepatic ischemia/reperfusion injury. The authors found that hypoxia and nutrient deficiency activated autophagy, which reduced the extent of hepatic reperfusion injury and restored the function of the damaged mitochondria (49). In another study, the researchers found that knock out of autophagy-associated protein cysteine protease ATG4B aggravated hepatic damage in mice due to increased sensitivity to ischemia/reperfusion injury (50). Studies on the association between autophagy and alcoholic and foodborne liver damage have suggested that autophagy protects liver cells by selectively removing harmful substances and repairing damaged mitochondria (51,52). Betsuyaku et al (51) reported that, to maintain
immune homeostasis, the levels of both apoptosis and autophagy were increased in rats with acute ethanol-induced damage to the thymus. Regarding the association between autophagy and drug-induced liver damage, Ni et al (52) reported that the excessive application of acetaminophen could cause mitochondrial damage and liver cells necrosis. Inhibition of autophagy by chloroquine could aggravate the necrosis of liver cells, whereas induction of autophagy with rapamycin could reduce or even reverse liver damage (53). However, a previous study indicated that ATG5-defective mice exhibited higher tolerance to acetaminophen and increased resistance to pathological stimuli by reducing autophagy (54).

In the present study, expression levels of MAP1LC3B and beclin 1 were low in the control group, and mTOR was highly expressed; MAP1LC3B expression indicated autophagy level in these cells. In the model group on day 30, following administration of arecoline, the expression of MAP1LC3B and beclin 1 in hepatocytes increased significantly compared with the control group, and the expression of mTOR decreased. One study has indicated that carboxylesterase, the main metabolic enzyme of arecoline, is highly active in liver mitochondria (55). When the accumulation of arecoline in the body exceeds the metabolic capacity, it can damage mitochondria and activate autophagy to ameliorate damage and ensure the survival of liver cells (32). Increased expression of MAP1LC3B and beclin 1 can indicate the increased autophagic activity (22). Jung et al (56) reported that mTOR complex 1 localized near mitochondria and was inhibited by oxidative stress and mitochondrial dysfunction. Furthermore, the expression of mTOR primarily in necrotic cells may imply that severely damaged cells cannot survive by activating autophagy (57). Therefore, the authors of the present study hypothesized that autophagy may be induced by an interaction between mTOR and beclin 1 (PI3K-VPS34/beclin 1), and via the destruction of mitochondria (56). Previous studies suggested that the phosphorylation levels of autophagy regulatory genes were closely associated with autophagy activity (58,59). Wang et al (58) indicated that PKB-mediated phosphorylation of beclin 1 served a role in autophagy inhibition. The phosphorylation level of 4E-binding protein 1, the first downstream substrate of mTOR, can directly reflect the activated state of mTOR (59). Therefore, the following study should evaluate the phosphorylation levels of autophagy-associated proteins.

Furthermore, the present study demonstrated that the expression of caspase-3, a key executor of apoptosis, was upregulated in the process of liver damage. Zhu et al (60) reported that beclin 1 was a substrate of caspase-3 and the cleavage of beclin 1 may contribute to inactivation of autophagy leading to augmentation of apoptosis. Beclin1 protein includes a bcl-2-homology-3 domain, which can combine with bcl-2/bcl-xl and abrogate anti-apoptotic effects (51,61). In the present study, administration of L-glutathione decreased the necrosis of hepatocytes. Expression of MAP1LC3B in the high-dose glutathione group increased significantly compared with the low-dose and model groups. The expression of MAP1LC3B may be associated with the concentration of glutathione. When mitochondria undergo damage by arecoline, ROS accumulation may accelerate apoptosis and lead to necrosis (32). However, L-glutathione, a major component of the endogenous antioxidant defense system, may accelerate autophagy to generate energy, decrease ROS production and reduce liver damage (62). In addition, the reactive sulfur atoms of glutathione can protect hepatocytes by binding to a variety of chemicals and metabolites, and increase the membrane stability (63). However, the exact association between L-glutathione, apoptosis and liver injury remains to be further elucidated in the future.

In conclusion, L-glutathione can effectively protect against the hepatic injury caused by arecoline. Autophagy may be an important mechanism in the procession of hepatic injury and autophagic activity may be promoted by targeting autophagy-associated genes. The results of the present study may thus aid the treatment of drug-induced liver injury.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

PW and XW designed the experiments; JX and BW analyzed the data; PW and XW performed the experiments; JX and BW analyzed the data; PW and XW drafted the manuscript. All authors have read and approved the final version for publication.

Ethics approval and consent to participate

All experimental protocols involving animals and human tissue samples were approved by the Ethics Committee of Binzhou Medical University (Yantai, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Gupta PC and Warnakulasuriya S: Global epidemiology of areca nut usage. Addict Biol 7: 77-83, 2002.
2. Chu NS: Effects of betel chewing on the central and autonomic nervous system. J Biomed Sci 8: 229-236, 2001.

3. Niu X, Li W, Xu H, Liu X and Qi L: Simultaneous quantification of 12 salivary alkaloids in Cordyline impatiens (Pall.) Fisch by HPLC. J Sep Sci 36: 2090-2095, 2013.

4. Warnakulasuriya S, Trivedy C and Peters TJ: Areca nut use: An independent risk factor for oral cancer. BMJ 324: 799-800, 2002.

5. Green PW, Simmons MS and Blaney WM: Toxicity and behavioural effects of diet-borne alkaloids on larvae of the black blowfly, Phormia regina. Med Vet Entomol 16: 157-160, 2002.

6. Shan LM and Wang H: Pharmacological characteristics of the endothelial target for acetylated lowdensity lipoprotein. Life Sci 70: 1285-1298, 2002.

7. Ellinger-Egelhaeuer H, Stuart B, Wahle B, Bomann W and Ahr HJ: Characteristic expression profiles induced by genotoxic carcinogens in rat liver. Toxicol Sci 77: 19-34, 2004.

8. Lin KH, Lin CY, Liu CC, Chou MY and Lin JK: Arecoline N-oxide: Its mutagenicity and possible role as ultimate carcinogen in areca oral carcinogenesis. J Agric Food Chem 59: 3420-3428, 2011.

9. Cho CW, Gun JY, Tsai SF, Hwang CC, Chen HC, Huang JS, Yang YL, Hung WC and Chuang LY: Arecoline-induced growth arrest and p21WAF1 expression are dependent on p53 in rat hepatocytes. Toxicology 243: 1-10, 2008.

10. Zhou J, Sun Q, Yang Z and Zhang J: The hepatotoxicity and testicular toxicity induced by arecoline in mice and protective effects of vitamins C and E. Korean J Physiol Pharmacol 18: 143-148, 2014.

11. Gupta R, Saha I, Pal S, Bhattacharyya A, Sa G, Nag TC, Das T and Mahi BR: Immunosuppression, hepatotoxicity and depression of antioxidant status by arecoline in albino mice. Toxicology 227: 94-104, 2006.

12. Cheng HL, Su SJ, Huang LW, Hsieh BS, Hu YC, Hung TC and Chang KL: Arecoline induces HA22TV/VGH hepatoma cells to undergo anoikis-involvement of STAT3 and Rhoa activation. Mol Cancer 9: 126, 2010.

13. Kang SW, Haydar G, Taniane C, Farrell G, Arias IM, Wang RC, Wei Y, An Z, Zou Z, Xiao G, Bhagat G, White M, Kominami E, Ohsumi Y and Yoshimori T: LC3, a Caenorhabditis elegans homeostasis. FEBS J 277: 66-73, 2010.

14. Kominami E, Ohsumi Y and Yoshimori T: LC3, a developmentally regulated autophagy related genes expression and clinical features in tongue squamous cell carcinoma cell lines. Oral Oncol 50: 343-349, 2014.

15. Kang R, Zeh HJ, Wang RC, Wei Y, An Z, Zou Z, Xiao G, Bhagat G, White M, Noda T, Kominami E, Ohsumi Y and Yoshimori T: Beclin1-mediated autophagy and tumorigenesis through Beclin 1 phosphorylation. J Biomed Sci 18: 229-236, 2011.

16. Wang X, Sun K, Xiao CY, Li SN, Tian YP and Wang CY: Effect of autophagy on glutathione protecting against arecoline-induced hepatic injury. Chin J Clin Exp Pathol 6: 660-664, 2016 (In Chinese).

17. Saha I, Chatterjee A, Mondal A, Matia BR and Chatterji U: Arecoline augments cellular proliferation in the prostate gland of male Wistar rats. Toxicol Appl Pharmacol 255: 160-168, 2011.

18. Qu XS, Shi H, Cao XL, Dong XF, Li T, Jiao QJ, Qi WS and Wu MN: Effects of adipocytokine on the anxiety and memory impairment in triple transgenic Alzheimer's disease model mice. Zhongguo Ying Yong Shen Li Xue Za Zhi 33: 405-409, 2017 (In Chinese).

19. honoré PH, Basnet A, Eljaja L, Kristensen P, Andersen LM, Neustrup S, Møllgaard P and Bjerrum OJ: Neuropathic pain models in the development of analgesic drugs. Scand J Pain 2: 167-177, 2012.

20. Kimura H, Weisz A, Kurashima Y, Hashimoto K, Ogura T, D’Acquisto F, Addeo R, Mukauchi M and Esumi H: Hypoxia response element of the human vascular endothelial growth factor gene mediates transcriptional regulation by nitric oxide: Control of hypoxia-inducible factor-I activity by nitric oxide. Blood 95: 189-197, 2000.

21. Giordano C, Rivas J and Zervos X: An update on treatment of drug-induced liver injury. J Clin Transl Hepatol 2: 74-79, 2014.

22. Björnsson ES, Bergmann OM, Björnsson HK, Kvaran RB and Oldfors S: Incidence and toxicogenomics in patients with drug-induced liver injury in the general population of Iceland. Gastroenterology 144: 1419-1425, 2013.

23. Au JS and Pockro PF: Drug-induced liver injury from antiepileptic drugs. Clin Liver Dis 17: 687-697, 2013.

24. Takenaka Y, Miyazaki T, Higashi S, Tsuyama K, Endo S, Tsutsumi T, Toyoda Y, Fujiura T, Nakanishi M and Yokoi T: Mechanisms of the hepatoprotective effects of tamoxifen against drug-induced and chemically-induced acute liver injuries. Toxicol Appl Pharmacol 264: 42-50, 2012.

25. Eskelinen EL: The dual role of autophagy in cancer. Curr Opin Pharmacol 11: 294-300, 2011.

26. Chen N and Karantza-Wadsworth V: Role and regulation of autophagy in cancer. Biochim Biophys Acta 1793: 1516-1523, 2009.

27. Ding W, Li M, Chen X, Ni HM, Lin CW, Gao W, Lu B, Stolz DB, Clemens DL and Yin XM: Autophagy reduces acute ethanol-induced hepatic toxicity and steatosis in mice. Gastroenterology 139: 1740-1752, 2010.

28. Kan C, Liu A, Fang H, Dirsch O, Dahmen U and Boetcker M: Induction of autophagy reduces ischemia/reperfusion injury in steatotic rat livers. J Surg Res 216: 207-218, 2017.

29. Wu G, Yang Q, Yu Y, Lin S, Feng Y, Lv Q, Yang J and Hu J: taurine inhibits kupffer cells activation induced by lipopolysaccharide in alcoholic liver damaged rats. Adv Exp Med Biol 795: 789-800, 2014.

30. Albani D and Friedman SL: Antifibrotic agents for liver disease. J Clin Transl Hepatol 2: 1982-1986, 2015.

31. Chen J, Yu Y, Li S, Liu Y, Zhou S, Cao S, Yin J and Li G: MicroRNA-30a ameliorates hepatic fibrosis by inhibiting Beclin1-mediated autophagy. J Cell Mol Med 21: 3679-3692, 2017.

32. Mao YQ and Fan XM: Autophagy: A new therapeutic target for liver fibrosis. World J Hepatol 7: 1892-1896, 2015.

33. Chaturvedi P, Vaishampayan SS, Nair S, Nair D, Agarwal JP, Chatterjee A and Mondal A: Odds ratio of risk factors for oral submucous fibrosis in a case control model. Br J Oral Maxillofac Surg 51: e169-e173, 2013.

34. Thangjam GS and Kondaiah P: Regulation of oxidative-stress responsive genes by arecoline in human keratinocytes. J Periodontal Res 44: 673-682, 2009.

35. Yen CY, Lin MH, Liu SW, Chiang WF, Hsieh WF, Cheng YC, Hsu KC and Liu CY: Arecoline ameliorates hepatic fibrosis by inhibiting TGF-β1 pathway in vivo. J Cell Mol Med 20: 1892-1899, 2016.

36. Wang X, Wang XY, Zou YH and Li S: Correlation between autophagy related genes expression and clinical features in carcinogenesis of oral squamous cell carcinoma. Int J Clin Exp Pathol 9: S59-S65, 2016.

37. Yoshikawa A, Hori K, Doki Y, Yamasaki M, Sohma I, Gotoh K, Takiguchi S, Fujisawa Y, Uchida Y and Monden M: LC3, an autophagosome marker, is highly expressed in gastrointestinal cancers. Int J Oncol 33: 461-468, 2008.

38. Dunning S, Ur Rehman A, Tiebosch MH, Hannivoort RA, Hairer FW, Woudenberg J, van den Heuvel FA, Buist-Homan M, Faber KN, Goedhart J, Cande and Koomen M: Glutathione and antioxidant enzymes serve complementary roles in protecting activated hepatic stellate cells against hydrogen peroxide-induced cell death. Biochim Biophys Acta 1832: 2027-2034, 2013.
48. Menon S, Dibble CC, Talbott G, Hoxhaj G, Valvezan AJ, Takahashi H, Cantley LC and Manning BD: Spatial control of the TSC complex integrates insulin and nutrient regulation of mTORC1 at the lysosome. Cell 156: 771-785, 2014.

49. Cursio R, Colosetti P and Gugenheim J: Autophagy and liver ischemia-reperfusion injury. Biomed Res Int 2015: 417590, 2015.

50. Wang JH, Ahn IS, Fischer TD, Byeon JI, Dunn WA Jr, Behrns KE, Leeuwenburgh C and Kim JS: Autophagy suppresses age-dependent ischemia and reperfusion injury in livers of mice. Gastroenterology 141: 2188-2199.e5, 2011.

51. Betsuyaku T, Eid N, Ito Y, Tanaka Y, Otsuki Y and Kondo Y: Ethanol enhances thymocyte apoptosis and autophagy in macrophages of rat thymi. Histol Histopathol 32: 963-975, 2017.

52. Ni HM, Boggess N, McGill MR, Lebofsky M, Borade P, Apte U, Jaeschke H and Ding WX: Liver-specific loss of Atg5 causes persistent activation of Nrf2 and protects against acetaminophen-induced liver injury. Toxicol Sci 127: 438-450, 2012.

53. Fang H, Liu A, Dahmen U and Dirsch O: Dual role of chloroquine in liver ischemia reperfusion injury: Reduction of liver damage in early phase, but aggravation in late phase. Cell Death Dis 4: e694, 2013.

54. Ni HM, Bockus A, Boggess N, Jaeschke H and Ding WX: Activation of autophagy protects against acetaminophen-induced hepatotoxicity. Hepatology 55: 222-232, 2012.

55. Patterson TA and Kosh JW: Elucidation of the rapid in vivo metabolism of arecoline. Gen Pharmacol 24: 641-647, 1993.

56. Jung CH, Ro SH, Cao J, Otto NM and Kim DH: mTOR regulation of autophagy. FEBS Lett 584: 1287-1295, 2010.

57. Codogno P, Mehrpour M and Proikas-Cezanne T: Canonical and non-canonical autophagy: Variations on a common theme of self-eating? Nat Rev Mol Cell Biol 13: 7-12, 2011.

58. Wang RC, Wei Y, An Z, Zou Z, Xiao G, Bhagat G, White M, Reichelt J and Levine B: Akt-mediated regulation of autophagy and tumorigenesis through Beclin 1 phosphorylation. Science 338: 956-959, 2012.

59. Fingar DC, Salama S, Tsou C, Harlow E and Blenis J: Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4E-BP1/eIF4E. Genes Dev 16: 1472-1487, 2002.

60. Zhu Y, Zhao L, Liu L, Gao P, Tian W, Wang X, Jin H, Xu H and Chen Q: Beclin 1 cleavage by caspase-3 inactivates autophagy and promotes apoptosis. Protein Cell 1: 468-477, 2010.

61. Huang W, Choi W, Hu W, Mi N, Guo Q, Ma M, Liu M, Tian Y, Lu P, Wang FL, et al.: Crystal structure and biochemical analyses reveal Beclin 1 as a novel membrane binding protein. Cell Res 22: 473-489, 2012.

62. Diaz de Barboza G, Guizzardi S, Moine L and Tolosa de Talamoni N: Oxidative stress, antioxidants and intestinal calcium absorption. World J Gastroenterol 23: 2841-2853, 2017.

63. Circu ML and Aw TY: Glutathione and modulation of cell apoptosis. Biochim Biophys Acta 1823: 1767-1777, 2012.

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