Functional Teas from *Penthorum chinense* Pursh Alleviates Ethanol-Induced Hepatic Oxidative Stress and Autophagy Impairment in Zebrafish via Modulating the AMPK/p62/Nrf2/mTOR Signaling Axis

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Abstract

*Penthorum chinense* Pursh (PCP), a medicinal and edible plant, is widely used in many clinical liver diseases. Oxidative stress and autophagy impairment play crucial roles in the pathophysiology of alcoholic liver disease (ALD). Therefore, the aim of this study was to elucidate the mechanism of PCP in attenuating ethanol-induced liver injury. The liver-specific transgenic zebrafish larvae (lfabp: EGFP) at three days post-fertilization (3 dpf) were treated with different concentrations of PCP (100, 50 and 25 μg/mL) for 48 h, after soaked in a 350 mM ethanol for 32 h. Whole-mount oil red O, H&E staining and biochemical kits were used to detect fatty liver function and fat accumulation, western blot (WB) and immunofluorescence were used to determine proteins expression, and RT-qPCR was used to further verify the related gene expression. PCP restored zebrafish liver function. Additionally, PCP (as dose-dependent) blocked the expression of cytochrome P450 2E1 (CYP2E1), the production of intracellular reactive oxygen species (ROS) and alleviated liver fat accumulation and oxidative damage. PCP exerted its hepatoprotective function by downregulating the expression of kelch-like ECH-associated protein 1 (Keap1), up-regulating the expression of nucleus factor-E2-related factor 2 (Nrf2) (transferring to the nucleus), and attenuating systemic oxidative stress. Furthermore, PCP reduced the expression of sequestosome 1 (p62/SQSTM1, p62), Atg13, and Beclin 1, up-regulating autophagy signaling pathway. Taken together, the molecular evidence that PCP protected the ethanol-induced hepatic oxidative stress and autophagy impairment through activating AMPK/p62/Nrf2/mTOR signaling axis.

Keywords *Penthorum chinense* Pursh · Zebrafish · Liver injury · Oxidative stress · Autophagy · AMPK/p62/Nrf2/mTOR signaling axis

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| ACC1         | Acetyl-CoA carboxylase 1 |
| ADH          | Alcohol dehydrogenase |
| ALD          | Alcoholic liver disease |
| ALT          | Alanine transaminase |
| AMPK         | Activated protein kinase |
| AST          | Aspartate aminotransferase |
| CAT          | Catalase |
| DCF-DA       | 2′,7′-Dichlorodihydrofluorescein diacetate |
| CYP450       | Cytochrome P450 |
| ER           | Endoplasmic reticulum |
| IOD          | Integral optical density |
| GSH          | Glutathione |
| Keap1        | Kelch-like ECH-associated protein 1 |
| Lfabp: EGFP  | Liver-specific transgenic zebrafish larvae |
| FAS          | Fatty acid synthetase |
| MDA          | Malondialdehyde |
| MEOS         | Microsomal ethanol oxidation system |
| Nrf2         | Nuclear factor-E2-related factor 2 |
| PCP          | *Penthorum chinense* Pursh |
| ROS          | Reactive oxygen species |
| RT-qPCR      | Quantitative reverse transcriptase-polymerase chain reaction |
| p62/SQSTM1   | Sequestosome 1 |
| SOD          | Superoxide dismutase |
| SREBP-1C     | Sterol regulatory component binding protein 1C |

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**Introduction**

Chinese herbal tea has a long history in China and is one of the most consumed beverage products [1] with a wide range of lipid lowering [2] and antioxidant effects [3–5]. *Penthorum chinense* Pursh (PCP) (Penthoraceae, Ganhuangcao in Chinese) is a medicinal and edible plant [6] and often used as a functional tea to prevent viral hepatitis, alcoholic liver, even liver fibrosis [7–9]. However, the mechanism of its function in ALD remains unclear.

Alcoholic liver disease (ALD) is a worldwide problem that requires much attention [10, 11]. The common drug (glucocorticoids) used for treating ALD in humans has adverse effects and are not suitable for long-term treatment [12]. Therefore, collective efforts are needed to identify natural drugs that effectively cure ALD [13].

Oxidative stress has been considered a key causing factor of ALD [14, 15], and Nrf2 is a key transcription factor that controls cellular defense responses against ALD-induced oxidative stress [16]. Autophagy is a catabolic pathway activated in response to different cellular stressors, such as accumulation of ROS [17, 18] and p62 serves as an essential adaptor to autophagosomes for degradation and is a multifunctional scaffolding protein involved in the regulation of various signaling pathways [19, 20]. Recently, these two cellular pathways were shown to intersect through the direct interaction between p62 and Nrf2 [21]. Induction of the p62 gene by oxidative stress is mediated by Nrf2 and, at the same time, p62 protein contributes to the activation of Nrf2 [22, 23], but hitherto the mechanisms involved were not known. Therefore, the aim of this study was to investigate the protective effects of PCP on ethanol-induced hepatic oxidative stress and autophagy impairment in zebrafish.

**Materials and Methods**

The Materials and Methods section is presented as supplementary material and drug administration were shown in Fig. 1A.

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**Fig. 1 A** Schematic diagram of experimental drug administration. Effects of different concentrations of PCP on the serum biochemical indicators of liver function. The following four liver function markers in the tissue were assayed: **B** ALT; **C** AST; **D** TG; and **E** TC. **F** The images are the representative of H&E experiments (the black circle indicates the liver, the blue arrow indicates fat vacuoles and red arrow indicates hepatocyte cord disorder, 200× original magnification), scale bar: 1 mm. **G** The images are the representative of whole-mount oil red O staining experiments (the white circle indicates the liver, 200× original magnification), scale bar: 1 mm. **H** The fatty changes score was analyzed using the liver injury scoring system. **I** the percentage of IOD of the area.

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*P* < 0.001 compared with control group; ***P* < 0.001, **P* < 0.01, *P* < 0.05 compared with the ethanol group.
Fig. 2 Effects of PCP on lipid homeostasis related enzymes, quantitation of mRNA expression of A SREBP1c, B FAS, C ACC1. And effects of PCP on ethanol metabolism related enzymes, quantitation of mRNA expression of D ADH8a, E ADH8b, F CYP1A1, G CYP2A3, H CYP2P6, I ALDH2 on larvae zebrafish; J ELISA kits measured zebrafish cytochrome P450 2E1 (CYP2E1) activity. **P < 0.01 compared with control group; ***P < 0.01, *P < 0.05 compared with ethanol group.

Fig. 3 A Fluorescence micrographs of ROS in zebrafish larvae, scale bar:1 mm. B The distribution and amounts of superoxide anions were quantified according to the fluorescence intensity. The following six liver function markers in the serum were assayed: C MDA, D SOD, E GSH, F CAT. **P < 0.001 compared with control group; ***P < 0.001, **P < 0.01, *P < 0.05 compared with the ethanol group.
Statistical Analyses

All experimental data were evaluated statistically using One-Way ANOVA. All values were expressed as means ± SD. Results were considered to be statistically significant when \( p < 0.05 \).

Results and Discussion

PCP Ameliorated Ethanol-Induced Liver Function Injury and Fat Accumulation

Excessive ethanol consumption increases every year globally, posing a significant medical, social and economic burden [24]. PCP have been used to treat liver-related diseases with no side effects in clinical use [25]. Our data (Supplementary Fig. 1) indicated that PCP contained a valuable source of phenolic compounds with bioactive potential as foods and as promising source of natural ingredients [26]. The higher levels of quercetin with antioxidant potential [27] have previously been reported to be significant to mitigate ethanol-induced liver injury [16].

After ethanol treatment, ALT and AST levels increased significantly \( (P < 0.001) \) (Fig. 1B, C) and caused a significant accumulation of the TG and TC concentrations \( (P < 0.001) \) (Fig. 1D, E). And H&E staining images showed a significant increase in fat vacuoles in the liver tissues \( (P < 0.01) \), accompanied by steatosis, lobular inflammation, hepatocyte necrosis, hepatocyte cord disorder, and hepatocyte ballooning (Fig. 1F, H). PCP definitely alleviated the liver failure with reduced hepatocyte necrosis and liver cell degeneration \( (P < 0.05) \), which were consistent with the whole-mount oil red O staining (Fig. 1G, I). These findings are consistent with those of other previous reports [8, 28, 29] that PCP ameliorated ethanol-induced liver function injury and fat accumulation.

PCP Modulated Lipid Homeostasis and Reduced CYP2E1-Mediated Ethanol Metabolism

The liver is an important organ for ethanol metabolism. Some studies have shown that CYP450, the main component of microsomal ethanol oxidation system (MEOs), plays an important role in the process of ethanol conversion to acetaldehyde [30].

Fig. 4 Effects of PCP on proteins and genes expression profiles. Quantitation of WB analysis of A Keap1, B Nrf2, C p-AMPK/AMPK, and quantitation of mRNA expression of D Keap1, E Nrf2, F HO-1, G AMPK on larvae zebrafish; ***\( P < 0.001 \) compared with the control group; ****\( P < 0.001 \), **\( P < 0.01 \), *\( P < 0.05 \) compared with the ethanol group.
Subsequently, we determined the lipogenesis-related genes expression levels at first. Sterol regulatory element-binding protein 1c (SREBP1c) regulates the expression of several genes related to fatty acid and triglyceride synthesis, fatty acid synthetase (FAS) and acetyl-CoA carboxylase 1 (ACC1). As shown in Fig. 2A-C, PCP obviously cancelled the increase of SREBP1c, FAS genes expression ($P < 0.001$) and the decrease of ACC1 gene expression ($P < 0.001$) triggered by ethanol. Different from previous studies [31], ADH8a and ADH8b were significantly increased after ethanol stimulation ($P < 0.001$), while only 100 $\mu$g/mL PCP had minimally affected ADH levels ($P < 0.05$) (Fig. 2D, E). Further, we assessed mRNA expression levels of CYP1A1, CYP2A3, CYP2p6 enzymes (Fig. 2G–I). Similarly, CYP2E1 activity increased nearly 10 times after ethanol stimulation, while PCP effectively reversed the result (Fig. 2J), which suggesting PCP was mainly involved in CYP2E1-dominated ethanol metabolism, instead of ADH.

**PCP Reduced Oxidative Stress in Ethanol-Induced Liver Injury**

CYP2E1 is also a ROS activity generator [32], leading to oxidative stress and liver damage [33]. Our results showed in Fig. 3A, a bright and strong fluorescent image that was observed in the ethanol treatment groups. PCP concentration-dependently decreased intracellular ROS production ($P < 0.001$) (Fig. 3B). Compared with the control group, the MDA levels of the ethanol-treated groups were significantly elevated ($P < 0.001$) (Fig. 3C). Conversely, the levels of SOD, CAT and GSH markedly declined ($P < 0.001$). Whereas, the PCP pretreatment reversed the process in a concentration-dependent manner (Fig. 3D, E, F). Thus, PCP alleviated ethanol-induced hepatosteatosis by inhibiting oxidative stress.

**PCP Decreased the Expression of Keap1 and Promoted Nrf2 Transferring Into the Nucleus**

Moreover, excessive CYP2E1 leads to Nrf2-mediated depletion of the antioxidant system in vivo [34]. WB and RT-qPCR experiments were conducted to detect the expression of oxidative stress related proteins and genes. Consistent with previous studies [35], PCP downregulated the expression of Keap1 protein ($P < 0.001$) and upregulated the transfer of Nrf2 into the nucleus ($P < 0.01$) (Fig. 4D, E). RT-qPCR results showed it regulated detoxifying enzymes (HO-1) ($P < 0.01$) (Fig. 4F) and maintained homeostasis.
Additionally, AMPK is an energy sensor that is an upstream molecule of autophagy [36]. Compared with ethanol treatment group (Fig. 4C, G), PCP has no significant effect on the total AMPK protein expression, whereas it could enhance the p-AMPK (P < 0.01), suggesting AMPK-mediated ethanol-induced liver injury by regulating p62 and autophagy crosstalk with the Keap1/Nrf2 pathway.

PCP Alleviated Ethanol-Induced Liver Injury via Activating p62/mTOR Pathway

Recently, autophagy was found to be protective against an acute alcohol/CYPE1-dependent liver injury model [37]. p62 is a specific autophagy marker, and the increase of p62 protein level indicates that autophagy is inhibited and autophagy flux is blocked. Consistent with the results of WB analysis, in ethanol treatment group, the protein expression of p62 was upregulated (P < 0.01). Meanwhile this result was reversed in the high-dose PCP group, improving autophagy flux (P < 0.05).

Subsequently, the immunofluorescence results (Fig. 5A, B) showed that the protein expression of LC3B in the ethanol group decreased compared with the control group (P < 0.01), which indicated that autophagy was inhibited and autophagy flux was blocked. Moreover, it has been found that Nrf2 can also regulate the expression of p62 gene, thus affecting the occurrence of autophagy and reducing lipid accumulation [23]. Consistent with these previous studies [35], our results indicated that PCP effectively change the autophagy flux in ethanol-induced oxidative impairment.

In addition, the activation of p-AMPK leads to inhibition of mechanistic target of rapamycin (mTOR), the main inhibitors of autophagy, which promotes autophagy [15]. And it has been found that upregulation of p62 activates mTORC1 by directly acting on the mTOR regulatory protein, thereby inhibiting autophagy [19]. We further investigated the effects of PCP in activating the mTOR-mediated autophagy pathway. A decreased in the p-mTOR expressions were observed in the ethanol treatment group (P < 0.001) (Fig. 5D), whereas, the expression of Atg13 and Beclin 1 decreased significantly (P < 0.001) (Fig. 5F, G) in the ethanol treatment group. Moreover, our data showed that 100 μg/mL PCP pretreatment significantly reversed the results (P < 0.001), which means that autophagy flux can be restored.

Conclusion

In summary, PCP could attenuate ethanol-induced liver injury in zebrafish by activating AMPK/p62/Nrf2/mTOR signaling pathway (Fig. 6), which identified a druggable pathway for the development of novel therapies in ALD.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11130-022-01010-0.

Declarations

All data generated or analyzed during this study are included in this article and its supplementary materials files and all authors declare that there are no conflicts of interests and consent to submit.

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Conflict of Interest No conflict of interest exits in the submission of this manuscript, and manuscript is approved by all authors for publication.

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