Journal of Ethnopharmacology 281 (2021) 114489

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Role of MicroRNA-155 in Triptolide-induced hepatotoxicity via the Nrf2-Dependent pathway

Yao Li [a,1], Lin Guo [a,1], Zhenyan Hou [b], Hui Gong [a], Miao Yan [a,*], Bikui Zhang [a,**]

[a] Department of Pharmacy, The Second Xiangya Hospital, Central South University, Changsha, Hunan, 410011, China
[b] Department of Pharmacy, The Affiliated Yantai Yuhuangding Hospital of Qingdao University, Yantai, Shandong, 264000, China

ARTICLE INFO

Keywords:
Triptolide
miR-155
Hepatotoxicity
Nrf2

ABSTRACT

Ethnopharmacological relevance: Triptolide (TP), the main bioactive and toxic ingredient of *Tripterygium wilfordii* Hook F, causes severe toxicity, particularly for hepatotoxicity. However, the underlying mechanisms for its hepatotoxicity are not entirely clear.

Aim of the study: The purpose of the study was to explore the role of miR-155, a microRNA closely related to various liver injuries and a regulator of the nuclear factor erythroid 2-related factor 2 (Nrf2) antioxidant pathway, in TP-induced liver injury *in vitro* and *in vivo*.

Materials and methods: First, *in vitro* L02 cells were treated with different concentrations of TP. The protein levels of Nrf2 and its downstream genes Heme oxygenase1 (HO-1) were determined by Western blot. The mRNA expression of miR-155, Nrf2, NAD(P)H: quinone oxidoreductase 1 (NQO1) and HO-1 were measured using qRT-PCR. And we transfected miR-155 inhibitor and mimic before TP treatment to determine the mRNA and/or protein levels of miR-155, Nrf2 and HO-1. Then, we further confirmed the interaction between miR-155 and Nrf2 pathway in TP-induced hepatic injury in BALB/C mice. The degree of liver injury was determined by HE staining and serum biochemical. The mRNA expression of miR-155 was examined with qRT-PCR and Nrf2 and HO-1 gene expression in liver were evaluated by immunohistochemistry and/or Western blot.

Results: The results showed that TP significantly induced the expression of miR-155 both in L02 cells and in rodents liver tissue, and the inhibition of miR-155 could mitigate the hepatic damages caused by TP. Further experiments demonstrated that the inhibition of miR-155 reversed the down-regulation of Nrf2 and HO-1 by TP, while the miR-155 mimic enhanced the effects of TP. Animal experiments also showed that the inhibition of miR-155 by miR-155 antagomir reversed the decrease of Nrf2 induced by TP administration.

Conclusions: These results indicated that miR-155 played an important role in TP-induced hepatotoxicity by regulating the Nrf2 signaling pathway.

1. Introduction

Natural products have long been part of traditional folk medicine, the role of which in the treatment of various diseases has become gradually clear and approved for clinical treatment in the last few decades (Al-Hrout et al., 2018; Ashktorab et al., 2019). Triptolide (TP) is one of the main bioactive and effective components of *Tripterygium wilfordii* Hook F, which has found increasingly wide applications against various diseases, including nephrotic syndrome, cancer, rheumatoid arthritis, and other autoimmune inflammatory diseases (Li et al., 2014, 2015). However, the widespread applications of TP remain restricted by narrow therapeutic windows and multi-organ injuries on the heart, liver, kidney, gastrointestinal tract, reproductive organs and bone marrow (Li et al., 2015; Xi et al., 2017). Therefore, it is important and necessary to understand the mechanisms involved in order to reduce the toxicity of TP.

To date, the mechanisms of hepatotoxicity caused by TP have not been fully articulated. some research have suggested that TP-induced hepatic toxicity was mainly related to oxidative stress (X.J. Li et al., 2014), metabolic enzymes and transporters (Hou et al., 2018; Lu et al., 2017), mitochondrial function (Hasnat et al., 2020), and immune

* Corresponding authors. Department of Pharmacy, the Second Xiangya Hospital, Central South University, Changsha, 410011, China.
** Corresponding author. Department of Pharmacy, the Second Xiangya Hospital, Central South University, Changsha, Hunan, 410011, China.
E-mail addresses: yanmiao@csu.edu.cn (M. Yan), zhbk68@163.com (B. Zhang).
[1] Equal contribution: Yao Li, Lin Guo.

https://doi.org/10.1016/j.jep.2021.114489
Received 16 May 2021; Received in revised form 3 August 2021; Accepted 3 August 2021
Available online 4 August 2021

Abbreviations

TP	Triptolide
Nrf2	erythroid 2-related factor 2
ARE	antioxidant reaction element
OD	Optical density
ALT	alanine aminotransferase
ALP	alkaline phosphatase
qRT-PCR	Quantitative real-time PCR
PFOS	perfluorooctane sulfonate
miR-155	microRNA-155
HO-1	Heme oxygenase1
miRNAs	MicroRNAs
H&E	hematoxylin and eosin
AST	aspartate aminotransferase
LDH	lactate dehydrogenase
NQO1	quinone oxidoreductase 1
SFN	Sulforaphane

response (Wang et al., 2016). Nuclear factor erythroid 2-related factor 2 (Nrf2) is a crucial defense factor against oxidative stress that regulates a series of downstream antioxidant genes, metabolic enzymes and transporters, subsequently accelerating the metabolism and efflux of toxic metabolites (Klaassen and Reisman, 2010). Our previous research revealed that Nrf2 played a crucial role in the occurrence and development of TP-induced hepatic toxicity by regulating downstream antioxidant genes and transporters in L02 cells, HepG2 cells, and ICR mice (Cao et al., 2016; Hou et al., 2018). Nevertheless, whether TP induced hepatotoxicity by directly targeting Nrf2 or by first affecting an upstream factor, and then inhibiting the Nrf2 pathway has not been thoroughly studied.

In the past decades, more attentions have been paid to MicroRNAs (miRNAs), endogenous non-coding small RNAs (21–25 nt) that regulated target genes at the post-transcriptional level. MiRNAs have been considered to be novel participants in regulating the Nrf2/antioxidant reaction element (ARE) pathway in redox homeostasis (Cheng et al., 2013). Many pieces of research have also confirmed that miRNAs participated in the development, diagnosis, treatment, and prognosis of various liver diseases (Schueller et al., 2018; Torres et al., 2018). Furthermore, it has been demonstrated that the aberrant expression of microRNA-155 (miR-155), a miRNA family, is related to viral hepatitis, alcoholic and non-alcoholic liver disease, liver fibrosis, hepatocellular carcinoma, and drug-induced liver injury (Bala et al., 2016; Chen et al., 2020; Cho et al., 2017; Torres et al., 2018; Wang et al., 2009).

MiR-155 can target multiple components, such as suppressor of cytokine signaling-1 (Socs1) and src homology-2 domain-containing inositol 5-phosphatase 1 (Ship1), to mainly influence the occurrence and development of inflammation and immunity (Wang et al., 2018). Kinds of research have reported that miR-155 played a vital role in hepatic damage and cell apoptosis by regulating Nrf2 and the downstream antioxidant genes NAD(P)H: quinone oxidoreductase 1 (NQO1) and hemeoxygenase1 (HO1) (Gu et al., 2017; Wan et al., 2016). However, the underlying mechanism of miR-155 participating in TP-induced liver injury has not been reported. Therefore, in the present study, we proposed a hypothesis that TP significantly altered the expression of miR-155, then inhibited the expression of the Nrf2 and downstream antioxidant genes, and thus triggered hepatotoxicity.

2. Materials and methods

2.1. Reagents

TP (purity≥98 %) were acquired from Hua Teng Pharmaceutical Co., Ltd (Hunan, China), DMSO and CCK8 were separately purchased from Sigma-Aldrich (St. Louis, MO, U.S.A) and Meilun Biotechnology Co., LTD (Dalian, China). TP was dissolved in an appropriate amount of DMSO, and stored separately at −20 °C and kept away from light. siRNA-mate, miR-155 mimic, mimic control, miR-155 inhibitor, inhibitor control, miR-155 antagomir and antagomir control were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). The primers were as follows: miR-155 mimic sense 5′-UUAAUGCUAAUCGUGAUAGGGGUU-3′ and antisense 5′-CCCCUAUCACGAUUAGCAUUAAUU-3’; mimic control sense 5′-UUCUCCGAACGUGUCACGUTT-3′ and antisense 5′-ACGUGACACGUUCGGAGAATT-3’; miR-155 inhibitor 5′-AACCCCUAUCACGAUUAGCAUUAA-3’; inhibitor control 5′-CAGUACUUUUGUGUAGUACAA-3’; miR-155 antagomir 5′-ACCCCUAUCACAAUUAGCAUUAA-3’; antagomir control 5′-CAGUACUUUUGUGUAGUACAA-3’; Lipofectamine 2000 kit was from Invitrogen (11,668–027, Carlsbad, CA, U.S.A.).

2.2. Cell culture

L02 cells (human normal hepatocyte) acquired from Zhong Qiao Xin Zhou Biotechnology Co., Ltd (ZQ0031, Shanghai, China) were cultured in RPMI 1640 (Gibco, Grand Island, NY, United States) supplemented with 10 % (v/v) FBS (BI, Israel) and 1 % antibiotics (100 μg/mL streptomycin/100 U/mL penicillin mix) (Gibco, Grand Island, NY, U.S.A) in a humidified 5 % CO_2 at 37 °C. When the L02 cell's growth density reached about 80 %, cells in good condition were taken for seeding.

2.3. CCK8 assay

Cell viability was detected using the CCK8 assay. Firstly, L02 cells (8×10^4/mL) were seeded in 96-well plates, then treated with TP (0, 30, 60, 90, 120, 150, 180 nM) for 24 h. The absorbance of each well was measured using a microplate reader (Thermo, United States) at 450 nm, and then the relative cell viability (%) was the ratio of the absorbance of the administration cells to that of the untreated cells.

2.4. Transfection for L02 cells

After L02 cells were seeded in 6-well plates (3×10^5/mL) and incubated to a confluence degree that reached 60 %, cells were transiently transfected with a mixture of siRNA-mate and miR-155 inhibitor (200 nM), inhibitor control (200 nM), or miR-155 mimic (50 nM), mimic control for 24 h according to the manufacturer instructions. The cell experimental groups were as follows: (1) inhibitor control, (2) miR-155 inhibitor, (3) inhibitor control + TP, (4) miR-155 inhibitor + TP, (5) mimic control, (6) miR-155 mimic, (7) mimic control + TP, and (8) miR-155 mimic + TP. After TP treatment (100 nM) for 24 h, we collected the cells for following RNA and protein extraction.

2.5. Animals treatments and induction of hepatotoxicity with TP

The animal experiment was reviewed and approved by the Department of the Laboratory Animal Management and Ethics Committee of Central South University (Number: 2018sydw0217). All operations were in compliance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of China, and all experimenters have passed the examination and obtained the animal experimental permissions.

Male BALB/C mice (20 ± 2 g) were purchased from Hunan SJA Laboratory Animal Co. Ltd. (Changsha, China). The mice were acclimatized for 5 days in a standard conditioned environment and had free access to food and water. Then, 32 mice were randomly divided into four groups (n = 8 per group) and were respectively given the following administration: (1) control, (2) TP (1.2 mg/kg), (3) antagomir control + TP (1.2 mg/kg), and (4) miR-155 antagomir + TP (1.2 mg/kg). The transfection mixture of miR-155 antagomir (or antagomir control) and

Lipofectamine 2000, prepared following the instructions on the Lipofectamine 2000 kit, were intravenously injected into mice through the tail vein 5 optical density (OD) per mouse a day for 3 consecutive days. 1 OD_{260} is about 33 μg RNA Oligo, 5 OD_{260} is 165 μg RNA Oligo. Following the manufacturer's instructions, we prepared the working solution by dissolving 40 OD miR-155 antagomir (or antagomir control) in 1 mL of DEPC H2O and then adding 1 mL of Lipofectamine 2000). Each 250 μL aliquot of working solution contained 165 μg of miR-155 antagomir (or antagomir control). 24 h after the final injection, mice were intraperitoneally injected with a single dosage of TP or normal saline. TP was dissolved in normal saline containing 0.1 % DMSO. Then all mice were euthanized on the fifth day, blood samples for biochemical analyses were collected to measure alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) on an automatic analyzer (7600, HITACHI Ltd., Tokyo, Japan). And liver samples were harvested, some of which were fixed with 4 % paraformaldehyde for histological analysis, others of which were frozen at −80 °C for further analysis.

2.6. Immunohistochemistry

Liver tissue slices were deparaffinized and rehydrated through xylene and graded alcohols. After treated with citrate buffer (PH 6.0, Servicebio, China) for antigen retrieval, we incubated the slides with 3 % H_2O_2 solution for 25 min. Then, the sections were incubated with diluted Nrf2 primary antibody (1:200) at 4 °C overnight, followed by incubation with biotinylated secondary antibody at 37 °C and then, coloration with 3,3-diaminobenzidine (DAB, Servicebio, China) at room temperature for 5–20 min. Finally, hematoxylin was used to counterstain nuclei of all sections. The expression of Nrf2 in the respective groups was scored by the ImageJ software. The score of tissue damage was stratified as follows: 1 (negative), 2 (low positive), 3 (positive), 4 (high positive).

2.7. Quantitative real-time PCR (qRT-PCR)

Total RNA was obtained with an RNAiso Plus reagent (9108, TaKaRa, Japan) according to the manufacturer's protocol. We synthesized cDNA with miRNA and mRNA using the PrimeScript™ RT reagent Kit (Perfect Real Time) (RR047A, TaKaRa, Japan), except for the difference that miRNA was reversed with specific stem-loop primers such as has-miR-155–5p RT-PCR primer (MQPS0000684-1), mmu-miR-155–5p RT-PCR primer (MQPS0002476-1), and U6 RT-PCR primer (MQPS0000002-1) (RiboBio, Co., Ltd., Changsha, China). The cDNAs were analyzed with LightCycler 96 (Roche Diagnostics, Germany) using the TB Green™ Premix DimerEraser™ (Perfect Real Time) (TaKaRa, Japan) and the qRT-PCR primers (TsingKe Biotech Co., Ltd., Changsha, China). The relative quantities were measured using the $2^{-\Delta\Delta Ct}$ method and were normalized to GAPDH for Nrf2, NQO1 and HO-1 mRNA and to U6 for miR-155 expression. The primers were as follows: human Nrf2 forward 5′-GGGGTAAGAATAAAGTGGCTGCTC-3′ and reverse 5′-ACA TTGCCATCTCTTGTTTGCTG-3'; human NQO1 forward 5′- AGTATCCTG CCGAGTCTGTTCTGG-3′ and reverse 5′-AATATCACAAGGTCTGCGGC TTCC-3'; human HO-1 forward 5′-CCTCCCTGTACCACATCTATGT-3′ and reverse 5′- GCTCTTCTGGGAAGTAGACAG-3'.

2.8. Western blot analysis

Liver tissue were homogenized with enhanced RIPA buffer (P0013B, Beyotime, Shanghai, China) to obtain total protein fractions. Nuclear and cytoplasmic proteins were extracted using nuclear and cytoplasmic extraction reagents (AR0106, Boster, Hubei, China) according to the manufacturer's instructions. After denaturation, the same amounts of protein (15–30 μg) were electrophoresed on 8–12 % SDS-PAGE and transferred to PVDF membranes. The membranes were incubated overnight at 4 °C with primary antibodies, including anti-Nrf2 (sc-722, Santa Cruz, 1:1000), anti–HO–1 (10701-1-AP, proteintech, 1:1000), anti-Histone H3 (AF0863, Affinity Bioscience, United States, 1:5000) and anti-β-actin (AF7018, Affinity Bioscience, United States, 1:5000). Subsequently, membranes were washed and incubated with HRP-conjugated secondary antibodies (BA1050, BA1054, Boster, Hubei, China). Enhanced chemiluminescence was visualized with an ECL kit (AR1170, Boster, Hubei, China) according to the manufacturer's protocol.

2.9. Statistical analysis

The data were expressed as mean±SD**.** Comparisons between three or more groups were evaluated with one-way ANOVA followed by Dunnett's *t*-test or LSD test. Statistical analyses were performed with GraphPad prism 8 software, and a value of $P \leq 0.05$ was considered significantly different.

3. Results

3.1. TP promotes miR-155 expression and inhibits Nrf2 and its downstream genes expressions in L02 cells

We first determined the cell viability of L02 cells treated by different concentrations of TP. The result showed that the toxicity of TP treatment alone for 24 h to L02 cells was concentration-dependent (Fig. 1A). Half maximal inhibitory concentration (IC_{50}) is approximately 100 nM, thus we used 50, 75, 100, 125 nM TP in subsequent experiments. As is shown in Fig. 1B, TP significantly increased the miR-155 expression in a dose-dependent manner. We then determined the protein and mRNA expressions of Nrf2. TP 100 nM and 125 nM dramatically decreased the total Nrf2 protein and Nrf2 mRNA expressions (Fig. 1C, G), the nuclear protein level of Nrf2 was down-regulated by TP treatment as well (Fig. 1E). Moreover, both the protein and mRNA expressions of HO-1, the downstream genes of Nrf2, were also decreased after treated with gradient concentrations of TP, and the NQO1 mRNA level was declined when treated with TP 125 nM (Fig. 1F, H, I). These results revealed that TP can promote the miR-155 levels and inhibit the Nrf2 and its downstream gene expressions in L02 cells.

3.2. Downregulation of Nrf2 caused by TP is miR-155 dependent in L02 cells

To further explore the interaction between miR-155 and Nrf2 pathway in TP-induced hepatic injury, we transfected L02 cells with miR-155 inhibitor and mimic before TP treatment. As is presented in Fig. 2A, compared with the inhibitor control group, the miR-155 level was significantly down-regulated after transfected with the miR-155 inhibitor, while the miR-155 level was upregulated in the inhibitor control + TP group. However, after transfected with miR-155 inhibitor, the miR-155 level was conspicuously decreased compared to the inhibitor control + TP group. Conversely, the miR-155 level was markedly increased after transfected with miR-155 mimic, and more upregulation was shown in the miR-155 mimic + TP group (Fig. 2B).

We then measured the total, cytoplasmic, and nuclear proteins expressions of Nrf2 using the immunoblotting assay. The results indicated that the inhibition of miR-155 reversed the down-regulation of the total, cytoplasmic, and nuclear Nrf2 protein levels induced by TP treatment (Fig. 3A–C), while the miR-155 mimic enhanced the effects of TP on Nrf2 (Fig. 3D–F). Unexpectedly, treatment with the miR-155 inhibitor or the miR-155 mimic in the absence of TP did not directly affect the expression of Nrf2. From Fig. 4A and B, the HO-1 protein and mRNA expressions were induced with the miR-155 inhibitor as compared with the inhibitor control, and the down-regulation of HO-1 treated by TP was also reversed by the miR-155 inhibitor. However, it seemed that there was no obvious effect on HO-1 when transfected with the miR-155 mimic (Fig. 4C and D). Based on these findings, we supposed that miR-

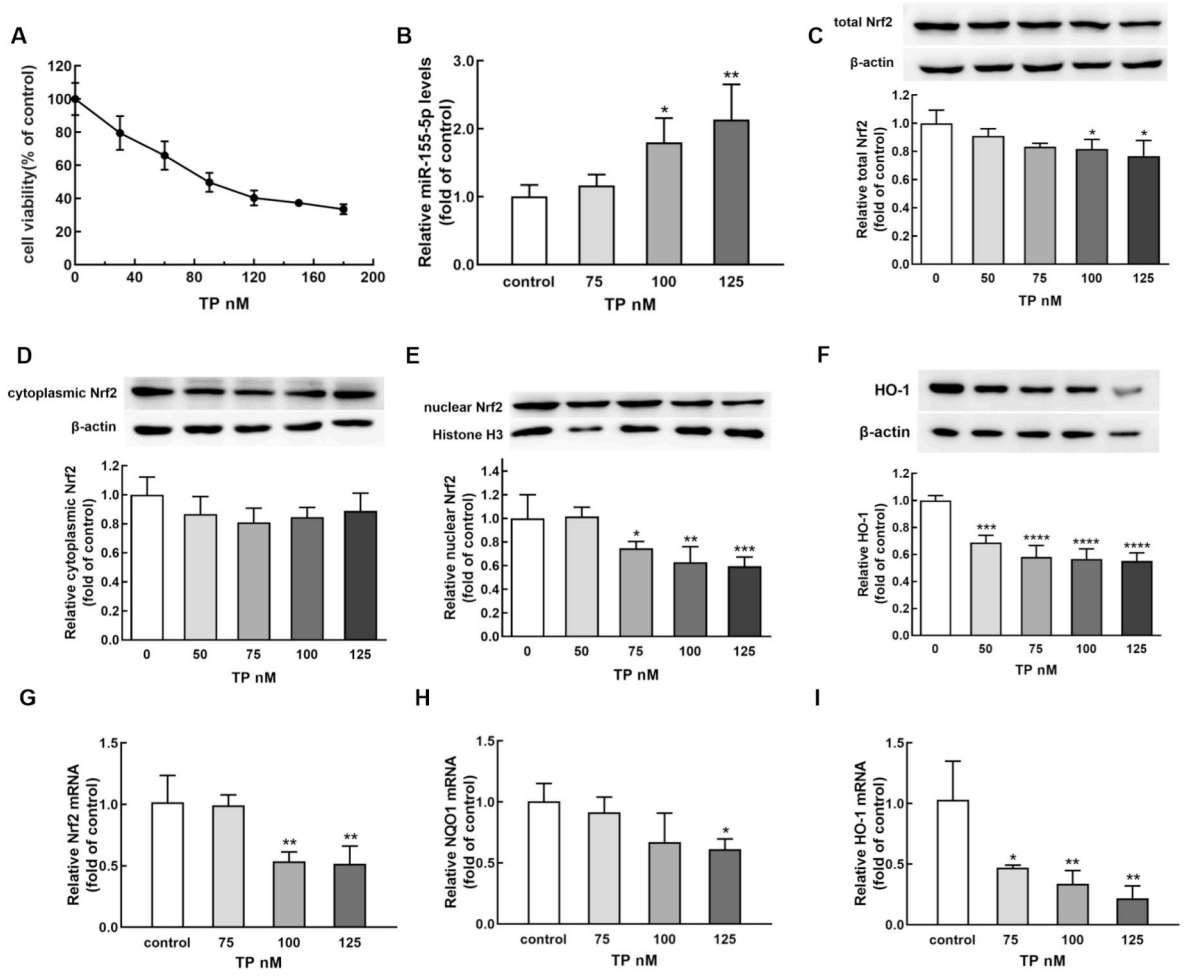

Fig. 1. Effects of TP on the expressions of miR-155 and the Nrf2 pathway in L02 cells. (**A**) Effects of TP on the viability of L02 cells (n = 6). **(B)** The mRNA level of miR-155 (n = 3). **(C, D, E and F)** The protein levels of total, cytoplasmic and nuclear Nrf2, and HO-1 determined by Western blot (n = 3). **(G, H and I)** The mRNA levels of Nrf2, NQO1 and HO-1 determined by qPCR (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ vs. the control group.

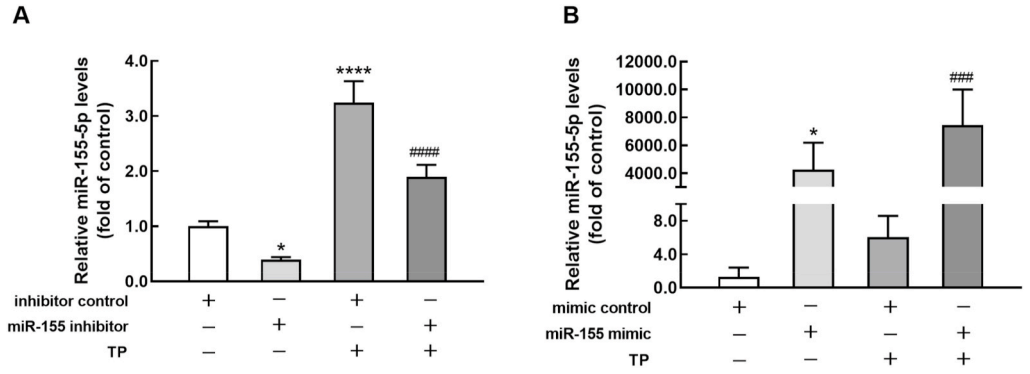

Fig. 2. Effects of TP on the expression of miR-155 after transfected with miR-155 inhibitor **(A)** and mimic **(B)** (n = 3). *P<0.05 and ****P<0.0001 vs. the inhibitor/mimic control group, $^{\#\#\#}$P< 0.001 and $^{\#\#\#\#}$P<0.0001 vs. the inhibitor/mimic control + TP group.

155 was possibly involved in the restraint of Nrf2 induced by TP.

3.3. miR-155 is involved in TP-induced acute hepatotoxicity in mice

Experiments *in vivo* were conducted to further validate *in vitro* experimental results. To evaluate the potentially vital role of miR-155 on hepatotoxicity induced by TP, first, some biochemical indicators were analyzed in all treated groups. Compared with the controls, the serum ALT and AST levels of the TP-treated mice were increased saliently. Inversely, the content of ALT, AST, and LDH in the miR-155 antagomir + TP group was decreased in comparison with the antagomir control + TP group (Fig. 5A–D). Histopathological analysis showed that TP alone treatment triggered abnormal ultra-structural changes in the liver tissue, including pale cytoplasm, hydropic degeneration, and inflammatory cellular infiltration accompanied by bleeding and necrosis. However, with the inhibition of miR-155 by miR-155 antagomir, the

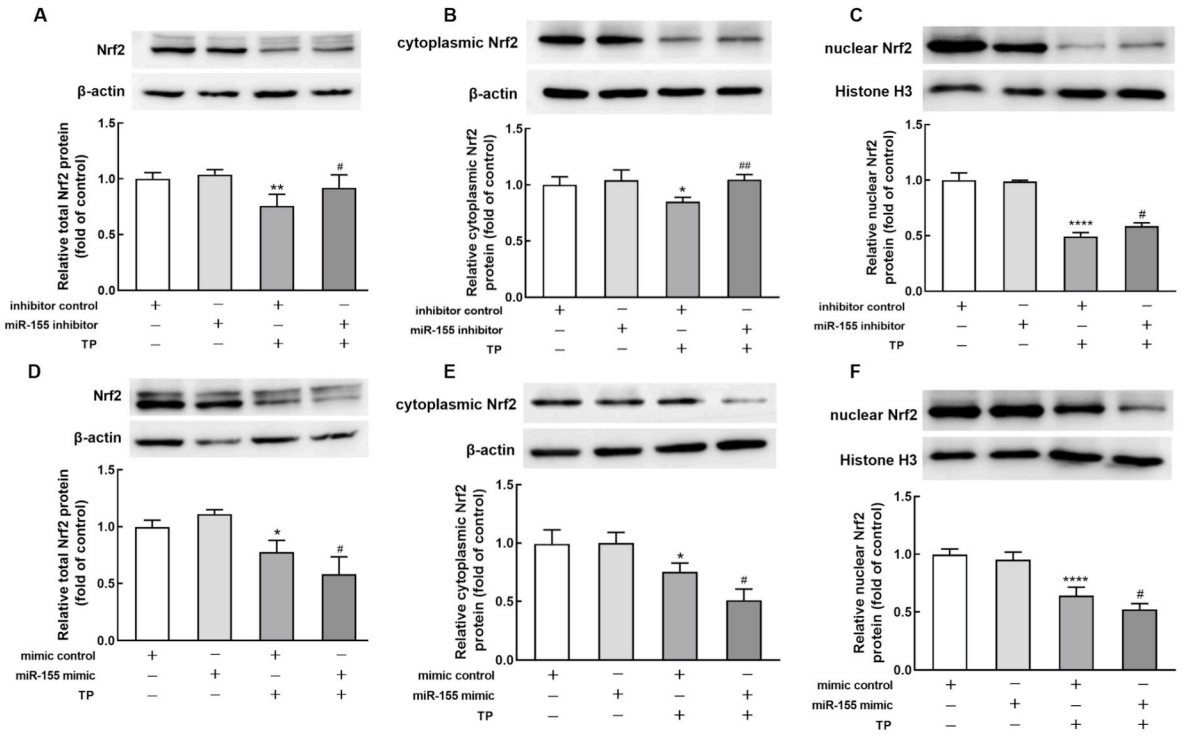

Fig. 3. Effects of TP on the protein expression of Nrf2 after transfected with miR-155 mimic and inhibitor ($n = 3$). The protein levels of total (**A and D**), cytoplasmic (**B and E**) and nuclear (**C and F**) Nrf2. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ vs. the inhibitor/mimic control group. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. the inhibitor/mimic control + TP group.

liver injuries induced by TP were alleviated (Fig. 5E).

3.4. MiR-155 mediates the effect of TP on Nrf2 expression in mice

Our result *in vivo* also showed that the miR-155 antagomir diminished the upregulation of miR-155 induced by TP (Fig. 6A). Subsequently, we determined the Nrf2 protein expression by using Western blot and immunohistochemistry, which showed that inhibition of miR-155 restored the expression of Nrf2 (Fig. 6B, D, E). And the downstream genes HO-1 expression after inhibiting the miR-155 was restored (Fig. 6C).

4. Discussion

MiRNAs regulate various biological processes, including cell development, proliferation, differentiation, apoptosis, as well as organ development and the maintenance of organ physiology (Bandiera et al., 2015). Aberrant expression of miRNAs was found in various diseases such as liver and kidney injuries, and inflammatory diseases, indicating the importance of miRNAs (Petejova et al., 2020; Sheedy et al., 2015; Wang et al., 2021). It has been reported that TP exerts its antineoplastic and anti-inflammatory activity through a miRNA-mediated mechanism (Feng et al., 2019; Liu et al., 2019). Nevertheless, the role of miRNAs in TP-induced liver damage has rarely been studied. Vliegenthart and colleagues (Vliegenthart et al., 2017) found that with the increasing severity of hepatic damage caused by TP, the number of miR-122 copies per zebrafish larva reduced. In fact, a large number of previous studies have shown that miRNAs are closely related to liver diseases, especially miR-155. Consequently, we explored the role of miR-155 in TP-triggered hepatotoxicity *in vivo* and *in vitro*.

Our results suggested that miR-155 was significantly upregulated in a concentration-dependent manner. The activity of hepatic L02 cells decreased with TP treatment, and the results of serum biochemistry and H&E staining showed that inhibition of miR-155 diminished TP-triggered liver injuries in mice, which was similar to the results that perfluorooctane sulfonate (PFOS) could upregulate miR-155 expression, thus inducing hepatotoxicity (Wan et al., 2016). Another study also revealed that knockout of miR-155 restrained Fas-caused hepatotoxicity (Chen et al., 2015). In contrast to these studies, lots of other studies concerning the anti-inflammatory effect of TP have suggested the miR-155 expression was down-regulated (Feng et al., 2019; Matta et al., 2009; Peng et al., 2014; Wu et al., 2013). MiR-155 has been reported to mediate inflammation by altering the expression of multiple target genes, but it remains controversial whether miR-155 is a pro-inflammatory factor or an anti-inflammatory factor. This may be due to different *in vivo* and *in vitro* models to investigate the role of miR-155 in diverse studies (Yuan et al., 2016). Therefore, the expression of miR-155 is different in different disease models. Another reason is believed to be related to the different dosages of TP. TP exerted anti-inflammatory effects in low doses, while TP induced hepatotoxicity in larger doses (Chen et al., 2018; Hou et al., 2018; Song et al., 2019; Yuan et al., 2019; Zhao et al., 2005).

Then, we found that TP decreased the protein and mRNA expressions of Nrf2 and its downstream genes HO-1 and NQO1, and the same results had been reported in our previous researches (Cao et al., 2016; Hou et al., 2018). MiRNAs are the potential upstream regulators of the Nrf2 pathway and have the ability to modulate the Nrf2 pathway at multiple stages (Cheng et al., 2013; Kabaria et al., 2015). The miRNAs may regulate the Nrf2 pathway by acting on kelch-like ECH-associated protein 1 (Keap1), by directly altering the expression of Nrf2, by affecting the nuclear translocation of Nrf2 and by regulating upstream mediators (Ashrafizadeh et al., 2020). Wagner and colleagues (Wagner et al., 2012) found that allyl-isothiocyanate (AITC) could restore the expression of miR-155 in lipopolysaccharide (LPS)-stimulated RAW264.7 cells, accompanied by the activation of the Nrf2 pathway. Sulforaphane (SFN) also alleviated LPS-induced upregulation of miR-155 levels and activated Nrf2 to inhibit inflammatory responses on microglia (Eren et al., 2018). However, these researches did not further clarify the potential interactions between miR-155 and the Nrf2 signaling pathway. Recently, some researches have demonstrated that miR-155 could

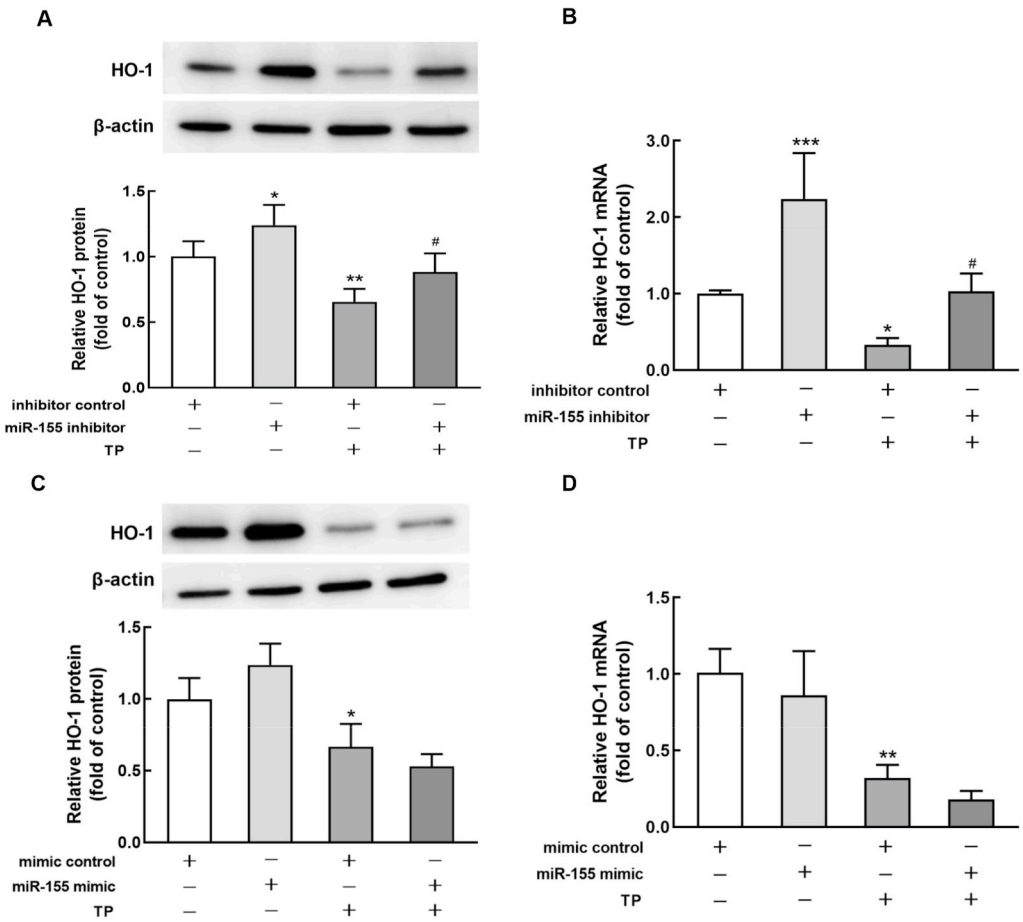

Fig. 4. Effects of TP on the expressions of HO-1 after transfected with miR-155 mimic and inhibitor (n = 3). **(A and C)** The protein level of HO-1. **(B and D)** The mRNA levels of HO-1. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. the inhibitor control or mimic control group, $^{\#}P < 0.05$ vs. the inhibitor control + TP group.

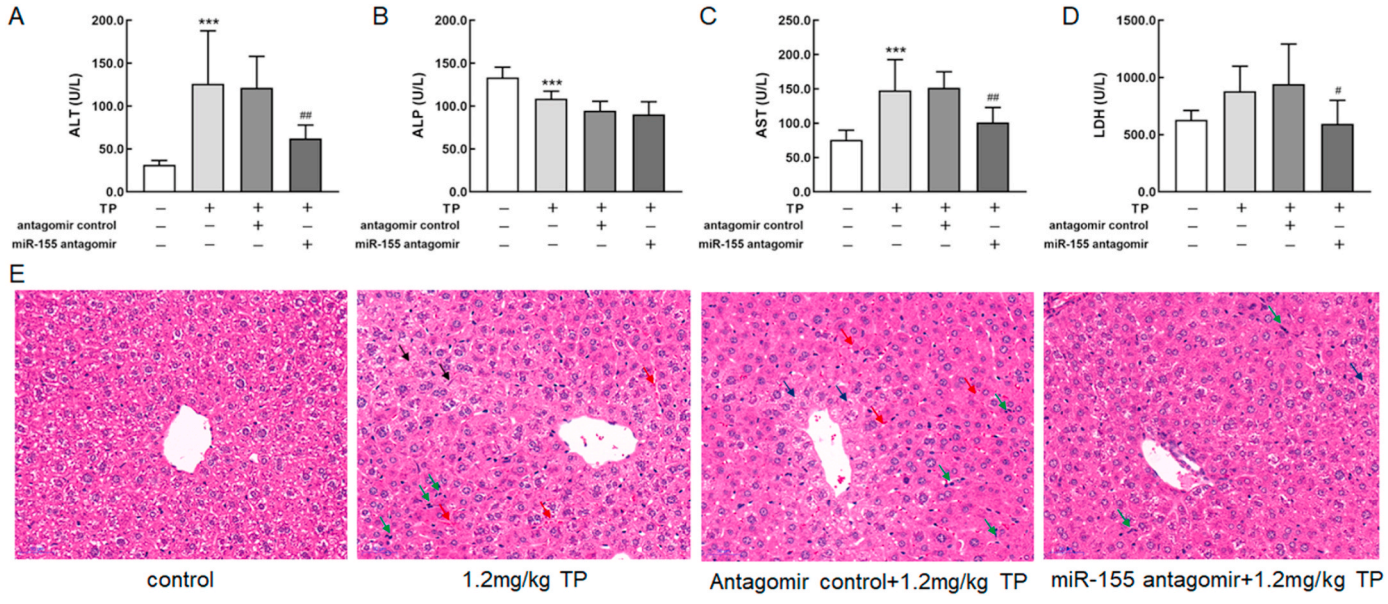

Fig. 5. Liver injuries in the all groups were detected by blood chemistry (n = 6–8) and H&E. **(A)** ALT, alanine aminotransferase; **(B)** ALP, aspartate aminotransferase; **(C)** AST, alkaline phosphatase; **(D)** LDH, lactate dehydrogenase. **(E)** Photomicrographs (20 ×) of H&E-stained liver sections. Green arrows indicate inflammatory cell infiltration, black arrows indicate pale cytoplasm, and red arrows indicate bleeding and necrosis. $^{***}P < 0.001$ vs. the control group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. the antagomir control+1.2 mg/kg TP group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

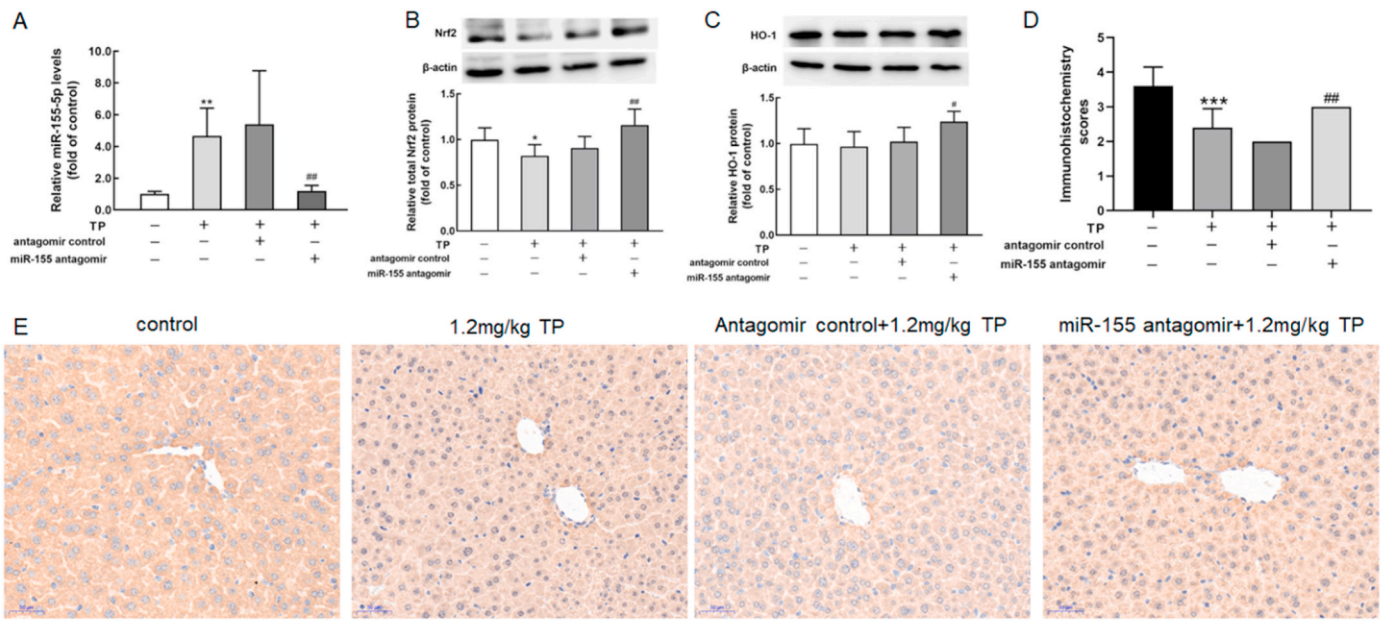

Fig. 6. Effects of different treatments on the mRNA level of miR-155. **(A)** and the protein levels of Nrf2 **(B)** and HO-1 **(C)** (n = 6). **(D)** Statistical results of immunohistochemistry of Nrf2 expression in each group. **(E)** Representative photomicrographs (20 ×) of immunohistochemistry of liver sections of Nrf2 expression obtained from the control, TP (1.2 mg/kg), antagomir control+1.2 mg/kg TP, and miR-155 antagomir+1.2 mg/kg TP groups. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. the control group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. the antagomir control+1.2 mg/kg TP group.

directly negatively regulate the expression of Nrf2 by utilizing the miR-155 inhibitor and miR-155 mimic, and by dual-luciferase reporter gene assay in some cell lines, such as human bronchial epithelial cells, rat Schwann cells and HepG2 cells (Chen et al., 2017, 2019; Wan et al., 2016). Based on those above, we further investigated the association between miR-155 and the Nrf2 signaling pathway in TP-induced hepatotoxicity. In this study, we observed the changes of Nrf2 and its downstream genes in TP-stimulated hepatotoxicity in L02 cells via inducing or inhibiting the expression of miR-155. Our results indicated that the inhibition of miR-155 after transfected with miR-155 inhibitor reversed the reduction of expressions of total, cytoplasmic and nuclear Nrf2 induced by TP, while the induction of miR-155 enhanced the effects of TP on Nrf2, indicating that miR-155 was involved in TP-induced liver damage via regulating Nrf2. But transfecting with miR-155 inhibitor or mimic without TP treatment in L02 cells showed no significant effect on Nrf2, which may be explained by direct and indirect targeting of miR-155 on Nrf2. For instance, it has been clarified that BTB and CNC homologous protein 1 (Bach1) is a target gene of miR-155, and changes in miR-155 expression may have no effect on Nrf2 expression (Pulkkinen et al., 2011). The miR-155 inhibitor also induced the protein and mRNA expressions of HO-1 and partially eliminated the decrease of HO-1 by TP, while the miR-155 mimic tended to enhance the down-regulation effect of TP on HO-1 though it has no statistical significance. Some studies found that Bach1 is an HO-1-specific transcriptional repressor (Li et al., 2017; Pulkkinen et al., 2011). Therefore, an increase of miR-155 might suppress the target gene Bach1 and promote the production of HO-1, and meanwhile, the down-regulation of Nrf2 can restrain the HO-1 expression, thus leading to the insignificant changes in HO-1. Besides, studies on mice indicated that inhibition of miR-155 reversed the down-regulation of Nrf2 and increased the HO-1 protein expression.

In short, our data found that TP induced miR-155 expression and inhibited the Nrf2 signaling pathway in hepatocytes and liver tissue. The inhibition of miR-155 could alleviate TP-induced liver damage in mice and partly restored the down-regulation of the Nrf2 signaling pathway. These findings revealed that miR-155 participated in TP-induced hepatotoxicity via regulating the Nrf2 signaling pathway.

CRediT authorship contribution statement

Yao Li: Formal analysis, Writing – original draft, Writing – review & editing, conceive and design animal experiments, extracted and analyzed the experimental data, drafted the manuscript, reviewed and revised the paper. **Lin Guo:** Formal analysis, Writing – original draft, Writing – review & editing, conceive and design animal experiments, conducted experiments, extracted and analyzed the experimental data, drafted the manuscript, reviewed and revised the paper. **Zhenyan Hou:** Formal analysis, conducted experiments, extracted and analyzed the experimental data. **Hui Gong:** Writing – original draft, Writing – review & editing, conducted experiments, drafted the manuscript, reviewed and revised the paper. **Miao Yan:** Formal analysis, Writing – original draft, conceive and design animal experiments, extracted and analyzed the experimental data, drafted the manuscript. **Bikui Zhang:** Formal analysis, Writing – review & editing, conceive and design animal experiments, extracted and analyzed the experimental data, reviewed and revised the paper.

Declaration of competing interest

The authors declare no conflicts of interests.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grant No. 81974532, No. 81903904 and No. 81803830) and the Natural Science Foundation of Hunan Province, China (Grant No. 2020JJ4130).

References

Al-Hrout, A., Chaiboonchoe, A., Khraiwesh, B., Murali, C., Baig, B., El-Awady, R., Tarazi, H., Alzahmi, A., Nelson, D.R., Greish, Y.E., Ramadan, W., Salehi-Ashtiani, K., Amin, A., 2018. Safranal induces DNA double-strand breakage and ER-stress-mediated cell death in hepatocellular carcinoma cells. Sci. Rep. 8 (16951) https://doi.org/10.1038/s41598-018-34855-0.

Ashktorab, H., Soleimani, A., Singh, G., Amin, A., Tabtabaei, S., Latella, G., Stein, U., Akhondzadeh, S., Solanki, N., Gondré-Lewis, M.C., Habtezion, A., Brim, H., 2019.

Saffron: the golden spice with therapeutic properties on digestive diseases. Nutrients 11 (943). https://doi.org/10.3390/nu11050943.
Ashrafizadeh, M., Ahmadi, Z., Samarghandian, S., Mohammadinejad, R., Yaribeygi, H., Sathyapalan, T., Sahebkar, A., 2020. MicroRNA-mediated regulation of Nrf2 signaling pathway: implications in disease therapy and protection against oxidative stress. Life Sci. 244 (117329) https://doi.org/10.1016/j.lfs.2020.117329.
Bala, S., Csak, T., Saha, B., Zatsiorsky, J., Kodys, K., Catalano, D., Satishchandran, A., Szabo, G., 2016. The pro-inflammatory effects of miR-155 promote liver fibrosis and alcohol-induced steatohepatitis. J. Hepatol 64, 1378–1387. https://doi.org/10.1016/j.jhep.2016.01.035.
Bandiera, S., Pfeffer, S., Baumert, T.F., Zeisel, M.B., 2015. miR-122–a key factor and therapeutic target in liver disease. J. Hepatol 62, 448–457. https://doi.org/10.1016/j.jhep.2014.10.004.
Cao, L.J., Li, H.D., Yan, M., Li, Z.H., Gong, H., Jiang, P., Deng, Y., Fang, P.F., Zhang, B.K., 2016. The protective effects of isoliquiritigenin and glycyrrhetinic acid against triptolide-induced oxidative stress in HepG2 cells involve Nrf2 activation. Evid. Based complement alternat. Med 2016 (8912184). https://doi.org/10.1155/2016/8912184.
Chen, C.Z., Jiang, X.J., Gu, S.Y., Zhang, Z.Z., 2017. MicroRNA-155 regulates arsenite-induced malignant transformation by targeting Nrf2-mediated oxidative damage in human bronchial epithelial cells. Toxicol. Lett. 278, 38–47. https://doi.org/10.1016/j.toxlet.2017.07.215.
Chen, J., Li, C., Liu, W.J., Yan, B., Hu, X.L., Yang, F.R., 2019. microRNA-155 silencing reduces sciatic nerve injury in diabetic peripheral neuropathy. J. Mol. Endocrinol. 63, 227–238. https://doi.org/10.1530/jme-19-0067.
Chen, L.Y., Ming, X.Y., Li, W.S., Bi, M.R., Yan, B.Z., Wang, X.R., Yang, P.F., Yang, B.S., 2020. The microRNA-155 mediates hepatitis B virus replication by reinforcing SOCS1 signalling-induced autophagy. Cell Biochem. Funct 38, 436–442. https://doi.org/10.1002/cbf.3488.
Chen, S.R., Dai, Y., Zhao, J., Lin, L., Wang, Y., Wang, Y., 2018. A mechanistic overview of triptolide and celastrol, natural products from *Tripterygium wilfordii* Hook F. Front. Pharmacol 9 (104). https://doi.org/10.3389/fphar.2018.00104.
Chen, W.N., Han, C., Zhang, J.Q., Song, K., Wang, Y., Wu, T., 2015. Deletion of Mir155 prevents fas-induced liver injury through up-regulation of mcl-1. Am. J. Pathol. 185, 1033–1044. https://doi.org/10.1016/j.ajpath.2014.12.020.
Cheng, X.H., Ku, C.H., Siow, R.C., 2013. Regulation of the Nrf2 antioxidant pathway by microRNAs: new players in micromanaging redox homeostasis. Free Radic. Biol. Med. 64, 4–11. https://doi.org/10.1016/j.freeradbiomed.2013.07.025.
Cho, Y.E., Kim, S.H., Lee, B.H., Baek, M.C., 2017. Circulating plasma and exosomal microRNAs as indicators of drug-induced organ injury in rodent models. Biomol. Ther (Seoul) 25, 367–373. https://doi.org/10.4062/biomolther.2016.174.
Eren, E., Tufekci, K.U., Isci, K.B., Tastan, B., Genc, K., Genc, S., 2018. Sulforaphane inhibits lipopolysaccharide-induced inflammation, cytotoxicity, oxidative stress, and miR-155 expression and switches to mox phenotype through activating extracellular signal-regulated kinase 1/2-nuclear factor erythroid 2-related factor 2/antioxidant response element pathway in murine microglial cells. Front. Immunol. 9 (36) https://doi.org/10.3389/fimmu.2018.00036.
Feng, Y., Zheng, C.Y., Zhang, Y.J., Xing, C.Y., Cai, W.B., Li, R.R., Chen, J.Z., Duan, Y.Y., 2019. Triptolide inhibits preformed fibril-induced microglial activation by targeting the MicroRNA155-5p/SHIP1 pathway. Oxid. Med. Cell Longev 2019 (6527638). https://doi.org/10.1155/2019/6527638.
Gu, S.Y., Lai, Y.H., Chen, H.Y., Liu, Y., Zhang, Z.Z., 2017. miR-155 mediates arsenic trioxide resistance by activating Nrf2 and suppressing apoptosis in lung cancer cells. Sci. Rep. 7 (12155) https://doi.org/10.1038/s41598-017-06061-x.
Hasnat, M., Yuan, Z.Q., Ullah, A., Naveed, M., Raza, F., Baig, M.M., Khan, A., Xu, D.Q., Su, Y.W., Sun, L.X., Zhang, L.Y., Jiang, Z.Z., 2020. Mitochondria-dependent apoptosis in triptolide-induced hepatotoxicity is associated with the Drp1 activation. Toxicol. Mech. Methods 30, 124–133. https://doi.org/10.1080/15376516.2019.1669247.
Hou, Z.Y., Chen, L., Fang, P.F., Cai, H.L., Tang, H.B., Peng, Y.B., Deng, Y., Cao, L.J., Li, H. D., Zhang, B.K., Yan, M., 2018. Mechanisms of triptolide-induced hepatotoxicity and protective effect of combined use of isoliquiritigenin: possible roles of Nrf2 and hepatic transporters. Front. Pharmacol 9 (226). https://doi.org/10.3389/fphar.2018.00226.
Kabaria, S., Choi, D.C., Chaudhuri, A.D., Jain, M.R., Li, H., Junn, E., 2015. MicroRNA-7 activates Nrf2 pathway by targeting Keap1 expression. Free Radic. Biol. Med. 89, 548–556. https://doi.org/10.1016/j.freeradbiomed.2015.09.010.
Klaassen, C.D., Reisman, S.A., 2010. Nrf2 the rescue: effects of the antioxidative/electrophilic response on the liver. Toxicol. Appl. Pharmacol. 244, 57–65. https://doi.org/10.1016/j.taap.2010.01.013.
Li, J., Shen, F.H., Guan, C.W., Wang, W.W., Sun, X.Z., Fu, X.L., Huang, M., Jin, J., Huang, Z.Y., 2014. Activation of Nrf2 protects against triptolide-induced hepatotoxicity. PloS One 9, e100685. https://doi.org/10.1371/journal.pone.0100685.
Li, X.J., Jiang, Z.Z., Zhang, L.Y., 2014. Triptolide: progress on research in pharmacodynamics and toxicology. J. Ethnopharmacol. 155, 67–79. https://doi.org/10.1016/j.jep.2014.06.006.
Li, X.X., Du, F.Y., Liu, H.X., Ji, J.B., Xing, J., 2015. Investigation of the active components in Tripterygium wilfordii leading to its acute hepatotoxicty and nephrotoxicity. J. Ethnopharmacol. 162, 238–243. https://doi.org/10.1016/j.jep.2015.01.004.
Li, Z.Z., Wang, Y., Zeng, G., Zheng, X.W., Wang, W.B., Ling, Y., Tang, H.M., Zhang, J.F., 2017. Increased miR-155 and heme oxygenase-1 expression is involved in the protective effects of formononetin in traumatic brain injury in rats. Am. J. Transl. Res. 9, 5653–5661.
Liu, Q., Wang, W., Li, F.Q., Yu, D.Y., Xu, C.F., Hu, H.B., 2019. Triptolide inhibits breast cancer cell metastasis through inducing the expression of miR-146a, a negative regulator of rho GTPase. Oncol. Res. 27, 1043–1050. https://doi.org/10.3727/096504019x15560124931900.
Lu, Y., Xie, T., Zhang, Y.J., Zhou, F.Q., Ruan, J., Zhu, W.N., Zhu, H.X., Feng, Z., Zhou, X. P., 2017. Triptolide induces hepatotoxicity via inhibition of CYP450s in rat liver microsomes. BMC complement altern. Med 17 (15). https://doi.org/10.1186/s12906-016-1504-3.
Matta, R.Y., Wang, X.X., Ge, H., Ray, W., Nelin, L.D., Liu, Y.S., 2009. Triptolide induces anti-inflammatory cellular responses. Am. J. Transl. Res. 1, 267–282.
Petejova, N., Martinek, A., Zadrazil, J., Kanova, M., Klementa, V., Sigutova, R., Kacirova, I., Hrabovsky, V., Svagera, Z., Stejskal, D., 2020. Acute Kidney Injury in Septic Patients Treated by Selected Nephrotoxic Antibiotic Agents-Pathophysiology and Biomarkers-A Review 21 (7115). https://doi.org/10.3390/ijms21197115.
Peng, A.P., Huang, X.Z., Liu, R.P., Wang, X.Y., Zhuang, J.H., 2014. [Triptolide inhibits the inflammatory response of monocytes from rheumatoid arthritis patients by regulating miR-155]. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 30, 635–638.
Pulkkinen, K.H., Yla-Herttuala, S., Levonen, A.L., 2011. Heme oxygenase 1 is induced by miR-155 via reduced BACH1 translation in endothelial cells. Free Radic. Biol. Med. 51, 2124–2131. https://doi.org/10.1016/j.freeradbiomed.2011.09.014.
Schueller, F., Roy, S., Vucur, M., Trautwein, C., Luedde, T., Roderburg, C., 2018. The role of miRNAs in the pathophysiology of liver diseases and toxicity. Int. J. Mol. Sci. 19 (261) https://doi.org/10.3390/ijms19010261.
Sheedy, F.J., 2015. Turning 21: induction of miR-21 as a key switch in the inflammatory response. Front. Immunol. 6 (19) https://doi.org/10.3389/fimmu.2015.00019.
Song, W., Liu, M.L., Wu, J.J., Zhai, H., Chen, Y., Peng, Z.H., 2019. Preclinical pharmacokinetics of triptolide: a potential antitumor drug. Curr. Drug Metab 20, 147–154. https://doi.org/10.2174/1389200219666180816141506.
Torres, J.L., Novo-Veleiro, I., Manzanedo, L., Alvela-Suarez, L., Macias, R., Laso, F.J., Marcos, M., 2018. Role of microRNAs in alcohol-induced liver disorders and non-alcoholic fatty liver disease. World J. Gastroenterol. 24, 4104–4118.
Vliegenthart, A.D.B., Wei, C.M., Buckley, C., Berends, C., de Potter, C.M.J., Schneemann, S., Del, P.J., Tucker, C., Mullins, J.J., Webb, D.J., Dear, J.W., 2017. Characterization of triptolide-induced hepatotoxicity by imaging and transcriptomics in a novel zebrafish model. Toxicol. Sci. 159, 380–391. https://doi.org/10.1093/toxsci/kfx144.
Wagner, A.E., Boesch-Saadatmandi, C., Dose, J., Schultheiss, G., Rimbach, G., 2012. Anti-inflammatory potential of allyl-isothiocyanate–role of Nrf2, NF-(kappa) B and microRNA-155. J. Cell Mol. Med. 16, 836–843. https://doi.org/10.1111/j.1582-4934.2011.01367.x.
Wan, C., Han, R., Liu, L.M., Zhang, F., Li, F., Xiang, M.D., Ding, W.J., 2016. Role of miR-155 in fluorooctane sulfonate-induced oxidative hepatic damage via the Nrf2-dependent pathway. Toxicol. Appl. Pharmacol. 295, 85–93. https://doi.org/10.1016/j.taap.2016.01.023.
Wang, B., Majumder, S., Nuovo, G., Kutay, H., Volinia, S., Patel, T., Schmittgen, T.D., Croce, C., Ghoshal, K., Jacob, S.T., 2009. Role of microRNA-155 at early stages of hepatocarcinogenesis induced by choline-deficient and amino acid-defined diet in C57BL/6 mice. Hepatology 50, 1152–1161. https://doi.org/10.1002/hep.23100.
Wang, W.W., Bian, H.J., Li, F.F., Li, X., Zhang, D., Sun, S.H., Song, S.Y., Zhu, Q., Ren, W. H., Qin, C.Y., Qi, J.N., 2018. HBeAg induces the expression of macrophage miR-155 to accelerate liver injury via promoting production of inflammatory cytokines. Cell. Mol. Life Sci. 75, 2627–2641. https://doi.org/10.1007/s00018-018-2753-8.
Wang, X.L., He, Y., Mackowiak, B., Gao, B., 2021. MicroRNAs as regulators, biomarkers and therapeutic targets in liver diseases. Gut 70, 784–795. https://doi.org/10.1136/gutjnl-2020-322526.
Wang, X.Z., Sun, L.X., Zhang, L.Y., Jiang, Z.Z., 2016. Effect of adoptive transfer or depletion of regulatory T cells on triptolide-induced liver injury. Front. Pharmacol 7 (99). https://doi.org/10.3389/fphar.2016.00099.
Wu, R., Li, Y., Guo, Z., Gong, J.F., Zhu, W.M., Li, N., Li, J.S., 2013. Triptolide ameliorates ileocolonic anastomosis inflammation in IL-10 deficient mice by mechanism involving suppression of miR-155/SHIP-1 signaling pathway. Mol. Immunol. 56, 340–346. https://doi.org/10.1016/j.molimm.2013.05.006.
Xi, C., Peng, S.J., Wu, Z.P., Zhou, Q.P., Zhou, J., 2017. Toxicity of triptolide and the molecular mechanisms involved. Biomed. Pharmacother. 90, 531–541. https://doi.org/10.1016/j.biopha.2017.04.003.
Yuan, K., Li, X., Lu, Q., Zhu, Q., Jiang, H., Wang, T., Huang, G., Xu, A., 2019. Application and mechanisms of triptolide in the treatment of inflammatory diseases-A review. Front. Pharmacol 10 (1469). https://doi.org/10.3389/fphar.2019.01469.
Yuan, K., Zhang, X., Lv, L., Zhang, J., Liang, W., Wang, P., 2016. Fine-tuning the expression of microRNA-155 controls acetaminophen-induced liver inflammation. Int. Immunopharm. 40, 339–346. https://doi.org/10.1016/j.intimp.2016.09.011.
Zhao, Y.F., Zhai, W.L., Zhang, S.J., Chen, X.P., 2005. Protection effect of triptolide to liver injury in rats with severe acute pancreatitis. Hepatobiliary Pancreat Dis. Bar Int. 4, 604–608.