Is Aryl Hydrocarbon Receptor Antagonism After Ischemia Effective in Alleviating Hepatic Ischemia-Reperfusion Injury in Rats?

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Abstract

Aryl hydrocarbon receptors (AhRs) have been reported to be important mediators of ischemic injury in the brain. Furthermore, the pharmacological inhibition of AhR activation after ischemia has been shown to attenuate cerebral ischemia-reperfusion (IR) injury. Here, we investigated whether AhR antagonist administration after ischemia was also effective in ameliorating hepatic IR injury. A 70% partial hepatic IR (45-minute ischemia and 24-hour reperfusion) injury was induced in rats. We administered 6,2',4'-trimethoxyflavone (TMF, 5 mg/kg) intraperitoneally 10 minutes after ischemia. Hepatic IR injury was observed using serum, magnetic resonance imaging-based liver function indices, and liver samples. TMF-treated rats showed significantly lower relative enhancement (RE) values and serum alanine aminotransferase (ALT) and aspartate aminotransferase levels than did untreated rats at three hours after reperfusion. After 24 hours of reperfusion, TMF-treated rats had significantly lower RE values, $\Delta T1$ values, serum ALT levels, and necrotic area percentage than did untreated rats. The expression of the apoptosis-related proteins, Bax and cleaved caspase-3, was significantly lower in TMF-treated rats than in untreated rats. This study demonstrated that inhibition of AhR activation after ischemia was effective in ameliorating IR-induced liver injury in rats.

Introduction

Hepatic ischemia-reperfusion (IR) injury commonly occurs during liver transplantation or resection and is considered a leading cause of liver damage and dysfunction.^{1–5} The mechanisms of hepatic IR injury have been extensively investigated, but nevertheless remain largely unclear. Furthermore, although anaerobic metabolism, mitochondria, oxidative stress, intracellular calcium overload, liver Kupffer cells (KC), neutrophils, cytokines, and chemokines have been found to be involved in the hepatic IR injury process, effective prevention or treatment in clinical practice is still lacking.⁶

The aryl hydrocarbon receptor (AhR) is a ligand-activated basic helix-loop-helix/Per-ARNT-Sim transcription factor known to mediate toxic and carcinogenic effects of xenobiotics.^{7,8} Upon ligand bindings, AhR translocates to the nucleus to form an active heterodimeric complex and induces rapid transcriptional activations in various organs and cellular systems.⁹ Recent studies have suggested that cerebral ischemia induces AhR activation and exacerbates neuronal damage.^{10,11} This is because L-kynurenine (L-Kyn), an endogenous ligand of AhR, is accumulated in the brain during ischemia and triggers the activation of AhR.¹⁰ Moreover, the pharmacological manipulation of AhR activation after ischemia has been shown to modulate neuronal damage due to cerebral IR *in vivo*.¹² Observations in the brain raise the possibility that similar pathways can be involved in the liver. Because L-Kyn is produced in a significant amount in the liver through the degradation of L-tryptophan by tryptophan-2,3-dioxygenase (TDO),¹³ it is more likely to accumulate in the liver than in the brain due to ischemia. This means that ischemia-induced AhR activation and tissue damage after reperfusion could be greater in the liver than in the brain. Therefore, the inhibition of AhR activation after ischemia is considered to be effective in

suppressing hepatic IR injury. However, to the best of our knowledge, the effects of AhR antagonism on hepatic IR injury have not yet been reported.

In the present study, we investigated the protective effects of AhR antagonism after ischemia in a rat hepatic IR injury model. Moreover, we used *in vivo* magnetic resonance imaging (MRI) and molecular biological techniques to evaluate whether the administration of an AhR antagonist influences the hepatoprotective effects of AhR antagonism.

Results

Animal modelling

Visual changes in the color of the median and left lobes of the liver were observed in all rats with induced IR, and no rats died during surgery and the follow-up period.

MRI-based liver function indices

The signal intensities (SIs) of the liver in T1-weighted gradient echo images (WIs) obtained at 3 and 24 hours after reperfusion were higher in all three groups at 20 minutes after the administration than those before the administration of Gd-EOB-DTPA (Fig. 1A). Conversely, the T1 values of the liver in T1 maps were lower in all three groups 20 minutes after the administration than those before the administration of Gd-EOB-DTPA. The relative enhancement (RE) values at 24 hours after reperfusion were significantly higher in the control group than in the sham group ($P < 0.01$, Fig. 1B). The RE values at 3 and 24 hours after reperfusion were significantly lower in the 6,2',4'-trimethoxyflavone (TMF) group than in the control group ($P < 0.05$ and $P < 0.001$, respectively). The $\Delta T1$ values at three hours after reperfusion were significantly higher in the TMF group than in the sham group. The $\Delta T1$ values at 24 hours after reperfusion were significantly higher in the control group than in the sham group ($P < 0.001$). The $\Delta T1$ values at 24 hours after reperfusion were significantly lower in the TMF group than in the control group ($P < 0.01$). However, there were no significant differences in the liver-to-muscle ratio (LMR) and $T1_{\text{post}}$ values among the sham, control, and TMF groups ($P > 0.05$).

Changes in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels

Compared with the sham group, the control and TMF groups had significantly higher serum ALT and AST levels at 3 and 24 hours after reperfusion ($P < 0.05$, Fig. 2A, B). The serum ALT levels were significantly lower in the TMF group than in the control group at 3 ($P < 0.001$) and 24 ($P < 0.01$) hours after reperfusion. The serum AST levels were significantly lower in the TMF group than in the control group at 3 hours after reperfusion ($P < 0.001$), but no significant difference was observed between the control and TMF groups at 24 hours after reperfusion ($P = 0.422$).

Reduction of necrotic area by TMF treatment

Extensive hepatocellular necrosis was observed in the control and TMF groups at 24 hours after reperfusion (Fig. 2C). In contrast, no distinct areas of necrosis could be identified in the sham group. The necrotic area percentage in the TMF group was significantly lower than that in the control group (Fig. 2D).

Alleviation of apoptosis with TMF treatment

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive cells (round brown nuclei) were mainly observed in the control and TMF groups but rarely in the sham group (Fig. 3A). To quantify the expression of apoptosis-associated proteins, we measured Bcl-2, Bax, and cleaved caspase-3 in the liver tissues (Fig. 3B and Supplementary Figure 1). The expression of BCL2 protein was significantly lower in the control and TMF groups than in the sham group ($P < 0.001$), but there was no significant difference between the control and TMF groups ($P = 0.979$). The expression of Bax protein in the control group was significantly higher than in the sham ($P < 0.01$) and TMF ($P < 0.05$) groups. The expression of C-cas3 protein in the control group was also significantly higher than in the sham ($P < 0.01$) and TMF ($P < 0.01$) groups.

Suppression of AhR expression by TMF administration

AhR expression was mainly observed in the control and TMF groups but rarely in the sham group (Fig. 4A). AhR proteins in the liver tissues were significantly higher in the control and TMF groups than in the sham group (Fig. 4B and Supplementary Figure 2, $P < 0.01$). The TMF group showed a significant decrease in AhR proteins than did the control group ($P < 0.05$).

Discussion

In this study, we assessed the hepatoprotective effects of AhR antagonism on hepatic IR injury in rats. Rats treated with TMF at 10 minutes after ischemia had lower RE values and serum ALT and AST levels than did untreated rats at three hours after reperfusion. After 24 hours of reperfusion, TMF-treated rats had lower RE values, $\Delta T1$ values, serum ALT levels, and necrotic area percentage than did untreated rats. In addition, the expression of the apoptosis-related proteins Bax and cleaved caspase-3 was lower in the TMF-treated rats than in the untreated rats. These results suggest that inhibiting AhR activation after ischemia ameliorates hepatic IR injury.

Under normal conditions, AhR remains inactive in the cytoplasm and is activated by endogenous ligands, such as L-Kyn¹⁰, or exogenous ligands, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin.¹⁴ Recently, L-Kyn was found to accumulate in the brain during ischemia and induce the activation of AhR, exacerbating neuronal damage even after reperfusion.¹⁰ Because a significant amount of L-Kyn is produced by tryptophan metabolism under TDO, brain-like phenomena due to ischemia are likely to occur in the liver as well.¹³ However, to the best of our knowledge, there have yet to be studies on this. In this study, we investigated, for the first time, whether AhR antagonism after ischemia affects liver damage due to IR. Our results showed that AhR antagonism inhibited the progression of apoptosis and necrosis. This suggests that L-Kyn may accumulate owing to ischemia in the liver, as in the brain, and activate AhR,

possibly leading to hepatic IR injury. However, in the present study, the degree of L-Kyn accumulation and the inhibition of AhR activation by the regulation of L-Kyn accumulation were not studied. Due to the limited results of this study, additional research is needed in the future.

The present study showed the inhibitory effect of apoptosis in animals administrated with AhR antagonists after ischemia. However, it has not been elucidated which pathways downstream of AhR mediate hepatic IR injury. In the brain, several studies have reported that AhR inhibition reduces neuronal cell death and neurotoxicity.^{15,16} In particular, cAMP response element binding protein (CREB) signaling has been reported as one of the downstream pathways caused by AhR activation.¹⁰ The report suggests that AhR activation due to brain ischemia regresses CREB protein-dependent signaling and exacerbates brain damage. However, it is considered that AhR likely works with various sub-mechanisms. Therefore, further studies of sub-mechanisms, including alterations of CREB signaling induced by AhR activation in the liver, are needed.

Changes in serum ALT and AST levels in our study were used to evaluate changes in hepatic injury caused by IR. The serum ALT and AST levels are considered the representative markers of hepatocellular injury or necrosis.^{17,18} Hepatocellular injury or necrosis causes ALT and AST leakage from hepatocytes into the blood, and elevated ALT and AST in serum suggest liver damage.¹⁹ In this study, all rats with IR had higher serum ALT and AST levels at 3 and 24 hours after reperfusion than did the sham-operated rats. On the other hand, the TMF-treated rats had lower serum ALT and AST levels at 3 and 24 hours after reperfusion than did the untreated rats. These results indicate that TMF administration after ischemia contributes to the alleviation of IR-induced hepatocellular injury or necrosis. Furthermore, histological analysis also revealed a similar pattern of differences in the necrotic area. However, the mechanisms involved in the association between AhR activation and necrosis in this study have not been elucidated. Further research on this is needed.

We analyzed changes in liver function due to IR using Gd-EOB-DTPA, a paramagnetic hepatobiliary MR contrast agent. This was based on several reports that Gd-EOB-DTPA-enhanced MRI is suitable for evaluating liver function due to its organic anion transporting polypeptide (OATP1B1/B3)-dependent hepatocyte-specific uptake and paramagnetic properties.^{20,21} In particular, several reports have been demonstrated the effectiveness of liver function assessment using the SI-based indices of T1-WIs and the T1 relaxometry.^{22,23} In this study, as a result of analyzing RE and LMR values reported to be highly correlated with liver function among SI-based indices,²⁴ RE values were decreased in TMF-treated rats at 3 and 24 hours after reperfusion compared to those of untreated rats. These results suggested that liver function was improved in the TMF-treated group. Based on T1 relaxometry, we analyzed $T1_{\text{post}}$ and $\Delta T1$ values.^{22,24} At 24 hours after reperfusion, the TMF-treated rats had decreased $\Delta T1$ values compared to those of untreated rats. These results indicated that liver function was improved in the TMF-treated rats.

There were two limitations in this study. First, we only tested a single dose of TMF in our analysis (5 mg/kg of TMF) that had been suggested previously by Cuartero et al. and Kwon et al. .^{10,12} The

concentration of TMF in the blood may be different depending on the concentration of TMF administered; thus, the effect on hepatic IR injury may also vary. Further studies are needed to find the optimal concentration to reduce hepatic IR injury. Second, we only observed that AhR expression at 24 hours after reperfusion was significantly higher in the rats with hepatic IR injury than in the sham-operated and TMF-treated rats. This means that an increased expression of AhR may be one of the factors that play an important role in the progression of hepatic IR injury. However, in this study, the change in AhR expression was observed only 24 hours after reperfusion, and it was not determined how the change in AhR expression changed or how long it lasted after reperfusion. Additional studies are needed to find the optimal timing of TMF administration by tracking changes in AhR expression after reperfusion.

Our findings demonstrated that post-ischemia administration of AhR antagonists has hepatoprotective effects that ameliorate hepatic IR injury. We propose that adequate AhR antagonist activity is a potential therapeutic approach for hepatic I/R injury. Nevertheless, further studies are needed to elucidate the mechanisms underlying the hepatoprotective effect to assess potential clinical applications of AhR antagonist administration.

Methods

Ethics statements

The present study was performed following approval by the Institutional Animal Care and Use Committee of Asan Medical Center (IACUC Number: 2019-02-035), and all included procedures were performed in strict accordance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care. All involved experiments are reported in accordance with the ARRIVE guidelines (Animal Research: Reporting in Vivo Experiments) for how to REPORT animal experiments. All animal surgeries were performed under isoflurane anesthesia and were closely monitored by trained individuals capable of assessing the animals' pain-related behaviors. Euthanasia was planned if the animals exhibited persistent pain-related behaviors, although no animals exhibited severe pain-related behaviors during the experimental period.

70% partial hepatic IR injury model

Sprague-Dawley rats (male; 8 weeks old; weight, 290–310 g; Orient Bio, Pyeongtaek, Republic of Korea) were used in the experiments. All the rats were housed, three per cage, in a 12-hour light-dark cycle with consistent temperature at 24–25 °C and unrestricted access to food and water.

Based on previous studies, a 70% partial hepatic IR injury model was established in rats.^{4,25} Briefly, the abdominal cavity was exposed by a midline incision, and the portal triad (hepatic artery, portal vein, and bile duct), which supplied the left lateral and median lobes, was clamped (Fig. 5). The clamp was removed 45 minutes after ischemia developed, and the abdomen was closed in a single layer. Successful induction of hepatic ischemia was visually confirmed by observing the median and left lobes, which turned pale, denoting the establishment of ischemia compared to non-ischemic lobes (right and caudate

lobes). Successful reperfusion was also determined by the color of ischemic lobes turning back to that of the non-ischemic lobes. All rats were initially anesthetized using 5% isoflurane in 70% N₂O/30% O₂ (flow rate, 1.0 L/min), and anesthesia was maintained using 2% isoflurane during surgery.

Drug treatment

As the AhR antagonist, 5 mg/kg of TMF (Sigma-Aldrich, St. Louis, MO, USA) dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) was used¹². An equal volume of DMSO was used for the vehicle-treated control group. All treatments were administered via intraperitoneal injection.

Animal groups

The rats were randomly divided into the following: (1) a sham group with no IR modeling or vehicle injection (n = 8); (2) a control group with IR modelling and vehicle injection (n = 8); and (3) a TMF group with IR modeling and administration of the drug at 10 minutes after ischemia (n = 8).

MRI

MRI was performed on a 9.4 T MRI system (Agilent Technologies, Santa Clara, CA, USA) with a 63-mm transmit/receive volume coil. The animals were maintained under respiratory anesthesia with 2.0–2.5% isoflurane in a 1:2 mixture of O₂:N₂O and body temperature of 37.5 ± 0.5°C through an air heater system during image acquisition. These were continuously monitored for stable breathing.

MRI data were obtained at 3 and 24 hours after reperfusion. The MRI protocols included T1-WI and T1 mapping. The parameters for each sequence are shown in Supplementary Table S1. MRI images were obtained before and 20 minutes after administration of Gd-EOB-DTPA (25 µM/kg, Primovist, Bayer Korea, South Korea).²²

MRI analysis

All MRI data were analyzed using the ImageJ software (National Institutes of Health, Bethesda, MA, USA; <https://rsbweb.nih.gov/ij/>) by an observer blind to grouping information. The mean SI values and T1 relaxation time were measured on T1-WIs and T1 maps obtained before and 20 minutes after Gd-EOB-DTPA administration. Region of interests (ROIs) were manually placed at identical locations on the hepatic parenchyma and paravertebral muscle in T1-WIs and T1 maps. The ROIs on hepatic parenchyma were placed by avoiding visible blood vessels or imaging artifacts. Two ROIs were randomly placed in the liver and paravertebral muscles in the T1-WIs and T1 maps before and after Gd-EOB-DTPA administration (Fig. 3). The mean SI and T1 values for both ROIs in the liver were considered representative SI and T1 values of the whole liver, respectively. The mean SI values for both ROIs in the paravertebral muscle were regarded as the representative SI values for the entire paravertebral muscle. The size of the ROIs ranged from 10 to 11 mm² in liver parenchyma and 8 to 9 mm² in paravertebral muscles. MRI-based liver function indices were calculated from the SI measurements or T1 relaxation time before (SI_{pre}, T1_{pre})

and 20 minutes after (SI_{post} , $T1_{\text{post}}$) Gd-EOB-DTPA administration as follows: (1) relative enhancement (RE) of the liver = $(SI_{\text{post}} - SI_{\text{pre}})/SI_{\text{pre}}$; (2) LMR = SI_{post} of the liver/ SI_{post} of the muscle; (3) $T1_{\text{post}}$ = $T1_{\text{post}}$ values of the liver; and (4) $\Delta T1 = T1_{\text{pre}} - T1_{\text{post}}$ ^{23,24}.

Measurement of serum enzymes

Blood samples were obtained from the rats' jugular veins 3 and 24 hours after reperfusion. The samples were centrifuged at 2,000 r/minutes for 14 minutes to obtain serum for analysis. The activities of serum ALT and AST were determined with a biochemical analyzer (7180, Hitachi, Tokyo, Japan).

Hematoxylin and eosin (H&E) staining

Liver tissues were harvested at 24 hours after reperfusion and fixed in 4% paraformaldehyde (Biosesang, Seongnam, Republic of Korea). Fixed tissues were embedded in paraffin, and the tissues were sectioned to 3- μ m thickness. H&E staining was performed using an automatic stainer (Leica, Wetzlar, Germany). The percentages of necrotic areas in five random sections per slide were analyzed under a microscope at a magnification of $\times 50$ (Vectra, PerkinElmer, MA, USA).²⁶

TUNEL assay

A TUNEL assay was performed to detect apoptotic cells using a commercially available apoptosis detection kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Paraffin-embedded liver tissue sections were treated with a mixture of reaction buffer and enzyme (7:3) at 37 °C for 1 hour, and the anti-digoxigenin peroxidase conjugate was treated at room temperature for 30 minutes. Thereafter, the tissue was treated with DAB substrate (3,3' diaminobenzidine tetrahydrochloride hydrate, Sigma-Aldrich) (1:50) in the dark for five minutes, and then stained with hematoxylin (Sigma-Aldrich) for two minutes in the dark. TUNEL-positive cells were observed under a microscope at a magnification of $\times 200$ (Vectra, PerkinElmer, MA, USA).

Western blot

The tissues from the sham (5 mg), control (30 mg), and TMF groups (30 mg) were centrifuged in cold-PBS at 500 $\times$ g and at 4 °C for five minutes and homogenized in radio-immunoprecipitation (RIPA) buffer (Sigma-Aldrich, MO, USA) with 1 $\cdot$ protease inhibitor cocktail (Sigma-Aldrich) on ice. After 30 minutes, the lysate was centrifuged at 13,000 rpm and at 4 °C for 20 min. The supernatant was aliquoted into a 1.5-mL tube and quantified using a bicinchoninic acid assay (ThermoFisher Scientific, MA, USA). In total, 30- μ g protein was separated in 8% pre-made sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (ThermoFisher Scientific) and transferred to a polyvinylidene fluoride (PVDF) membrane (ThermoFisher Scientific). The membrane was blocked with 5% skim milk (Becton and Dickinson [BD], NJ, USA) in 1 $\cdot$ tris-buffered saline (Elpis-Biotech, Daejeon, Republic of Korea) with 20 (Sigma-Aldrich) (TBST) at room temperature for 1 hour and then incubated with mouse anti-AhR (1:1000, Santa Cruz Biotechnology, TX, USA), rabbit anti-Bcl-2 (1:1000, Novus Biologicals, CO, USA), rabbit anti-Bax (1:1000, Cell Signaling Technology, MA, USA), rabbit anti-cleaved caspase-3 (C-cas3, 1:1000, Cell Signaling Technology), and mouse anti-GAPDH (1:10000, ThermoFisher Scientific) at 4 °C overnight. The

antibodies were washed three times for 10 min in 1 × TBST, and the membrane was incubated with goat anti-mouse (1:5000, ThermoFisher Scientific) or rabbit (1:2000, ThermoFisher Scientific) immunoglobulin G secondary antibody-horseradish peroxidase (HRP) conjugate at room temperature for 1 hour. The membrane reacted with Lumi Femto Solution A:B (A:B = 1:1, DoGenBio, Seoul, Republic of Korea) of a chemi-luminescence (ECL) reagent after washing, and AhR was quantified using GAPDH via ImageJ software.

Immunohistochemistry (IHC) staining

IHC staining was conducted as indicated in the Ultra View Universal DAB Detection kit (Ventana Medical Systems, Inc., Oro Valley, AZ, USA). The slides were incubated at 60 °C for four minutes, Ezprep was treated for deparaffinization, and slides were rinsed with a Tris buffer (pH 7.6). After reacting the slides to the antigen retrieval buffer for one hour at 100 °C, they were treated with rabbit anti-AhR (1:1000, Enzo Life Sciences, NY, USA) for 36 minutes at 37 °C. The rinsed slides were processed using the DISCOVERY Ultra Map anti/Rabbit HRP for 12 minutes at 37 °C. Ultraview DAB and DAB H₂O₂ were treated for eight minutes at 37 °C, during which AhR-positive cells were stained brown, before Ultra View COPPER was used to treat washed slides for four minutes at 37 °C. After rinsing, the slides were incubated in hematoxylin for 4 minutes at 37 °C. Finally, the rinsed slides were exposed to BLUING REAGENT for four minutes at 37 °C before they were washed again. AhR-positive cells were observed under a microscope at a magnification of × 200 (Vectra, PerkinElmer, Waltham, MA, USA).

Statistics

Statistical analyses were performed using the IBM SPSS version 21.0 software (IBM Corp., Armonk, NY, USA). The AST, ALT, RE, LMR, T1_{post}, ΔT1, percentage of necrotic area, Bcl-2/GAPDH, Bax/GAPDH, and C-cas3/GAPDH in multiple groups were compared using a one-way analysis of variance with Tukey's post-hoc test. All the data are expressed as means ± standard deviations. Values of P < 0.05 were considered statistically significant.

Declarations

Data Availability

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Author Contributions

JI Kwon, DC Woo, and CW Woo conceived the study. JI Kwon, H Heo, YJ Chae, J Min, DW Lee, ST Kim, MY Choi, and YS Sung performed the experiments and analyzed the data. JI Kwon, KW Kim, Y Choi, DC Woo, and CW Woo interpreted the results. JI Kwon, DC Woo, and CW Woo wrote the manuscript. All the authors reviewed the manuscript, provided critical input, and approved the final version for submission.

Competing Interests

The authors declare no competing interests

References

1. Kokudo, N., Takemura, N., Ito, K. & Mihara, F. The history of liver surgery: Achievements over the past 50 years. *Ann Gastroenterol Surg* **4**, 109-117, doi:10.1002/ags3.12322 (2020).
2. Orci, L. A. *et al.* The role of hepatic ischemia-reperfusion injury and liver parenchymal quality on cancer recurrence. *Dig Dis Sci* **59**, 2058-2068, doi:10.1007/s10620-014-3182-7 (2014).
3. Yamazaki, S. & Takayama, T. Current topics in liver surgery. *Ann Gastroenterol Surg* **3**, 146-159, doi:10.1002/ags3.12233 (2019).
4. Zabala, V. *et al.* Transcriptional changes during hepatic ischemia-reperfusion in the rat. *PLoS One* **14**, e0227038, doi:10.1371/journal.pone.0227038 (2019).
5. Bi, J. *et al.* Irisin alleviates liver ischemia-reperfusion injury by inhibiting excessive mitochondrial fission, promoting mitochondrial biogenesis and decreasing oxidative stress. *Redox Biol* **20**, 296-306, doi:10.1016/j.redox.2018.10.019 (2019).
6. Guan, L. Y. *et al.* Mechanisms of hepatic ischemia-reperfusion injury and protective effects of nitric oxide. *World J Gastrointest Surg* **6**, 122-128, doi:10.4240/wjgs.v6.i7.122 (2014).
7. Opitz, C. A. *et al.* An endogenous tumour-promoting ligand of the human aryl hydrocarbon receptor. *Nature* **478**, 197-203, doi:10.1038/nature10491 (2011).
8. Denison, M. S. & Nagy, S. R. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Annu Rev Pharmacol Toxicol* **43**, 309-334, doi:10.1146/annurev.pharmtox.43.100901.135828 (2003).
9. Hankinson, O. The aryl hydrocarbon receptor complex. *Annu Rev Pharmacol Toxicol* **35**, 307-340, doi:10.1146/annurev.pa.35.040195.001515 (1995).
10. Cuartero, M. I. *et al.* L-kynurenine/aryl hydrocarbon receptor pathway mediates brain damage after experimental stroke. *Circulation* **130**, 2040-2051, doi:10.1161/CIRCULATIONAHA.114.011394 (2014).
11. Padmanabhan, A. & Haldar, S. M. Neuroprotection in ischemic stroke: AhR we making progress? *Circulation* **130**, 2002-2004, doi:10.1161/CIRCULATIONAHA.114.013533 (2014).

12. Kwon, J. I. *et al.* Aryl hydrocarbon receptor antagonism before reperfusion attenuates cerebral ischaemia/reperfusion injury in rats. *Sci Rep* **10**, 14906, doi:10.1038/s41598-020-72023-5 (2020).
13. Green, A. R., Woods, H. F. & Joseph, M. H. Tryptophan metabolism in the isolated perfused liver of the rat: effects of tryptophan concentration, hydrocortisone and allopurinol on tryptophan pyrrolase activity and kynurenine formation. *Br J Pharmacol* **57**, 103-114, doi:10.1111/j.1476-5381.1976.tb07660.x (1976).
14. Quintana, F. J. *et al.* Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. *Nature* **453**, 65-71, doi:10.1038/nature06880 (2008).
15. Kajta, M., Wojtowicz, A. K., Mackowiak, M. & Lason, W. Aryl hydrocarbon receptor-mediated apoptosis of neuronal cells: a possible interaction with estrogen receptor signaling. *Neuroscience* **158**, 811-822, doi:10.1016/j.neuroscience.2008.10.045 (2009).
16. Sanchez-Martin, F. J., Fernandez-Salguero, P. M. & Merino, J. M. Aryl hydrocarbon receptor-dependent induction of apoptosis by 2,3,7,8-tetrachlorodibenzo-p-dioxin in cerebellar granule cells from mouse. *J Neurochem* **118**, 153-162, doi:10.1111/j.1471-4159.2011.07291.x (2011).
17. Jo, K. M. *et al.* Serum Aminotransferase Level in Rhabdomyolysis according to Concurrent Liver Disease. *Korean J Gastroenterol* **74**, 205-211, doi:10.4166/kjg.2019.74.4.205 (2019).
18. Giboney, P. T. Mildly elevated liver transaminase levels in the asymptomatic patient (vol 71, pg 1105, 2005). *American Family Physician* **72**, 41-41 (2005).
19. Young, T. H. *et al.* Quantitative rat liver function test by galactose single point method. *Lab Anim* **42**, 495-504, doi:10.1258/la.2007.06040e (2008).
20. Meier, P. J., Eckhardt, U., Schroeder, A., Hagenbuch, B. & Stieger, B. Substrate specificity of sinusoidal bile acid and organic anion uptake systems in rat and human liver. *Hepatology* **26**, 1667-1677, doi:10.1002/hep.510260641 (1997).
21. Nassif, A. *et al.* Visualization of hepatic uptake transporter function in healthy subjects by using gadoxetic acid-enhanced MR imaging. *Radiology* **264**, 741-750, doi:10.1148/radiol.12112061 (2012).
22. Ma, C. *et al.* The hepatocyte phase of Gd-EOB-DTPA-enhanced MRI in the evaluation of hepatic fibrosis and early liver cirrhosis in a rat model: an experimental study. *Life Sci* **108**, 104-108, doi:10.1016/j.lfs.2014.05.016 (2014).
23. Haimerl, M. *et al.* Gd-EOB-DTPA-enhanced MRI for evaluation of liver function: Comparison between signal-intensity-based indices and T1 relaxometry. *Sci Rep* **7**, 43347, doi:10.1038/srep43347 (2017).
24. Nakagawa, M. *et al.* Measuring hepatic functional reserve using T1 mapping of Gd-EOB-DTPA enhanced 3T MR imaging: A preliminary study comparing with (99m)Tc GSA scintigraphy and signal intensity based parameters. *Eur J Radiol* **92**, 116-123, doi:10.1016/j.ejrad.2017.05.011 (2017).
25. Karatzas, T., Neri, A. A., Baibaki, M. E. & Dontas, I. A. Rodent models of hepatic ischemia-reperfusion injury: time and percentage-related pathophysiological mechanisms. *J Surg Res* **191**, 399-412, doi:10.1016/j.jss.2014.06.024 (2014).
26. Bae, U. J. *et al.* SPA0355 attenuates ischemia/reperfusion-induced liver injury in mice. *Exp Mol Med* **46**, e109, doi:10.1038/emm.2014.48 (2014).

Figures

Figure 1

Effect of 6,2',4'-trimethoxyflavone (TMF) treatment on magnetic resonance imaging-based liver function indices (A) Representative T1-weighted gradient echo images and T1 maps at 3 and 24 hours after reperfusion. Mean signal intensity (SI) and T1 values were measured in the liver (blue circles). Mean SI values were measured in the paravertebral muscle (white circles). (B) The relative enhancement (RE) values at 24 hours after reperfusion were significantly higher in the control group than in the sham group. At 3 and 24 hours after reperfusion, the RE values were significantly decreased in the TMF group than in the control group. The $\Delta T1$ values at three hours after reperfusion were significantly higher in the TMF group than in the sham group. The $\Delta T1$ values at 24 hours after reperfusion were significantly higher in the control group than in the sham group. At 24 hours after reperfusion, the $\Delta T1$ values were significantly decreased in the TMF group than in the control group. Data are represented as means $\pm$ standard deviations (n = eight rats in each group). # $P < 0.05$ vs. sham group; ## $P < 0.01$ vs. sham group; ### $P < 0.001$ vs. sham group; * $P < 0.05$ vs. control group; ** $P < 0.01$ vs. control group; *** $P < 0.001$ vs. control group.

Figure 2

Reduction of serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and necrotic area by 6,2',4'-trimethoxyflavone (TMF) treatment (A) The control and TMF groups had significantly higher serum ALT levels than did the sham group at 3 and 24 hours after reperfusion. Serum ALT levels were significantly lower in the TMF group than in the control group at 3 and 24 hours after reperfusion. (B) The control and TMF groups had significantly higher serum AST levels than did the sham group at 3 and 24 hours after reperfusion. Serum AST levels were significantly lower in the TMF group than in the control group at three hours after reperfusion. (C) Representative photomicrographs of hematoxylin and eosin staining 24 hours after reperfusion (magnification $\times 50$, scale bar = 200 μ m). The under row shows enlarged versions of the black boxes in the upper row (scale bar = 50 μ m). (D) The percentage of necrotic area in the TMF group was significantly lower than that in the control group. Data are represented as means $\pm$ standard deviations (n = eight rats in each group). # $P < 0.05$ vs. sham group; ## $P < 0.01$ vs. sham group; ### $P < 0.001$ vs. sham group; * $P < 0.05$ vs. control group; ** $P < 0.01$ vs. control group; *** $P < 0.001$ vs. control group.

Figure 3

Reduction of apoptosis after 6,2',4'-trimethoxyflavone (TMF) administration (A) Representative photomicrographs of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining 24 hours after reperfusion (magnification $\times 200$, scale bar = 50 μm). Red arrows indicate apoptotic cells; blue arrows indicate viable cells. (B) The expression of BCL2 protein was significantly lower in the control and TMF groups than in the sham group. The expression of Bax protein in the control group was significantly higher than in the sham and TMF groups. The expression of C-cas3 protein in the control group was significantly higher than in the sham and TMF groups. Data are represented as means $\pm$ standard deviations ($n =$ eight rats in each group). ## $P < 0.01$ vs. sham group; ### $P < 0.001$ vs. sham group; * $P < 0.05$ vs. control group; ** $P < 0.01$ vs. control group. The full-length blots/gels are presented in Supplementary Figure 1.

Figure 4

Suppression of Aryl hydrocarbon receptor (AhR) expression by 6,2',4'-trimethoxyflavone (TMF) treatment (A) Representative photomicrographs of AhR staining 24 hours after reperfusion (magnification $\times 200$, scale bar = 50 μ m). (B) AhR proteins were significantly higher in the control and TMF groups than in the sham group. The TMF group showed a significant decrease in AhR proteins than did the control group. Data are represented as means $\pm$ standard deviations ($n =$ eight rats in each group). ## $P < 0.01$ vs. sham group; ### $P < 0.001$ vs. sham group; # $P < 0.05$ vs. control group. The full-length blots/gels are presented in Supplementary Figure 2.

Figure 5

Schematic diagram of the experimental procedure Hepatic ischemia was induced by clamping the portal triad for 45 minutes followed by reperfusion. After, 6,2',4'-trimethoxyflavone was administered 10 minutes after ischemia. Blood samples and magnetic resonance imaging data were obtained at 3 and 24 hours after reperfusion. Liver tissues were harvested at 24 hours after reperfusion.

Supplementary Files

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- [SupplementaryFiguresandTable.docx](#)