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Original article

The natural product fucoidan ameliorates hepatic ischemia–reperfusion injury in mice

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ABSTRACT

Fucoidan is a sulfated polysaccharide based predominantly on L-fucose, and has several biologic functions. Reactive oxygen species-mediated apoptosis and autophagy and release of related inflammatory factors have important roles in hepatic ischemia–reperfusion injury (IR). Here, the effect of fucoidan on hepatic IR was investigated. Mice were randomized into sham, IR, and fucoidan (20, 40 mg/kg for 14 days) groups. Samples were collected to assess biochemical indicators, hepatocyte damage and levels of proteins related to signaling pathways at different time points. Fucoidan had no effect on normal liver tissue, but inhibited the increases in alanine aminotransferase, aspartate transaminase, inflammatory factors, and the hepatocyte damage caused by IR. Also, apoptosis and autophagy via the activated JAK2/STAT1 pathway were attenuated by fucoidan to protect against hepatic injury. In conclusion, fucoidan ameliorates hepatic IR injury in mice via JAK2/STAT1-mediated apoptosis and autophagy. Inhibition of this pathway may be associated with reduced release of related inflammatory cytokines, especially interferon-γ.

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1. Introduction

Fucoidan is an L-fucose-based sulfated polysaccharide produced by various species of brown seaweed and brown algae, such as *Fucus vesiculosus, Sargassum kjellmanianum* and *Cladosiphon okamuranus Tokida* [6]. Recent studies have reported that natural moieties from marine resources possess antiviral and anti-tumor [7], antioxidant and anti-inflammatory properties [4,8,9], as well as scavenge free radicals, thereby enhancing the immune response [10]. In 1997, Preobrazhenskaya et al. showed that fucoidan inhibited leukocyte recruitment in a rat model of inflammation and blocked interaction of P-selectin with its carbohydrate ligand [11]. Kim et al. and Mizumo et al. [12–14] showed that oxidative stress and inflammation can be suppressed by regulation of the release of inflammatory factors such as interleukin (IL)-8, IL-6, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ. Li et al. and Li et al. showed that fucoidan reduced the inflammatory response in a rat model of hepatic injury and myocardial ischemia–reperfusion (IR) injury, but the mechanism of action remains unknown [6,15]. Other studies have shown that fucoidan can interrupt many cell-signaling pathways, including mitogen-activated protein kinase (MAPK), phosphatidylinositide 3-kinase (PI3K)/Akt, janus kinase (JAK)/signal transducer and activator of transcription (STAT), mammalian target of rapamycin (mTOR)/ULK, and transforming growth factor (TGF)β1/smads [16–20]. Therefore, we hypothesized that fucoidan may affect expression of inflammatory cytokines through these pathways.

IR after liver transplantation/resection increases the risk of postoperative complications and mortality through a mechanism of injury related to Kupffer cell activation and subsequent cytokine release (e.g., TNF-α, IFN-γ), excessive expression of reactive oxygen species (ROS) and calcium overload [21]. In response, various other apoptotic pathways are activated, including JAK/STAT, MAPK family, Fas/TRAIL and NF-κB, which mediate cell death and have roles in IR injury. Among these, the JAK/STAT pathway plays an important part in IR. Hence, several drugs have been developed to modulate JAK/STAT pathway to protect against IR injury to the heart [22,23]. And, hippocampus ischemia reperfusion after liver transplantation could be protected by JAK/STAT

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pathway [24]. ROS-mediated endoplasmic reticulum stress during IR has been studied extensively. The related inflammatory factors released by Kupffer cells warrant further exploration due to their important role in IR onset.

We hypothesized that fucoidan from *F. vesiculosus* pretreatment can attenuate IR-induced production of inflammatory cytokines to protect hepatic function and that the mechanism of action may be associated with inhibition of apoptosis and autophagy *via* the JAK2/STAT1 pathway.

2. Materials and methods

2.1. Mice and induction of IR

Male BALB/C mice (body weight, 20–25 g) were purchased from Shanghai Laboratory Animal Co., Ltd. (Shanghai, China) and maintained under a 12-h light–dark cycle at a constant temperature (22 °C–25 °C) with free access to food and water and monitored every day. The study protocol was approved by the Animal Care and Use Committee of Tongji University (Shanghai, China) and conducted in compliance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. No animals died or became severely ill prior to the experimental endpoint. Throughout the course of this study, all efforts were made to minimize animal suffering. A 70% hepatic warm ischemia mouse model was created as previously reported [25]. In brief, the abdominal cavity was opened along a longitudinal incision after the response to pain stimulation had disappeared. Wet swabs were used to identify the anatomy and free the porta hepatis. The common blood vessels of the left hepatic lobe and middle lobe were clipped using a vascular clamp to elicit ischemia. An albescent liver as visualized by the naked eye denoted ischemia. The incision was covered with humid saline-moisened gauze for 60 min and then sewn without clamps. Intraoperative application of a meter (ZS Dichuang, Beijing, China) permitted maintenance of a constant body temperature. The sham-operated group underwent no action apart from opening of the abdominal cavity and stitching.

2.2. Reagents

Fucoidan from F. *vesiculosus* was purchased from Sigma–Aldrich Corporation(St. Louis, MO, USA) and stored at room temperature. Antibodies (Bcl-2, LC3, caspase-3, caspase-8, caspase-9, JAK2, p-JAK2, STAT1, and p-STAT1) were obtained from Cell Signaling Technology (Danvers, MA, USA). P62, Beclin-1, Bax, and IFN-γ were obtained from Proteintech (Chicago, IL, USA). An RNA polymerase chain reaction (PCR) kit was purchased from TaKaRa Biotechnology (Dalian, China). Testing of liver function was based using microplate test kits (Jancheng Biotech, Nanjing, China). Enzyme-linked immunosorbent assay (ELISA) kits for measurements of cytokines were acquired from eBioscience (San Diego, CA, USA). A terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) apoptosis assay kit was purchased from Roche (Basel, Switzerland).

2.3. Experimental program

In a preliminary study, 32 mice were divided randomly into four groups: control (no treatment), sham, fucoidan (20 mg/kg body weight) and fucoidan (40 mg/kg). Fucoidan diluted in physiologic (0.9%) saline was administered (p.o.) at daily doses of 20 mg/kg and 40 mg/kg once a day for 2 weeks before surgery. The selection of fucoidan dose is based on the previous researches [4,26,27]. The mice were euthanized and sera and liver tissues were collected for analysis of liver enzymes and cytokines levels as well as the degree of necrosis.

An additional 96 mice were distributed randomly into one of four groups of 24: sham-operation without fucoidan; IR without fucoidan pretreatment; IR + pretreatment with fucoidan (20 mg/kg) by gavage: and IR + pretreatment with fucoidan (40 mg/kg) by gavage.Eight mice were selected from each group and killed at 2, 8 and 24 h after IR. Sera and liver samples were collected for further study.

2.4. Biochemical detection and pathologic evaluation

Levels of alanine aminotransferase (ALT), aspartate transaminase (AST) and cytokines were determined using commercially available kits in accordance with the manufacturers' protocols. Liver tissues were fixed by 4% paraformaldehyde, embedded in paraffin (thickness, 5 μm), stained with hematoxylin and eosin, and then pathologically assessed under a light microscopy equipped with a digital camera (Leica Microsystems, Wetzlar, Germany). Image Pro Plus v6.0 image processing, enhancement, and analysis software (Media Cybernetics, Silver Spring, MD, USA) was used to calculate the area of necrosis and edema on sections.

2.5. Immunohistochemical staining

Paraffin sections were dried for 2 h at 60 °C and then dewaxed using dimethyl benzene. Sections were washed with phosphate-buffered saline (PBS) after dehydration with a series of graded ethanol solutions. Antigens immersed in citrate buffer were recovered by heating to 95 °C for 10 min and cooling to room temperature for four cycles. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide solution for 20 min at 37 °C. After blockade with 5% bovine serum albumin, the liver tissues were incubated overnight with the following primary antibodies at 4 °C: caspase-8 (dilution, 1:100), caspase-9 (1:100), LC3 (1:50), beclin-1 (1:50), P62 (1:100), IFN-γ (1:50), p-JAK2 (1:100), p-STAT1 (1:100). Samples containing brown-colored substances after staining with a diaminobenzidine kit were considered positive.

2.6. Detection of mRNA by real-time quantitative PCR

Total RNA was extracted from approximately 100 mg of tissue using TRIzol^{TM} reagent, chloroform, and isopropyl alcohol, then reverse transcribed into cDNA using a commercial kit (TaKaRa biotechnology (Dalian)Co., Ltd) and conformed to possess an appropriate concentration and purity. A 10-μL reaction volume

Table 1
Nucleotide sequences of primers used for qRT-PCR.

Gene		Primer sequence (5′—3′)
TNF-α	Forward	CAGGCGGTGCCTATGTCTC
	Reverse	CGATCACCCCGAAGTTCAGTAG
IFN-γ	Forward	GCCACGGCACAGTCATTGA
	Reverse	TGCTGATGGCCTGATTGTCTT
IL-1β	Forward	CGATCGCGCAGGGGCTGGGCGG
	Reverse	AGGAACTGACGGTACTGATGGA
IL-6	Forward	CTGCAAGAGACTTCCATCCAG
	Reverse	AGTGGTATAGACAGGTCTGTTGG
LC3-II	Forward	GACCGCTGTAAGGAGGTGC
	Reverse	AGAAGCCGAAGGTTTCTTGGG
Beclin-1	Forward	ATGGAGGGGTCTAAGGCGTC
	Reverse	TGGGCTGTGGTAAGTAATGGA
P62	Forward	GAGGCACCCCGAAACATGG
	Reverse	ACTTATAGCGAGTTCCCACCA
Bax	Forward	AGACAGGGGCCTTTTTGCTAC
	Reverse	AATTCGCCGGAGACACTCG
Bcl-2	Forward	GCTACCGTCGTCGTGACTTCGC
	Reverse	CCCCACCGAACTCAAAGAAGG
β-actin	Forward	GGCTGTATTCCCCTCCATCG
		CCAGTTGGTAACAATGCCATGT

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