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Diatom frustule silica extracted from *Melosira nummuloides* ameliorates acetaminophen-induced acute liver injury in mice

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Abstract

Melosira nummuloides is a type of diatom in the family Melosiraceae. Diatoms are unicellular microalgae widely distributed in aquatic environments. Diatoms are known to be suitable for many industrial and biomedical applications because of their high biocompatibility and ease of use. In this study, we investigated the hepatoprotective effect of diatom frustule silica (DFS) extracted from *Melosira nummuloides* on hepatotoxicant-induced liver injury. Hepatoprotective effects of DFS were examined using acetaminophen-induced acute liver injury (ALI) mouse model. We evaluated the hepatoprotective effects through hepatotoxicity, pro-inflammatory cytokines, transcriptional factors, upstream signaling pathways, and histopathological analysis by DFS in an animal model of acetaminophen-induced ALI. Our results showed serum alanine aminotransferase/aspartate aminotransferase activity and hepatic malondialdehyde formation were significantly attenuated upon DFS administration. DFS also ameliorated glutathione depletion and down-regulated acetaminophen-induced CYP2E1. DFS administration also down-regulated expressions of pro-inflammatory cytokines through preventing NF-κB activation by JNK1/2 phosphorylation inhibition. These findings demonstrate that the hepatoprotective effect of DFS is associated with suppression of inflammatory responses in an animal model of acetaminophen-induced ALI.

Keyword: Acute liver injury (ALI), Diatom Frustule Silica (DFS), Hepatotoxicity, Melosira nummuloides, Pro-inflammatory cytokines

Introduction

The liver is an important organ responsible for metabolism and drug detoxification in the body, but various causes such as indiscriminate drug intake, excessive alcohol consumption, and viral infection can damage the liver. Excluding genetic and environmental factors, most liver damage is caused by drug ingestion [1, 2]. Acute liver injury (ALI) is a common pathological feature of many liver diseases and is associated with hepatitis, liver fibrosis, liver cirrhosis, and liver cancer [3]. Drug-induced hepatotoxicity is a major cause of ALI and is known to cause high morbidity and mortality worldwide [4]. Acetaminophen, the main component of Tylenol, can be purchased without a prescription as an analgesic and antipyretic; however, it can cause ALI through inflammation induction, hepatic cell damage, and liver failure when acetaminophen is used more than indicated [5]. Hepatic damage due to acetaminophen overdose accounts for 50% of all ALI cases in the United States and for 40–70% in the United Kingdom and Europe [6, 7].

ALI is induced when acetaminophen is metabolized, through oxidation by CYP2E1, to form N-acetyl-*p*-benzoquinone imine (NAPQI), a highly electrophilic

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metabolite. NAPQI causes hepatocellular damage by forming lipid peroxide and depleting glutathione (GSH), causing oxidative stress, which activates upstream signaling pathways leading to mitochondrial toxicity [8–11]. In response to acetaminophen-induced hepatocellular damage, antioxidant enzymes scavenge mitochondria-generated reactive oxygen species (ROS) to maintain hepatocellular homeostasis [12]. Therefore, the induction of antioxidant enzymes is an important strategy for protecting or ameliorating acetaminophen-induced liver injury.

Natural resources are less toxic and can be used as an effective remedy for diseases. There is increasing interest in their application to protect the liver from damage caused by drugs or alcohol intake [13]. Diatoms are unicellular eukaryotic microalgae with porous silica micro shells that can replace synthetic silica. Diatom cells are surrounded by nanostructured silicon cell walls to protect them against environmental stress. This silica is a biocompatible and non-toxic substance collected from living algae or fossil deposits of diatomaceous soil and has medical uses, as micro/nano-carrier materials or drug delivery systems, and theranostic uses [14]. A recent study reported that Diatom Frustule Silica (DFS) extracted from *Melosira nummuloides* in Lava seawater from Jeju Island exhibited hemostatic reactions both in vivo and in vitro [15]. However, the hepatoprotective effect of DFS extracted from *Melosira nummuloides* on hepatotoxicant-induced ALI has been unknown. In this study, we aimed to investigate the potential hepatopreventive effects of DFS extracted from *Melosira nummuloides* on acetaminophen-induced ALI in mice model.

Materials and methods

Reagents

Acetaminophen and silymarin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies against cytochrome P450 family 2 subfamily E member 1 (CYP2E1), phospho-nuclear factor kappa B (NF-κB) p65, NF-κB p65, phospho- IkappaB-alpha (IκBα), IκBα, phospho-JNK1/2, JNK1/2, β-actin, HRP-linked anti-mouse IgG, and HRP-linked anti-rabbit IgG were obtained from Abcam, Inc. (Cambridge, MA, USA), Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), and Cell Signaling Technology, Inc. (Danvers, MA, USA). Nuclease-free water was obtained from Invitrogen (Carlsbad, CA, USA).

Preparation of DFS

Melosira nummuloides was collected from Lava seawater from Jeju Island. Cells were enriched at 18 °C for two weeks using Jeju Lava seawater and then dried at JNC Bio Fisheries Co., Ltd. (Jeju, Korea). Cells are ignited in a muffle furnace for 6 h at 950 °C to remove organic matters. Using NaOH, pH was adjusted to 12 to make it water-soluble. HCl was then added to neutralize the pH to 7. Inorganic composition of DFS was measured using inductively coupled plasma optical emission spectroscopy (ICP-OES) at Korea institute of ceramic engineering & technology (Jinju, Korea) (Additional file 1: Table S3). Previously, the properties of DFS were characterized by scanning electron microscope (Inspect F50, FEI Company Inc., OR, USA), x-ray photoelectron spectroscopy (Sigma probe, Thermo VG Scientific, MA, USA), pH meter (Orion Star A211, Thermo Fisher Scientific, MA, USA), attenuated total reflection-Fourier transform infrared spectroscopy (Nicolet iS50, Thermo Fisher Scientific Instrument, MA, USA), and zeta potential and particle size analyzer (ELSZ-2000, Otsuka Electronics Co., Osaka, Japan) [15]. This DFS was supplied by JDKBIO Inc (Jeju, Korea).

Animals and experimental design

SPF 8 week-old, male ICR mice were purchased from DBL Co., Ltd. (Daejeon, Korea). The mice were allowed ad libitum access to rodent chow diet (Orientbio, Gyeonggi-do, Korea) and tap water. The mice were maintained in a controlled environment at 22 ± 2 °C and 50 ± 5% relative humidity under a 12 h dark/light cycle. The mice were randomly divided into five groups to set up a dose–response model (n = 6 mice/group): (1) control group, (2) 300 mg/kg acetaminophen group, (3) 300 mg/kg acetaminophen + 7.5 mg/kg DFS group, (4) 300 mg/kg acetaminophen + 30 mg/kg DFS group, and (5) 300 mg/kg acetaminophen + 50 mg/kg silymarin group. DFS and silymarin were orally administered once daily for seven consecutive days. Mice in the control and acetaminophen groups were given saline. After administration of treatment on the seventh day, the mice fasted for 12 h were subsequently injected intraperitoneally with acetaminophen solution and euthanized after 8 h. Blood was collected from the vena cava, and the right liver lobe was removed, subjected to histopathological analysis, and stored at − 80 °C until required for hepatic functional analyses [10]. All experimental protocols were performed according to the rules and regulations of the Animal Ethics Committee of Jeju National University (The IACUC of Jeju National University; Approval Number 2021-0029).

Histopathological examination

Liver samples damaged by acetaminophen were fixed in 10% neutral buffered formalin solution for 7 days. Briefly, the fixed liver tissues were embedded in paraffin followed by a dehydration process with increasing ethanol concentration to remove water. The embedded tissue was sliced into small Sects. (5 μm) using a

microtome (Leica Biosystems, IL, USA). For histological evaluation, each liver sections were mounted on plain glass slides and generally stained with hematoxylin and eosin staining methods (Histoire, Seoul, Korea). Histopathological observations of each section were observed at 100 ×magnification under a microscope (Leica Microsystems, Wetzlar, Germany).

Hepatotoxicity determination

Hepatotoxicity was determined by measuring serum ALT and AST activity levels, hepatic MDA content, and GSH levels according to the manufacturer's instructions. Serum ALT/AST activity was analyzed using GPT/GOT diagnostic kits (Asan Pharmaceutical Co., Seoul, Korea) [10]. Hepatic MDA formation was measured by OxiTec™ TBARS Assay Kit (Biomax Co, Ltd., Seoul, Korea) in colorimetric at 532 nm. Briefly, 100 mg liver tissue was washed with cold PBS, homogenized, and centrifuged to collected the supernatant. Indicator solution was added to the prepared supernatant and standard solution microcentrifuge tube. After incubation at 65 °C for 45 min, 150 µL of each sample was dispensed into a 96-well microplate, and absorbance was measured at 532 nm with a microplate reader (Agilent, CA, USA). Hepatic GSH contents were measured by OxiTec™ Glutathione Assay Kit (Biomax Co, Ltd., Seoul, Korea) in colorimetric at 412 nm. Briefly, 100 mg liver tissue was washed with cold PBS, homogenized by 5% MPA, and centrifuged to collect the supernatant. After carefully placing 50 µL of sample at the bottom of the Eppendorf tube, cold 5% MPA solution was added to the Eppendorf tube, vortexed for 15–20 s and centrifuged. The supernatant was mixed with Assay Buffer I, and absorbance was measured at 412 nm with a microplate reader (Agilent, CA, USA). The information of evaluation kits for hepatotoxicity is summarized in Additional file 1: Table S1.

RNA extraction and qPCR

Total RNA was extracted from the left liver tissues using RNAiso Plus reagent (Takara Korea Biomedical Inc., Seoul, Korea). Total RNA concentration was measured using a spectrophotometer DS-11 plus (DENOVIX Inc., DE, USA). cDNA was synthesized from 1 µg of RNA using the BioFACT™ RT Kit (BioFACT Inc., Daejeon, Korea) with oligo dT primers for reverse transcription. The PCR primers were synthesized by Macrogen Inc. (Seoul, Korea), and the primer sequences and annealing temperatures are summarized in Table 1. qPCR was performed using a Thermal Cycler Dice® Real Time System Lite (Takara Bio Inc., Shiga, Japan) with 40 cycles of denaturation (94 °C, 60 s), annealing (60 s), and elongation (72 °C, 60 s).

Western Blot

Total protein was extracted from the leftover liver tissues using CETi lysis buffer with inhibitors containing protease and phosphatase inhibitors (TransLab, Daejeon, Korea). Total protein was quantified using the Bradford method, separated on 10% or 12% polyacrylamide gels, transferred to nitrocellulose membranes (Bio-Rad, CA, USA), and probed with the appropriate 1st and 2nd antibodies. For western blot, each primary antibody was incubated on overnight at 4°C, and each secondary antibody was incubated on 1–2 h at room temperature. The western blot antibodies were obtained by Abcam, Inc., Santa Cruz Biotechnology, Inc., and Cell Signaling Technology, Inc., and antibodies information are summarized in Additional file 1: Table S2. The membrane was made luminescent with ECL Plus Detection Kit (BioFACT Inc., Daejeon, Korea) solution and visualized using the ImageQuant™ LAS 4000 mini (GE Healthcare Japan Corporation, Tokyo, Japan). Protein expression and phosphorylation for specific antibodies were quantified using by densitometry ImageJ software (National Institutes of Health, Bethesda, MD, USA). The information of

Table 1 The primer sequences and annealing temperature for qPCR

Gene	Direction	Sequences	NCBI Number	Annealing temperature
TNF-α	Forward	TTGTCTACTCCCAGGTTCTCTT	NM_001278601.1	58
	Reverse	ACTTTCTCCTGGTATGAGATAGC		
IL-1β	Forward	TGACTCATGGGATGATGATGATAAC	NM_008361.4	55
	Reverse	TGAGGTGGAGAGCTTTCAG		
IL-6	Forward	CTCTCTGCAAGAGACTTCCAT	NM_001314054.1	58
	Reverse	CCGACTTGTGAAGTGGTATAG		
β-actin	Forward	CCACCAGTTCGCCATGGAT	NM_007393.5	56
	Reverse	CCACGATGGAGGGGAATACA		

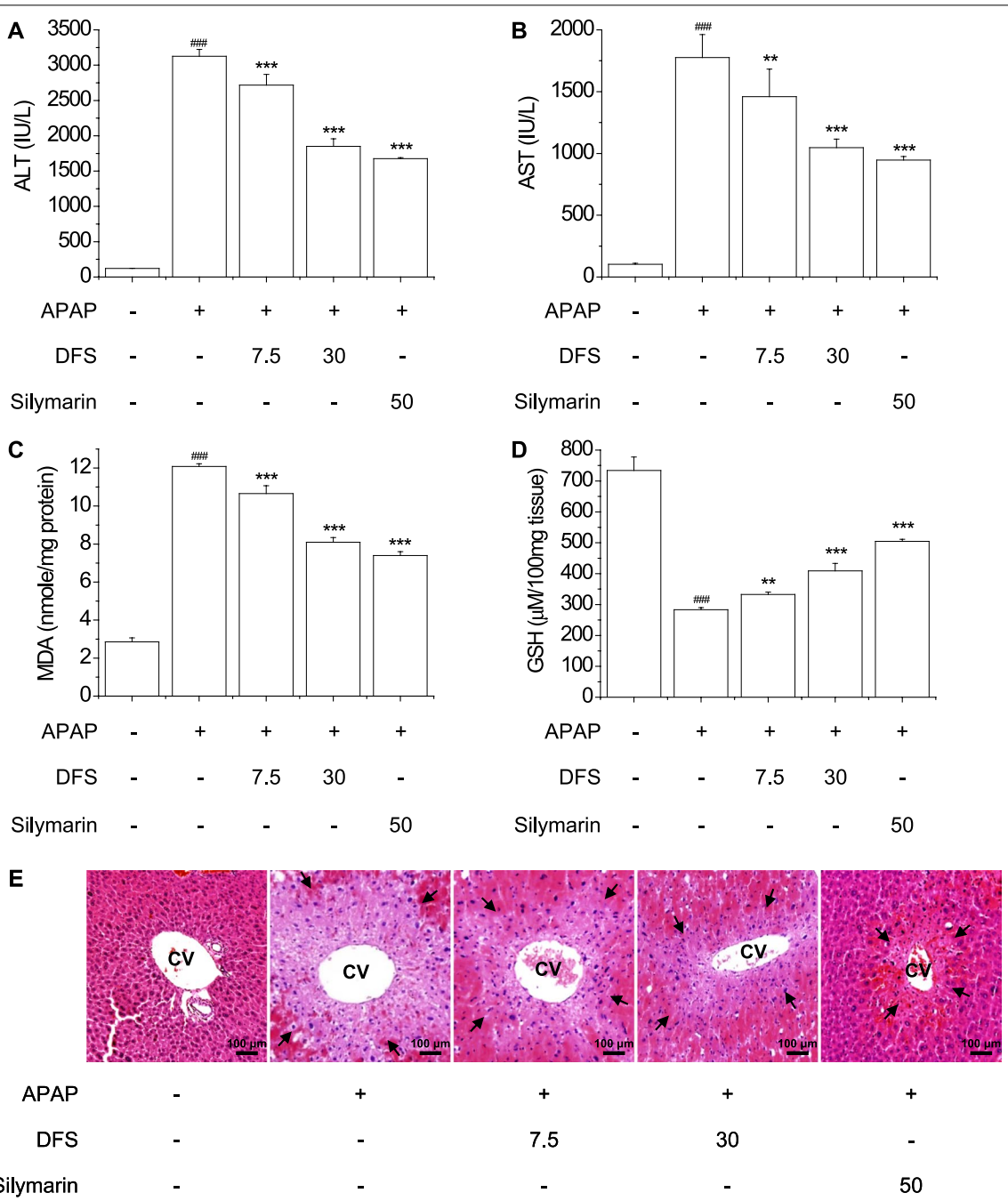

Fig. 1 Preventive effects of Diatom Frustule Silica (DFS) extracted from *Melosira nummuloides* on acetaminophen-induced acute liver injury (ALI) in mice. Mice were orally administered with 7.5 or 30 mg/kg DFS or 50 mg/kg silymarin once daily for seven consecutive days. Control and acetaminophen-treated groups were orally administered with saline. After oral administration of the last chemical and fasting for 12 h, mice were intraperitoneally injected with 300 mg/kg acetaminophen and sacrificed after 8 h. ALI was evaluated by measuring **A** serum alanine aminotransferase (ALT) and **B** aspartate aminotransferase (AST) activity levels, **C** hepatic malondialdehyde (MDA) content, and **D** glutathione (GSH) levels. **E** Representative liver tissues of each group for histopathological analysis were stained with hematoxylin and eosin at 100 × magnification. The results are presented as the mean ± standard deviation (SD) (n = 6). ###Significantly different from the control group ($p < 0.001$). **Significantly different from the acetaminophen-treated group ($p < 0.01$). ***Significantly different from the acetaminophen-treated group ($p < 0.001$)

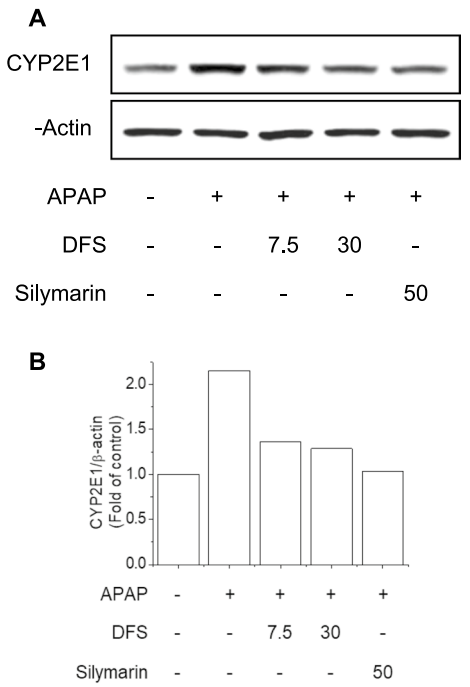

Fig. 2 Preventive effect of DFS on acetaminophen-induced hepatic CYP2E1 expression in mice. **A** The hepatic protein level of CYP2E1 and β-actin was determined by western blot. **B** Protein expression was evaluated using ImageJ software. The relative expression of target protein was compared using β-actin as a control

antibodies for western blot is summarized in Additional file 1: Table S3.

Statistical analysis

The results are presented as the mean ± standard deviation. Statistical analyses were performed using GraphPad InStat (GraphPad Software Inc., San Diego, CA, USA) using a one-way of variance (ANOVA) as indicated. Statistical significance was evaluated using the Tukey–Kramer test by ANOVA with $p < 0.01$ and $p < 0.001$ as the levels of significance.

Results and discussion

DFS administration prevented acetaminophen-induced ALI by suppressing CYP2E1 expression

Hepatitis, alcohol, obesity, and drugs are known causes of liver damage. Among these, excessive drug use is the most frequent cause of liver damage. Drug-induced hepatotoxicity is the major cause of acute liver damage, and an overdose of acetaminophen has been frequently reported [16]. Acetaminophen overdose is known to cause liver inflammation, hepatocellular damage, and liver failure. In fact, liver dysfunction due to acetaminophen overdose is the most well-known cause of drug-induced liver damage worldwide [17] thus, it is important to prevent drug-induced acute liver injuries. Therefore, we first evaluated the hepatopreventive effects of DFS on acetaminophen-induced hepatotoxicity in mice.

The indicators of acetaminophen-induced hepatotoxicity are shown in Fig. 1A–D. Serum alanine aminotransferase/aspartate aminotransferase (ALT/AST) activity levels and hepatic malondialdehyde (MDA) formation increased, whereas hepatic GSH content decreased by acetaminophen. Furthermore, representative images from each group were evaluated based on the degree of hepatocellular damages caused by acetaminophen in histopathological observations stained with hematoxylin and eosin (Fig. 1E). Acetaminophen-treated group was observed serious hepatocellular damages around the central venous areas compared with the control group. However, the administration of DFS reduced hepatocellular injury in a dose-dependent manner.

CYP2E1 plays an important role in the biological activation of toxic substances that cause liver damages by converting acetaminophen to NAPQI [18, 19]. Acetaminophen-induced ALI is initiated by NAPQI, a metabolite formed by the drug-metabolizing enzyme CYP2E1. NAPQI is a toxic metabolite that reacts with GSH and is safely excreted. When GSH content is depleted, excess free NAPQI reacts with in tracellular proteins to induce cell disturbance, which gradually induces liver dysfunction, hepatocyte damage, and acute liver injuries [4]. In this study, we observed that hepatic CYP2E1 expression was inhibited in mice that were pre-administered with DFS in a dose dependent manner (Fig. 2), suggesting that DFS has preventive effects on acetaminophen-induced hepatotoxicity through suppression of CYP2E1 expression.

DFS administration decreased acetaminophen-induced pro-inflammatory cytokine expression by suppressing NF-κB activation

Overproduction of pro-inflammatory cytokines is a symptom of hepatic injury, and an inflammatory response

(See figure on next page.)

Fig. 3 Preventive effect of DFS on acetaminophen-induced hepatic pro-inflammatory cytokine expression levels and NF-κB activation in mice. The hepatic mRNA expression levels of **A** TNF-α, **B** IL-1β, and **C** IL-6 was determined by quantitative PCR. **D** The hepatic protein expression level of phospho-NF-κB p65, NF-κB p65, phospho-IκBα, IκBα, and β-actin was determined by western blot. (E) Protein expression was evaluated using ImageJ software. The relative expression of target protein was compared using β-actin as a control. The results are presented as the means ± SD ($n = 6$). ### Significantly different from the control group ($p < 0.001$). *** Significantly different from the acetaminophen-treated group ($p < 0.001$)

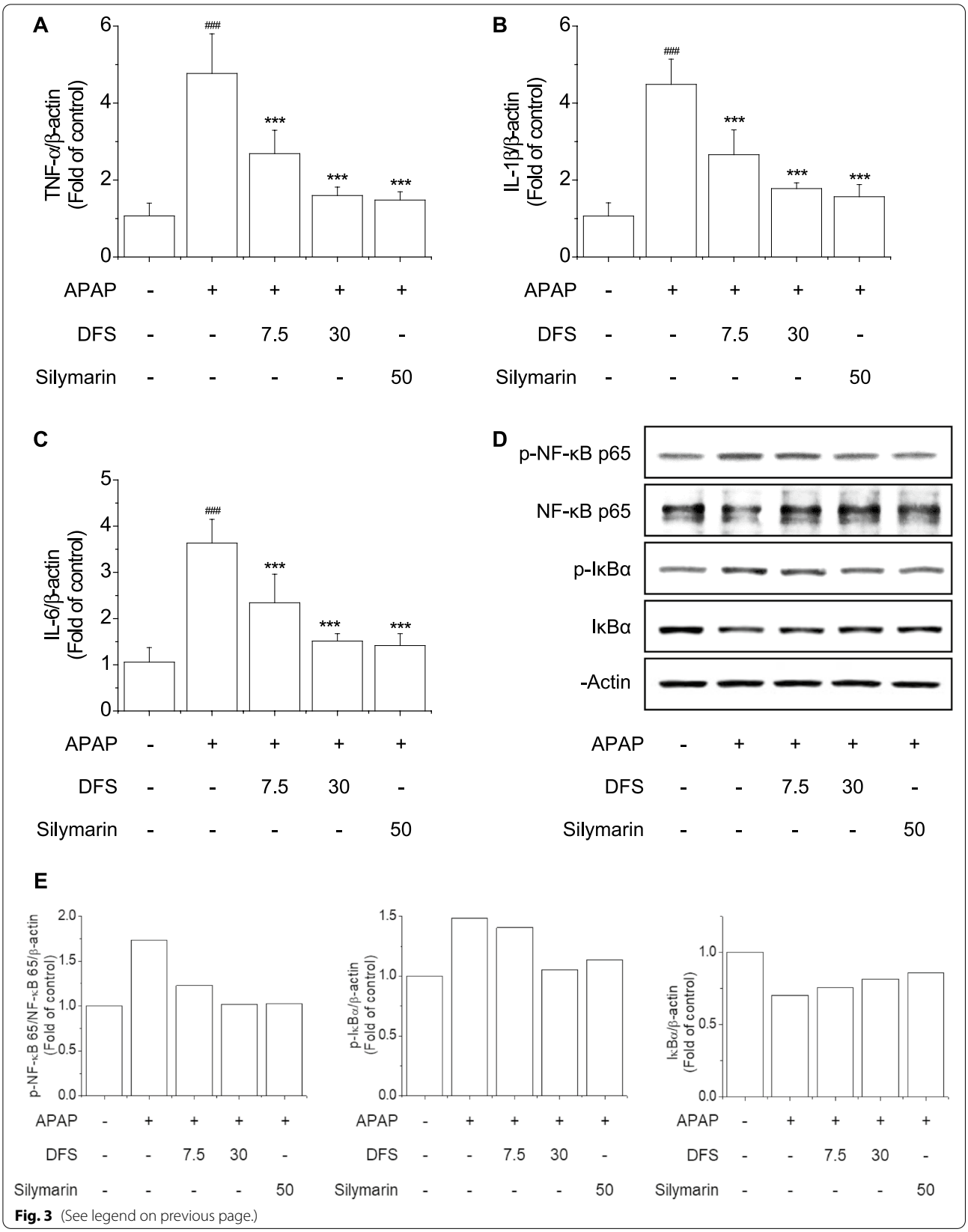

Fig. 3 (See legend on previous page.)

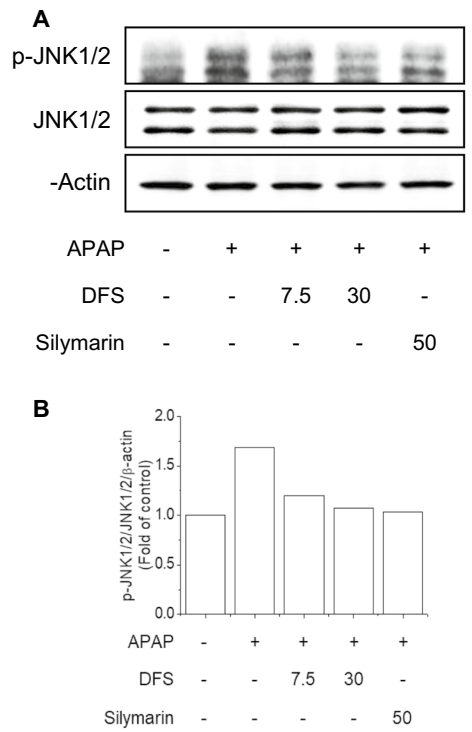

Fig. 4 Preventive effect of DFS on acetaminophen-induced hepatic JNK1/2 activation in mice. **A** The hepatic protein level of phospho-JNK1/2, JNK1/2, and β-actin was determined by western blot. **B** Protein expression was evaluated using ImageJ software. The relative expression of target protein was compared using β-actin as a control

is related to the pathogenesis of acetaminophen-induced hepatotoxicity [3, 20]. In particular, the increased expression of pro-inflammatory cytokines is due to the generation of ROS by NAPQI, causing an inflammatory response in the liver [21]. Moreover, ROS are known to induce the activity of NF-κB, a transcriptional regulator of pro-inflammatory cytokine expression [22]. In this study, we evaluated the preventive effect of DFS on acetaminophen-induced pro-inflammatory cytokine expression using quantitative PCR (qPCR). As shown in Fig. 3A, B, C, the acetaminophen-induced pro-inflammatory cytokine expression level was decreased upon DFS administration in a dose-dependent manner. Moreover, acetaminophen-induced phosphorylation of NF-κB p65 and IκBα was also decreased upon DFS administration in a dose-dependent manner (Fig. 3D). Additionally, DFS administration restored the degradation of IκBα, reduced by acetaminophen, in a dose-dependent manner. These results suggest that the inhibition of NF-κB activity by DFS administration is closely related to the suppression of pro-inflammatory cytokine expression. NF-κB is known to regulate the inflammatory response and increase the expression of related target genes in liver tissue damaged by drugs [23, 24]. A previous study has reported that inflammatory responses mediated by NF-κB are associated with the pathogenesis of acute liver injuries caused by acetaminophen overdose [25]. Here, we demonstrated that DFS administration down-regulated the expression of pro-inflammatory cytokines by inhibiting NF-κB activation, suggesting that DFS may prevent acetaminophen-induced ALI by inhibiting the NF-κB pathway.

DFS administration decreased acetaminophen-induced hepatotoxicity by suppressing JNK1/2 pathway

NAPQI induces oxidative stress by mitochondrial ROS generation, which increases hepatic injury by increasing the continuous activation of JNK1/2 [26, 27]. In addition, an overdose of acetaminophen activates JNK1/2, which induces an inflammatory response by inducing hepatocellular death [28]. In this study, we examined the preventive effect of DFS on acetaminophen-induced JNK1/2 phosphorylation by western blot analysis. Figure 4 shows that the acetaminophen-induced phosphorylation of JNK1/2 was inhibited by DFS administration in a dose-dependent manner. Moreover, it has been reported that certain herbs improve the inhibitory pathway of JNK1/2, which significantly attenuates ALI induced by acetaminophen overdose [29, 30]. These results suggest that the administration of DFS reduced the inflammatory response by inhibiting the activation of both JNK1/2 and NF-κB, which ultimately prevented acetaminophen-induced ALI.

Conclusion

Many studies have reported that natural products, such as extracts of various marine biological resources, protect the liver in the early stages of ALI caused by acetaminophen. However, few studies have reported the hepatoprotective effects of diatom extracts. To our knowledge, no studies have reported the hepatoprotective effects of Diatom Frustule Silica (DFS) extracted from *Melosira nummuloides*. In this study, we demonstrated the preventive effects of DFS on acetaminophen-induced ALI. In conclusion, we suggest that DFS administration suppresses acetaminophen-induced hepatotoxicity and hepatic inflammation and ultimately ameliorates ALI.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13765-022-00741-7.

Additional file 1: Table S1. The information of evaluation kits for hepatotoxicity. **Table S2.** The information of antibodies for western blot. **Table S3.** Relative abundance of minerals observed in DFS used in this study.

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Author contributions
JHC conducted experiments and wrote draft of the manuscript, GMG and TU designed the study and edited manuscript, TU received the fund for the study. All authors read and approved the final manuscript.

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Data availability
All data are included within the manuscript.

Declarations

Ethics approval and consent to participate
All experimental protocols were approved by the rules and regulations of the Animal Ethics Committee of Jeju National University (The IACUC of Jeju National University; Approval number 2021–0029).

Competing interests
The authors declare that there is no conflict of interest regarding the publication of this article.

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