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Inhibition of Oxidative Stress and Inflammation by Fisetin Ameliorates Heat Stress-Induced Intestinal Injury in Rats

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Key words
Fisetin, Heat stress, Inflammation, Intestine, Oxidative stress

ABSTRACT

Intestinal injury and dysfunctions play an important role in the pathophysiology of heat stress. The objective of this study was to determine whether fisetin could ameliorate heat stress-induced intestinal oxidative stress and inflammation, and explore the possible mechanisms at transcriptional levels. Twenty-four male Sprague-Dawley rats aged 8 weeks were randomized to 3 groups, namely, control, heat stress, and heat stress + fisetin (HS-FIS). The experiment lasted for 3 days with daily 1.5 h of heat treatment (40°C) for the heat stress and HS-FIS groups. Rats of the HS-FIS group were orally given 100 mg fisetin /kg body weight/day before the heat treatment. The results showed that fisetin restored the heat stress-induced jejunum morphological damage and increased intestinal permeability, which may be attributed to the improved redox status, the decreased myeloperoxidase activity, the suppressed toll-like receptor 4 signaling pathway mediated expression of pro-inflammatory cytokine tumor necrosis factor alpha at translational and transcriptional level, and the increased gene expression of interleukin 10 in the jejunum. In conclusion, fisetin alleviated the intestinal injury in rats caused by heat stress through inhibiting of oxidative stress and inflammation. This may offer a useful nutritional strategy for improving the health status of individuals exposed to heat stress.

INTRODUCTION

With climate change and global warming, the adverse effects of heat stress (HS) on human and animal health are becoming serious. The HS causes damage to multiple organs and high rate of mortality. Individuals exposed to HS are vulnerable to intestinal injury and dysfunction, as indicated by morphological alteration (He *et al*., 2015), reduced absorption and digestion of nutrients (Song *et al*., 2018; Wu *et al*., 2021), enhanced oxidative stress (Yun *et al*., 2012), over activation of inflammation and increased paracellular permeability (Song *et al*., 2017). Importantly, intestinal injury and dysfunctions play a pivotal role in the pathophysiology of HS, which were observed in clinical studies (Snipe, 2019) and animal models (Ye *et al*., 2019). Therefore, the search for preventive or/and therapeutic strategies that could alleviate the adverse effects of HS on intestine is a main concern.

Fisetin (FIS, 3, 3′, 4′, 7-tetrahydroxyflavone, Fig. 1) is a dietary flavonoid and can be found in many fruits (e.g., strawberries and apples), vegetables (e.g., tomatoes and onions), nuts and wine (Pal *et al*., 2016). In addition, it is also widely present in various acacias trees and shrubs (Pal *et al*., 2016). Substantial amount of evidence exists in the literature to indicate that FIS is capable of preventing or/and treating various diseases associated with inflammation and oxidative stress, such as diabetes (Kim *et al*., 2012; Prasath and Subramanian, 2013), obesity (Shi *et al*., 2018), atherosclerosis (Lian *et al*., 2008) and cancers (Seo and Jeong, 2015; Kashyap *et al*., 2018). Hepatoprotective, neuroprotective and cardioprotective roles of FIS have been demonstrated in different vitro and animal models (Maher *et al*., 2011; Prasath and Subramanian, 2013; Currais *et al*., 2014; Kwak *et al*., 2014; Yonesaka *et al*., 2014). In recent research, Sahu *et al*. (2016) reported that

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oral FIS administration elevated glutathione (GSH) level, suppressed the infiltration of inflammatory cells, production of pro-inflammatory cytokines (e.g., tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, and IL-1β), reactive oxygen species (ROS) and reactive nitrogen species in the colon tissues of colitis mice exposed to dextran sulphate sodium. However, data related to FIS modulation of intestinal health are limited. We hypothesized that FIS could attenuate the HS-induced intestinal damage in rats due to its excellent antioxidant and anti-inflammatory properties. In the present study, the beneficial effects of FIS on intestinal morphology, oxidative and immune status in heat-stressed rats, as well as the possible mechanisms at transcriptional levels were explored.

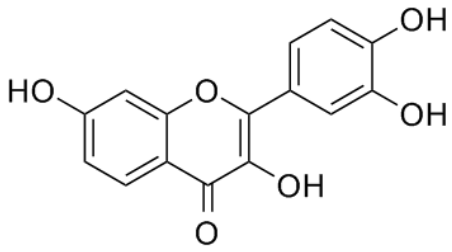

Fig. 1. Structure of fisetin.

MATERIALS AND METHODS

Animals and treatments

Male Sprague-Dawley rats, aged 8 weeks, weighing 200±20 g, were acclimated to the environment (temperature, 20-24 °C; humidity, 40-60%; 12 h light/dark cycle) for 1 week. During the entire experimental period including acclimation, rats were provided with tap water and standard chow diet *ad libitum* under the normal condition. And then, rats were allocated into 3 groups (n=8): (1) the control (CON) group: rats were fed with 0.5% carboxymethylcellulose sodium (CMC-Na, diluted in 0.86% normal saline; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) by oral gavage administration for 3 days; (2) the HS group: rats were fed with 0.5% CMC-Na by oral gavage administration for 3 days under HS environment (1.5 h per day at 40 °C from 11:30 am to 1:00 pm for 3 consecutive days); and (3) the HS-FIS group: rats were fed with 100 mg FIS/kg body weight/day (purity 98%; diluted in 0.5% CMC-Na; Yuanye Biotechnology Co. Ltd, Shanghai, China) by oral gavage administration for 3 days under HS environment. The CMC-Na or FIS were provided for 3 consecutive days at 2 h before HS treatment. The FIS dose in the present study was selected according Lee *et al*. (2015).

Sample collection

After heat treatment for third day, all rats were anesthetized and sacrificed quickly. Blood was collected through eyeball of each rat and centrifuged at 2000 g (15 min, 4 °C) to harvest serum. The serum was stored at -80 °C until subsequent analysis. The procedure of jejunum sample collection was performed according to the method of Lu *et al*. (2011). Part of the jejunum was fixed in 4% buffered paraformaldehyde for histological analysis, and another part was immediately snap-frozen in liquid nitrogen for further analysis.

Diamine oxidase (DAO) activity

The activity of DAO (catalog No. A088-1) in the serum was determined using a commercial kit purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Histology analysis

The fixed jejunum sample was dehydrated and embedded in paraffin. Five-μm sections were cut and then stained with hematoxylin and eosin (HE). Ten well-oriented, intact villi and their associated crypts per rat were selected, and images were recorded using an optical binocular microscope (Olympus BX5; Olympus Optical Co. Ltd, Tokyo, Japan) equipped with a digital camera (Nikon H550L; Nikon, Tokyo, Japan). Measurements of the villus length, crypt depth, and villus width of the jejunum were detected using the Image-Pro Plus software (version 6.0, Media Cybernetics, Inc., Rockville, MD, USA). According to the method described in the previous study (Dong *et al*., 2014), the villus: crypt ratio and villus surface area were calculated.

Oxidative status assay

As described in the previous study (Cheng *et al*., 2017), the jejunal malondialdehyde (MDA, catalog No. A003-1) concentration, total superoxide dismutase (T-SOD, catalog No. A001-1) and glutathione peroxidase (GPX, catalog No. A005) activities, as well as total antioxidant capacity (T-AOC, catalog No. A015-1) and GSH (catalog No. A006-2) levels were determined using assay kits according to the guidelines of manufacturer (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). All results were normalized to total protein concentration in each sample for inter-sample comparison. The jejunal total protein concentration was determined according to the guidelines of the manufacturer (catalog No. A045-3, Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Table I. Primer sequences used for qRT-PCR.

Gene	Gene bank ID	Primer sequence, sense/antisense	Length (bp)
Villin	NM_001108224.2	GGCTTGGGATCCCTTCAAGT	107
		GGCTCATAACCTCATCAGCAA	
MMP3	NM_133523.3	AATCCCTCTATGGACCTCCC	185
		GGTCCTGAGAGATTTTCGCCA	
I-FABP	NM_013068.1	CGGCGTCGACTTTGCCTAT	173
		CTCCTTCATATGTGTAGGTTTGGAT	
HSP70	NM_153629.1	TCAGAGCTGCTATGTCGCTG	73
		GCAGCGGTCGCTATACTCAT	
TNF-α	NM_012675.3	AACACACGAGACGCTGAAGT	93
		TCCAGTGAGTTCCGAAAGCC	
IFN-γ	NM_138880.2	ATCCATGAGTGCTACACGCC	197
		TCGTGTTACCGTCCTTTTGC	
IL6	NM_012589.2	ACAAGTCCGGAGAGGAGACT	172
		TTCTGACAGTGCATCATCGC	
IL10	NM_012854.2	TGCGACGCTGTCATCGATTT	186
		GTAGATGCCGGGTGGTTCAA	
TLR4	NM_019178.1	TCCACAAGAGCCGGAAAGTT	126
		TGAAGATGATGCCAGAGCGG	
SOD1	NM_017050.1	GCATGGGTTCCATGTCCATC	127
		CAGGTCTCCAACATGCCTCTC	
GPX1	NM_030826.4	GCTCACCCGCTCTTTACCTT	162
		TGGAACACCGTCTGGACCTA	
Nrf2	NM_031789.2	TTTGTAGATGACCATGAGTCGC	142
		TGTCCTGCTGTATGCTGCTT	
Keap1	NM_057152.2	TGTGCTGCATGTGATGAACG	198
		AAGAACTCCTCCTCCCCGAA	
β-actin	NM_031144.3	GCAGGAGTACGATGAGTCCG	74
		ACGCAGCTCAGTAACAGTCC	

GPX1, glutathione peroxidase 1; *HSP70*, heat shock protein 70; *I-FABP*, intestinal fatty acid-binding protein; *IFN-γ*, interferon γ; *IL6*, interleukin 6; *IL10*, interleukin 10; *MMP3*, matrix metalloproteinase 3; *Nrf2*, nuclear factor, erythroid 2-like 2; *SOD1,* superoxide dismutase 1; *TLR4*, toll-like receptor 4; *TNF-α*, tumor necrosis factor alpha; *Keap1*, Kelch-like ECH-associated protein 1; *β-actin*, beta actin.

Cytokine assays by ELISA

A commercial ELISA kit (catalog No. EK382/3-96) purchased from Multisciences Biotech Co., Ltd (Hangzhou, China) was employed to analyze the jejunal TNF-αconcentration following the manufacturer's instructions. The detection limit was 0.43 pg/mL; the inter- and intra-assay coefficients of variation were less than 7% and 9%, respectively. All results were normalized to total protein concentration in each sample for inter-sample comparison. The jejunal total protein concentration was determined according to the guidelines of the manufacturer (catalog No. A045-3, Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Myeloperoxidase (MPO) activity assay

The MPO activity (catalog No. A044) and total protein concentration (catalog No. A045-3) in the jejunum were analyzed using a commercial kit following the instructions of the manufacturer (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). All results were normalized to total protein concentration in each sample for inter-sample comparison.

Table II. Effects of fisetin on the jejunal morphology in heat-stressed rats.

Item	CON	HS	HS-FIS
Villus height, μm	199.18±3.16	159.72±5.78*	207.47±12.23#
Crypt depth, μm	70.62±3.34	79.92±4.74	73.99±3.40
Villus height/crypt depth, μm/μm	2.86±0.14	2.02±0.08*	2.82±0.15#
Villus width, μm	45.51±3.25	40.79±3.80	53.22±1.49#
Villus surface area, $\times 10^3 \mu m^2$	14.34±1.10	10.42±1.20	17.55±1.31#

CON, rats were orally fed with 0.5% carboxymethylcellulose sodium; HS, rats were orally fed with 0.5% carboxymethylcellulose sodium and then subjected to heat treatment; HS-FIS, rats were orally fed with 100 mg FIS /kg body weight/day and then subjected to heat treatment. Results are expressed as mean and standard error (n = 6). *P<0.05 was compared with the CON group; #P<0.05 was compared with the HS group.

Table III. Effects of fisetin on the jejunal redox status in heat-stressed rats.

Item	CON	HS	HS-FIS
MDA, nmol/mg protein	0.35±0.02	0.43±0.03*	0.28±0.01#
GPX, U/mg protein	99.96±12.37	155.55±11.84*	98.28±12.31#
T-SOD, U/mg protein	120.93±4.26	235.61±5.25*	115.38±4.63#
GSH, mg/g protein	5.26±1.15	4.22±0.81	4.78±0.95
T-AOC, U/mg protein	1.26±0.06	1.12±0.04	1.22±0.08

MDA, malondialdehyde (n=6); GPX, glutathione peroxidase (n=5); T-SOD, total superoxide dismutase (n=8); T-AOC, total antioxidant capacity (n=8); CON, rats were orally fed with 0.5% carboxymethylcellulose sodium; HS, rats were orally fed with 0.5% carboxymethylcellulose sodium and then subjected to heat treatment; HS-FIS, rats were orally fed with 100 mg FIS /kg body weight/day t and hen subjected to heat treatment. Results are expressed as mean and standard error. *P<0.05 was compared with the CON group; #P<0.05 was compared with the HS group.

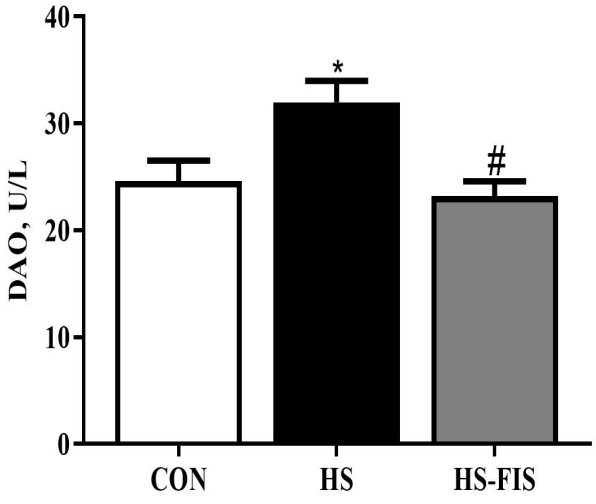

Fig. 2. Effects of fisetin on the jejunal diamine oxidase (DAO) activity in heat-stressed rats. CON, rats were orally fed with 0.5% carboxymethylcellulose sodium; HS, rats were orally fed with 0.5% carboxymethylcellulose sodium and then subjected to heat treatment; HS-FIS, rats were orally fed with 100 mg FIS /kg body weight/day and then subjected to heat treatment. Results are expressed as mean and standard error (n=8). *P<0.05 was compared with the CON group; #P<0.05 was compared with the HS group.

Quantitative RT-PCR analysis

Total RNA from liver samples were extracted using TRIzol Reagent (TaKaRa, Dalian, China) according to the guidelines of the manufacturer. The integrity, concentration, and purity of RNA, reverse transcription, as well as qRT-PCR were performed according to the previous studies (Cheng *et al*., 2016, 2018). The primer sequences of genes used in this study are presented in Table I. The target genes expression levels were normalized by the housekeeping gene β-actin, and then were calculated via the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The values of CON group were used as a calibrator.

Statistical analysis

Results are expressed as mean and standard error and analyzed by SPSS 17.0. The individual rat was used as the experimental unit. Statistical differences between different groups were determined via one-way analysis of variance (ANOVA) and Tukey's post hoc test for multiple comparisons. Significant difference was accepted at $P < 0.05$.

RESULTS

FIS reduces the severity of intestinal injury in rats subjected to HS

In Figure 2, the DAO activity in the serum was

significantly higher in rats exposed to HS compared with the CON group (P<0.05). Also, the jejunal matrix metalloproteinase 3 (*MMP3*, P=0.079) and heat shock protein 70 (*HSP70*, P<0.05) genes expression were increased in the HS group compared with the CON group (Fig. 3A). Rats in the HS group exhibited jejunal villus atrophy and shedding (Fig. 4). The jejunal villus height and villus height: crypt depth ratio were significantly lower (Table II, P<0.05) in rats exposed to HS compared with the CON group. Administration of FIS to rats exposed to HS significantly decreased (P<0.05) the serum DAO activity and the jejunal *HSP70* gene expression, improved jejunum morphology, increased (P<0.05) jejunal villus height, villus surface area, villus width and villus height: Crypt depth ratio in the HS-FIS group compared with the HS group (P<0.05). However, crypt depth, the *IFABP* and *villin* mRNA expression in the jejunum of rats were not affected among the 3 groups (P>0.05).

FIS attenuates the jejunal oxidative stress in rats subjected to HS

The higher MDA content, T-SOD activity, GPX activity in the jejunum of heat-stressed rats were reduced by FIS administration (Table III, P<0.05). However, the T-AOC and GSH levels in the jejunum were comparable among the 3 groups (P>0.05). At transcriptional level, administration of FIS to rats exposed to HS alleviated the increased nuclear factor, erythroid 2-like 2 (*Nrf2*) and *GPX1* expression compared with the HS group (Fig. 3B, P<0.05). The genes expression of *SOD1* and Kelch-like ECH-associated protein 1 (*Keap1*) in the jejunum were not influenced by HS and FIS treatment (P>0.05).

FIS relieves the jejunal inflammation in rats subjected to HS

The TNF-α concentration was higher in the jejunum of the HS group compared with the CON group (Fig. 5A, P<0.05). However, FIS treatment to heat-stressed rats caused decreases in jejunal TNF-α concentration and MPO activity (Fig. 5B) compared with the HS group (P<0.05). At transcriptional level, rats in the HS group exhibited higher jejunal *TNF-α*, *IL10* and toll-like receptor 4 (*TLR4*) mRNA expression compared with the CON group (Fig. 3C, P<0.05). Expectedly, the increased *IL10*, decreased *TNF-α* and *TLR4* genes expression were observed in the jejunum of the HS-FIS group compared with the HS group (P < 0.05). The jejunal *IL6* and interferon γ (*IFN-γ*) genes expression in rats were not affected (P > 0.05) among the 3 groups.

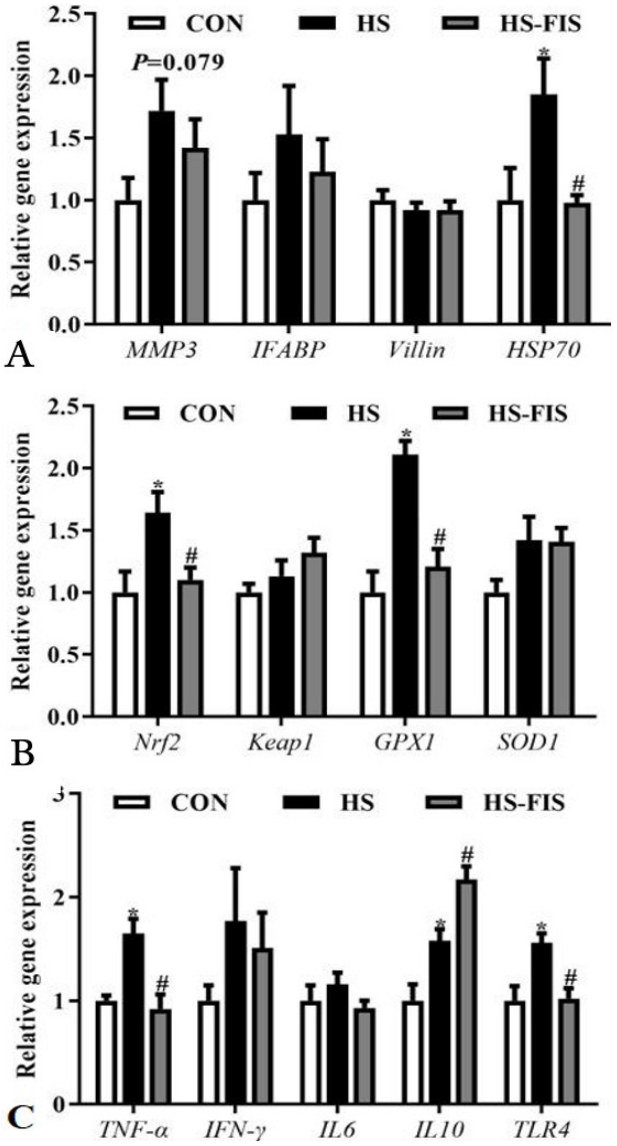

Fig. 3. Effects of fisetin on the genes expression related to jejunal injury markers (A), redox status (B) and inlammation (C) in heat-stressed rats. *HSP70*, heat shock protein 70; *I-FABP*, intestinal fatty acid-binding protein; *MMP3*, matrix metalloproteinase 3; *GPX1*, glutathione peroxidase 1; *Nrf2*, nuclear factor, erythroid 2-like 2; *SOD1*, superoxide dismutase 1; *Keap1*, Kelch-like ECH-associated protein 1; *IL6*, interleukin 6; *IL10*, interleukin 10; *TLR4*, toll-like receptor 4; *TNF-α*, tumor necrosis factor alpha; *IFN-γ*, interferon γ; CON, rats were orally fed with 0.5% carboxymethylcellulose sodium; HS, rats were orally fed with 0.5% carboxymethylcellulose sodium and then subjected to heat treatment; HS-FIS, rats were orally fed with 100 mg FIS /kg body weight/day and then subjected to heat treatment. Results are expressed as mean and standard error (n=8). $^{*}P$<0.05 was compared with the CON group; $^{\#}P$<0.05 was compared with the HS group.

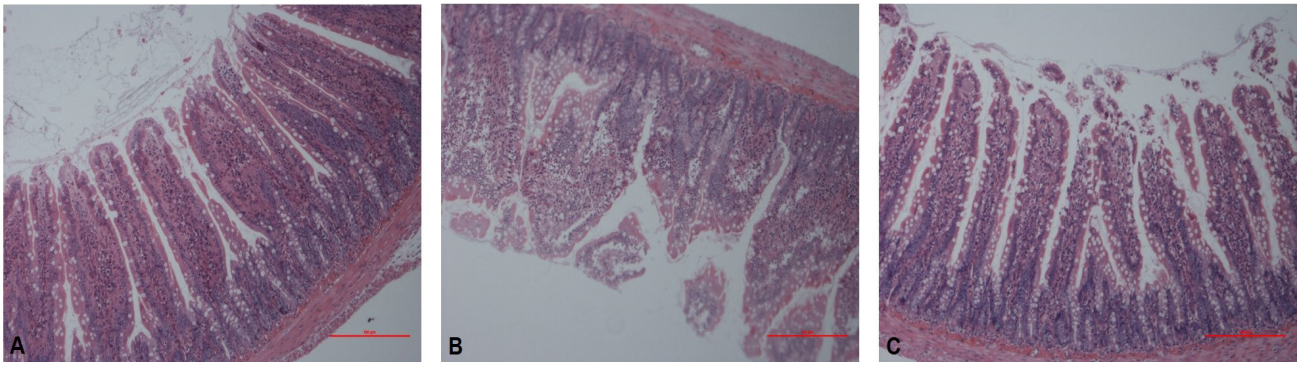

Fig. 4. The jejunal histological appearance (hematoxylin and eosin). (A) CON, rats were orally fed with 0.5% carboxymethylcellulose sodium; (B) HS, rats were orally fed with 0.5% carboxymethylcellulose sodium and then subjected to heat treatment; (C) HS-FIS, rats were orally fed with 100 mg FIS /kg body weight/day and then subjected to heat treatment. Original magnification 100 ×, Scale bars = 100 μm.

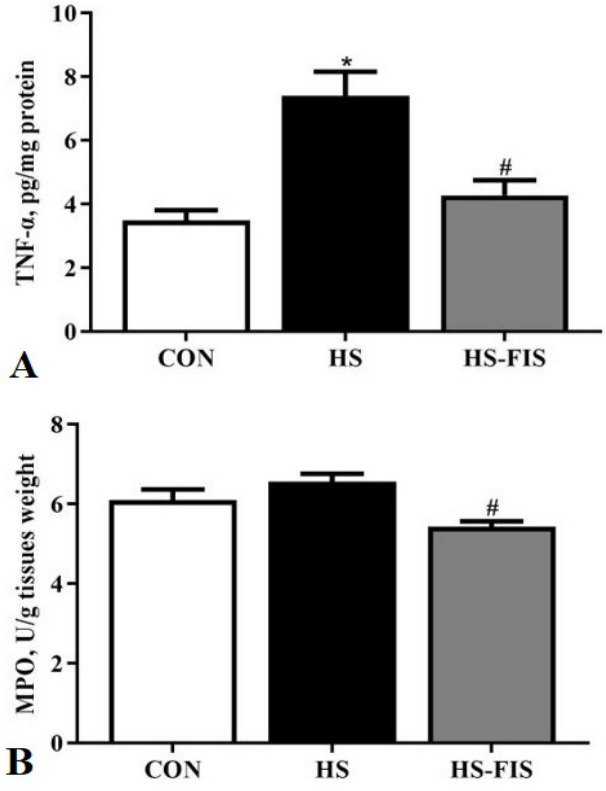

Fig. 5. Effects of fisetin on the jejunal tumor necrosis factor alpha (TNF-α) level (A) and myeloperoxidase (MPO) activity (B) in heat-stressed rats. CON, rats were orally fed with 0.5% carboxymethylcellulose sodium; HS, rats were orally fed with 0.5% carboxymethylcellulose sodium and then subjected to heat treatment; HS-FIS, rats were orally fed with 100 mg FIS /kg body weight/day and then subjected to heat treatment. Results are expressed as mean and standard error (n=6). $^{*}P<0.05$ was compared with the CON group; $^{\#}P<0.05$ was compared with the HS group.

DISCUSSION

In the present study, the effects of oral FIS administration on the jejunum of rats subjected to HS were investigated for the first time. As expected, FIS relieved the heat stress-induced intestinal damage demonstrated by the decreased serum DAO activity, improved histologic structure, inhibited oxidative stress and inflammation. In this study, the beneficial effects of FIS on intestine of heat-stressed rats may be attributed to the decreased gene expression of *TLR4*, reduced TNF-α expression, increased *IL10* gene expression, and suppressed MPO activity.

The intestine is one of the first and more susceptible organs negatively affected by hyperthermia challenges due to the fact that animals redistribute blood to the periphery to maximize radiant heat dissipation (Pearce *et al*., 2014). When exposed to HS, the synthesis of most proteins is delayed, but HSP is rapidly synthesized (Al-Aqil and Zulkifli, 2009). Among the HSP, HSP70 is the most conserved and most common family, which is abundant in various tissues in most organism. There is ample evidence that the transcription of HSP70 is rapidly induced by high temperature (Tedeschi *et al*., 2015; Song *et al*., 2017; Cheng *et al*., 2019). Therefore, the expression of HSP70 in intestine is a reliable biomarker for measuring a thermotolerance response. Similarly, in this study, heat exposure led to an increase in the jejunual HSP70 mRNA expression. As expected, FIS restored the heat stress-induced upregulated jejunal *HSP70* gene expression, suggesting that FIS reduced the heat responses of rats exposed to high temperature. Similar results observed in scrotal hyperthermia model showed that administration of FIS decreased the gene expression of HSP72 (Pirani *et al*., 2021).

Heat stress results in the increased intestinal permeability in animals. The serum DAO activity is

recognized as a sensitive marker for monitoring the alteration of intestinal barrier permeability (Song *et al*., 2017; Cheng *et al*., 2019). In the present study, the serum DAO activity was increased during HS, suggesting that the intestinal barrier function was compromised. In addition, MMP3 expression was analyzed as an indicator of intestinal damage, as has been reported by other authors (Wu *et al*., 2018; Yi *et al*., 2018), results in this study showed that this parameter was affected by HS, which further confirmed heat stress-induced intestinal injury. As expected, FIS treatment could attenuate the intestinal morphologic damage of heat-stressed rats as indicated by the increased villus height, villus width, the ratio of villus height to crypt depth and villous surface area. Meantime, FIS significantly decreased circulating DAO activity in response to HS exposure in this study. Thus, our results showed that FIS could be used as a potential regulator in improving intestinal morphologic damage and permeability of rats under HS.

Emerging evidence revealed that an increase in the generation of ROS such as superoxide anions, hydrogen peroxide and hydroxyl radicals was observed in individuals exposed to HS, which eventually led to intestinal damage (Yun *et al*., 2012). As it is known, the enzymatic antioxidant defense against excessive ROS has an important function on maintaining redox homeostasis; SOD catalyzes superoxide radicals to molecular oxygen and hydrogen peroxide, which is decomposed by CAT and GPX to harmless compounds such as water and oxygen. However, the overwhelming ROS will harm DNA, proteins and lipids, even leading to cellular injury and death. The results in our study presented that HS induced increases in the activities of GPX and T-SOD; nevertheless, these increases were shown to be inadequate to counteract the oxidative damage in the intestine of rats as indicated by the increased MDA concentration, which supported the findings of Cheng *et al*. (2019). In addition, Nrf2 and its target antioxidant enzyme GPX genes expression were upregulated in the intestine of heat-stressed rats, which may explain the increased GPX activity. Accumulating studies have confirmed that HS can result in the increased adaption of Nrf2 and its target antioxidants genes (Zhang *et al*., 2002; Bhusari *et al*., 2008). However, the decreased T-SOD activity in the jejunum of heat-stressed rats was not parallel with its gene expression, which need to be further investigated. In this study, FIS administration alleviated the increased GPX and T-SOD activities, MDA content, Nrf2 and GPX mRNA expression, suggesting that FIS can attenuate intestinal oxidative damage induced by HS. Our data in rats are consistent with the experiment in HS-induced oxidative stress broilers, in which FIS supplementation improved the circulating redox status (Ogbuagu *et al*., 2018). The beneficial effects of FIS on redox status of heat-stressed rats in the present study were attributed to its hydroxyl groups and anti-inflammation property rather than enhancing antioxidant defense systems.

Previous studies have shown that the overproduction of pro-inflammatory cytokines such as TNF-α induced by HS contributed significantly to the intestine tissues necrosis and dysfunction (Cheng *et al*., 2019). Similarly, results observed in our study also demonstrated that the protein and mRNA levels of jejunal TNF-α were increased in heat-stressed rats, which may be attributed to the up-regulation of TLR4 gene expression. The TLR4, a well-known pattern recognition receptor, simulation of which triggers the biosynthesis and release of inflammatory cytokines, including IL6 and TNF-α (Shi *et al*., 2006). Our results were in accordance with the previous studies in different animal models such as broilers (Song *et al*., 2017), mice (Mohyuddin *et al*., 2021) and rats (Cheng *et al*., 2019) in which HS increased TLR4 mRNA abundance and its targeted inflammatory cytokines production in intestine. In addition, in the present study, the upregulated expression of jejunal IL10 mRNA during heat exposure may be due to the fact that heat stress-induced inflammation and the cells undergoing inflammation will respond by producing anti-inflammatory cytokines. These results in this study further supported the notion that heat stress-induced jejunal inflammation could be due to the increased TLR4 mRNA expression. Expectedly, FIS counteracted the increased TNF-α concentration, and the upregulated genes expression of TLR4 and TNF-α in the jejunum of heat-stressed rats, suggesting that FIS could play a positive role in inhibiting jejunal inflammation. Likewise, FIS has been reported to reduce the colonic the protein expression of pro-inflammatory cytokines, TNF-α, IL6, and IL-1β, in colitis mice subjected to dextran sulphate sodium (Sahu *et al*., 2016). On the other hand, in this work, FIS treatment upregulated the transcription of IL10 gene in the jejunum of rats exposed to HS. The IL10 is considered a more potent inhibitor of many pro-inflammatory cytokines produced by monocytes and dampens many inflammatory responses (Patel and Davidson, 2014). Moreover, in the current study, FIS administration also inhibited the MPO activity in the jejunum of heat-stressed rats. The MPO system of neutrophil plays a critical role in intestinal mucosal inflammation. Additionally, MPO has been implicated as a participant in intestinal damage under many inflammation conditions, which catalyzes the production of cytotoxic oxidant hypochlorous acid (Hampton *et al*., 1998; Nicholls and Hazen, 2005). Hypochlorous acid can react avidly with cellular bio-macromolecules such as proteins, lipids and DNA, which consequently contributes to oxidative

stress and inflammation in tissues including small intestine (Smith, 1994). Thus, the antioxidant property of FIS is partly attributable to the decreased MPO activity. Taken together, the FIS anti-inflammation property probably results from the downregulated TLR4 mRNA expression, upregulated IL10 mRNA abundance and inhibited MPO system of neutrophil.

CONCLUSION

The data obtained from the present study indicates that FIS administration confers protection against heat stress-induced intestinal damage partly by mitigating oxidative stress and inflammation via the downregulated TLR4 mRNA expression, upregulated IL10 mRNA abundance and inhibited MPO system of neutrophil. This study offers a useful nutritional strategy for improving the health status of individuals exposed to HS.

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IRB approval and ethical statement

All procedures involving animals were allowed by the Institutional Animal Care and Use Committee of Henan University of Technology (Zhengzhou, China).

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Al-Aqil, A. and Zulkifli, I., 2009. Changes in heat shock protein 70 expression and blood characteristics in transported broiler chickens as affected by housing and early age feed restriction. *Poult. Sci.*, **88**: 1358-1364. https://doi.org/10.3382/ps.2008-00554

Bhusari, S., Hearne, L.B., Spiers, D.E., Lamberson, W.R. and Antoniou, E., 2008. Transcriptional profiling of mouse liver in response to chronic heat stress. *J. Therm. Biol.*, **33**: 157-167. https://doi.org/10.1016/j.jtherbio.2008.01.001

Cheng, K., Niu, Y., Zheng, X.C., Zhang, H., Chen, Y.P., Zhang, M., Huang, X.X., Zhang, L.L., Zhou, Y.M. and Wang, T., 2016. A comparison of natural (D-α-tocopherol) and synthetic (DL-α-tocopherol acetate) vitamin E supplementation on the growth performance, meat quality and oxidative status of broilers. *Asian-Australas. J. Anim. Sci.,* **29**: 681-688. https://doi.org/10.5713/ajas.15.0819

Cheng, K., Song, Z., Chen, Y., Li, S., Zhang, Y., Zhang, H., Zhang, L., Wang, C. and Wang, T., 2018. Resveratrol protects against renal damage via attenuation of inflammation and oxidative stress in high fat diet induced obese mice. *Inflammation,* **42**: 937-945. https://doi.org/10.1007/s10753-018-0948-7

Cheng, K., Song, Z., Li, S., Yan, E., Zhang, H., Zhang, L., Wang, C. and Wang, T., 2019. Effects of resveratrol on intestinal oxidative status and inflammation in heat-stressed rats. *J. Therm. Biol.,* **85**: 102415. https://doi.org/10.1016/j.jtherbio.2019.102415

Cheng, K., Zhang, M., Huang, X., Zheng, X., Song, Z., Zhang, L. and Wang, T., 2017. An evaluation of natural and synthetic vitamin E supplementation on growth performance and antioxidant capacity of broilers in early age. *Can. J. Anim. Sci.,* **98**: 187-193. https://doi.org/10.1139/CJAS-2017-0040

Currais, A., Prior, M., Dargusch, R., Armando, A., Ehren, J., Schubert, D., Quehenberger, O. and Maher, P., 2014. Modulation of p25 and inflammatory pathways by fisetin maintains cognitive function in Alzheimer's disease transgenic mice. *Aging Cell,* **13**: 379-390. https://doi.org/10.1111/acel.12185

Dong, L., Zhong, X., Ahmad, H., Li, W., Wang, Y., Zhang, L. and Wang, T., 2014. Intrauterine growth restriction impairs small intestinal mucosal immunity in neonatal piglets. *J. Histochem. Cytochem.,* **62**: 510-518. https://doi.org/10.1369/0022155414532655

Hampton, M.B., Kettle, A.J. and Winterbourn, C.C., 1998. Inside the neutrophil phagosome: Oxidants, myeloperoxidase, and bacterial killing. *Blood*, **92**: 3007-3017. https://doi.org/10.1182/blood.V92.9.3007.421k47_3007_3017

He, S., Hou, X., Xu, X., Wan, C., Yin, P., Liu, X., Chen, Y., Shu, B., Liu, F. and Xu, J., 2015. Quantitative proteomic analysis reveals heat stress-induced injury in rat small intestine via activation of the MAPK and NF-κB signaling pathways. *Mol. Biosyst.,* **11**: 826-834. https://doi.org/10.1039/C4MB00495G

Kashyap, D., Sharma, A., Sak, K., Tuli, H.S., Buttar, H.S. and Bishayee, A., 2018. Fisetin: A bioactive phytochemical with potential for cancer prevention and pharmacotherapy. *Life Sci.,* **194**: 75-87. https://

doi.org/10.1016/j.lfs.2017.12.005

Kim, H.J., Kim, S.H. and Yun, J.M., 2012. Fisetin inhibits hyperglycemia-induced proinflammatory cytokine production by epigenetic mechanisms. *Evid. Based Complement. Altern.,* **2012**: 639469. https://doi.org/10.1155/2012/639469

Kwak, S., Ku, S.K. and Bae, J.S., 2014. Fisetin inhibits high-glucose-induced vascular inflammation *in vitro* and *in vivo*. *Inflamm. Res.,* **63**: 779-787. https://doi.org/10.1007/s00011-014-0750-4

Lee, J.H., Kim, M., Chang, K.H., Hong, C.Y., Na, C.S., Dong, M.S., Lee, D. and Lee, M.Y., 2015. Antiplatelet effects of rhus verniciflua stokes heartwood and its active constituents-fisetin, butein, and sulfuretin-in rats. *J. Med. Fd.,* **18**: 21-30. https://doi.org/10.1089/jmf.2013.3116

Lian, T.W., Wang, L., Lo, Y.H., Huang, I.J. and Wu, M.J., 2008. Fisetin, morin and myricetin attenuate CD36 expression and oxLDL uptake in U937-derived macrophages. *BBA-Mol. Cell Biol. L.,* **1781**: 601-609. https://doi.org/10.1016/j.bbalip.2008.06.009

Livak, K.J. and Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods*, **25**: 402-408. https://doi.org/10.1006/meth.2001.1262

Lu, A., Wang, H., Hou, X., Li, H., Cheng, G., Wang, N., Zhu, X., Yu, J., Luan, W., Liu, F. and Xu, J., 2011. Microarray analysis of gene expression profiles of rat small intestine in response to heat stress. *J. Biomol. Screen.,* **16**: 655-667. https://doi.org/10.1177/1087057111403928

Maher, P., Dargusch, R., Bodai, L., Gerard, P.E., Purcell, J.M. and Marsh, J.L., 2011. ERK activation by the polyphenols fisetin and resveratrol provides neuroprotection in multiple models of Huntington's disease. *Hum. mol. Genet.,* **20**: 261-270. https://doi.org/10.1093/hmg/ddq460

Mohyuddin, S.G., Qamar, A., Hu, C.Y., Li, Y., Chen, S.W., Wen, J.Y., Bao, M.L. and Ju, X.H., 2021. Terpinen4-ol inhibits heat stress induced inflammation in colonic tissue by activating occludin, claudin-2 and TLR4/NF-κB signaling pathway. *Int. Immunopharmacol.,* **99**: 107727. https://doi.org/10.1016/j.intimp.2021.107727

Nicholls, S.J. and Hazen, S.L., 2005. Myeloperoxidase and cardiovascular disease. *Atertio. Thromb. Vasc. Biol.,* **25**: 1102-1111. https://doi.org/10.1161/01.ATV.0000163262.83456.6d

Ogbuagu, N.E., Aluwong, T., Ayo, J.O. and Sumanu, V.O., 2018. Effect of fisetin and probiotic supplementation on erythrocyte osmotic fragility, malondialdehyde concentration and superoxide dismutase activity in broiler chickens exposed to heat stress. *J. Vet. med. Sci.,* **80**: 1895-1900. https://doi.org/10.1292/jvms.18-0477

Pal, H.C., Pearlman, R.L. and Afaq, F., 2016. Fisetin and its role in chronic diseases. *Adv. exp. med. Biol.,* **928**: 213-244. https://doi.org/10.1007/978-3-319-41334-1_10

Patel, H. and Davidson, D., 2014. Control of pro-inflammatory cytokine release from human monocytes with the use of an interleukin-10 monoclonal antibody. *Methods mol. Biol.,* **1172**: 99-106. https://doi.org/10.1007/978-1-4939-0928-5_8

Pearce, S.C., Sanz-Fernandez, M.V., Hollis, J.H., Baumgard, L.H. and Gabler, N.K., 2014. Short-term exposure to heat stress attenuates appetite and intestinal integrity in growing pigs. *J. Anim. Sci.,* **92**: 5444-5454. https://doi.org/10.2527/jas.2014-8407

Pirani, M., Novin, M.G., Abdollahifar, M.A., Piryaei, A., Kuroshli, Z. and Mofarahe, Z.S., 2021. Protective effects of fisetin in the mice induced by long-term scrotal hyperthermia. *Reprod. Sci.,* **28**: 3123-3136. https://doi.org/10.1007/s43032-021-00615-1

Prasath, G.S. and Subramanian, S.P., 2013. Fisetin, a tetra hydroxy flavone recuperates antioxidant status and protects hepatocellular ultrastructure from hyperglycemia mediated oxidative stress in streptozotocin induced experimental diabetes in rats. *Fd. Chem. Toxicol.,* **59**: 249-255. https://doi.org/10.1016/j.fct.2013.05.062

Sahu, B.D., Kumar, J.M. and Sistla, R., 2016. Fisetin, a dietary flavonoid, ameliorates experimental colitis in mice: Relevance of NF-κB signaling. *J. Nutr. Biochem.,* **28**: 171-182. https://doi.org/10.1016/j.jnutbio.2015.10.004

Seo, S.H. and Jeong, G.S., 2015. Fisetin inhibits TNF-α-induced inflammatory action and hydrogen peroxide-induced oxidative damage in human keratinocyte HaCaT cells through PI3K/AKT/Nrf-2-mediated heme oxygenase-1 expression. *Int. Immunopharmacol.,* **29**: 246-253. https://doi.org/10.1016/j.intimp.2015.11.014

Shi, H., Kokoeva, M.V., Inouye, K., Tzameli, I., Yin, H. and Flier, J.S., 2006. TLR4 links innate immunity and fatty acid-induced insulin resistance. *J. clin. Invest.,* **116**: 3015-3025. https://doi.org/10.1172/JCI28898

Shi, Y.S., Li, C.B., Li, X.Y., Wu, J., Li, Y., Fu, X., Zhang, Y. and Hu, W.Z., 2018. Fisetin attenuates metabolic dysfunction in mice challenged with a

high-fructose diet. *J. Agric. Fd. Chem.,* **66**: 8291-8298. https://doi.org/10.1021/acs.jafc.8b02140

Smith, J.A., 1994. Neutrophils, host defense, and inflammation: A double-edged sword. *J. Leukocyte Biol.,* **56**: 672-686. https://doi.org/10.1002/jlb.56.6.672

Snipe, R.M.J., 2019. Exertional heat stress-induced gastrointestinal perturbations: Prevention and management strategies. *Br. J. Sports Med.,* **53**: 1312-1313. https://doi.org/10.1136/bjsports-2018-099660

Song, Z., Cheng, K., Zhang, L. and Wang, T., 2017. Dietary supplementation of enzymatically treated artemisia annua could alleviate the intestinal inflammatory response in heat-stressed broilers. *J. Therm. Biol.,* **69**: 184-190. https://doi.org/10.1016/j.jtherbio.2017.07.015

Song, Z.H., Cheng, K., Zheng, X.C., Ahmad, H., Zhang, L.L. and Wang, T., 2018. Effects of dietary supplementation with enzymatically treated artemisia annua on growth performance, intestinal morphology, digestive enzyme activities, immunity, and antioxidant capacity of heat-stressed broilers. *Poult. Sci.,* **97**: 430-437. https://doi.org/10.3382/ps/pex312

Tedeschi, J.N., Kennington, W.J., Berry, O., Whiting, S., Meekan, M. and Mitchell, N.J., 2015. Increased expression of Hsp70 and Hsp90 mRNA as biomarkers of thermal stress in loggerhead turtle embryos (Caretta Caretta). *J. Therm. Biol.,* **47**: 42-50. https://doi.org/10.1016/j.jtherbio.2014.11.006

Wu, Q.J., Jiao, C., Liu, Z.H., Cheng, B.Y., Liao, J.H., Zhu, D.D., Ma, Y., Li, Y.X. and Li, W., 2021. Effect of glutamine on the growth performance, digestive enzyme activity, absorption function, and mRNA expression of intestinal transporters in heat-stressed chickens. *Res. Vet. Sci.,* **134**: 51-57. https://doi.org/10.1016/j.rvsc.2020.12.002

Wu, T., Lv, Y., Li, X., Zhao, D., Yi, D., Wang, L., Li, P., Chen, H., Hou, Y., Gong, J. and Wu, G., 2018. Establishment of a recombinant *Escherichia coli*-induced piglet diarrhea model. *Front. Biosci.,* **23**: 1517-1534. https://doi.org/10.2741/4658

Ye, N., Yu, T., Guo, H. and Li, J., 2019. Intestinal injury in heat stroke. *J. Emerg. Med.,* **57**: 791-797. https://doi.org/10.1016/j.jemermed.2019.08.033

Yi, D., Liu, W., Hou, Y., Wang, L., Zhao, D., Wu, T., Ding, B. and Wu, G., 2018. Establishment of a porcine model of indomethacin-induced intestinal injury. *Front. Biosci.,* **23**: 2166-2176. https://doi.org/10.2741/4697

Yonesaka, T., Yoshida, K., Iizuka, S. and Hagiwara, H., 2014. Effects of fisetin on mouse lipid metabolism *in vitro* and *in vivo*. *Funct. Fd. Hlth. D.,* **4**: 429-441. https://doi.org/10.31989/ffhd.v4i10.152

Yun, S.H., Moon, Y.S., Sohn, S.H. and Jang, I.S., 2012. Effects of cyclic heat stress or vitamin C supplementation during cyclic heat stress on HSP70, inflammatory cytokines, and the antioxidant defense system in Sprague Dawley rats. *Exp. Anim.,* **61**: 543-553. https://doi.org/10.1538/expanim.61.543

Zhang, H.J., Drake, V.J., Morrison, J.P., Oberley, L.W. and Kregel, K.C., 2002. Selected contribution: Differential expression of stress-related genes with aging and hyperthermia. *J. appl. Physiol.,* **92**: 1762-1769. https://doi.org/10.1152/japplphysiol.00733.2001