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Original article

Zingerone attenuates intestinal injury and colitis caused by a high-fat diet through Nrf2 signaling regulation

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ABSTRACT

This study examined the protective effect of Zingerone against a high-fat diet (HFD)-induced intestinal damage. Control and HFD rats were treated with the vehicle or Zingerone (100 mg/kg, orally) (n = 8 rats/-groups). An extra group, HFD + Zingerone + brusatol (an Nrf2 inhibitor). This study treatment lasted four weeks. Zingerone reduced the nuclear levels of NF-B p65 in control and HFD-fed rats while increasing SOD, CAT, GSH, levels of mRNA, cytoplasmic levels, and Nrf2 nuclear levels. Zingerone treatment attenuated the duodenal epithelial damage and maintained the mucosal barrier by reducing plasma FITC-DX and serum LPS in rats fed with HFD. Concomitantly, it lowered the duodenal MDA, TNF-α, IL-6, and IL-1β levels. These impacts included changes in body weight, duodenal lipid levels, and Keap-1 expression, a natural Nrf2 inhibitor. We concluded that Zingerone reduces HFD-induced duodenal injury. These findings support Zingerone's clinical applicability against various inflammatory diseases of the intestine.

1. Introduction

As the body's largest and most significant organ, the intestine is in charge of immunity, pathogen detection, commensal tolerance, and the digestion and absorption of nutrients (Segrist & Cherry, 2020). The intestine has a robust mucosal firewall to maintain its functionality, consisting of an intact epithelial mucosal barrier, mucus, antibodies, and a range of related immune cells and lymphoid organs (Macpherson et al., 2009). The role of the mucosal barrier in maintaining the health of the intestine is well-documented in the literature, where it prevents the luminal antigen, toxins, and bacteria invasion of the lower mucosal immune cells. Tight and adherent junction proteins such as claudin, occluding, and E-cadherin keep these mucosal barriers intact (Xu et al., 2019). However, disturbing the integrity of this barrier by epithelial cell apoptosis or loss of the junction proteins was associated with a type of intestinal inflammation, including celiac disease, inflammatory bowel disease (IBD), and irritable bowel syndrome (IBS) (McDaniel et al., 2016; Merga et al., 2014).

Newly, research has revealed that food and oxidative stress play a key role in intestinal inflammatory disorders development (Wang et al., 2020). Increased formation of reactive oxygen species (ROS) as compared to removal is referred to as oxidative stress, which has been confirmed to be a significant contributor to the development of several intestinal inflammatory illnesses, ischemia, and cancers (Wang et al., 2020; Bhattacharyya et al., 2014). ROS high can induce intestinal damage and inflammation by altering the microbiota population, inducing epithelial cell apoptosis, tight junction protein loss, disruption of mucosal membrane integrity, and promotion of mucosal inflammation (Han, 2016; Wang et al., 2020). Current studies have indicated that a high-calorie diet is a main factor in the IBD and Crohn's disease developing (McDaniel et al., 2016) and ulcerative colitis (Cani et al., 2009), mainly through generating high quantities of ROS and promoting oxidative stress, which subsequently led to disturbing the mucosal barrier damage, mucosal inflammation, and intestinal fibrosis (Fan-Jiang et al., 2021; Mahmood et al., 2019). Using HFD-animal models, the reported mechanisms ROS include: 1) Tight junction proteins are downregulated when myosin light chain kinase (MLCK) is activated; 2); depletion of antioxidants; 3) prompting the epithelial endoplasmic reticulum (ER) stress; 4) activation of the fibrotic transforming growth factor-β1 fibrotic protein (TGF-β1; and 5) reducing the differentiation of the goblet cells (Fan-Jiang et al., 2021; Gil-Cardoso et al., 2017; Li et al., 2019; Zhao et al., 2020).

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On the other hand, the intestine has a well-developed antioxidant system to combat the oxidation caused by stress and sustain the intestinal role. The main antioxidant transcription factor that supports glutathione (GSH) as well as other antioxidant enzymes in the gut is Nrf2. Furthermore, Nrf2 functions as an anti-inflammatory and anti-apoptotic molecule, suppressing NF-B and stimulating anti-apoptotic genes. Furthermore, Nrf2 is essential for gut and overall body function by regulating smooth muscle function and the growth of intestinal cells (Piotrowska et al., 2021). Notably, the majority of intestinal inflammatory and non-inflammatory illnesses have considerably reduced Nrf2 levels and activity, an effect that was described to be a major mechanism for the progression of the disorders through promoting oxidative stress-mediated damage (Lu et al., 2016; Piotrowska et al., 2021; Wang et al., 2021). However, those authors have also indicated that transgenic or pharmacological Nrf2 activation is a protective therapy to alleviate IBD, ulcerative colitis UC, and other intestinal disorders. Yet, it is still largely unknown if the damaging effect of HFD on intestinal cells and mucosal membrane integrity involves suppression of Nrf2, which requires further investigation.

Zingerone (4-(4-Hydroxy-3-methoxyphenyl) butan-2-one) is a primary ketone extracted from ginger which is used in food as a flavoring agent (Muhammad et al., 2021). Several health benefits of Zingerone have been reported, including antiobesity, antidiabetic, and anti-cancer (Leal et al., 2019; Li et al., 2012). Furthermore, Zingerone prevented hepatic, cardiac renal, neural, and pulmonary injuries, as well as rheumatoid arthritis and hepatic steatosis by inhibiting the effect of ROS, inducing the action of antioxidants, activation of Nrf2, and suppressing the rate of production of NF-κB and inflammatory cytokines (Bashir et al., 2021; Gehad E. Elshopakey et al., 2021; Lee et al., 2020; Muhammad et al., 2021; Muniandy Narayanan and Jesudoss, 2016; Soliman et al., 2018; Türk et al., 2020). Zingerone, also as an antidiarrhoeic agent, can act directly on the intestinal smooth muscle and intestinal bacteria (Chen et al., 2007; Iwami et al., 2011). Zingerone was recently described as an important intestinal regulator that inhibits the activity of pacemaker cells, cells of Cajal (Kim et al., 2018). In addition, Zingerone attenuated stress-induced IBD, neurobehavioral perturbations, and dysregulation of the intestinal barrier by quelling the peroxidation of lipids and increasing the rate of GSH and other antioxidant enzymes (Banji et al., 2014). Zingerone prevented the production of inflammatory cytokines and the activation of NF-B, which protected experimentally generated colitis (Hsiang et al., 2013). It is also protects against ethanol-caused gastric ulcers by scavenging ROS and lowering the rate of nitric oxide production and peroxidation of lipids (Sistani et al., 2019).

However, the protective effect of Zingerone, as well as the mechanism of protection on HFD-induced intestinal injury and was never tested before. Therefore, in this study, we assumed that HFD-mediated duodenal damage involves the suppression of Nrf2. In addition, we tested the hypothesis that regular zingerone administration at a previous therapeutic dose could alleviate HFD-induced duodenal and mucosal barrier disturbance by suppressing oxidative stress and inflammation, mainly through activating Nrf2 signaling.

2. Materials and methods

2.1. Animals

Adult male Wistar rats weighing (200 ± 20 g) were supplied from the Experimental Animal Care Center at King Saud University, KSA. Throughout the trial, the experimental animals were kept under autonomously controlled settings. The animal methods were conducted following the Local Committee of Research Ethics at Shaqra University, Saudi Arabia (Ethics No. ERC_SU_20230022).

2.2. Diets and drugs

Standard diet (STD) and HFD were purchased commercially from Research Diets, NJ, USA (# D12450B and # HY-N0102, respectively. The ingredients of both diets are available on the supplier site. The STD contained 67.3%, 19.2%, and 4.3% carbohydrates, proteins, and fats, providing 70%, 20%, and 10% Kcal, respectively, and total energy of 4057 Kcal/kg. Conversely, The HFD contained 35%, 20%, and 40% carbohydrates, proteins, and fats, providing 35%, 20%, and 45% Kcal, respectively, and total energy of 4057 Kcal/kg. More information about the precise composition of both diets can be located on the supplier's website. MedChemExpress LLC, NJ, USA, supplied the zingerone powder (# HY-14621). Sigma Aldrich, MO, USA, provided the Nrf2-specific inhibitor Brusatol (# SML1868) and the low-viscosity carboxymethylcellulose (CMC) (# C5678). To obtain final concentrations of fresh drugs, they mixed with 0.5% CMC.

2.3. Experimental design

After adaption (1 week), the rats were grouped into five groups (8 each) and treated for four weeks:1) Control: fed the STD and had an oral dose daily of the vehicle (0.5% CMC); 2) Zingerone-treated rats: fed the SFD and received an oral dose of Zingerone solution (100 mg per kg); 3) HFD-model rats: fed the HFD but received an oral dose daily of 0.5% CMC; 4) HFD + Zingerone-treated group: fed the HFD and received an oral dose of Zingerone solution (100 mg/kg); 5) HFD + Zingerone-treated + brusatol group: fed the HFD, received an oral dose of Zingerone solution (100 mg/kg) and brusatol; changes in daily food consumption and the weight of body were documented.

2.4. Dose selection

Authors have indicated that 4 weeks of feeding of HFD can induce intestinal colitis, independent of obesity (Luo et al., 2019; Zhu et al., 2019). Zingerone at the selected dose (1/10 or the LD50) was shown to be a therapeutic dose in rats that protected against HFD-induced NAFLD and hepatic oxidative damage, alloxan-induced diabetic complications, inflammation, oxidative stress, ethanol-induced gastric ulcer, carbon tetrachloride (CCl4)-induced nephropathy (Ahmad et al., 2018; Safhi, 2018). The regimen of brusatol was used by many authors to suppress tissue activation of Nrf2 in vivo (Bovilla et al., 2021; Shatoor et al., 2021).

2.5. Intestinal permeability testing

This was performed using the fluorescein isothiocyanate dextran (FITC-DX) assay (Cani et al., 2009; Du et al., 2020). On the last day of the experiment, the animals fasted for 10 h and orally administered FITC-dextran 4000 (Cat. No. 46944Sigma, St. Louis, MO) (500 mg/kg; 125 mg/ml). After 4 h, blood (0.3 ml) was collected from the tail of each rat into EDTA-anticoagulated tubes and centrifuged (11000 × g/10 min, room temperature). The serum of each sample was collected and diluted with phosphate-buffered saline (PBS/pH = 7.4) at a ratio of 1:1. The fluorescent signal of each sample was read at 490 excitation/530 nm emission and recorded. The concentrations of FITC-DX in each sample were determined using a standard curve derived from PBS-diluted non-treated plasma samples.

Table 1
Primers characteristics of the real-time PCR.

Gene	Primers (5′→3′)	accession #	BP
Occludin	F: CTCCAACGGCAAAGTGAATG R: CTCCAACGGCAAAGTGAATG	NM_031329.2	104
ZO-1	F: GCGAGGCATCGTTCCTAATAAG R: TCGCCACCTGCTGTCTTTG	NM_001106266.1	81
Nrf2	F: -AAAATCATTAACCTCCCTGTTGAT R: R: ′-CGGCGACTTTATTCTTACCTCTC	NM_031789	118
B-actin	F: GACCTCTATGCCAACACAGT R: CACCAATCCACACAGAGTAC	NM_031144	154

2.6. Serum collection and biochemical analysis

Two days after the permeability assay, all rats were anesthetized by an intraperitoneal dose of ketamine/hydrochloride mix (90:10 mg/kg, v/v), and blood samples were collected from the heart directly into plain tubes. All samples were centrifuged at 1500 × g for 10 min at room temperature to collect serum. The sera samples of all groups were preserved at −20℃ and used later to measure levels of lipopolysaccharides (LPS) using an ELISA (Cat. No MBS268498, CA, USA) for n = 8 samples/group and as per the manufacturer's instructions.

2.7. Intestinal collection and preparations

The rats were authenticated by neck dislocation, and their abdomen was opened. The duodenal part was identified and isolated in ice. The duodenum of each rat was washed with ice-cold PBS (pH = 7.4) and cut on ice into smaller parts. Some parts were snap-frozen at −80℃ and used later to prepare tissue homogenates and protein extracts. Other parts were directly placed in 10% buffered formalin and sent to the pathology laboratory for pathological evaluation. To prepare tissue homogenates, duodenal parts were homogenized in 10 volumes PBS (pH = 7.4), centrifuged (12000 × g/4℃/15 min), and the supernatant was collected and kept at −80℃ until use. On the other hand, cytoplasmic/nuclear fractions were prepared from frozen duodenal tissues using a commercial kit (Cat No. 78833, ThermoFisher Scientific, USA). Also, total protein homogenates for the western blotting procedure were prepared by homogenizing the duodenal tissues (40 mg) in 0.5 ml radioimmune-precipitation assay (RIPA) buffer (Cat. No. 156034; Abcam, Cambridge, UK) containing protease inhibitor cocktail. Once needed, protein concentration in any homogenate was determined using a commercial kit (Cat. No. 23225, ThermoFisher, TX, USA).

2.8. Hepatic and stool extraction

As detailed by Folch et al. (1957), some components were used directly to extract lipids using the methanol/chloroform method. Similarly, animal feces were collected and dried in a 60 °C oven for two days throughout the experiment before being crushed and exposed to lipid extraction.

2.9. Biochemical measurements

The duodenal levels of total cholesterol (CHOL) were measured using an assay kit (# ECCH-100, BioAssay Systems, CA, USA). Total cholesterol levels in the duodenal homogenates were prepared using the Cayman Chemical assay kit (#10010303, MI, USA). Total malondialdehyde levels (MDA) were measured using assay kits (# MBS268427, MyBioSource, CA, USA). Levels of interleukin-1β (IL-1β) were measured by ELISA (#ab255730). Total homogenate levels of total glutathione (GSH), tumor necrosis factor-alpha (TNFα), superoxide dismutase, interleukine-6 (IL-6), and catalase (Li Y) were measured using ELISA kits (# MBS265966; # MBS2507393# MBS036924; # MBS175908; MBS726781 respectively; MyBioSource, CA, USA). The cytoplasmic and nuclear levels of Nrf2 and NF-κB in all tissue homogenates were measured using special ELISA kits (# MBS2505513 and # MBS752046, respectively, MyBiosources, CA). All analyses were performed for n = 8 sample/-group and as per each kit instruction.

2.10. Real-time PCR (qPCR) application

Real-time PCR was conducted to measure the expression of some targets (Table 1). All used primer pairs were purchased from ThermoFisher, and their sequences are shown in Table 1. RNA isolation and the first-strand cDNA synthesis were performed using commercial Roche diagnostic kits purchased from Sigma Aldrich (MO, USA) (# 12,033,674,001 and # GE27-9261-01, respectively). The amplification protocol used a CFX69 touch real-time PCR system (Biorad, CA, USA). Detection was used using the Sofas Evergreen master mix kit describing all ingredients and amplification steps (# 172–5200, Biorad, CA, USA). In this view, each PCR sample well contained 20 µl reaction mixture composed of 2 µl cDNA (50 ng/well), 10 µl of the master mix reagent, 0.4 µl of both primers (0.2 µl (500 nM/each), and 7.6 µl nuclease-free water. The amplification was conducted using the following steps: heating (1 cycle/98℃/30 sec; denaturation (40 cycles/98℃/5 sec), annealing (40 cycles/60℃/5 sec), and melting (1 cycle/5 sec/95℃). The relative expression of all targets was normalized to the expression of the reference gene, β-actin.

2.11. Western blotting of the protein

Protein concentrations in the nuclear extracts were diluted in the loading buffer at a final 2 µg/µl concentration. Equal protein concentrations of each sample (40 µg/well) were separated using the SDS-PAGE and transferred to the nitrocellulose membrane. The membranes were then blocked with 5% skimmed milk prepared in the washing buffer Tris-buffered saline with 0.1% Tween (TBST) for 1 h with shaking. All membranes were then washed with 1X TBST (3 times/each of 10 min). These membranes were then incubated with the primary antibody against keap-1 (#4678, 60 kDa, 1:1000), Nrf2 (#12721, 100 kDa: 1:500), β-actin (# 4970, 45 kDa), and lamine A (nuclear loading control) (#, 86846, 74 kDa, 1:1000) (Cell Signaling Technology). The membranes were then washed with TBST again 3 times each of 10 min and then incubated with the secondary horseradish peroxidase-conjugated antibody (1:10000) again. The dilution of all antibodies was done in 1X TBST. After washing again, the membranes were incubated with mixed reagents A and B of the enhanced Chemiluminescence reagent (# 34577, ThermoFisher Scientific, USA) for 5 min, and all developed bands were scanned using the C-Di Git blot scanner (LI-COR, NE, USA). The relative expression of kepa1 and cytoplasmic levels of Nrf2 were expressed

to that of β-actin, whereas the relative expression of the nuclear levels of Nrf2 was expressed to that of lamin A (ALTamimi et al., 2021).

2.12. Tissue histology

Freshly collected duodena were fixed in 10% buffered formalin for 24 h. All samples were rehydrated using descending alcohol (100%, 90%, and 70%). All tissues were fixed in paraffin, sliced into 3 μM sections with a rotary microtome, mounted on labeled glass slides, and routinely stained with Harris hematoxylin/glacial acetic acid solution (Segrist & Cherry, 2020). Destining was followed using 1:400 v/v HCL/70% ethanol mix. The slides were then stained by adding 1–2 drops of Eosin. All slides were then dehydrated with ethanol and xylene. Amounting media was added to each slide which was then covered with a coverslip and left in the dark for the next day to dry. All slides were evaluated and captured under

Table 2
Final body weights, fasting glucose, and insulin, and intestinal and fecal lipid levels in all groups of rats.

	Control	Zingerone	HFD	HFD + Zingerone	HFD + Zingerone + brusatol + brusatol
Final body weight (g)	318.2 ± 22	311.4 ± 18	374.6 ± 24^{ab}	315.2 ± 20^{c}	368.6 ± 26^{abd}
Weekly food intake (g/rat)	221.5 ± 17	226.4 ± 21	291.8 ± 26^{ab}	286.3 ± 24^{ab}	297.7 ± 31^{ab}
Plasma Pasting glucose (mg/dl)	93.2 ± 4.7	96.1 ± 6.1	146.8 ± 5.4^{ab}	105.3 ± 6.0^{ac}	145.2 ± 5.7^{abd}
Plasma fasting insulin (μIU/mL)	4.8 ± 0.54	4.7 ± 0.63^{a}	6.9 ± 0.31^{ab}	5.1 ± 0.47^{c}	7.2 ± 0.44^{abd}
HOMA-IR	1.19 ± 0.12	1.14 ± 0.19^{a}	2.51 ± 0.19^{ab}	1.31 ± 0.17^{c}	2.64 ± 0.24^{abd}
Intestinal TGs (mg/g)	1.67 ± 0.47	1.19 ± 0.49	3.66 ± 0.82^{abc}	3.19 ± 0.78^{abc}	3.11 ± 1.3^{abc}
Intestinal CHOL (μg/g)	612 ± 43	638 ± 51	1465 ± 112^{abc}	1319 ± 129^{abc}	1482 ± 123^{abc}
Stool TGs (mg/g)	4.72 ± 0.64	5.1 ± 0.72	11.6 ± 1.91^{abc}	12.1 ± 2.14^{abc}	11.4 ± 2.3^{abc}
Stool CHOL (mg/kg)	5.79 ± 0.84	5.67 ± 0.91	13.2 ± 2.9^{abc}	12.6 ± 2.3^{abc}	12.9 ± 3.1^{abc}

Data were analyzed by 2-way ANOVA. Values were considered significantly different at $p < 0.05$ for n = 8 rats/group. [a]:vs. STD-fed rats, [b]: vs. Zingerone-treated rats. [C]: vs. HFD-fed rats, and [d]: vs. HFD + Zingerone-treated rats.

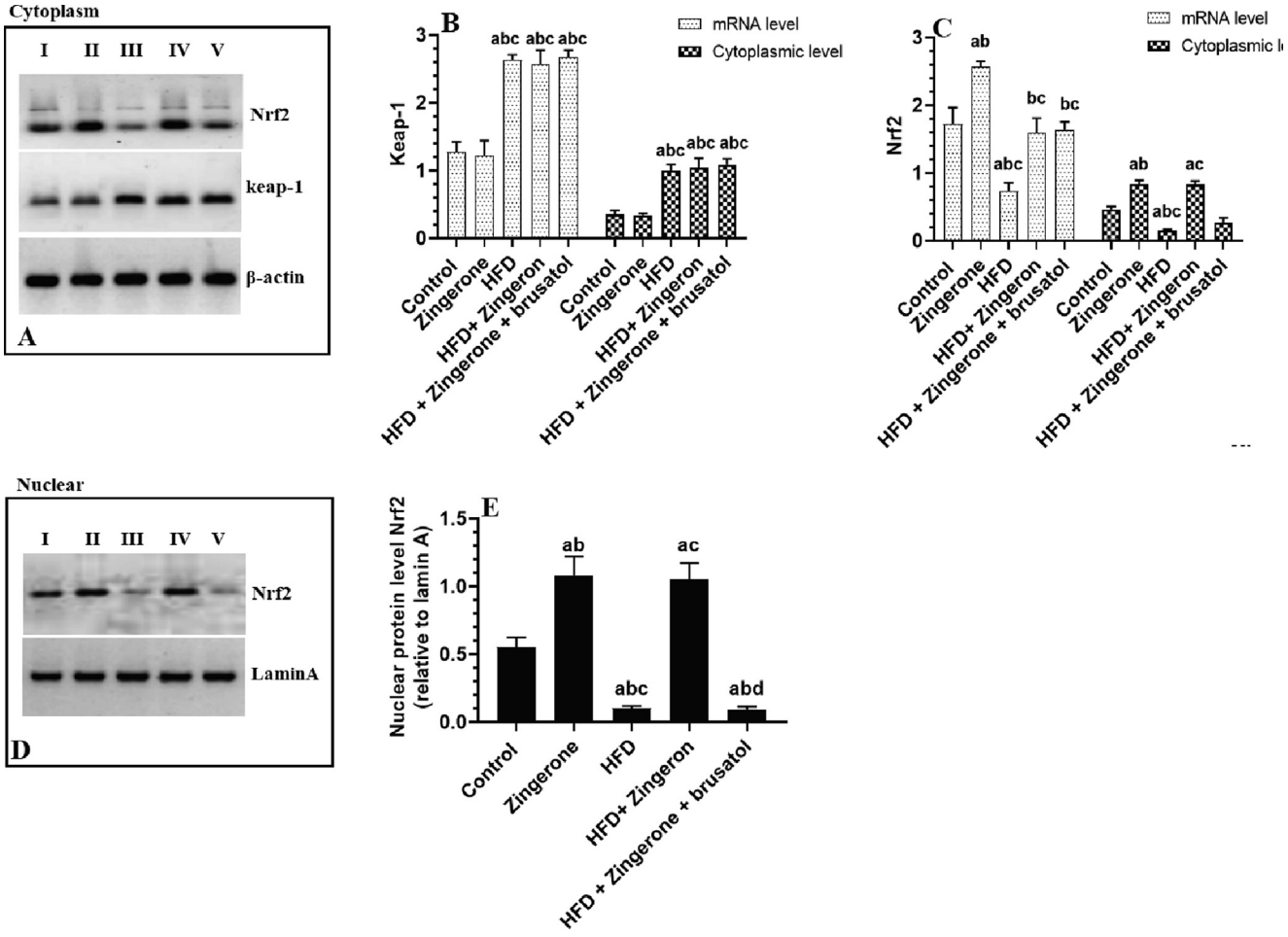

Fig. 1. Zingerone stimulates the transcription, translation, and nuclear activation of Nrf2, independent of modulating keap-1. Data were analyzed by 2-way ANOVA. Values were considered significantly different at $p < 0.05$ for n = 8 rats/group. [a]: vs. STD-fed rats, b: vs. Zingerone-treated rats. C: vs. HFD-fed rats, and d: vs. HFD + Zingerone-treated rats. FITC-A: total cytoplasmic levels of keap-1, Nrf2, and β-actin (cytoplasmic loading reference). D: Nuclear levels of Nrf2 and lamin A (nuclear loading control). Brusatol: a selective Nrf2 inhibitor.

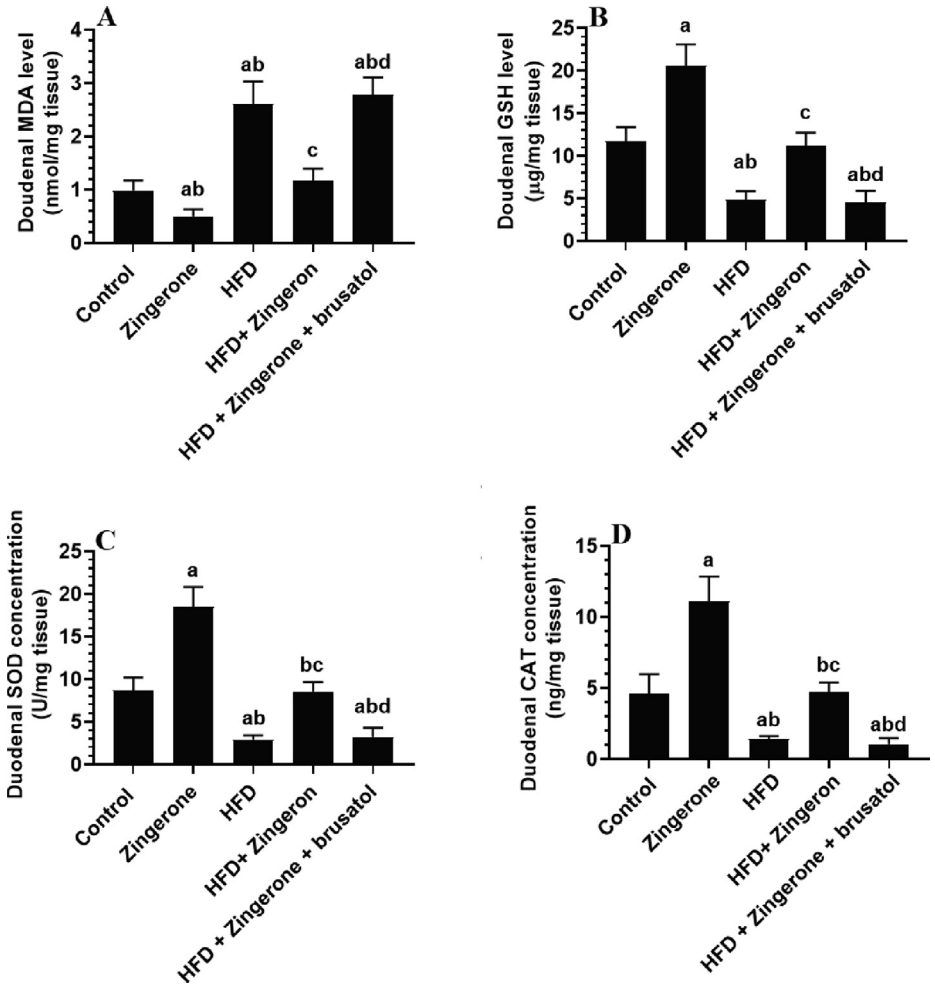

Fig. 2. Zingerone attenuates duodenal lipid peroxidation and increases antioxidant levels in HFD-fed rats in an Nrf2-dependent mechanism. Data were analyzed by 2-way ANOVA. Values were considered significantly different at $p < 0.05$ for n = 8 rats/group. a: vs. STD-fed rats, b: vs. Zingerone-treated rats. C: vs. HFD-fed rats, and d: vs. HFD + Zingerone-treated rats. MDA: malondialdehyde; GSH: total glutathione; SOD: superoxide dismutase; and CAT: catalase. Brusatol: A selective Nrf2 inhibitor.

a light microscope by a blind pathologist unaware of the experimental groups.

2.13. Statistical analysis

GraphPad Prism software (Version 8) was used for all analyses. The level of significance between different groups was determined by ANOVA (one-way analysis) and Tukey's test as a post hoc test. P level at 0.05 was deemed to be significant statistically. The data were given as average values with SD.

3. Results

Only fasting glucose and Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) were significantly reduced in Zingerone-treated rats fed STD as compared to control rats (Table 2). At the end of the experiment, HFD-fed animals gained significantly greater body weights, weekly food consumption, fasting glucose and insulin levels, HOMA-IR index, and intestinal and stool levels of CHOL and TGs than control rats (Table 2). All of these parameters were significantly lower in HFD + Zingerone-treated compared to HFD-fed animals (Table 2). HFD + Zingerone + brusatol-treated rats, conversely, exhibited significantly higher final weight of the rats body, food consumption per week, levels of fasting glucose and insulin, HOMA-IR index, and intestinal and stool levels of both CHOL and TGs (Table 2).

Keap-1 mRNA and cytoplasmic protein levels were significantly higher in HFD-fed rats, but Nrf2 mRNA, cytoplasmic protein, and nuclear protein levels were significantly lower (Fig. 1A-E). Zingerone administration to STD or HFD-fed rats did not affect duodenal mRNA or keap-1 levels but significantly elevated Nrf2 mRNA, total protein, and nuclear protein production rate compared to the STD and HFD untreated groups (Fig. 1A-E). In the HFD + Zingerone + brusatol-treated rats, the duodenal mRNA and protein levels of keap-1, and the mRNA levels of Nrf2, did not significantly change, but the Nrf2 duodenal and nuclear proteins were reduced significantly (Fig. 1A-E).

MDA levels were significantly higher in the duodena of rats who took the HFD compared to control rats fed the STD (Fig. 2A-D). GSH, SOD, and CAT levels were significantly lower (Fig. 2A-D). For duodena of Zingerone-treated control animals and HFD + Zingerone-fed animals, the levels of MDA were considerably lower than in control or HFD-fed rats, but the levels of the antioxidants were observed to be significantly high (Fig. 2A-D). These effects seen in HFD + Zingerone-treated animals were reversed in HFD + Zingerone-treated rats (Fig. 2A-D). STD-fed Zingerone rats did not have considerably high TNF-α, IL-1, or IL-6 levels in the duodenum than STD-fed control rats, but they did have considerably lower nuclear levels of NF-B p65 (Fig. 3A-D). The levels of each

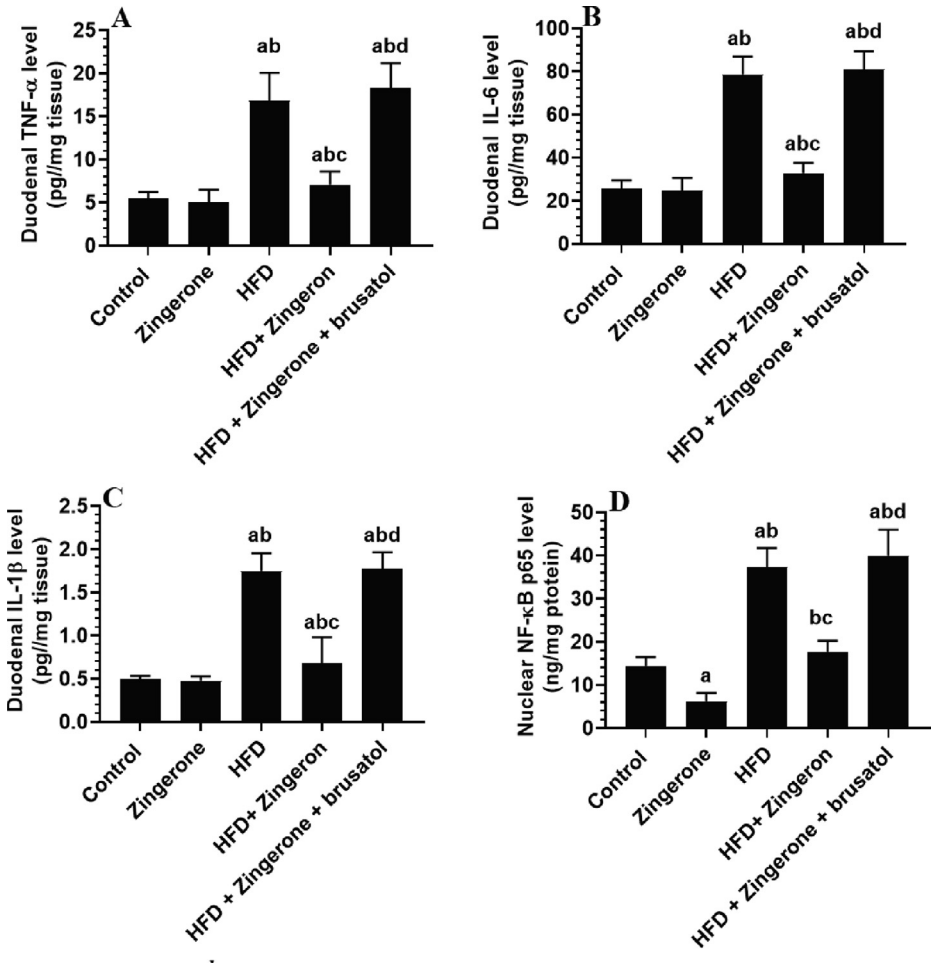

Fig. 3. Zingerone suppresses duodenal inflammatory cytokines production and the nuclear activation of NF-κB in an Nrf2-dependent mechanism. Data were analyzed by 2-way ANOVA. Values were considered significantly different at $p < 0.05$ for n = 8 rats/group. a:vs. STD-fed rats, b: vs. Zingerone-treated rats. C: vs. HFD-fed rats, and d: vs. HFD + Zingerone-treated rats. TNF-α: tumor necrosis factor-α; IL-1β: interluekine-1β; IL-6: interluekine-6. Brusatol: a selective Nrf2 inhibitor.

of these inflammatory markers in the duodenum of HFD-fed rats were observed to be significantly higher than those in control animals. In rats given HFD + Zingerone treatment, all were noticeably decreased (Fig. 3A-D). However, TNF-α, IL-1, and IL-6 levels in the duodenum. The nuclear NF-B p65 levels were considerably greater in the HFD + Zingerone + brusatol-treated animals compared to HFD + Zingerone-treated rats (Fig. 3A-D). Indicators of the integrity of the intestinal mucosal barrier, FITC-DX, and LPS, as well as the mRNA of the tight junction proteins occludin and ZO-1, were not significantly different between the control and Zingerone-treated rats (Fig. 4A–D). Rats given the HFD had significantly higher FITC-DX and LPS concentrations than the control group but lower concentrations of occludin and ZO-1 (Fig. 4A–D). When HFD + Zingerone + brusatol-treated rats were contrasted with HFD + Zingerone-treated rats, the results were the opposite (Fig. 5).

4. Discussion

Zingerone, as a major component of ginger with numerous biological activities, has never been shown to prevent HFD-caused intestinal rupture and inflammation. The study's main finding was that Zingerone has a new ability to reduce HFD-induced duodenal rupture by reducing inflammation as well as stress result-oxidation and increasing mucosal barrier integrity by upregulating the tight junction proteins expression. Furthermore, we also show that Zingerone's protection mechanism is mainly mediated by the keap-1/Nrf2/antioxidant signaling pathways. A full mechanism of action is shown in the graphical abstract (Fig. 6).

In general, diet type, composition, and calorie content are known to affect intestinal health and significantly function in intestinal disorders development like IBD, CD, and UC (Knight-Sepulveda et al., 2015; Wang et al., 2021). Therefore, dietary intervention could be a cause or a potential therapy for these disorders (Knight-Sepulveda et al., 2015; Lewis & Abreu, 2017). Increased uptake of a rich-calorie diet, particularly HFD, is linked with obesity, metabolic disorders, intestinal injury, and IBD and UC (Zhao et al., 2020). HFD's impact on developing intestinal inflammatory disorders could be a direct effect or secondary to obesity and insulin resistance (Mahmood et al., 2019). Obesity has been observed as a dangerous independent factor for both IBD and UC by causing slight inflammation and subsequent intestinal oxidative stress and inflammation (Ding et al., 2011; Pavelock, 2019; Rahmani, 2019). Several other studies have confirmed that HFD alone can induce these intestinal disorders through various mechanisms like promoting oxidative stress, dysbiosis, and producing damaging metabolites (Hildebrandt et al., 2009; Zhao et al., 2020). In support, increased dietary intake of CHOL alone increased the risk of UC in the general population by 20%. Furthermore, HFD increased intestinal oxidative stress and IBD in $TNF^{\Delta\Delta}RE/WT$ and $Mdr1a^{-/-}$ mice, which do not ordinarily acquire obesity (Gruber & Haller, 2013; Paik, 2013; van der Logt, 2013). In addition, HFD-induced CD-like ileitis is independent of obesity (Gruber & Haller, 2013).

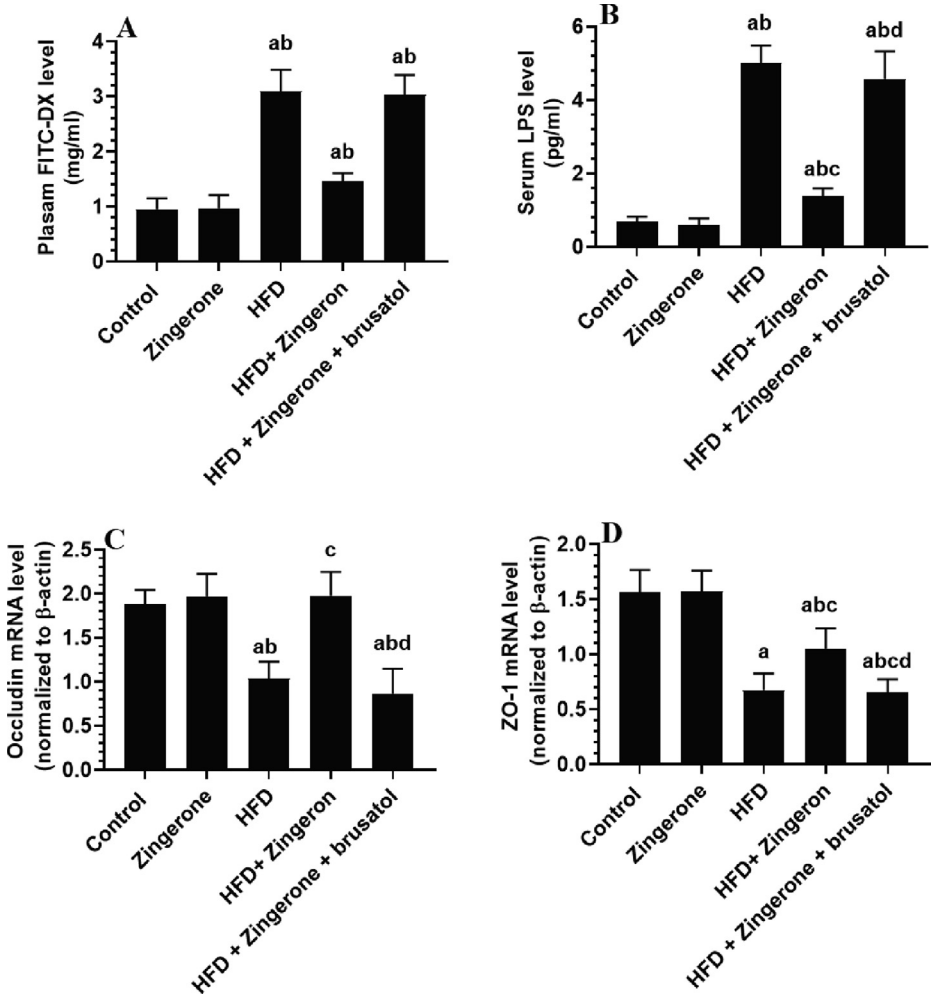

Fig. 4. Zingerone attenuates duodenal permeability in HFD-fed rats through upregulating tight junction proteins and in an Nrf2-dependent mechanism. Data were analyzed by 2-way ANOVA. Values were considered significantly different at $p < 0.05$ for n = 8 rats/group. [a]: vs. STD-fed rats, b: vs. Zingerone-treated rats. C: vs. HFD-fed rats, and d: vs. HFD + Zingerone-treated rats. FITC-DX: fluorescein isothiocyanate dextran; LPS: lipopolysaccharides. ZO-1: zona occludens-1. Brusatol: a selective Nrf2 inhibitor.

In this study, HFD feeding for 4 weeks induced intestinal damage and epithelial ulceration that coincided with marked obesity and IR. This was evidenced by a considerable gain in the weight of the rats' bodies, which was associated with hyperglycemia and hyperinsulinemia in rats fed HFD. These results support the claims of several other authors who have shown that short (4 weeks) or long-term (17 weeks) HFD feeding induced ulcerative colitis and obesity (Gil-Cardoso et al., 2017). However, although Zingerone lessened the control and HFD-fed rats' fasting glucose, it failed to reduce the increase in fasting insulin levels and body weights in the HFD-fed rats. Despite this, it was able to but was able to suppress HFD-induced duodenal damage. The hypoglycemic effect of Zingerone is well-reported in the literature (Ahmad et al., 2018). These findings imply that Zingerone's demonstrated gut protection against HFD is not dependent on obesity or IR.

Nonetheless, HFD induces IBD, CD, and UC mainly by promoting oxidative damage, which acts as a signal that initiates barrier damage and submucosal inflammation (Aviello & Knaus, 2017). In the gut, ROS are regularly produced in response to micronutrient metabolisms and various cells, including epithelial, immune, and endothelial cells (Brown, 2014). Other resources of ROS include external foreign substances and pathogenic bacteria (Bhattacharyya et al., 2014). In the case of HFD feeding, the accumulation of CHOL, TGs, and free fatty acids, as well as oxidized fats, trigger ROS by alerting the microbiota population, inducing lipid peroxidation, scavenging antioxidants, promoting endoplasmic reticulum (ER) stress, and generating toxic mediators such as bile acids (Gruber & Haller, 2013; Hildebrandt et al., 2009; Schulz et al., 2014; Zhao et al., 2020). These ROS not only damage the membrane of the cell by peroxidation of lipids, DNA, and proteins but also alter membrane fluidity and impair the mucosal membrane barrier, thus stimulating the invasion of the bacteria and subsequently activation of mucosal resident leukocytes (Brown, 2014; Li et al., 2012; Wang et al., 2020). These inflammatory cells further increase ROS production, leading to positive activation feedback and sustained stress due to oxidation as well as inflammation (Wang et al., 2020). Additionally, the majority of inflammatory illnesses include NF-B activation, a transcription factor that primarily promotes inflammation by increasing a number of inflammatory cytokines such as TNF-α, IL-1, and IL-6 (Liu et al., 2017). ROS and LPS are the potent activators of NF-κB in the gut (Anderson et al., 1994; De Plaen et al., 2000; Luo et al., 2019).

Higher levels of ROS, MDA, TNF-, IL-6, and IL-1 in this investigation, as well as increased nuclear NF-B activation and lower levels of GSH, SOD, and CAT, suggested that oxidative stress and inflammation played separate roles in mediating duodenal injury after 4 weeks of HFD. These findings are in agreement with many other studies that found increased levels of oxidative stress and inflammatory markers, low antioxidant levels, and improved NF-B activation in the saliva, serum, and various intestinal tissues (such as the

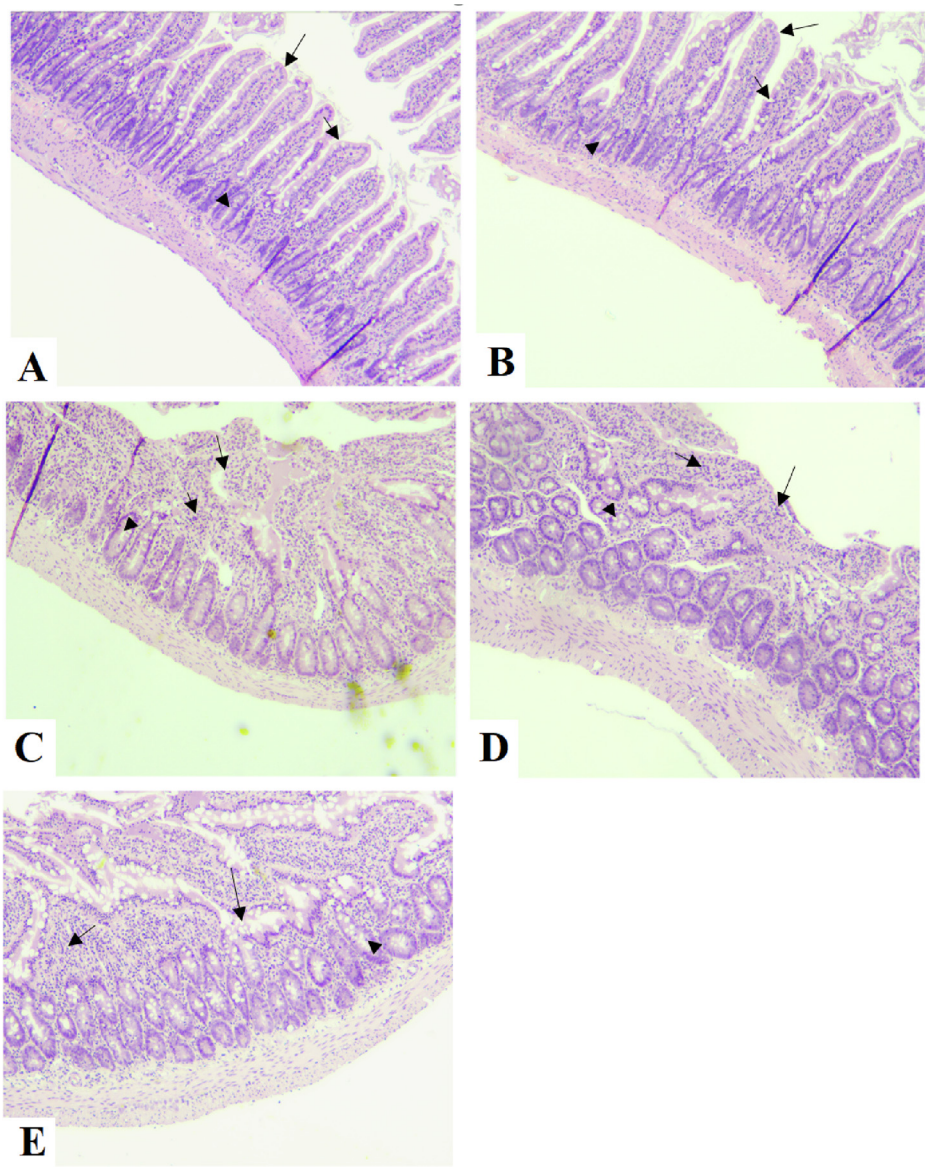

Fig. 5. The duodenal histology for all experimental groups. **A and B:** photomicrographs were taken from control and Zingerone-treated animals and demonstrated normal duodenal histology of the epithelia with obvious intact microvilli (long arrow) at the top of the mucosa, goblet cells (short arrow), and intestinal crypts (arrowhead). **C:** was taken for an HFD-fed rat exhibiting severe ulcers in the intestinal mucosal layers where most villi and goblet cells were absent (long arrow). These tissues also showed increased inflammatory cell infiltration (short arrow) and crypt abscess (arrowhead). **D:** was taken from an HFD + Zingerone-treated rat and demonstrated much improvement in the structure of the mucosa villi (long arrow), abundant goblet cells (short arrow), and normal size and features of the intestinal crypt (arrowhead). **E:** was obtained from an HFD + Zingerone + brusatol-treated rat and showed similar changes to those in the HFD-fed rats with the same marks. H&E, 200 X.

duodenum, jejunum, and colon) of HFD-fed animals as well as in people with IBD and UC (Brown, 2014; Fan-Jiang et al., 2021; Li et al., 2012; Luo et al., 2019; Mahmood et al., 2019; McDaniel et al., 2016). Moreover, HFD stimulated LPS levels of fed animals serum indicating impairment in membrane integrity and endotoxemia, explaining the observed inflammatory response strongly. Indeed, LPS from invading bacteria can promote intestinal inflammation by acting on CD14 and TLR4 of the submucosal immune cells and activating NF-κB p65 (Luo et al., 2019; Plociennikowska et al., 2015). Furthermore, our data confirm that Zingerone treatment could prevent HFD-induced duodenal damage and endotoxemia by inhibiting the production of ROS, suppressing inflammation, and upregulating antioxidants. An intriguing finding in this study is that Zingerone lowered ROS and MDA levels and NF-κB p65 activation but enhanced GSH, CAT, and SOD levels not only in the duodena of HFD-fed rats but also in untreated animals. Thus, confirming that the protecting effect of Zingerone against HFD-mediated duodenal injury involves a complex mechanism that includes scavenging ROS, activation of endogenous antioxidants, and inhibiting NF-κB.

Similar to these findings, Ginger and Zingerone inhibited LPS-induced systemic organ inflammation in mice by reducing NF-κB and IL-1β production, with the jejunum showing the greatest effect (Hsiang et al., 2013). It also suppressed trinitrobenzene sulphonic acid-caused colitis in animals by preventing the production of NF-κB p65 (Hsiang et al., 2013). Zingerone also inhibited experimentally-induced colon cancer, diabetic nephropathy, cisplatin-induced ototoxicity, Adriamycin-induced liver damage, alloxan-induced organ damage, and antibiotic-induced endotoxemia by suppressing NF-κB p65 and reducing the TNF-α, IL-1β, and IL-6 (Elshopakey et al., 2021; Ganaie & Wani, 2019; Lee et al., 2020; Singh et al., 2020). Moreover, Zingerone suppressed

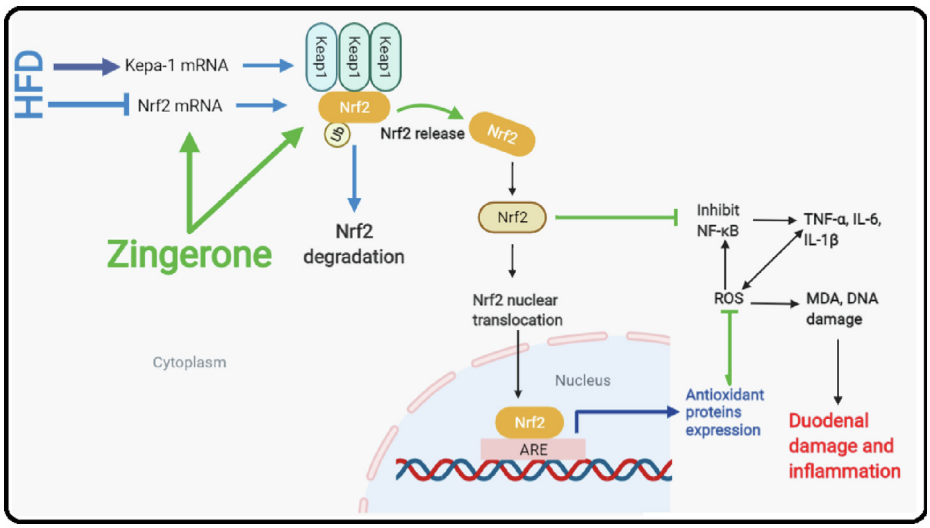

Fig. 6. An abstract graphical shows the protective effect of Zingerone against HFD-induced duodenal damage involving upregulation and activation of Nrf2-mediated suppression of oxidative stress and inflammation. HFD stimulates the transcription/translation of keap-1 and inhibits those of Nrf2. This stimulates the proteasome degradation of Nrf2 in the cytoplasm. Zingerone contradicts this by upregulating Nrf2 without affecting levels of keap-1, thus stimulating Nrf2-induced inhibition of NF-κB p65 and the nuclear translocation of Nrf2. In turn, the increased transcriptional activities of Nrf2 stimulate the transcription of glutathione (GSH) and other antioxidant enzymes such as superoxide dismutase (SOD) and catalase. All these events suppress HFD-induced duodenal generation of reactive oxygen species (ROS) and other inflammatory cytokines.

levels of lipid peroxides and activities of NF-κB p65 and concomitantly stimulated levels of SOD, GSH, and CAT, GPx in the serum of alloxan-induced diabetic rats, as well as in the kidneys of cisplatin-treated female rats and mice model of sepsis (Ahmad et al., 2018; Kandemir et al., 2019; Lee et al., 2019). It also reduced lead-induced liver and kidney damage as well as bleomycin-induced lung fibrosis by decreasing lipid peroxidation and increasing CAT, SOD, and GPx activity (Segrist & Cherry, 2020). Furthermore, Zingerone reduced the generation of ROS in septic mice (Lee et al., 2020).

Associated with these changes, HFD feeding for four weeks also caused a considerable increment in the permeability of the intestine, as shown by the significant enhancement in the serum levels of FITC-DX, which may explain the significantly higher LPS level in the serum of rats. In addition, HFD- reduced the expression of claudin-1, a major protein forming the tight junction of the mucosal barrier. A similar increase in membrane permeability with a reduction in occludin expression was also reported in mice fed HFD for 4 weeks (Li et al., 2012). Also, intestinal levels of all these tight junction proteins are significantly lowered in HFD-fed animals and in patients with IBD and CD, an effect that was attributed to ROS and inflammatory cytokines (Cani et al., 2009; Gulhane et al., 2016; Kiatprasert et al., 2015; Xei et al., 2020; Xu et al., 2019). Indeed, numerous lines of evidence have indicated a stimulatory role of ROS and TNF-α on the activities of the MLCK enzyme, which normally suppresses the junctional protein expression (Gulhane et al., 2016; Li et al., 2012). Hence, it could be reasonable that the decrease in the mRNA expression in the intestine of HFD-fed rats is secondary to the activation of this pathway. However, because Zingerone reduced FITC and LPS levels in the serum of HFD-fat rats while increasing mRNA levels of claudin-1 and occludin in the duodenum of control rats, these findings suggest that Zingerone improves intestinal mucosal barrier integrity by reducing ROS and inflammatory cytokines production.

On the other hand, our data also indicated an emerging role of the keap-1/Nrf2 axis in mediating HFD-induced duodenal damage in rats. Accordingly, HFD feeding increased keap-1′s total protein and mRNA levels while decreasing Nrf2′s mRNA, protein levels, and nuclear accumulation. Although the previous study's investigation of this effect was inadequate, it is consistent with numerous other studies that have revealed decreased Nrf2 expression and activation in IBD, CD, and UC people and animals (Piotrowska et al., 2021; Wang et al., 2021). Kepa-1 is the major cytoplasmic protein that promotes cytoplasmic protostome Nrf2 degradation without oxidative stress (Hennig et al., 2018). Conversely, ROS causes phosphorylation of keap-1 and subsequent dissociation and nuclear localization of Nrf2 to stimulate the transcriptional machinery through binding to the antioxidant response element (Al-Qahtani et al., 2022; Nezu et al., 2017). As a result, Nrf2 is described as an antioxidant and anti-inflammatory molecule that can stimulate the synthesis of GSH and antioxidant enzymes and inhibit NRLP3 inflammasome and NF-κB p65 (Hennig et al., 2018; Li et al., 2008; Serafini et al., 2020; Wardyn et al., 2015).

On the other hand, activation of the keap-1/Nrf2 axis is a possible protective mechanism to treat IBD and UC by suppressing intestinal oxidative cellular and barrier damage and reducing the associated inflammation and fibrosis (Piotrowska et al., 2021; Wang et al., 2021). Zingerone restored normal Nrf2 levels and encouraged Nrf2 nuclear localization. It also increased Nrf2 expression and nuclear levels in control rats. Interestingly, Zingerone did not affect keap-1 mRNA or protein expression in the control or HFD-fed rats. These findings indicate that Zingerone is a strong inducer of Nrf2 in the gut. Furthermore, our findings suggest that Zingerone may promote the dissociation between keap-1 and Nrf2 by raising keap-1 phosphorylation. Unfortunately, this is still a theory that requires further investigation.

Notably, Nrf2 is also under the control of NF-κB, which can inhibit Nrf2 through upregulating keap-1 (Wardyn et al., 2015). This led us to question whether activation/upregulation of Nrf2 is the major pathway by which Zingerone acts. In addition, we want to investigate if Nrf2 is located upstream or downstream NF-κB. Therefore, we have suppressed Nrf2 in the tissue of HFD rats by co-administration of brusatol, which increases the dissociation between keap-1 and Nrf2 without altering their expression. Brusatol co-administration completely abolished the protection afforded by Zingerone on intestinal histology and all measured markers, including the suppression of NF-κB. These findings led us to the

conclusion that Zingerone's inhibition of NF-B p65 is indirectly mediated via activating Nrf2. These findings imply that the primary mechanism driving Zingerone's antioxidant and anti-inflammatory effects is Nrf2 activation.

Although the stimulatory effect of Zingerone on the expression of the duodenal tight junction protein of HFD-fed rats of this study could be attributed to Nrf2-induced suppressing of ROS and inflammatory cytokines, some authors have suggested that this could be a direct effect of Nrf2, and independent of its antioxidant and anti-inflammatory effects (Piotrowska et al., 2021). However, this seems unlikely in our study as normal levels of occludin and ZO-1 were observed in the duodena of control rats that received Zingerone, despite the significant increase in the mRNA expression, total cytoplasmic levels, and nuclear levels of Nrf2. Therefore, we could propose that Zingerone's antioxidant and anti-inflammatory actions come before its stimulatory effect on tight junction proteins.

While numerous additional research may corroborate our findings, our data are the first to show Zingerone's impact on the keap-1/Nrf2 pathway in mice receiving HFD. For instance, Zingerone increased Nrf2 activity and reduced cardiac remodeling in rats (Liu et al., 2019). According to Zhu et al. (2021), Zingerone alleviated Asthma by upregulating and activating Nrf2 signaling in animal models. Further, they have also shown that Zingerone reduces cecal ligation puncture-induced organ damage in the polymicrobial sepsis model by acting through the same mechanism (Zhu et al., 2022). Zingerone's inhibitory effect on adriamycin-induced renal oxidative stress, inflammation, apoptosis, and NF-κB p65 activation was also linked to Nrf2 activation (Elshopakey et al., 2021). However, this study did not further examine the Zingerone mechanism to stimulate Nrf2, which is considered one limitation. In this regard, some authors have shown that Zingerone stimulates Nrf2 by modulating the activities of AMPK and eNOS (Zhu et al., 2021). Further investigations employing transgenic methods to knock down Nrf2 in vitro and in vivo may be necessary to support our findings.

In conclusion, the study's findings implicate keap-1/Nrf2 suppression in mediating the toxic effect of FFD on rat duodenum and show that activation of this pathway by Zingerone is a potentially effective therapy that can inhibit oxidative stress and inflammation, as well as their detrimental effect on mucosal membrane permeability and tight junction protein expression. Given Zingerone's safety, these findings support pre-clinical and clinical research to treat diverse intestinal inflammatory disorders in people.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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