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Article

6-Gingerol Slightly Reduces Hepatic Endoplasmic Reticulum Stress Markers in Rats with High-Fat, High-Fructose Diet-Induced Metabolic Syndrome

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Abstract. Metabolic syndrome (MetS) is linked to hepatic endoplasmic reticulum (ER) stress. This study evaluated 6-gingerol's potential to alleviate ER stress in a high-fat high-fructose (HFHF)-induced MetS rat model. Male Sprague-Dawley rats (8 weeks, 180–220 g) were assigned to five groups: Normal, HFHF, and HFHF with 6-gingerol (50, 100, or 200 mg/kg). The Normal group received a standard diet, while others had HFHF for 16 weeks. From Week 8, intervention groups received 6-gingerol daily. Except for Normal, other groups also received Streptozotocin (22mg/kg, i.p.) at Week 8. At Week 16, rats were euthanized, and liver tissues collected to assess ER stress markers (GRP78, IRE1, TRAF2, PERK, CHOP) via qPCR and apoptotic markers (Bax, Bcl-2) via ELISA. 6-Gingerol slightly reduced liver ER stress markers, including GRP78 (P=0.392), CHOP (P=0.798), IRE1 (P=0.419), TRAF2 (P=0.470), and PERK (P=0.357), but these changes were not significant. Similarly, apoptotic markers Bax and Bcl-2 showed no significant differences, though the Bax/Bcl-2 ratio decreased (P=0.186). These results indicate that 6-gingerol had only a slight effect on ER stress and apoptosis within the parameters of this experiment.

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1. Introduction

Metabolic syndrome (MetS), often tied to obesity, increases the risk of developing non-alcoholic fatty liver disease (NAFLD). This condition is characterized by endoplasmic reticulum (ER) stress, which activates the unfolded protein response (UPR) and exacerbates the progression of liver disorders [1]. MetS has a very high global prevalence ranging from 12.5% to 31.4%, representing a significant public health concern due to its close links with other non-communicable diseases i.e. cardiovascular diseases and diabetes, which contribute to severe health risks [2-3] The global rise in MetS incidence, driven by shifts in diet and lifestyle, has led to a parallel increase in NAFLD. This has contributed to increased morbidity and mortality from metabolic, hepatic, and cardiovascular complications [4-5]. NAFLD and MetS share a bidirectional relationship, with visceral adiposity playing a pivotal role in both conditions by promoting insulin resistance and hepatic fat accumulation [6].

Endoplasmic reticulum stress plays a pivotal role in the regulation of metabolic syndrome and the pathogenesis of NAFLD by promoting lipid accumulation and activating the unfolded protein response (UPR), leading to hepatic steatosis, inflammation, hepatocyte death, and fibrosis. This highlights a potential therapeutic target for NAFLD [7-8]. In a high-fat diet, the IRE1α/TRAF2 complex of the UPR plays a key role, along with the IKK/IκB/NF-κB and ASK1/JNK1 signaling pathways, in the progression of Nonalcoholic steatohepatitis [9]. TRIB3, which mediates HNF4α degradation, also plays a key role in progression of NAFLD by triggering the UPR [10]. UPR maintains cellular homeostasis through three key sensor pathways: IRE1, PERK, and ATF6. These sensors initiates downstream signaling for homeostasis [11]. The downstream mediators of these sensors include GRP78, PERK, IRE1, TRAF2, and CHOP [12-13]. Thus targeting these sensors and mediators has been proposed as a therapeutic strategy to mitigate liver damage associated with different disorders.

6-Gingerol has gained attention for its strong antioxidant and anti-inflammatory effects. Gingerol mitigated endoplasmic reticulum stress-induced hepatic steatosis in HepG2 cells by downregulating IRE1, GRP78, PERK, and CHOP levels [14]. It has also shown potential in mitigating hepatic steatosis, alleviating oxidative stress, and regulating crucial signaling pathways linked to liver health, including the LKB1/AMPK and Nrf2 pathways [15-16]. 6-Gingerol also mitigates ectopic lipid accumulation and mitochondrial dysfunction linked to metabolic disorders [17]. These mechanisms prevent liver damage while enhancing overall metabolic health. In dietary models of metabolic syndrome, 6-Gingerol has shown to reduce oxidative stress and inflammation and to dose-dependently reverse lipid accumulation in the liver, highlighting its potential to address liver-related complications [18-19]. However, its role in modulating hepatic ER stress and apoptosis in the context of metabolic syndrome remains underexplored. This study investigated the effects of 6-gingerol on ER stress markers and apoptotic pathways in the livers of rats fed an HFHF diet with induced metabolic syndrome.

2. Materials and Method

2.1 Animal Model

Male Sprague-Dawley rats (Aged 8 weeks; 180–220 g) were obtained from the National Agency of Drug and Food Control, Jakarta, Indonesia. The animals were kept in plastic cages with top grills in compliance with the Animal Research Facility standards of our institution. Environmental conditions were maintained at 65%–75% humidity with a 12-hour light-dark cycle. All animal procedures followed the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. The study was approved by the Institutional Animal Care and Use Committee of the University of Indonesia (Approval number: KET-945/UN2.F1/ETIK/PPM.00.02/2021).

2.2 Experimental Design

Twenty-five male Sprague-Dawley rats were randomly assigned to five experimental groups: The Normal group (standard chow diet), the HFHF Control group (high-fat, high-fructose diet), and three 6-Gingerol-treated groups receiving HFHF supplemented with 6-gingerol at doses of 50 mg/kg, 100 mg/kg, and 200 mg/kg. The dosing regimen for 6-gingerol in this study was established based on prior research and acute toxicity data. The highest dose was set at 1/10th of the reported LD50, which exceeds 2000 mg/kg in rats [20]. The intermediate and lowest doses were determined using a geometric progression relative to the maximum dose. The HFHF diet (Table 1) was administered over a 16-week period to induce metabolic changes, consistent with methodologies described in previous studies [19], [21]. Diet was obtained from the University of Brawijaya Laboratory in Malang, Indonesia. The method flowchart is depicted below (Figure 1)

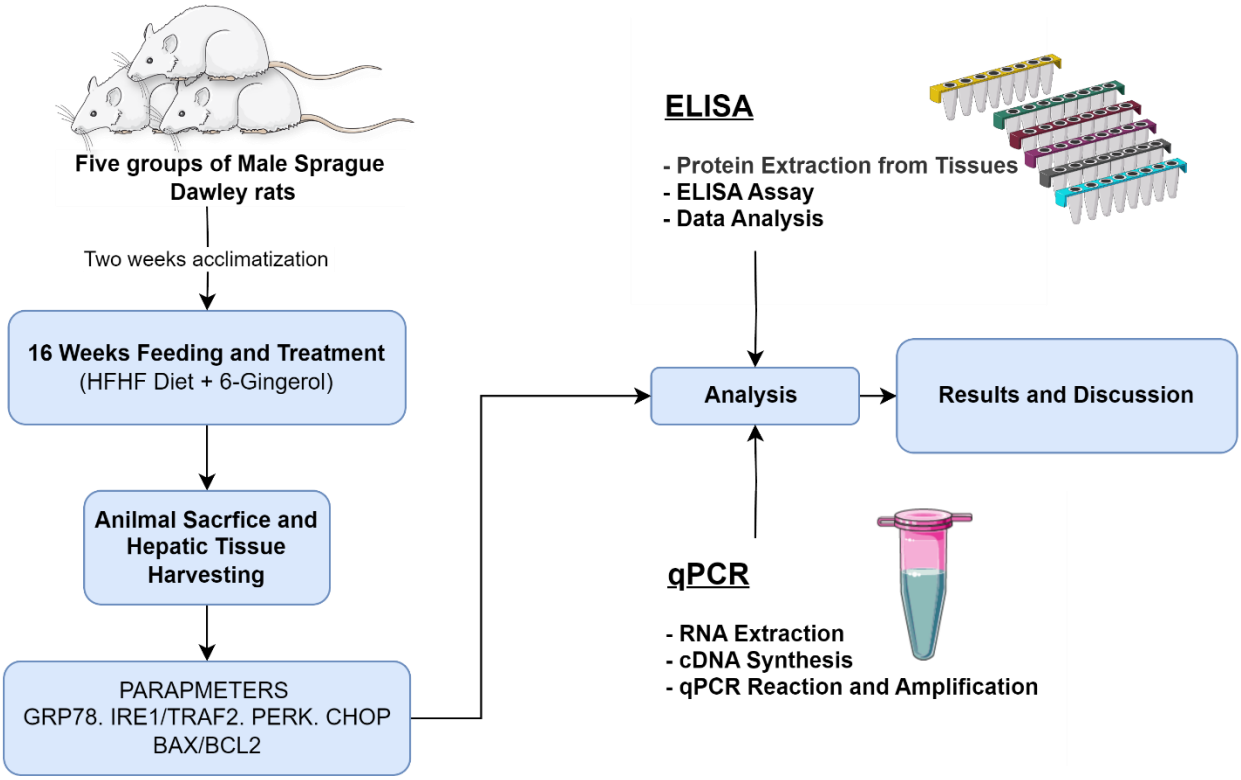

Figure 1. Flowchart depicting the study selection process

6-Gingerol was administered to the treatment groups via daily oral gavage at doses of 50, 100, and 200 mg/kg, suspended in 1.5 mL/kg of corn oil, starting in the 9th week after 8 weeks of HFHF feeding and continuing for the remaining 8 weeks of the 16-week experiment. Additionally, at the onset of this treatment phase, the HFHF groups, including the non-gingerol HFHF group (Control group), were administered a single intraperitoneal dose of streptozotocin (CAS 18883-66-4; Santa Cruz Biotechnology Texas, USA) at 22 mg/kg to further exacerbate the induction of metabolic syndrome. At Week 16, following a 12-h fast, the rats were anesthetized with ketamine and euthanized via exsanguination. Hepatic tissues were collected for subsequent analysis.

Table 1. Rat food composition

Ingredients	Percentage Composition	
	Chow Diet	HFHF Diet
Carbohydrates	47%	31.92%
Fats	4%	29.02%
Proteins	20%	21.31%
Water	12%	12.6%
Minerals/Ash	17%	5.15%
Total Energy	304 kcal/100g	474 kcal/100g

HFHF; High-Fat High-Fructose

2.3 Quantitative Assessment of ER Stress Response Gene Expression

Total RNA was extracted from preserved rat hepatic tissue using the Quick-RNA™ Miniprep Plus Kit, and its concentration was measured at 260 nm using a Nanodrop 2000 Spectrophotometer (Thermo Scientific). The A260:A280 ratios of the mRNA samples ranged from 2.05 to 2.14. cDNA was synthesized using the ReverTra Ace™ qPCR RT Master Mix with gDNA Remover, and qPCR was conducted on the MiniOpticon™ RT-PCR System (48-well Thermal Cycler; Bio-Rad, Hercules, CA, USA) using the THUNDERBIRD™ SYBR® qPCR Mix.

Primers specific to target genes (GRP78, IRE1, TRAF2, PERK, CHOP, and GAPDH) were sourced from Integrated DNA Technologies (Singapore) and used for amplification. Primer sequences were designed at the Integrated Laboratory of the Faculty of Medicine, University of Indonesia (Table 2). The qRT-PCR reactions involved initial denaturation at 95 °C for 2 min, followed by 45 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 10 s, and extension at 72°C for 20 s. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method, with GAPDH as the reference gene. This assessment of ER stress response gene expression, including GRP78, IRE1, TRAF2, PERK, and CHOP expression, provides insights into the cellular stress response under metabolic conditions.

Table 2. Sense and antisense primers

Gene	Direction	Sequence	Amplicon Size (bp)
GADPH	Forward	GAATGGGAAGCTGGTCATCAA	111
	Reverse	CCAGTAGACTCCACGACATACT	
GRP78	Forward	CCAAGGATGCTGGCACTATT	108
	Reverse	TGTTCTTCTCTCCCTCTCTCTT	
CHOP	Forward	TCACAAGCACCTCCCAAAG	108
	Reverse	CACTCTGTTTCCGTTTCCTAGT	
PERK	Forward	CCAGGCATCGTGAGGTATTT	100
	Reverse	AGTCTGTGCTTTCGTCCTTC	
IRE1	Forward	CCGAATGTGATCCGCTACTT	105
	Reverse	GTCCTTCTGCTCCACATACTC	
TRAF2	Forward	GTCTCTCTTCTTCGTGGTGATG	112
	Reverse	GATCACATGCTCTCGGTTGT	

GAPDH; Glyceraldehyde-3-phosphate dehydrogenase, GRP78; Glucose Regulated Protein 78, CHOP; C/-EBP homologous protein, PERK; protein kinase RNA-like endoplasmic reticulum kinase, IRE1; inositol-requiring enzyme 1, TRAF2; TNF receptor-associated factor 2

2.4 Apoptotic Markers

Bax and Bcl-2 levels in liver tissue were quantified using commercially available enzyme-linked immunosorbent assay kits (Fine Biotech Co. Hubei, China) following the manufacturer's instructions. Each sample was measured in duplicate to ensure data reliability. A 100 μL of the appropriately diluted sample and standard was added to each well of the microplate and incubated at 37°C for 90 minutes to allow specific binding. Next, 100 μL of biotin-conjugated anti-Bax or anti-Bcl-2 antibody was added to each well, the plate was sealed, and the plate was incubated statically for 60 min at 37°C. After incubation, the wells were washed multiple times with wash buffer to minimize nonspecific binding. The detection was achieved by adding an HRP-conjugated secondary antibody followed by TMB substrate solution. After color development, 50 μL of stop solution was added to each well. The absorbance was then measured at 450 nm using an xMark™ Microplate Absorbance Spectrophotometer (BIO-RAD). Bax and Bcl-2 concentrations (ng/mL) were calculated based on the standard curve and were normalized to the total protein content in the tissue, expressed as ng/mg protein.

2.5 Statistical Analysis

Nonparametric methods were applied to present the results as medians (interquartile range, IQR) for data that did not follow a normal distribution, as determined by the Shapiro-Wilk test. Data that met the normality assumptions are expressed as mean ± standard deviation (SD). One-way ANOVA was used to compare groups, followed by Tukey's post hoc test for pairwise comparisons. Statistical significance was set at $p < 0.05$. Analyses were conducted using SPSS (version 25) and GraphPad Prism (version 9.5.1).

3. Results and Discussion

3.1 Effect of 6-Gingerol on ER Stress Markers

The relative mRNA expression levels of key ER stress markers—GRP78, CHOP, IRE1, PERK, and TRAF2—were analyzed across five experimental groups: Normal (chow diet), HFHF Control (high-fat, high-fructose diet), and 6-Gingerol-treated groups (6G-50, 6G-100, and 6G-200). As shown in Figure 1 (A–E), the HFHF Control group showed no significant elevation in the expression of ER stress markers compared with the normal group.

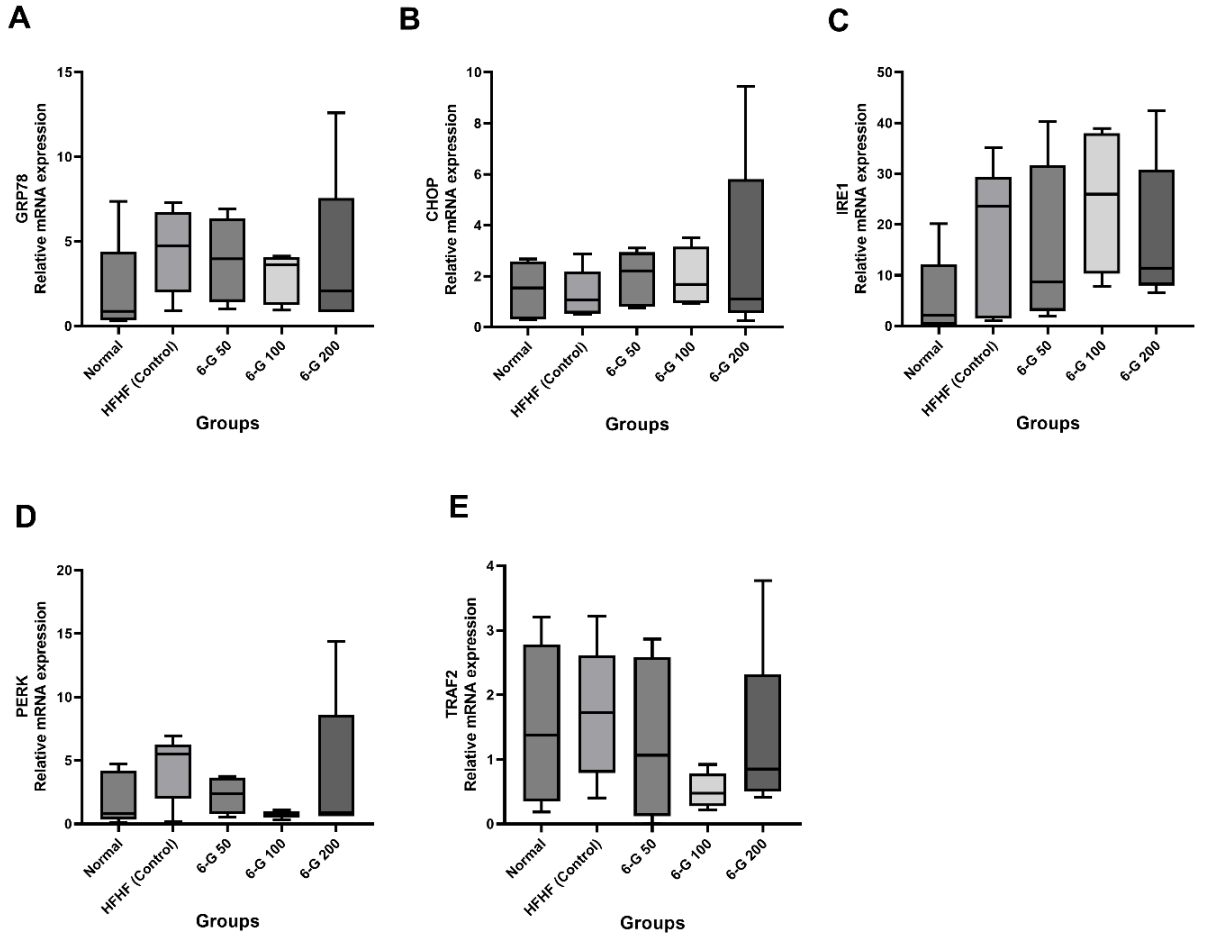

Figure 2. Effect of 6-Gingerol on the relative mRNA expression of GRP78 (A), CHOP (B), IRE1 (C), PERK (D), and TRAF2 (E). The groups include Normal (chow diet), HFHF Control (high-fat, high-fructose diet), and 6-Gingerol-treated groups (6G-50, 6G-100, and 6G-200, corresponding to 50, 100, and 200 mg/kg, respectively). Data are presented as median ± interquartile range (IQR). The Kruskal-Wallis test showed no statistically significant differences in the expression levels of any marker among the groups, with statistical significance set at $p < 0.05$

The expression levels of ER stress markers demonstrated varying effects across the 6-gingerol-treated groups, though no statistically significant differences were observed. GRP78 levels were decreased in all the treatment group compared to the HFHF group, with the greatest reduction seen in the 100 mg/kg group; however, these changes did not reach statistical significance (p=0.392). CHOP expression remained inconsistent across the treatment groups, showing no discernible pattern or significant change (p=0.798) except the 200 mg/kg group with a slight decrease. For IRE1, notable variability was observed, with the 50 mg/kg group exhibiting a small decline relative to the HFHF control, though this difference was not significant (p=0.419). PERK expression was decreased in all treatment groups, with significant reductions in the 50 and 100 mg/kg groups; however, statistical analysis indicated no overall significance (p=0.357). Similarly, TRAF2 expression was also reduced in all groups, with most decrease in the 100 mg/kg group, but the changes among groups did not achieve significance (p=0.470). These findings suggest that while 6-gingerol influences ER stress marker expression, its impact under these experimental conditions requires further investigation.

The results shown above can also be observed in the fold change table (Table 3) below, which provides the relative fold changes of various markers in the three treatment groups (50 mg/kg, 100 mg/kg, and 200 mg/kg) compared to the HFHF group.

Table 3. Fold changes relative to HFHF group

Marker	HFHF Group Mean $2^{\Delta\Delta Ct}$	Relative Fold Change		
		50 mg/kg	100 mg/kg	200 mg/kg
GRP78	4.45	0.70	0.64	0.85
IRE1	4.54	0.91	1.44	1.04
CHOP	1.29	1.50	1.53	0.85
PERK	4.40	0.51	0.17	0.88
TRAF2	1.71	0.76	0.30	0.76

HFHF; High Fat High Fructose

3.2 Effects of 6-Gingerol on Apoptotic Markers

Figure 2 illustrates the effect of 6-Gingerol treatment on Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic) protein concentrations in hepatic tissue. The HFHF control group showed no significant elevation in the expression of Bax and Bcl-2 compared with the normal group.

Bax levels (Figure 2A) showed no statistically significant differences among the groups ($p=0.071$, one-way ANOVA), although a decrease was observed in the 6G-100 group, compared to the Normal and HFHF groups. For Bcl-2 concentrations (Figure 2B), Welch's ANOVA revealed significant differences among the groups ($p=0.041$). Post hoc Games-Howell analysis found no significant differences between the normal and HFHF control groups or between the HFHF control and any treatment groups. However, a slight increase in Bcl-2 levels was observed in the 6G-200 group compared to HFHF group. This increase was not statistically significant but was consistent with our hypothesis.

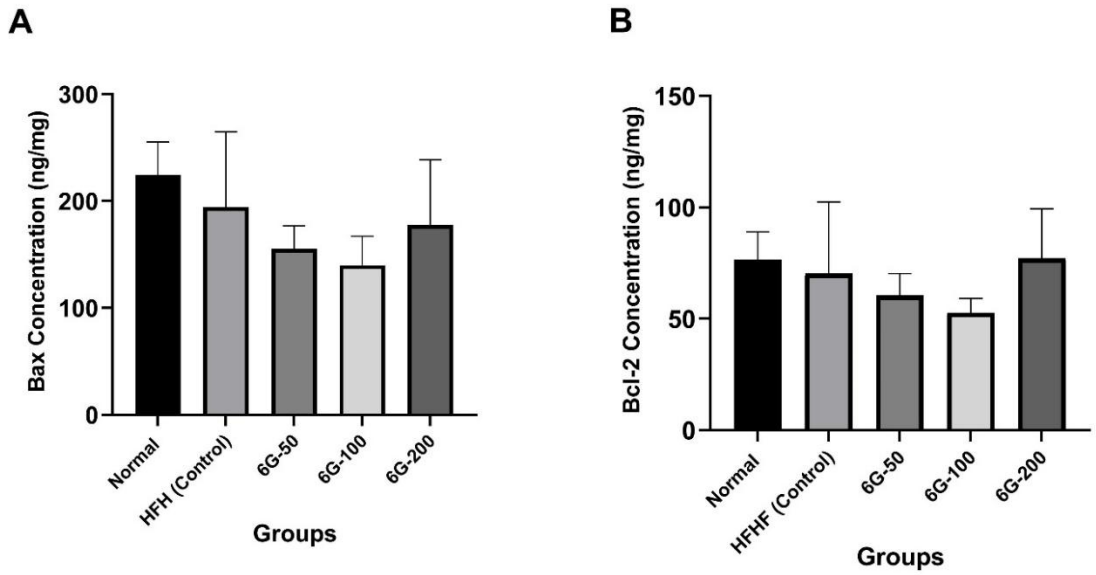

Figure 3. Effect of 6-Gingerol on Bax (A) and Bcl-2 (B) Protein Concentrations (ng/mg) in Hepatic Tissue. Groups include Normal (Chow diet), Control (HFHF diet), and 6-Gingerol-treated groups (6G-50, 6G-100, and 6G-200, corresponding to 50, 100, and 200 mg/kg, respectively). Data are expressed as mean ± SEM. One-way ANOVA revealed no statistically significant differences in Bax levels among groups (p=0.071). For Bcl-2, statistical significance was observed (p=0.041), but pairwise comparisons showed no significant differences.

The Bax/Bcl-2 ratio was analyzed (Kruskal-Wallis Test) among the five groups (Figure 3) and expressed as the median ± interquartile range (IQR). No statistically significant differences were found in the Bax/Bcl-2 ratio among the groups (p=0.186). But the ratio was decreased in all three treatment groups with 6G-100 group showing the highest decrease.

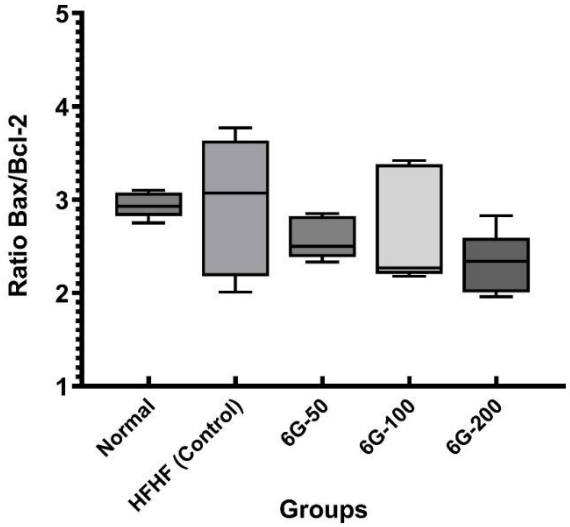

Figure 4. Bax/Bcl-2 Ratio. Groups include Normal (Chow diet), Control (HFHF diet), and 6-gingerol-treated groups (6G-50, 6G-100, and 6G-200, representing 50, 100, and 200 mg/kg, respectively). Data are presented as median ± IQR. Kruskal-Wallis test did not show statistically significant differences between groups (p = 0.186).

This study assessed the effects of 6-gingerol on hepatic ER stress markers (GRP78, CHOP, IRE1, PERK, and TRAF2) and apoptotic proteins (Bax and Bcl-2) in rats fed an HFHF diet. 6-Gingerol exhibited variable effects on ER stress markers at varying doses across the treatment groups. While some decreasing levels, including reduced GRP78, IRE1, PERK and TRAF2 expression, were observed in some groups compared to the HFHF control group, these differences were not statistically significant. Similarly, no significant changes were observed in Bax or Bcl-2 levels. However, Bcl-2 levels showed a slight increase in the 6G-200 group, leading to a decrease in the Bax/Bcl-2 ratio across all three treatment groups.

Variability in the progression of metabolic syndrome and ER stress induction has been reported in previous studies, particularly concerning the duration of high-fat-diet exposure. For instance, CHOP expression, an ER stress marker, was found to be insignificant at 16 weeks but showed a pronounced change by 20 weeks in liver tissue [22]. On the contrary, a 16-week high-fructose, high-fat diet significantly upregulated the expression of all ER stress markers, including GRP78, PERK, IRE1α, XBP1, and CHOP [23] whereas only the high-fructose diet showed increase in the expression of GRP78 and IRE1α in another study [24]. This shows that dietary selection and duration influences ER stress manifestation. But other studies showed that while eIF2α phosphorylation, a hallmark of ER stress, was evident in NAFLD, downstream UPR pathways, including ATF4, CHOP, and GADD34 activation, were not upregulated [25]. A good candidate for ER stress induction in hepatic tissue is exposure to heavy metals like cadmium or tunicamycin, which upregulates all ER stress markers [14],[26].

Although 6-gingerol did not produce a statistically significant or consistent reduction in ER stress markers in this model, it demonstrated mitigation of ER stress markers in the experimental groups across varying concentrations. Previous studies have demonstrated the impact of 6-gingerol in a tunicamycin-induced hepatotoxicity model, where it significantly decreased Endoplasmic Reticulum stress markers and the Bax/Bcl-2 ratio [14], [27]. Another derivative of ginger has shown selective effects on ER stress markers, showing regulation of the PERK pathway while exhibiting no impact on IRE1 and ATF pathways [28]. Since our study did not give statistically significant results in terms of attenuating ER stress markers, and no differences were observed between the normal and positive control groups, we cannot conclusively comment on the effect of 6-gingerol on ER stress markers.

Although this study provided insights into the mRNA expression of ER stress markers, it did not assess protein translation using Western Blotting. Western blotting enables direct quantification of protein levels, providing insights into functional protein expression. In contrast, mRNA levels may not reliably indicate protein abundance due to post-transcriptional regulatory mechanisms [29-30]. Western blotting enables precise protein quantification using fluorescent detection systems that generate signals proportional to protein levels [31]. Future studies should include protein-level analyses, such as Western blotting, to confirm these findings. Besides, extending the study duration beyond 16 weeks, perhaps to 20 weeks, may help better induce and capture the effects of ER stress in the liver and evaluate the more pronounced impacts of 6-gingerol on ER stress and apoptotic markers.

4. Conclusion

This study observed a slight reduction in hepatic ER stress markers (GRP78, CHOP, IRE1, PERK, TRAF2) and the Bax/Bcl-2 ratio following 6-gingerol treatment at varying concentrations in HFHF diet-induced metabolic syndrome rats. However, these changes were not statistically significant ($P > 0.05$). While 6-gingerol did not exert a pronounced effect on hepatic ER stress or apoptosis in this model, further investigations with optimized experimental conditions are needed to determine its potential role in modulating cellular stress pathways. Future studies should explore alternate dosages and combination therapies to assess whether 6-gingerol could elicit a more pronounced response in hepatic ER stress regulation.

Several limitations may have influenced the findings of this study. The liver tissues were not preserved in RNAlater after harvesting, which may have affected RNA integrity and the reliability of gene expression analysis. Additionally, the study duration was 16 weeks; extending it to 20 weeks could potentially induce more pronounced ER stress, creating a better model to evaluate 6-gingerol's effects. Moreover, this study focused solely on mRNA expression levels, which may not directly reflect protein translation or functional outcomes. Addressing these limitations in future research will be essential for a more comprehensive understanding of 6-gingerol's impact on ER stress in metabolic syndrome.

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