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Original Research Article

Fucosterol improves palmitic acid-induced oxidative stress, lipid droplet formation and insulin resistance in liver cells by mediating Keap1-Nrf2-lipocalin 13 axis

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Abstract

Purpose: *To determine the possible effects of fucosterol (FST) on non-alcoholic fatty liver disease (NAFLD), and the mechanisms involved.*
Methods: *The NAFLD model was constructed using palmitic acid (PA) induction, and the expression of NF-E2-related factor 2 (Nrf2), lipocalin 13 (LCN13) and Keap1 were analyzed by immunoblot. The oxidative stress of hepatocytes was determined via ELISA assay. In addition, the role of FST on lipid content and metabolism were evaluated by Oil Red O staining and immunoblot, while the levels of p-AKT, p-IRS1, and p-PI3K were evaluated by immunoblot assay.*
Results: *The data revealed that FST significantly increased the viability of PA-induced hepatocytes, and the expression levels of Nrf2 and LCN13 ($p < 0.05$). Fucosterol enhanced Keap1-Nrf2 mediated LCN13 expression, and alleviated PA-induced oxidative stress by contributing to Keap1-Nrf2-LCN13 axis. In addition, it significantly reduced ($p < 0.05$) lipid droplet formation, promoted lipid metabolism, and lowered insulin resistance by enhancing Keap1- Nrf2-LCN13 axis.*
Conclusion: *Fucosterol regulates Keap1-Nrf2-mediated LCN13 to aid the ameliorate palmitic acid-induced oxidative stress, lipid droplet formation and insulin resistance in liver cells.*

Keywords: *Non-alcoholic fatty liver disease (NAFLD), Fucosterol (FST), Keap1-Nrf2-LCN13 axis, Insulin resistance*

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease worldwide [1,2]. Liver fat is physiologically balanced by several progressions including plasma non-esterified free fatty acids, dietary fat intake, fat regeneration, mitochondrial β - oxidation, and the inflow of peripheral fat from intracellular triglyceride (TG) lipolysis. Increased intake of fat in high-fat diet usually results in imbalance in fat metabolism which may lead to the accumulation of fat in the liver. This accumulation promotes fat formation by

enhancing lipogenic enzymes, acetyl-CoA carboxylase and fatty acid synthase (FAS) [3]. NAFLD is a range of histological abnormalities which is characterized by steatosis, lobular inflammation, and ballooning of hepatocytes, and is the result of multiple factors, such as insulin resistance, oxidative stress, impaired autophagy, lipid toxicity, and enterogenic lipopolysaccharides [4]. However, there are currently no approved drug treatments for NAFLD.

Ecklonia kurome is an important traditional Chinese medicine in *Kunzao Tiaozhi* capsule. It contains Fucosterol (FST) belonging to sterols and has a variety of biological attributes, including anti-osteoarthritis, anti-cancer, anti-inflammatory, anti-photoaging, immune regulation, and liver protection. The FST can effectively up-regulate the phosphorylation of ATP-activated protein kinase and acetyl-CoA carboxylase, and down-regulate the expression level of adipogenesis related factors [5]. In addition, FST can also play a role in alleviating macrophage inflammation and oxidative stress by regulating the Keap1-Nrf2-HO-1 pathway [6]. Previous studies have shown that lipocalin 13 (LCN13) is overexpressed in the liver of obese mice induced by a long-term high-fat diet [7]. It has been reported that LCN13 plays a non-negligible role in mediating glucose metabolism, fat production and insulin sensitivity [8]. However, the possible effects of FST on NAFLD and the possible mechanism are still unclear. In this study, the possible effects of FST on NAFLD and the regulatory mechanisms was investigated.

EXPERIMENTAL

Cell culture

HepG2 cells were obtained from American Type Culture Collection (ATCC). The Cells were cultured with Dulbecco's modified Eagle's medium containing L-glutamine, glucose, 15 mM Hepes, 200 U/mL penicillin, 270 μg/mL streptomycin and 10 % (v/v) fetal bovine serum (FBS) at 37 °C under a humidified atmosphere of 5 % CO_2. The PA stock solution was prepared by the soaping of PA and subsequently mixed with bovine serum albumin (BSA) blocking buffer. Palmitic acid (100 mM) was incubated in 0.1 N NaOH at 70 °C for 30 mins. A 50 μL volume of 100 mM PA was added to 950 μL of 10 % BSA blocking buffer at 55 °C, vortexed and incubated further for 15 min at 55 °C. Final working solution contained 5 mM PA. For shRNA experiments, oligonucleotides targeting LCN13 (5'-GCCGTGAGTTTAAATTCGTGA-3') were annealed and cloned into the pENTR/U6 shRNA vector (Invitrogen). Plasmids encoding LCN13-specific or non-specific shRNA were transfected.

Cell viability

The experiment was performed by following the procedure reported in a previous study [4]. The HepG2 cells were plated at a density of 3×10^3 cells/well into 96-well plates. Cell viability was assessed with the addition of CCK-8 solution, and the cells were incubated for another 4 h before the absorbance was measured at 520 nm wavelength.

Quantitative real time-polymerase chain reaction (qRT-PCR)

Total cellular RNA was extracted with TRIzol reagents (Thermo, Rockford, USA). The total RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega Corporation, Madison, WI, USA). The cDNA was amplified using the following primers in Table 1.

Table 1: Primer sequences used in PCR

Primer	Forward	Reverse
LCN13	GTCATTCGGGA TGGGAAAG	CTGTTGCAGAC CTGGGTA
GAPDH	AGAAGGCTGGG GCTCATTTG	AGGGGCCATCC ACAGTCTTC

Western blot

Proteins were extracted with RIPA buffer (Beyotime, Shanghai, China). Then, the samples were collected, electrophoresed by 10 % SDS-PAGE, transferred onto PVDF membranes, and then blocked with 5 % fat-free milk. Subsequently, the membranes were incubated with primary antibodies targeting the following antibodies for 1 h, including Keap1, p65, Nrf2, HO-1, NQO-1, and LCN-13 (all product for mouse, 1:1000, Abcam), as well as SREBP1c, SCD1, PPAR-α, p-AKT, AKT, p-PI3K, PI3K, p-IRS-1, and IRS-1 (for rabbit, 1:1000, Abcam), and β-actin (Mouse, 1:10000, Abcam). Then the membranes were conjugated with the anti-mouse IgG and anti-rabbit IgG antibodies (Abcam, Cambridge, UK) at room temperature for 1 h. Specific proteins were then visualized with enhanced chemiluminescence detection kit (ECL, Thermo, Rockford, USA).

Determination of superoxide dismutase (SOD), malondialdehyde (MDA), glutathione (GSH) and myeloperoxidase (MPO) levels

After the indicated treatment on HepG2 cells, these cells were collected to determine the levels of MDA, SOD, GSH, and MPO with relevant

commercial kits (Jiancheng Bioengineering Institute of Nanjing, China). The cells were centrifuged (1000 *g*) for 20 min and the supernatant was collected. Then the samples were gently shaken, mixed, and covered for reaction at 37 °C for 2 h. A microplate reader was then employed to determine the absorbance at wavelength of 450 nm immediately.

Oil Red O staining

The HepG2 cells were washed with phosphate-buffered saline (PBS) and fixed with methanol: acetic acid (3:1) solution overnight at 4 °C, and subsequently washed and incubated using PBS containing Oil Red O for 30 min at 37 °C. Images were then taken by a microscope (Nikon, Tokyo, Japan).

Statistical analysis

GraphPad 6.0 was used for statistical analysis. Triplicate determinations were performed for each experiment. One-way ANOVA and student's *t*- test were used for statistical comparisons. $P < 0.05$ was considered statistically significant.

RESULTS

Fucosterol promotes LCN13 expression mediated by Keap1-Nrf2

Fucosterol had no significant effect on cell viability. Palmitic acid treatment led to decreased cell viability in liver cells, and the addition of FST improved cell viability when stimulated by PA (Figure 1 A). The PA significantly reduced the mRNA level of LCN13, and FST treatment reversed the level of LCN13 ($p < 0.05$; Figure 1 B). In addition, PA upregulated the level of Keap1, but it reduced the levels of Nrf2, HO-1 and NQO-1. The effect of PA on these alterations were abrogated by FST treatment (Figure 1 C). In order to verify the involvement of Nrf2 in LCN13 induction, Nrf2 was depleted in the presence of FST, and it was observed that Nrf2 depletion blocked the induction of LCN13 by FST (Figure 1 D). Moreover, the upregulation of Nrf2, LVN13, HO-1 and NQO-1 by FST were significantly inhibited by Nrf2 depletion ($p < 0.05$, Figure 1 E). Fucosterol promoted the LCN13 expression mediated by Keap1-Nrf2 in liver cells.

Fucosterol relieves LCN13-induced oxidative stress in liver cells

To further reveal the role of LCN13 in liver cells, the depletion of LCN13 was performed and the silencing efficacy was confirmed in liver cells (Figure 2 A). The levels of SOD, MDA, GSH and MPO were analyzed in different groups. The upregulation of MDA and MPO, and downregulation of SOD and GSH in PA group were confirmed, and the treatment of FST significantly reversed the SOD, MDA, GSH and MPO levels caused by LCN13 depletion ($p < 0.05$) while, LCN13 knockdown abrogated the effect of FST in liver cells in oxidative stress (Figure 2 B). These results suggest that FST is associated with reduced oxidative stress and dependent on LCN13 in liver cells.

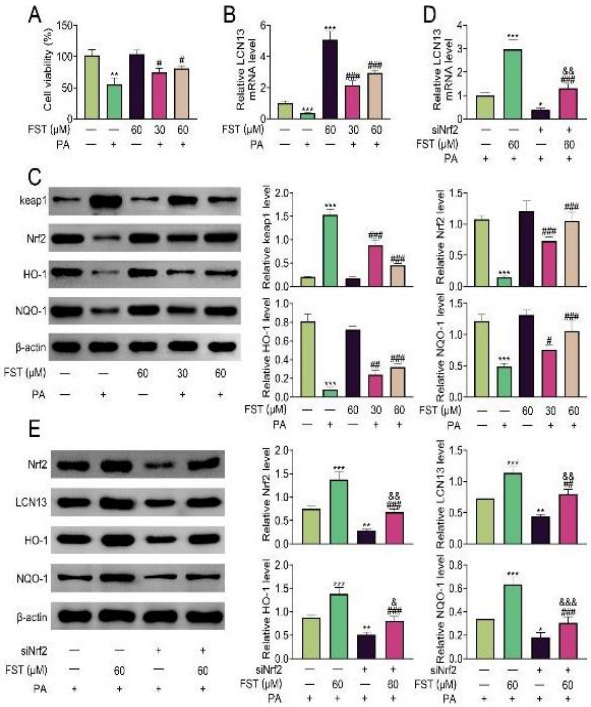

Figure 1: Fucosterol promotes LCN13 expression mediated by Keap1-Nrf2. (A) The cell viability of liver cells exposed to PA and FST. (B) The relative level of LCN13 of liver cells exposed to PA and FST. (C) The Expression levels of Keap1, Nrf2, HO-1, NQO-1 in liver cells exposed to PA and FST. (D) The level of LCN13 in PA, FST and Nrf2 depleted liver cells. (E) The Levels of Nrf2, LCN13, HO-1, NQO-1 in PA, FST and Nrf2 depleted liver cells. ***$P < 0.001$ vs control, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs PA

Fucosterol reduces lipid droplet formation and promotes lipid metabolism

As revealed by Oil Red O staining, the PA significantly resulted in lipid accumulation. Fucosterol treatment relieved the lipid content in the cells, whereas LCN13 knockdown abrogated the effects of FST treatment in lipid content in liver cells (Figure 3 A). Palmitic acid enhanced the levels of SREBP1c and SCD1, and reduced PPAR-α levels. The LCN13 depletion restored the effect of PA on lipid metabolism proteins (Figure 3 B). Therefore, FST reduced lipid

droplet formation and promoted lipid metabolism by enhancing Keap1-Nrf2-LCN13 axis.

Fucosterol reduces insulin resistance

Palmitic acid significantly repressed the level of p-AKT, p-PI3K and p-IRS-1, and fucosterol treatment alleviated these alterations. The improved insulin signaling pathway by fucosterol was impaired by LCN13 depletion (Figure 4). These results indicated that fucosterol reduced insulin resistance by enhancing Keap1-Nrf2-LCN13 axis.

DISCUSSION

Non-alcoholic fatty liver disease (NAFLD) is a clinicopathological syndrome characterized by excessive deposition of fat in liver cells, in the absence of alcohol and other clear liver damaging factors [9]. Simple fatty liver is a relatively benign stage of NAFLD, and it is easily reversed. About 10 – 20 % of simple fatty liver may progress to non-alcoholic steatohepatitis (NASH), which is an important step in the progression of NAFLD to end-stage liver diseases such as cirrhosis, hepatocellular carcinoma and liver failure [10]. Epidemiological investigation has shown that the incidence of NAFLD in China is about 15 %, while that in Europe and America is more than 20 %. Therefore, it is of great significance to explore effective therapies for NAFLD, and an in-depth study of the pathogenesis of NAFLD is crucial for the formulation of effective treatment strategies [11].

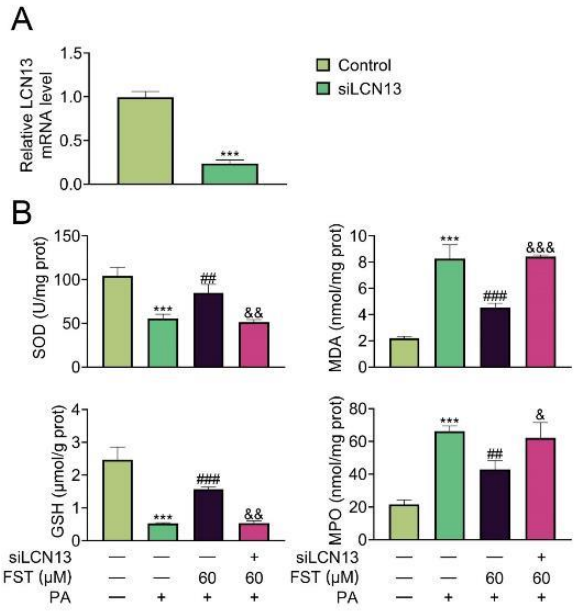

Figure 2: Fucosterol relieves LCN13-induced oxidative stress in liver cells. (A) The knockdown efficacy of LCN13 in liver cells. (B) The SOD, GSH, MDA and MPO levels in control, PA, FST+PA, and LCN13 depleted FST+PA cells. ***P < 0.001 vs control, $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01, $^{\#\#\#}p$ < 0.001 vs PA, $^{\&}p$ < 0.05, $^{\&\&}p$ < 0.01, $^{\&\&\&}p$ < 0.001 vs FST+PA

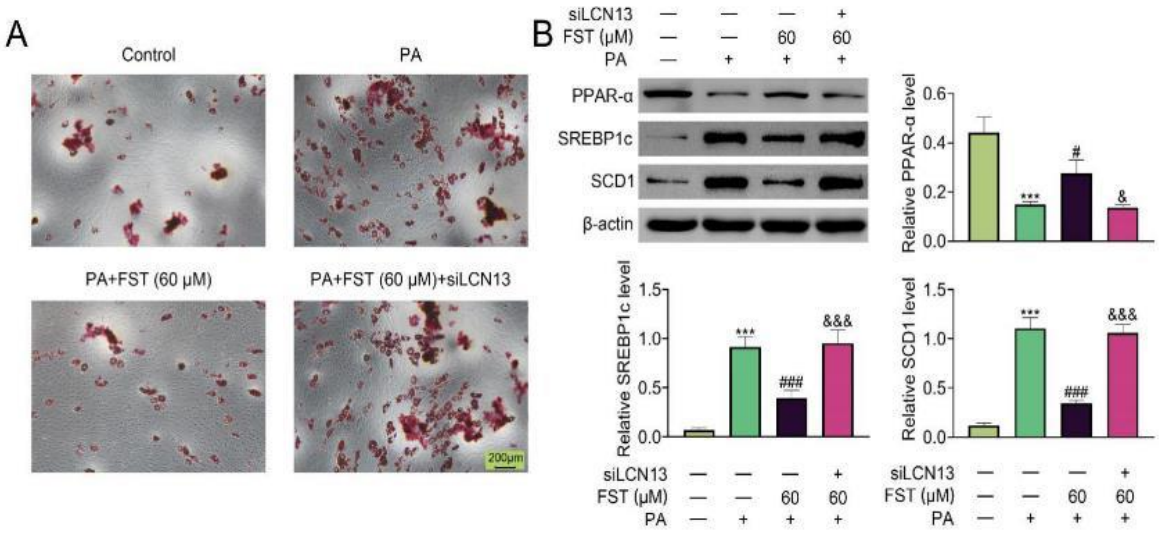

Figure 3: Fucosterol reduce lipid droplet formation and enhanced lipid metabolism by enhancing Keap1-Nrf2-mediated LCN13. (A) Oil Red O staining of liver cells in control, PA, FST+PA, and LCN13 depleted FST+PA cells. (B) The levels of PPAR-a, SREBP1c, and SCD1 in control, PA, FST+PA, and LCN13 depleted FST+PA cells. ***P < 0.001 vs control, $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01, $^{\#\#\#}p$ < 0.001 vs PA, $^{\&}p$ < 0.05, $^{\&\&}p$ < 0.01, $^{\&\&\&}p$ < 0.001 vs FST+PA

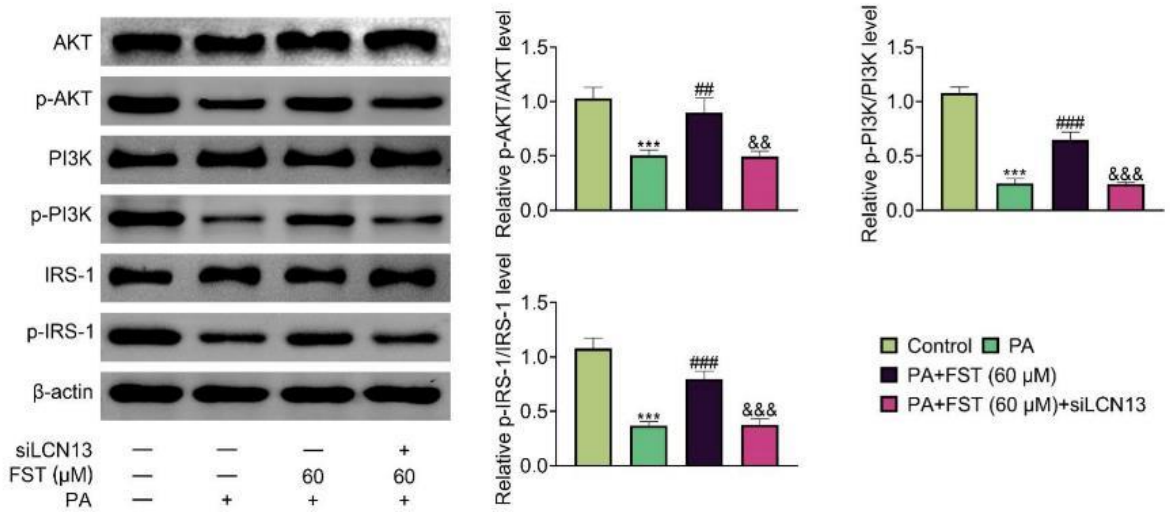

Figure 4: Fucosterol reduces insulin resistance by enhancing Keap1-Nrf2-mediated LCN13. The level of p-AKT, p-IRS-1, and p-PI3K in control, PA, FST+PA, and LCN13 depleted FST+PA cells. ***P < 0.001 vs control, $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01, $^{\#\#\#}p$ < 0.001 vs PA, $^{\&}p$ < 0.05, $^{\&\&}p$ < 0.01, $^{\&\&\&}p$ < 0.001 vs FST+PA

In this study, fucosterol improved palmitic acid-induced oxidative stress, lipid droplet formation, and insulin resistance in liver cells via mediating LCN13, suggesting that Fucosterol could serve as a promising drug of NAFLD. Through CCK-8, Immunoblot, and ELISA assays, fucosterol increased the activity of hepatocytes and the expression levels of Nrf2 and LCN13, and alleviated oxidative stress. Furthermore, through hematoxylin-eosin (HE) staining, Oil Red O staining and Immunoblot, fucosterol reduced lipid droplet formation, promoted lipid metabolism, and inhibited insulin resistance. These data indicate that fucosterol may serve as a drug for NAFLD. Fucosterol has a variety of biological activities such as anti-osteoarthritis, anti-cancer, anti-inflammatory, anti-photoaging, immune regulation, liver protection and other functions, which have been widely revealed [12]. It is known as a bioactive compound belonging to the sterol group which may be isolated from marine algae.

A previous study indicated that fucosterol suppressed ovarian cancer progression by stimulating mitochondrial dysfunction as well as endoplasmic reticulum stress [13]. Similarly, fucosterol alleviated oxidative stress in hepatocytes, and exhibited anti-proliferative effects on lung cancer cells by stimulating apoptosis and the arrest of cell cycle. In addition, it could protect against Aβ-induced neurotoxicity, and reduce Aβintracellular levels in Aβ-induced SH-SY5Y cells.

The effects of fucosterol on liver tissues have also been revealed [14]. Fucosterol caused small changes in the lipid storage in Atlantic salmon hepatocytes [15]. Another study indicated that fucosterol could protect against concanavalin A-induced acute liver injury (ALI) via the MAPK pathway [16]. In this study, fucosterol reduced lipid droplet formation, promoted lipid metabolism, and inhibited insulin resistance in hepatocytes.

Previous studies have also indicated that LCN13 was significantly down-regulated in the livers of Nrf2 knockout mice [17]. The LCN13 play a significant role in glucose metabolism regulation, adipogenesis, and insulin sensitivity [18]. In this study, fucosterol improved the expression of Keap1-Nrf2-LCN13 axis.

CONCLUSION

Fucosterol increases Keap1-Nrf2-mediated LCN13, and therefore reduces lipid droplet formation, promotes lipid metabolism, and inhibits insulin resistance. Thus, it can potentially be developed as a drug for the management of non-alcoholic fatty liver disease.

DECLARATIONS

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None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors contributed to the study conception and design. Material preparation and the experiments were performed by Zhekun Xiong and Liming Gan. Data collection and analysis were performed by Chuntian Sun, Hui Zhang and Yanshan He, and the first draft of the manuscript was written by Huixia Su, Yiyuan Zheng and Fanglian Liao. All authors read and approved the final draft of the manuscript.

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