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# Long Term Green Tea Intervention with Exercise Efficiently Ameliorate Hepatic Steatosis and Obesity Complication in High Fat Diet Induced Mice

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## Abstract

Dietary habits and exercise play important roles in wellbeing of human health. Currently, how long of drinking tea with exercise could be efficiently ameliorating hepatic steatosis and obesity complication still need to be investigated. Here, we comparatively explored short and long term green tea consumption plus exercise improve hepatic steatosis and obesity complication in high fat diet (HFD) mice. Our results showed that Yunkang 10 green tea (YKGT) plus exercise exhibited synergistic prevention effects on ameliorating hepatic steatosis and obesity complication at the 8-week intervention. Moreover, 22-week intervention with YKGT or exercise improved all symptoms of obesity complication, which indicated that long term intervention exhibited profound preventive effects than short term. All treatments inhibited NF-κB activation and pro-inflammatory cytokine expression at both 8- and 22-week intervention, indicated that inflammation may be a cause event for developing hepatic steatosis. The key molecules for regulating lipid and glucose metabolism SCD1 were obviously down-regulated, and GLU2 and PPARγ were significantly up-regulated by YKGT and exercise in the liver of HFD mice. This study demonstrated that long term intervention with YKGT and exercise effectively relieved hepatic steatosis and obesity complication by ameliorating hepatic inflammation, reducing lipid synthesis and accelerating glucose transport.

## 1 Introduction

Dietary habits and exercise play important roles in the well-being of human health. Currently, how long drinking tea with exercise could efficiently ameliorate hepatic steatosis and obesity complication still need to be investigated. Hepatic steatosis associated with obesity is the most common cause of chronic liver disease and may progress to nonalcoholic steatohepatitis and end-stage liver disease1. The risk for developing hepatic steatosis is closely related to diet and lifestyle. The rapid development of the economy and technology has resulted in increasing food supply and declining physical activity2, 3. Over the past decades, sedentary lifestyles and over eating habits are primary causes for the growing rate of obesity complications across the world4-6. Although great progress has been made in the treatment of obese complications, some potential side effects are inevitable6, 7. At present, lifestyle improvement, diet intervention, and exercise therapy are the basic strategies for the prevention of obesity complications. Regular exercise training increases oxidative capacity, lipid metabolism, reduces serum triglycerides, blood pressure8, 9. However, what types of diet and exercise would exhibit synergistic beneficial effects? How long intervention could perform profound beneficial effects? And what is the underlying molecular mechanism? All these questions need to be further investigated.

Tea is one of the most popular beverages because of its natural and healthy properties. Green tea has a high amount of monomeric catechin, and (-)-epigallocatechin gallate (EGCG) is the major component of catechin. Many reports have previously demonstrated that catechins, especially EGCG, promote fat oxidation, lower blood total cholesterol and triglycerides, and reduce body weight10. Yun Kang 10 (Camellia sinesis var. assamica cv. Yun Kang 10) is a widely cultivated tea cultivar in Southwestern China. The chemical profiling and volatile components of Yun Kang 10 have been reported11. In a previous paper, our group reported that YKGT has a higher amount of monomeric catechin, and a combination of YKGT supplement with aerobic exercise synergistically ameliorated existing metabolic syndrome12. Previous studies also reported that a combination of green tea and exercise facilitates sports performance and endurance capacity, and effectively prevents obesity13, 14. The goal of this study is to investigate whether a combination YKGT supplement and physical exercise exhibits synergistically preventive effects, and to compare how long intervention period (8 week vs. 22 week) will show effectively preventive effects on hepatic steatosis and obese complication induced by HFD in C57BL/6J Mice.

# 2 Material and methods

## 2.1 Tea sample

YKGT was produced by steaming the leaves collected from Yun Kang 10 tea trees (Camellia sinensis var. assamica cv. Yun Kang 10) in Menghai County of Yunnan province China following a standard protocol. YKGT was further crushed into powder by BLENDER 800S (Warning, Corp., Torrington, CT, USA), and was added into HFD at the concentration of 5% (w/w).

## 2.2 Animal experiments and ethics approval

The specific pathogen-free male C57BL/6J mice, age of five weeks, the weight of 18~21 g, were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The animals were housed in cages at the Animal Facility Center of Anhui Agricultural University, which was controlled with constant temperature (22±1°C) and humidity (50±5%) with a 12:12 h light-dark cycle falls on 8:00 a.m. to 8:00 p.m. The mice were provided with standard AIN93 food and water ad libitum. After 3-week of acclimation, the mice were fed with low-fat-diet (LFD, TP23303, 11% of energy derived from fat), high-fat-diet (HFD, TP23300, 60% of energy derived from fat), HFD with 5% YKGT (YKGT), HFD with treadmill exercise (Ex), HFD with YKGT plus Ex (YKGT+Ex), respectively. Each group has 12 mice. All diets were obtained from Trophic Animal Feed High-tech Co., Ltd (Nantong, China). The composition of diets was listed as Supplementary table S1. The exercise mice received treadmill running 6 days per week during the experimental period. A detailed treadmill running schedule was provided in Supplementary Table S2. Food intake and water drink were recorded daily. Body weight was monitored with a weight scale weekly.

## 2.3 Serum and tissue samples collection

After 8 weeks of treatment, or at the end of 22 weeks of intervention, upon overnight fasting, 6 mice from each group were anesthetized via injection with 4%

chloral hydrate (10 mL/kg, i.p.), and were then sacrificed. Peripheral blood was collected from the ophthalmic vein. Serum was obtained by centrifugation at 3000 rpm/min for 5 min at 4°C, and then stored at -80°C. Liver and abdominal fat weights were measured on a scale. A small piece of liver tissues was preserved in RNA stabilization solution (Thermo Fisher Scientific, Baltics, USA) for gene expression analysis, and was fixed in formaldehyde solution (Zhanyun, Jiangsu, China) for the histological experiment, respectively. The rest of liver tissues were immediately liquid nitrogen frozen and stored at -80°C for protein expression studies.

### 2.4 Serum biochemical parameter analysis

The serum LDL and TC were measured using micro test kits (Johnson medical equipment, Shanghai, China). The enzymatic activity of ALT was analyzed using enzyme kits (Jiancheng Biotechnology, Nanjing, China). Fasting blood glucose was measured using Nova StatStrip XpresstM Glucose CR Meter (Nova Biomedical, Waltham, UK) with Nova StatStrip XpresstM Glu-test Strips (Nova Biomedical, Waltham, UK).

### 2.5 Quantitative Real-Time PCR Assay

Real-time PCR was performed with SYBR Green Master Mix using Real-Time PCR Detection System (CFX96 Touch, Bio-RAD, USA) following the previous protocol15. Primer sequences used for this study are listed in supplementary table S3.

### 2.6 Western blot analysis

Western blot was performed following the method described previously16. The primary antibodies included total-IKKβ, phosphorylation-IKKα/β, total and phosphorylation -IκBα, total and phosphorylation P 65 (Cell Signaling Technology, MA, USA), SCD1, GLUT2, PPARγ and β-actin (Santa Cruz, CA, USA). Appropriate HRP conjugated secondary antibodies were from Santa Cruz (CA, USA). The intensities of protein expression were analyzed using Image J software. β-actin was used as an internal control.

### 2.7 Hematoxylin-eosin staining

Hematoxylin-eosin (HE) staining was performed following the published protocol17. All the images were obtained using microscope (LEICA DM500, USA). Hepatic adipose infiltration cells were counted manually using Image J software.

### 2.8 Statistical analysis

The statistic results were presented as mean ± S.E.M. Graph Pad Prism5 software was used for statistical analysis. Multiple groups were compared by one-way or two-way ANOVA with Tukey's test when appropriate. The student's t-test was conducted to determine significant differences between specific two groups. $P<0.05$ is considered a statistically significant difference.

## 3 Results

### 3.1 YKGT and Ex ameliorated obese complication in HFD mice

An 8-week HFD feeding significantly increased C57BL/6J mice body weight, abdominal fat weight to body weight ratio, serum glucose, TC, LDL, and activity of ALT **(Figure 1 A-G)**. Ex alone for 8-week just averted body weight and TC increase **(Figure 1 A, F).** YKGT alone for 8-week significantly prevented the increases of body weight, abdominal fat weight to body weight ratio, glucose, and ALT activity. However, intervention with YKGT+Ex for 8- week exhibited synergetic effects and prevented all these indexes increases (**Figure 1 A-G**). HFD feeding or YKGT, Ex, YKGT+Ex treatment for 8-week did not alter liver weight to body weight ratio (**Figure 1C**). While, 22-week intervention with YKGT, Ex, YKGT+Ex improved all indexes (**Figure 1A-G**). Our data indicated that long-term intervention exhibited more profound preventive effects than short-term. Liver tissue sections showed the same physiological structures (**Figure 2 A-E)** and existed little amount adipose infiltration cells **(Figure 2 F)** from all 8-week intervention groups of mice. However, 22-week HFD feeding mice displayed aberrantly fatty hepatocytes with high volumes of lipid droplets. Treatment with YKGT, Ex, or YKGT plus Ex persevered the normal liver architecture with minimal deposition of fat droplets in hepatocytes **(Figure 2G-L)**.

### 3.2 YKGT and Ex ameliorated liver inflammation in HFD mice

The mRNA expression of pro-inflammatory cytokines IL-6, TNF-α, and MCP-1 was significantly up-regulated in the liver of HFD group mice compared to that of LFD group mice. However, intervention with YKGT, Ex, or YKGT plus Ex for both 8-week and 22-week significantly prevented the increases of the cytokines genes expression in liver tissue (**Figure 3 A-C**). The phosphorylation of IKK and IκBα is a key process for activating of NF-κB pathway. The western blotting showed that the phosphorylation of KKα/β, IκBα, and P65 protein were all dramatically increased in the HFD mice liver compared to that of LFD mice. Intervention with YKGT, Ex, or YKGT+Ex for both 8-week and 22-week dramatically prevented these phosphorylated protein increases compared to HFD mice **(Figure 3 D-O)**.

The data showed that the phonotype of fatty liver was not altered when mice were fed HFD for 8-week **(Fig 2 A-F)**. However, pro-inflammatory cytokines IL-6, TNF-α, and MCP1 were significantly up-regulated by 8-week HFD feeding **(Fig 3 A-C)**. Feeding HFD for 8-week, the mRNA and protein expression of SCD1 did not change in the liver of HFD mice compared to that of LFD mice. However, in an 8-week intervention with YKGT, Ex, or YKGT+Ex, the mRNA and protein expression of SCD1 was significantly down-regulated **(Figure 4 A, B, D)**. HFD feeding for 22-week significantly up-regulated the SCD1 expressions in the liver of HFD group mice compared to LFD group mice **(Figure 4 A, C, E)**. However, the increase of SCD1gene and protein expression was significantly prevented by YKGT, Ex, or YKGT+Ex intervention **(Figure 4 A, C, E)**. Our data further found that either YKGT or Ex prevented both mRNA and protein expression of SCD1 in the liver of HFD mice **(Fig 4 A-E)**.

### 3.3 YKGT and Ex accelerated glucose metabolism in HFD mice

HFD feeding for 22-week significantly decreased GLUT2 gene and protein expression in the liver of HFD mice. However, a supplement of YKGT, or Ex or YKGT plus Ex, significantly increased GLUT2 gene and protein expression in the liver **(Figure 5 A, C, E)**. In addition, the gene and protein expression of PPARγ did not alter in the liver of HFD group mice compared to that of LFD group mice. While, YKGT, Ex, or YKGT plus Ex obliviously increased PPARγ gene and protein expression in the liver of treated group mice **(Figure 5 B, D, F)**. Our data further revealed that intervention with YKGT, Ex, or YKGT plus Ex for 22-week significantly up-regulated GLUT2 gene and protein expression in the liver of HFD mice **(Figure 5 A, C, E)**, which may contribute to ameliorates HFD induced hepatic steatosis. The results found that gene and protein expression of PPARγ was increased in the liver of HFD mice treated with YKGT, Ex, or YKGT plus Ex for 22-week **(Figure 5 B, D, F)**.

## 4 Discussion

Obesity complication includes obesity, dyslipidemia, diabetes, and fatty liver. Inflammation initiates a vicious cycle between obesity and nonalcoholic fatty liver disease18. Fatty liver disease is characterized by chronic hepatic inflammation. Numerous inflammatory cytokines are involved in the process of developing into hepatic steatosis. Martin et al. reported that short-term GT supplementation did not affect glucose kinetics, however, GT was associated with attenuated insulinemia19. Recently, Bagheri et al. found that the combination of GTE and exercise promotes a further decrease in weight, body mass index, and body fat percentage than exercise alone in inactive overweight women20. Khoo et al. also reported that the combination of decaffeinated green tea extract and voluntary exercise synergistically mitigated nonalcoholic fatty liver disease in HFD mice21. The current data indicated that hepatic inflammation is a preceding event for developing hepatic steatosis. YKGT supplement and treadmill exercise together only exert synergetic beneficial effects for short-term intervention. The long-term 22-week intervention exhibited profound preventive effect than that of the 8-week treatment. The schematic diagram for this study is shown in **Figure 6**.

Indeed, Ma et al found that HFD elevated plasma IL-6, and blockade of IL-6 signaling ameliorated systemic insulin resistance and improves hepatic steatosis in HFD fed mice22. The NF-κB contains multiple proteins complex that controls cytokine production. The phosphorylation of IKK and IκBα is a key factor for activation of NF-κB pathway23. Our results showed that YKGT, Ex or YKGT+Ex all decreased the phosphorylation of IKKα/β and IκBα, and thus inhibited the transcript of pro-inflammatory cytokines at both short and long term intervention. Moreover, the phosphorylated NFκB p65 subunit were significantly down-regulated by YKGT, Ex or YKGT+Ex at 8- and 22-week intervention. Li et al. reported that GTE decreased phosphorylation of the NFκB p65 subunit and alleviated nonalcoholic steatohepatitis NASH in HFD induced mice24. Our data further found that YKGT, exercise or YKGT drinking plus exercise ameliorated hepatic steatosis via inhibiting NFκB activation in liver of HFD mice.

The accumulation of excess fat in the liver is a primary cause of hepatic steatosis. SCD1 is a key regulator for de novo lipogenesis in the liver. SCD1 is also a central regulator of fuel metabolism and catalyzes the synthesis of monounsaturated fatty acids

(MUFA). Li et al. reported that hepatic SCD1 plays a key role in the prevention of steatohepatitis by partitioning excess lipid into MUFA25. Zhou et al. found that aqueous extract of post-fermented tea reversed the hepatic steatosis of hyperlipidemia rats by down-regulating the hepatic SCD1 genes expression in HFD fed rats26. Consistent with previous reports, this study showed that an increase of inflammatory cytokines is a preceding inducer in the development of hepatic steatosis, while YKGT and Ex inhibited the expression of SCD-1 in the liver of HFD mice. Moreover, the liver is an important organ for nutrient metabolism. GLUT2 is the predominant glucose transporter in hepatocytes and PPARγ is the master regulator of glucose metabolism which plays a significant role in protecting the liver from inflammation, oxidation, fibrosis, fatty liver27. Few studies have investigated the exact signaling molecules that may explain the mechanism following exercise training with or without longer-term GTE intake in human and animal studies28. The GLUT2 represents the main gate of glucose uptake by the liver. Ahmed et al. reported that genetic depletion of Soat2 diminished hepatic steatosis via genes regulating de novo lipogenesis and by GLUT2 in female mice29. Green tea polyphenol extract up-regulates the GLUT2 expression in the liver of rats fed a high fructose diet30. Importantly, we further extended our mechanistic exploration in this study and found that short-term drinking tea and exercise might perform synergetic health benefits, and long-term tea-drinking habit or regular exercise could produce the same beneficial effects.

Overall, this study demonstrated that YKGT supplement and exercise effectively relieved hepatic steatosis and obesity complication in HFD Mice by ameliorating hepatic inflammation, reducing lipid synthesis, and accelerating glucose transport and metabolism. Our results suggested that long-term green tea drinking and regular aerobic exercise might be a good habit for preventing hepatic steatosis and obesity complication for the human population.

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## Data Availability Statement

The original contributions presented in the study are included in the article or supplementary materials, further inquiries can be directed to the corresponding author.

## Author Contributions

Ruru Wang, Mingxing Gu, and Yanzhong Zhang performed the experiments and drafted the manuscript. Qinglin Zhong and Linbo Chen designed the experiments and performed the statistical analysis. Daxiang Li, Zhongwen Xie designed the study, edited the manuscript. All authors contributed to the article and approved the submitted version.

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## Ethics Statement

All the animal experimental procedures imposed in this study were by guidelines of institutional animal care and use committee (IACUC) of Anhui Agricultural University with ethical approval code AHAU 2016-002.

## Statement

The study is reported in accordance with ARRIVE guidelines.

## Additional information

## Conflict of 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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**Figure captions**

**Fig. 1.** YKGT and exercise improved symptoms of obesity complications in HFD C57BL/6J mice. Body weight (A), abdominal fat weight to body weight ratio (B) and liver weight to body weight ratio (C), glucose (D), LDL (E), TC (F), and ALT (G) were measured at 8 and 22-week intervention, respectively. * $P<0.05$, ** $P<0.01$, ***$P<0.001$, compared to LFD; and #$P<0.05$, ##$P<0.01$, ###$P<0.001$ compared to HFD. YKGT, Yunkang 10 Green Tea; LDL, low-density lipoprotein; TC, total cholesterol; ALT, alanine aminotransferase activity. (n=6, mean ± SEM).

**Fig. 2.** YKGT and exercise ameliorated fatty liver in HFD C57BL/6J mice. The HE staining of liver sections of LFD groups (A, G), HFD groups (B, H), HFD supplement YKGT groups (C, I), HFD with Ex groups (D, J), HFD with YKGT plus Ex groups (E, K), and statistic results of hepatic adipose infiltration cell (F, L) were presented at 8 and 22-week intervention, respectively. ***$P<0.001$, compared to LFD; and #$P<0.05$, ##$P<0.01$, compared to HFD (Note: 5 images per section and 3 sections per mice were randomly selected for statistical analysis, n=3, mean ± SEM).

**Fig. 3.** YKGT and exercise inhibited inflammation and NFκB activation in the liver of HFD C57BL/6J mice. The mRNA expression of *IL-6* (A), *TNFα* (B), *MCP1* (C) in the liver tissues were quantified by Real Time PCR at 8 and 22 weeks of intervention, respectively. The representing images of protein expression of total IKKβ and phosphorylated IKKα/β (D, E), and statistical results of D (F) and E (G); total and phosphorylated IκBα (H, I), and statistical results of H (J) and I (K); total and phosphorylated P65 (L, M), and statistical results of L (N) and M (O) in the liver tissues at 8 and 22 weeks intervention, respectively. *$P<0.05$, **$P<0.01$, ***$P<0.001$, compared to LFD; and #$P<0.05$, ##$P<0.01$, ###$P<0.001$ compared to HF. NF-κB, nuclear factor kappa light chain enhancer of activated B cells; IL-6, interleukin 6; TNFα, tumor necrosis factor α; MCP-1, monocyte chemotactic protein-1 (n=3-6, mean ± SEM).

**Fig. 4.** YKGT and exercise inhibited SCD1 gene and protein expression in the liver of HFD C57BL/6J mice. The mRNA expression of *SCD1* (A), representing images of protein expression of SCD1 (B, C), and statistical results of B (D) and C (E) were presented in the liver tissues at 8 and 22 weeks intervention, respectively. *$P<0.05$,

***P<0.001, compared to LF; and #P<0.05, ##P<0.01, ###P<0.001 compared to HF. SCD1, Stearoyl CoA desaturase 1 (n=3, mean ± SEM).

**Fig. 5.** YKGT and exercise increased GLU2 and PPARγ gene and protein expression in the liver of HFD C57BL/6 mice. The mRNA expression of *GLU2* mRNA (A) and *PPARγ* (B), representing images of protein expression of GLU2 (C) and PPARγ (D), statistical results of C (E) and D (F) were presented in the liver tissues at 22 weeks intervention, respectively. *P<0.05, compared to LF; and #P<0.05, ##P<0.01, compared to HF. GLU 2, glucose transporter 2; PPARγ, peroxisome proliferator-activated receptor γ (n=3, mean ± SEM).

**Fig. 6.** The schematic diagram for this study.

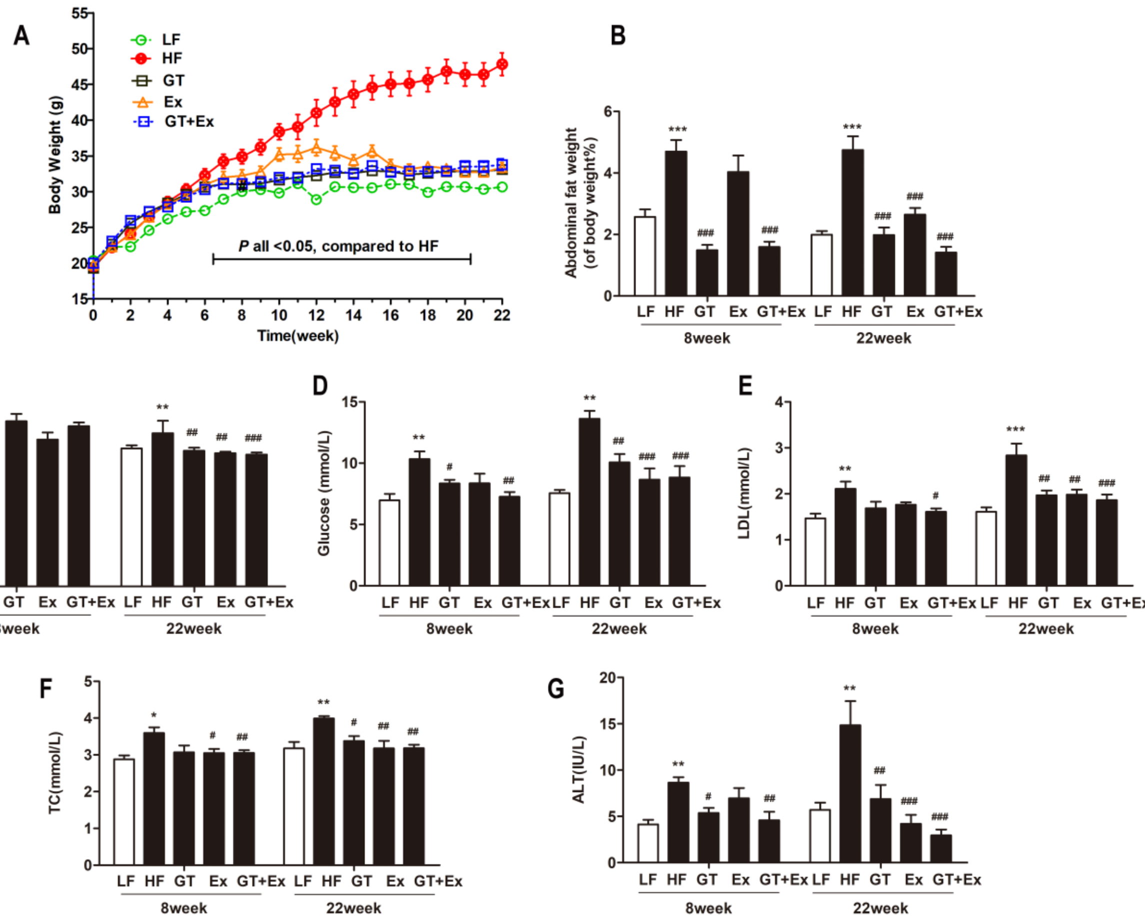
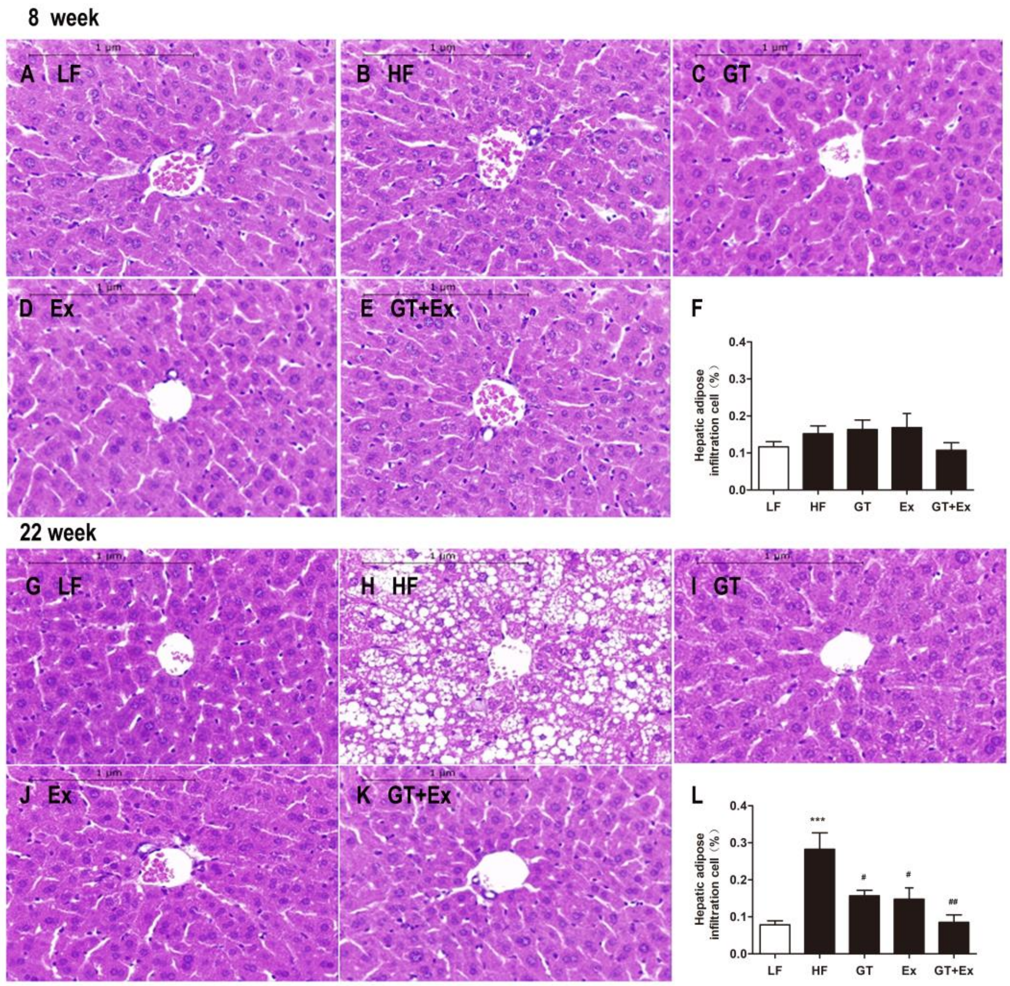



Fig. 1. YKGT and exercise improved symptoms of obesity complications in HFD C57BL/6J mice. Body weight (A), abdominal fat weight to body weight ratio (B) and liver weight to body weight ratio (C), glucose (D), LDL (E), TC (F), and ALT (G) were measured at 8 and 22-week intervention, respectively. * P<0.05, ** P<0.01, ***P<0.001, compared to LFD; and #P<0.05, ##P<0.01, ###P<0.001 compared to HFD. YKGT, Yunkang 10 Green Tea; LDL, low-density lipoprotein; TC, total cholesterol; ALT, alanine aminotransferase activity. (n=6, mean ± SEM).

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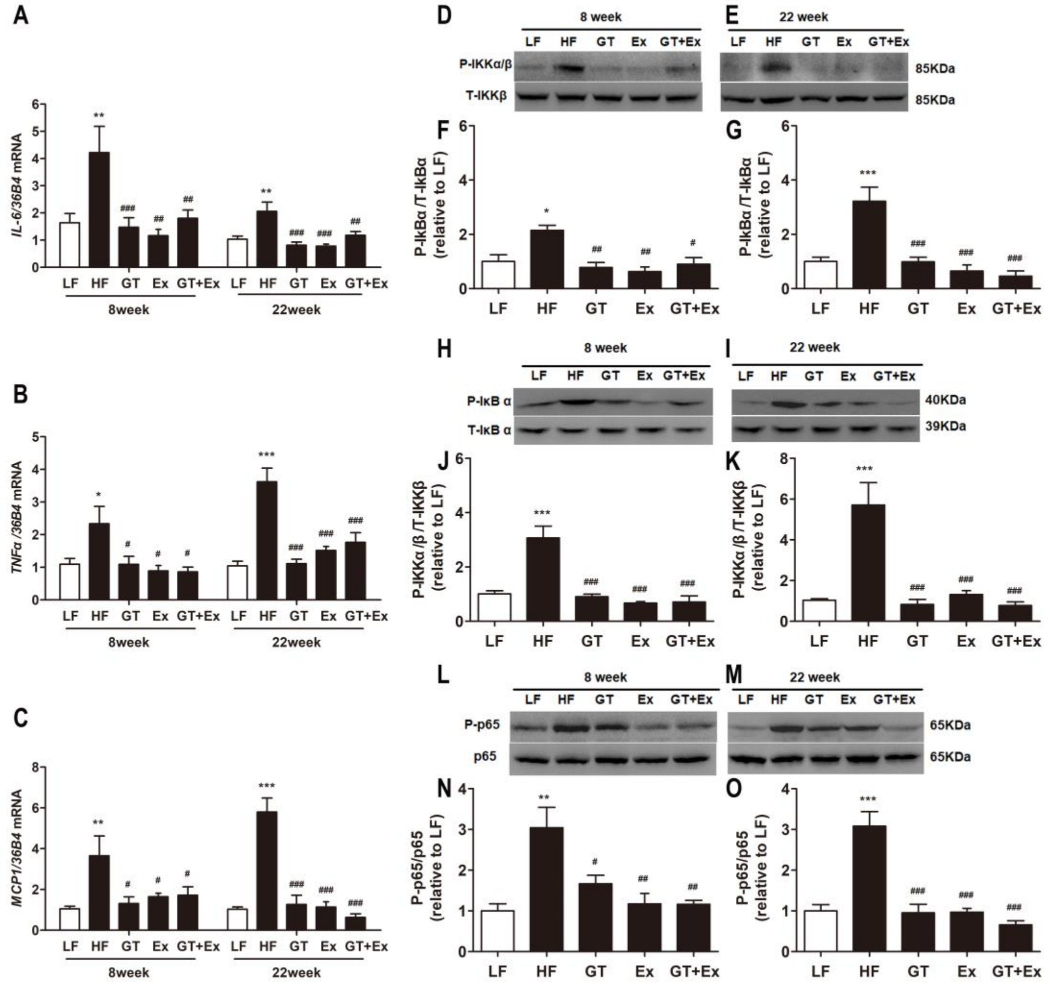


Fig. 3. YKGT and exercise inhibited inflammation and NFκB activation in the liver of HFD C57BL/6J mice. The mRNA expression of *IL-6* (A), *TNFα* (B), *MCP1* (C) in the liver tissues were quantified by Real Time PCR at 8 and 22 weeks of intervention, respectively. The representing images of protein expression of total IKKβ and phosphorylated IKKα/β (D, E), and statistical results of D (F) and E (G); total and phosphorylated IκBα (H, I), and statistical results of H (J) and I (K); total and phosphorylated P65 (L, M), and statistical results of L (N) and M (O) in the liver tissues at 8 and 22 weeks intervention, respectively. *P<0.05, **P<0.01, ***P<0.001, compared to LFD; and #P<0.05, ##P<0.01, ###P<0.001 compared to HF. NF-κB, nuclear factor kappa light chain enhancer of activated B cells; IL-6, interleukin 6; TNFα, tumor necrosis factor α; MCP-1, monocyte chemotactic protein-1 (n=3-6, mean ± SEM).

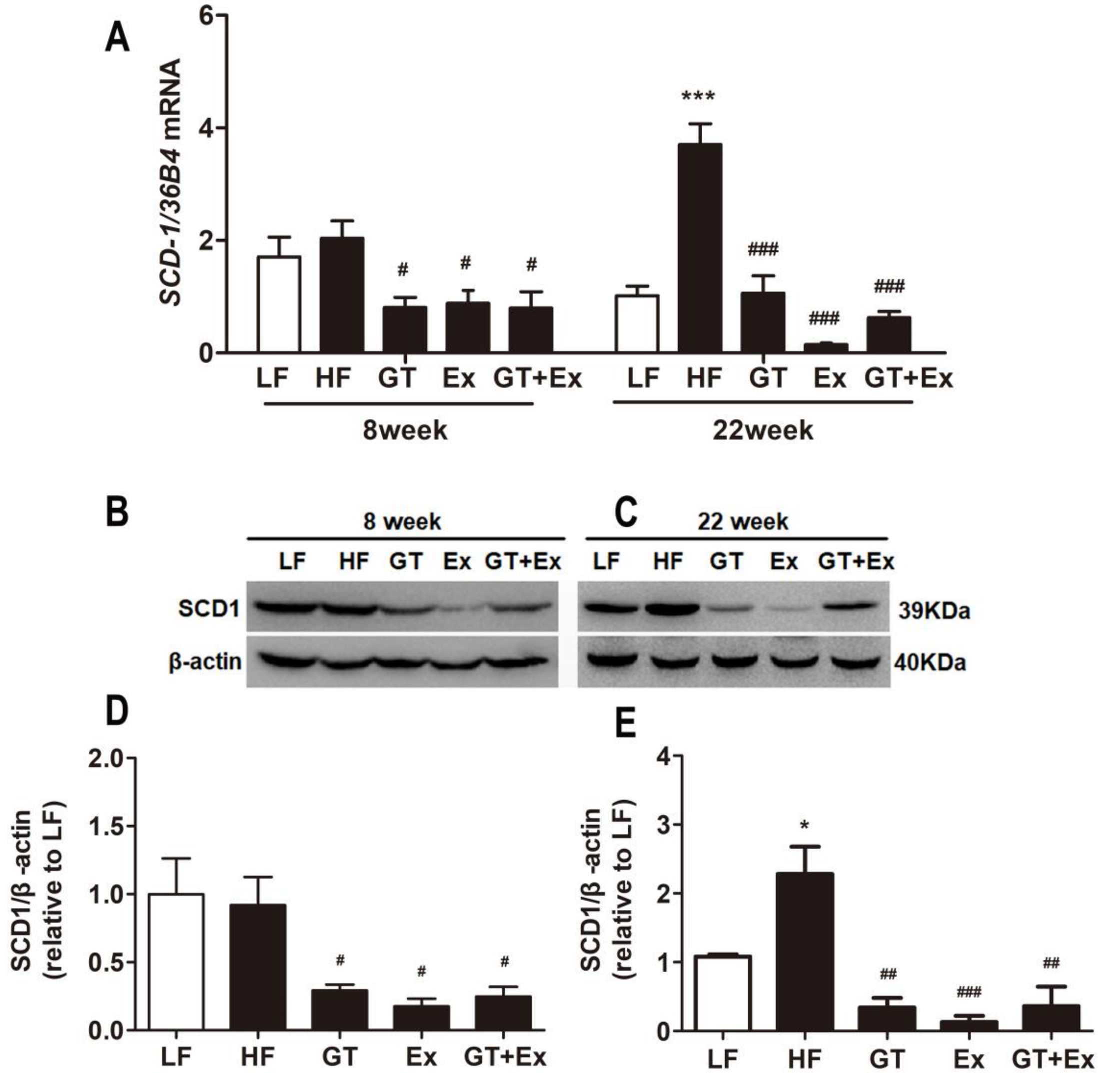


Fig. 4. YKGT and exercise inhibited SCD1 gene and protein expression in the liver of HFD C57BL/6J mice. The mRNA expression of *SCD1* (A), representing images of protein expression of SCD1 (B, C), and statistical results of B (D) and C (E) were presented in the liver tissues at 8 and 22 weeks intervention, respectively. *P<0.05, ***P<0.001, compared to LF; and #P<0.05, ##P<0.01, ###P<0.001 compared to HF. SCD1, Stearoyl CoA desaturase 1 (n=3, mean ± SEM).

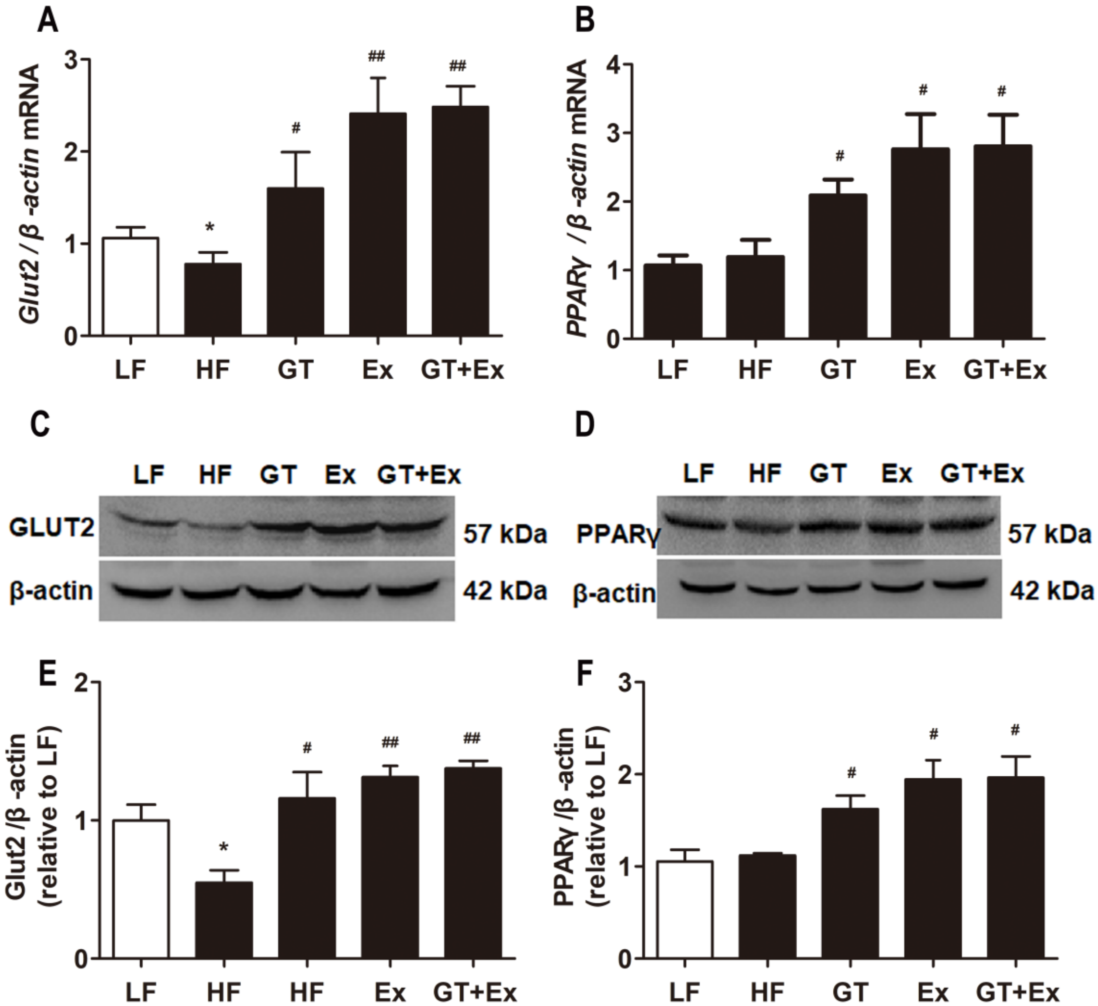


Fig. 5. YKGT and exercise increased GLU2 and PPARγ gene and protein expression in the liver of HFD C57BL/6 mice. The mRNA expression of *GLU2* mRNA (A) and *PPARγ* (B), representing images of protein expression of GLU2 (C) and PPARγ (D), statistical results of C (E) and D (F) were presented in the liver tissues at 22 weeks intervention, respectively. *P<0.05, compared to LF; and #P<0.05, ##P<0.01, compared to HF. GLU 2, glucose transporter 2; PPARγ, peroxisome proliferator-activated receptor γ (n=3, mean ± SEM).

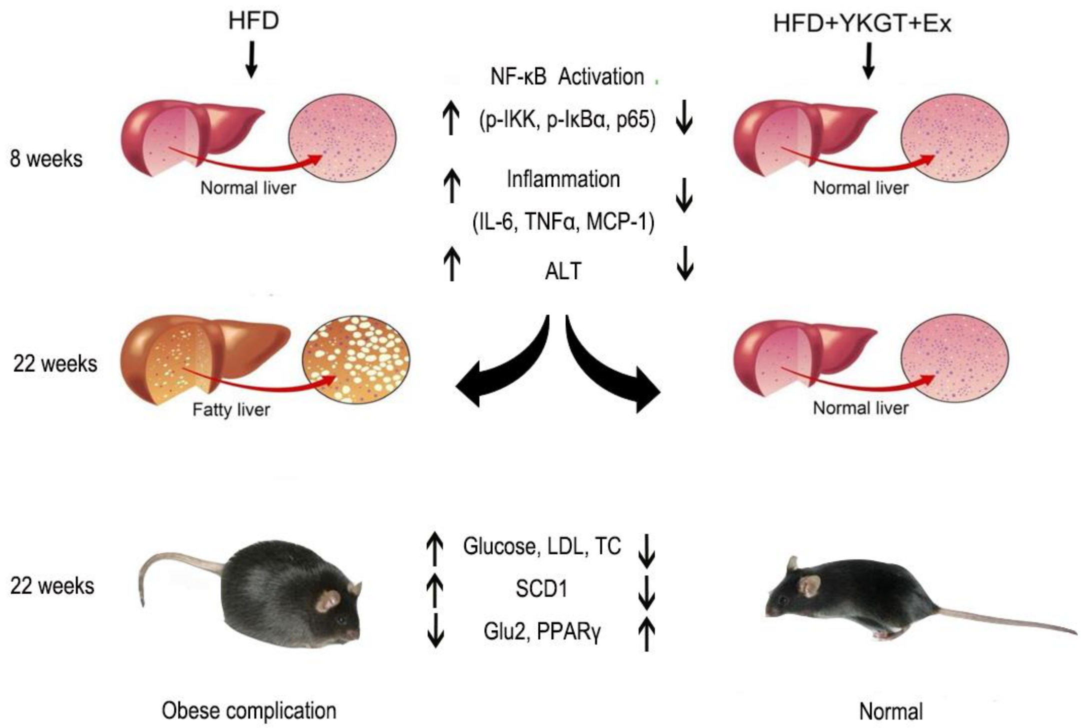


Fig. 6. The schematic diagram for this study.

# Supplementary Files

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- supplementarymaterial.pdf