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Original Article

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Genipin improves lipid metabolism and sperm parameters in obese mice via regulation of miR-132 expression

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Abstract

Obesity has now surpassed malnutrition and infectious diseases as the most significant contributor to health problems worldwide. In particular, obesity is associated with several metabolic disorders, including hyperlipidemia, hepatic steatosis, and subfertility. Genipin (GNP), the aglycone of geniposide, is isolated from the extract of the traditional Chinese medicine *Gardenia jasminoides* Ellis and has been used in traditional oriental medicine against several inflammation-driven diseases. However, the effect and molecular mechanism of GNP on obesity-associated dyslipidemia and sperm dysfunction still need to be explored. In this study, we detect the effects of GNP on hyperlipidemia, hepatic lipid accumulation and sperm function using a high-fat diet (HFD)-induced obese mouse model. We find that obese mice treated with GNP show an improvement in body weight, serum triglyceride levels, serum hormone levels, serum inflammatory cytokines, hepatic steatosis and sperm function. At the molecular level, HFD/GNP diversely regulates the expression of miR-132 in a tissue-specific manner. miR-132 further targets and regulates the expression of SREBP-1c in liver cells, as well as the expressions of SREBP-1c and StAR in Leydig cells in the testis, thus modifying lipogenesis and steroidogenesis, respectively. Collectively, our data demonstrate that GNP shows a broad effect on the improvement of HFD-induced metabolic disorder and sperm dysfunction in male mice by tissue-specific regulation of miR-132. Our findings reveal the function GNP in ameliorating hepatic lipid metabolism and sperm function and suggest that this compound is a versatile drug to treat metabolic disorders.

Key words metabolic disorder, sperm function, miR-132, obesity, genipin

Introduction

Obesity is rising globally and has become one of the most prevalent public health issues worldwide. In the last 40–50 years, a consistent upwards trend of obesity and overweight has emerged in the epidemic. As a key risk factor for many chronic health problems, obesity is associated with diabetes, coronary heart disease, hyperuricemia, sleep-breathing disorders, and certain forms of cancer [1,2]. Moreover, obesity has been reported to affect male fertility through alterations in endocrine profiles, spermatogenesis, and sperm function [3]. Although obesity is associated with aging, changes in lifestyles, such as unbalanced nutrition intake, lack of exercise, drug abuse and stress, have accelerated the occurrence of obesity symptoms in young people [2]. Long-term high-fat diet (HFD) feeding induces obesity in male mice associated with hyperlipidemia, hepatic steatosis, and fertility disorders [4,5].

Currently, there are three major strategies in the management of obesity: (1) behavioral modification, including exercise and dietary management, (2) pharmacologic therapy, and (3) bariatric surgery [6]. Behavioral self-management training, also referred to as lifestyle modification, has figured prominently in the treatment of obesity. However, most behavioral therapies are only temporarily successful in maintaining weight loss. Bariatric surgery is an effective intervention in the treatment of clinically severe obesity, which contributes to weight loss and the improvement of obesity-

related comorbidities [7]. This therapy is also potentially associated with a greater risk of substance misuse disorders, suicide, and nutritional deficiencies [8]. Pharmacological agents have been approved by the Federal Drug Administration (FDA) for the treatment of obesity, including several medications for the long-term treatment of obesity [9]. However, the applications of these medications are limited by their availability and harmful side effects. Therefore, there is an urgent need to develop safe, effective, economic and easily accessible entities. Plant-derived drugs are considered to be the first line of defense to maintain health by preventing and treating obesity and its complications [10]. Recently, many studies have focused on the effective ingredients from natural medicinal plants, such as herbal remedies [11].

Genipin (GNP) is a natural aglycon of geniposide produced by glucosidase hydrolysis and is an active constituent of the traditional Chinese medicine *Gardenia jasminoides* Ellis [12]. GNP is an excellent natural biological cross-linking agent that can be cross-linked with protein, collagen, gelatin and chitosan to make biomaterials [13]. Moreover, GNP has also been reported to possess multipurpose biological and pharmacological activities, such as antitumor, antioxidant, antimicrobial, anti-inflammatory, neuroprotective and hepatoprotective effects [14–16]. Our previous study showed that GNP treatment ameliorates sperm motility and reproductive health of male mice under circadian disruption conditions [12]. However, the effect and molecular mechanism of GNP on the improvement of metabolic disorder and subfertility in obese subjects induced by HFD still need to be explored.

Here, we reported that long-term HFD-fed-induced obesity in male mice is associated with hyperlipidemia, hepatic steatosis, serum hormone disorder and sperm dysfunction, which are ameliorated by GNP treatment. Using molecular and biochemical assays, we demonstrated that diversely regulated miR-132 is involved in HFD-induced metabolic disorder and sperm dysfunction in obese mice. HFD feeding inhibits miR-132 in liver tissues but upregulates miR-132 expression in Leydig cells of the testis. GNP treatment reverses the HFD-regulated miR-132 expression in both tissues. Moreover, we showed that miR-132 affects lipid metabolism in liver cells and regulates steroidogenesis in steroid-producing MLTC-1 cells. Our study identified key regulatory functions of miR-132 that contribute to GNP improvement of metabolic disorder and sperm dysfunction in HFD-induced obese mice.

Materials and Methods

Animals

All animal experiments were performed according to the procedures approved by the Laboratory Animal Care Committee at Nanjing Normal University and followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Six-week-old male C57/BL6 mice were purchased from GemPharmatech Co., Ltd (Nanjing, China). Mice were initially housed under a 12-h light/dark cycle (12:12 LD) at 25°C and had free access to water and food. After a 1-week quarantine period, the mice were randomly divided into two groups: 8 were given a normal laboratory diet (CD), and 10 were fed with a high-fat diet (HFD) (D12492; Research Diets, New Brunswick, USA). Six weeks later, these mice were assigned into four groups, with 4 or 5 mice per group: CS (CD + saline), CG (CD with 20 mg/kg/day GNP), HS (HFD + saline), and HG (HFD with 20 mg/kg/day GNP). GNP (genipin, $C_{11}H_{14}O_5$, HPLC ≥ 98%; Zelang, Nanjing, China) was dissolved in saline solution and given to mice every day by oral gavage for 6 weeks. The doses of GNP were selected based on a previous report [15]. Equal volumes of saline solution were infused into mice as a control. Mouse body weights and food intake were monitored every week.

Primary hepatocyte isolation

Primary hepatocytes were isolated from the livers of C57BL/6J mice as previously described [15]. Briefly, the mouse was anesthetized with 10% chloral hydrate (10 μL/g) (Sigma-Aldrich, St Louis, USA), and then hepatocytes were isolated through digestion using collagenase type II (Sigma-Aldrich). Hepatocytes were maintained in DMEM (Thermo Fisher Scientific, Waltham, USA) supplemented with 10% fetal bovine serum (FBS) (Wisent, St Bruno, Canada), 100 units/mL penicillin and 100 μg/mL streptomycin sulfate (Sigma-Aldrich).

Cell culture and treatment

HepG2 cells were obtained from the Cell Bank of the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science (Shanghai, China). MLTC-1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, USA). All cells were cultured in recommended medium supplemented with 10% FBS and antibiotics at 37°C in a humidified incubator in the presence of 5% CO_2/95% air. For treatment, HepG2 cells were plated and cultured in 6-well or 12-well plates and treated with vehicle, PA (0.4 mM, BSA-palmitate saturated fatty acid) (91763; Glpbio, Montclair, USA), GNP (20 μM, dissolved in DMSO) or PA + GNP for 36 h. For transfection, HepG2 cells, primary hepatocytes and MLTC-1 cells were plated and cultured for 12 h prior to transfection with Lipofectamine 3000 (Invitrogen, Thermo Fisher Scientific (China) Inc., Shanghai, China) according to the manufacturer's instructions. Cells were transfected with miR-132 mimic (sense: 5′-UAACAGUCUACAGCCAUGGUCG-3′; antisense: 5′-CGACCAUGGCUGUAGACUGUUA-3′), inhibitor (5′-CG ACCAUGGCUGUAGACUGUUA-3′) or scrambled oligonucleotide (negative control; sense: 5′-UUUGUACUACACAAAAGUACUG-3′; antisense: 5′-CAGUACUUUUGUGUAGUACAAA-3′) which were obtained from RiboBio (Guangzhou, China) for 48 h before carrying out the planned studies. The supernatants of cultured MLTC-1 media were collected for progesterone measurement, and cells were collected for RNA and protein extraction.

Sperm parameters

The sperm parameters of male mice were detected as previously described [12]. The animals were sacrificed by cervical dislocation. Both sides of the epididymis cauda were quickly removed and immediately crumbed by syringe needles within the 0.2 mL of physiological saline into a petri dish to release spermatozoa. Sperm motility was measured by computer-assisted semen analysis (CASA, WLJY-9000; Weili New Century Technology Development Co., Ltd, Beijing, China). A Macro sperm-counting chamber (Nanjing Yuancheng Company, Nanjing, China) was used to load the spermatozoa samples. Average path velocity (VAP), curvilinear velocity (VCL), straight-line velocity (VSL), amplitude of lateral head displacement (ALH) and beating cross frequency (BCF) were calculated for the sperm of each group of mice by analysing four or five recordings of at least 100 spermatozoa.

Histological analysis

Testicular tissues and liver tissues were collected from different

animals and fixed in 4% paraformaldehyde solution. Paraffin-embedded samples were cut into 5-μm transverse sections for routine hematoxylin & eosin (H&E) staining and oil red O staining (Service Biology, Wuhan, China). All stained sections were examined by light microscopy at × 200 magnification.

Immunofluorescence analysis

Paraffin-embedded sections from tissue specimens were deparaffinized and heated at 100°C in 10 mM citrate buffer (pH 6.0) for 15 min for antigen retrieval. Slides were incubated with anti-StAR primary antibody (A1035, 1:250; ABclonal, Wuhan, China) at 4°C overnight. After being washed with PBST three times, the slides were incubated with fluorescent goat anti-mouse IgG as secondary antibodies (1:200 dilution) in the Alexa Fluor™ 594 Tyramide SuperBoost™ Kit (B40925; Invitrogen, Thermo Fisher Scientific (China) Inc. for 2 h at room temperature. Subsequently, slides were stained with DAPI and visualized under a fluorescence microscope (80I; Nikon, Tokyo, Japan).

Fluorescence *in situ* hybridization (FISH)

The expression of miR-132 in the testis was detected by miRNA FISH using a commercial kit (GenePharma, Shanghai, China) following the manufacturer's manual. Briefly, testicular tissues were fixed in 4% paraformaldehyde solution and made into paraffin embedded sections. The paraffin sections were deparaffinized and then digested with Proteinase K (ST535; Beyotime Company, Suzhou, China) at 37°C for 20 min. The slides were hybridized with 8 ng/mL miR-132-3p FISH probe (sequences: 5′-CGACCATGGCT GTAGACTGTTA-3′) labelled with FAM for 12 h at 37°C. Subsequently, slides were stained with DAPI and visualized under a laser-scanning confocal microscope (Ti-E-A1R; Nikon).

Biochemical analysis

The supernatants of MLTC-1 cell cultures were collected for progesterone measurement using the PROG ELISA kit (#SBJ-M0440; SenBeiJia Biological Technology Co., Nanjing, China). Blood samples were collected from the right retroorbital plexus of different groups of mice and centrifuged at 4000 *g* for 10 min at 4°C. The serum levels of testosterone (#SBJ-M0439), follicle-stimulating hormone (FSH) (#SBJ-M0479), luteinizing hormone (LH) (#SBJ-M0408), thyroid-stimulating hormone (TSH) (#SBJ-M0445) and estradiol (E2) (#SBJ-M0444) were determined using the corresponding commercial kits (SenBeiJia Biological Technology Co.) according to the manufacturer's instructions. The triglyceride (TG) and total cholesterol (TC) levels in serum and in liver samples were assessed using commercial kits (Jiancheng Institute of Biotechnology, Nanjing, China).

Real-time quantitative PCR

Total RNA was extracted from testicular tissues or MLTC-1 cells using TRIzol reagent (Invitrogen, Thermo Fisher Scientific (China) Inc.). Total RNA (1 μg) samples were reverse-transcribed using superscript II reverse transcriptase (Life Technologies, Grand Island, USA). cDNA amplification was performed using a qPCR kit (RR420A; Takara Biomedical Technology (Beijing), Beijing, China) and gene-specific primers on an ABI StepOnePlus system (Applied Biosystems, Foster City, USA) according to the manufacturer's instructions [17]. Ribosomal protein lateral stalk subunit P0 (RPLP0/36B4) was used as an internal control. All primers for qPCR are shown in Table 1. For qRT-PCR of miRNA, total RNA was extracted from different tissues and cells using TRIzol reagent. Then, the expression of miR-132 was detected using a Bulge-loopTM miRNA qRT-PCR Primer Set (Bulge-Loop hsa-miR-132-3p Primer Set, MQPS0000604-1-100), which was specifically designed for miR-132 by RiboBio (Guangzhou, China). This primer set contained one RT primer and a pair of qPCR primers. The qPCR cycling conditions were as follows: denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 10 s and primer annealing and extension at 60°C for 30 s. The relative miRNA expression of miRNAs was normalized to that of the internal control *U6* (Bulge-Loop U6 qPCR Primer Set, MQPS0000002-1-100). Relative miRNA levels were calculated using the comparative threshold $2^{-\Delta\Delta Ct}$ method [18].

Table 1. Sequences of primers used for quantitative real-time PCR

Gene	Primer sequence
h-ACTIN β	Forward: 5′-ACATCCGCAAAGACCTGTAC-3′
	Reverse: 5′-TGATCTTCATTGTGCTGGGTG-3′
h-SREBP1	Forward: 5′-CACCAGCGTCTACCATAGC-3′
	Reverse: 5′-AAAGAGAAGCACCAAGGAGAC-3′
h-FASN	Forward: 5′-TGTTTGAGTTCGTGGAGCAG-3′
	Reverse: 5′-CATGAAGTAGGAGTGGAAGGC-3′
h-ACC1	Forward: 5′-ACAGTGGAGCAAGAATCGG-3′
	Reverse: 5′-AATGGACAGAGTTGAGAGCAC-3′
h-SCD1	Forward: 5′-GAATAGAAGCTGAGAAACTGG-3′
	Reverse: 5′-GCAAGAAAGTGGCAACGAAC-3′
h-ACLY	Forward: 5′-CTTTGACTATGTCTGCTCCCG-3′
	Reverse: 5′-ATCAGGATCTCTTTGTGCCC-3′
m-ACTIN β	Forward: 5′-GGCTGTATTCCCCTCCATCG-3′
	Reverse: 5′-CCAGTTGGTAACAATGCCATGT-3′
m-SREBP1	Forward: 5′-TGACCCGGCTATTCCGTGA-3′
	Reverse: 5′-CTGGGCTGAGCAATACAGTTC-3′
m-FASN	Forward: 5′-GGAAACACATTGGCAAAGTCC-3′
	Reverse: 5′-GATGTAACTCTTATGGGCTGGG-3′
m-ACC1	Forward: 5′-CGGCAGCAGTTACACCACAT-3′
	Reverse: 5′-CATAGCACTGGCCAGCAAAC-3′
m-SCD1	Forward: 5′-TTCTTGCGATACACTCTGGTGC-3′
	Reverse: 5′-CGGGATTGAATGTTCTTGTCGT-3′
m-ACLY	Forward: 5′-ACCCTTTCACTGGGGATCACA-3′
	Reverse: 5′-GACAGGGATCAGGATTTCCTTG-3′
m-STAR	Forward: 5′-CGGGTGGATGGGTCAAGTTC-3′
	Reverse: 5′-GCACTTCGTCCCCGTTCTC-3′

h, means the human genes; m, means the mouse genes.

Western blot analysis

Total proteins were isolated from HepG2 and MLTC-1 cells using RIPA buffer (P0013C; Beyotime Biotechnology, Suzhou, China). Equal amounts of protein (10–15 μg) were separated by 12% SDS-polyacrylamide gel electrophoresis and blotted onto PVDF membranes. The membranes were blocked with 5% skim milk in phosphate-buffered saline (PBS) with 0.1% Tween-20 for 1.5 h and incubated with anti-SREBP-1c (A15586, 1:1000; ABclonal, Wuhan, China), anti-StAR antibody (A1035, 1:1000; ABclonal), or anti-vin-

culin (TBB5) antibody (1:1000; Abways Technology, Shanghai, China) overnight at 4°C. Then, the membranes were washed with PBS for three times and probed with HRP goat anti-rabbit IgG (1:5000; ABclonal) for 1.5 h. The blots were developed via a Tanon-4500 luminescent imaging workstation (Tanon Science & Technology, Shanghai, China).

Statistical analysis

Statistical analysis was performed with GraphPad Prism 6.0 (GraphPad Software, San Diego, USA). Significant differences were identified using Student's *t*-test or analysis of variance (ANOVA) in the case of comparisons among more than two groups. Statistical analyses were performed using ANOVA followed by Bonferroni's post-test. $P < 0.05$ was considered as statistically significant. All data are expressed as the mean ± SD.

Results

Genipin alleviates hepatic steatosis in HFD-induced obese mice

Long-term HFD feeding has been demonstrated to induce hepatic steatosis and metabolic disorders in mice [18]. A previous study showed that GNP treatment decreases lipid accumulation in the liver of HFD-fed obese mice [15]. To detect the effect of GNP on hepatic steatosis, six-week-old C57BL/6 male mice were fed with an HFD for six weeks and fed with HFD + GNP or HFD + saline for another six weeks, saline was used as a control. HFD-fed mice had a significant increase in body weight (BW) compared with the control normal diet (CD) groups. After six weeks of treatment, the HFD-fed obese mice that received GNP (20 mg/kg/day) weighed less than the HFD group mice that received saline gavage (as a control) (Figure 1A,B). The HS and HG groups of mice consumed less HFD food than the other two groups of mice (CS and CG groups) which consumed normal chow diet food (Figure 1C). However, GNP did not show any apparent effect on food intake either in the two CD-fed groups or in the HFD-fed groups, suggesting that GNP did not affect appetite. As expected, HFD feeding led to increases in both hepatic and serum TG and TC levels, which were alleviated by GNP administration (Figure 1D–G). Histological analysis with H&E and Oil Red O staining further confirmed the attenuation of lipid accumulation (hepatic steatosis) in HFD + GNP (HG) mice compared with that in the HFD + saline group (HS) mice (Figure 1H). Furthermore, we assessed the effect of GNP on lipid accumulation in HepG2 cells incubated with palmitic acid (PA) to mimic hyperlipidemic conditions *in vivo*. Accordingly, GNP impeded lipid accumulation in HepG2 cells, which was obtained by PA treatment (Figure 1I). These data strongly suggest that GNP attenuates lipid accumulation

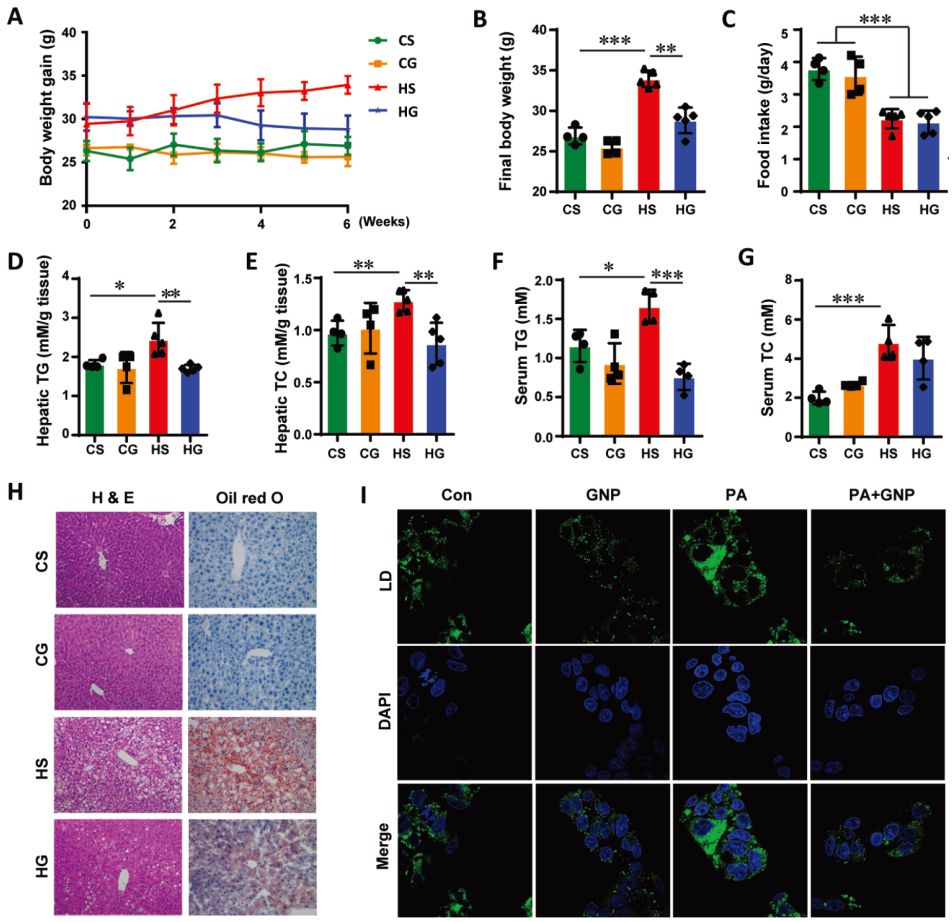

Figure 1. Genipin improves lipid metabolic disorders in HFD-fed obese mice Mice were fed with an HFD for six weeks and then treated with GNP (20 mg/kg/day) by gavage for another six weeks. (A) Body weight gain, (B) final body weight and (C) food intake were examined and are shown following GNP treatment. Food intake data were analysed by ANOVA. (D) Hepatic TG, (E) hepatic TC, (F) serum TG and (G) serum TC in mice with different treatments. (H) Liver tissue sections stained with H&E (left) or Oil red O (right) to visualize lipid contents. (I) Lipid accumulation in HepG2 cells labelled with BODIPY lipid probe. Data are presented as the mean ± SD, $n = 4$ (CS, CG) or $n = 5$ (HS, HG) mice per group. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

in liver cells and alleviates hepatic steatosis in HFD-induced obese mice.

Genipin improves sperm function in HFD-fed obese mice

Obesity is associated with a series of health disorders, including male fertility. Emerging evidence indicates that male obesity negatively impacts male fertility, not only impeding sperm quality but also in particular altering the physical and molecular structure of germ cells in the testes and ultimately mature sperm [19]. Our previous study showed that GNP improves sperm dysfunction and infertility caused by circadian disruption in male mice [12]. Thus, we detected the effect of GNP on the gross anatomy of testes and sperm function in HFD-fed mice. Following 12 weeks of HFD, the testis indexes were reduced, and treatment with GNP increased the testis indexes (Figure 2A). Histological analysis of the testes showed that the diameter of seminiferous tubules remained similar among the four groups, whereas atrophic seminiferous tubules and more vacuoles appeared in the HS group compared to the other three groups (CS, CG and HG) (Figure 2B). We further isolated sperm and analysed the concentration, motility and motion parameters of the sperm by CASA to evaluate the effect of GNP on sperm function in mice under HFD conditions. No differences in sperm concentrations were detected among the four groups (data not shown). Compared with that of male CS mice, sperm motility of male HS mice was significantly decreased, as characterized by lower progressive motility (PR) (7.83% vs 21.27%), lower nonprogressive motility (NP) (10.48% vs 25.47%), and higher immotility (IM) (81.69% vs 55.76%) in the HS group than in the CS group (Figure 2C–E). GNP treatment improved sperm motility after HFD feeding. Consistently, the motion parameters of mouse sperm, VCL, VSL, VAP and ALH, were decreased after 12 weeks of HFD feeding, and GNP treatment restored them (Figure 2F–I). Furthermore, HFD and GNP treatment did not change the BCF of sperm (Figure 2J). These data indicated that GNP treatment could improve sperm function in mice fed with HFD.

Genipin modulates serum hormone and inflammatory cytokine levels dysregulated by HFD

A long-term high-fat diet shows a profound effect on serum hormone levels, an imbalance that correlates with male infertility [4,20,21]. We collected blood from four experimental groups of mice to evaluate the effect of HFD and GNP on hormone balance *in vivo*. Our data showed that HFD feeding for 12 weeks markedly reduced the levels of two sex hormones, testosterone and estradiol, in mice (Figure 3A,B). GNP treatment improved the serum levels of both hormones, which were diminished by HFD. In contrast, HFD enhanced the anterior pituitary hormone FSH and TSH levels, which were restored by GNP treatment (Figure 3C,D). No significant changes were observed in LH levels among different mice under HFD and GNP treatment (data not shown). Furthermore, we detected serum inflammatory cytokines, including IL-6 and TNFα, which affect lipid metabolism and sperm function [22,23]. It was found that HFD feeding significantly promoted serum IL-6 and TNFα levels, which were recovered by GNP treatment (Figure 3E,F). These data indicated that GNP treatment improves both serum hormone levels and the inflammatory response in HFD-fed mice.

Tissue-specific dysregulation of miR-132 by HFD and GNP

Dysregulation of miRNAs has been reported to participate in obesity-associated metabolic syndrome and male fertility disorders

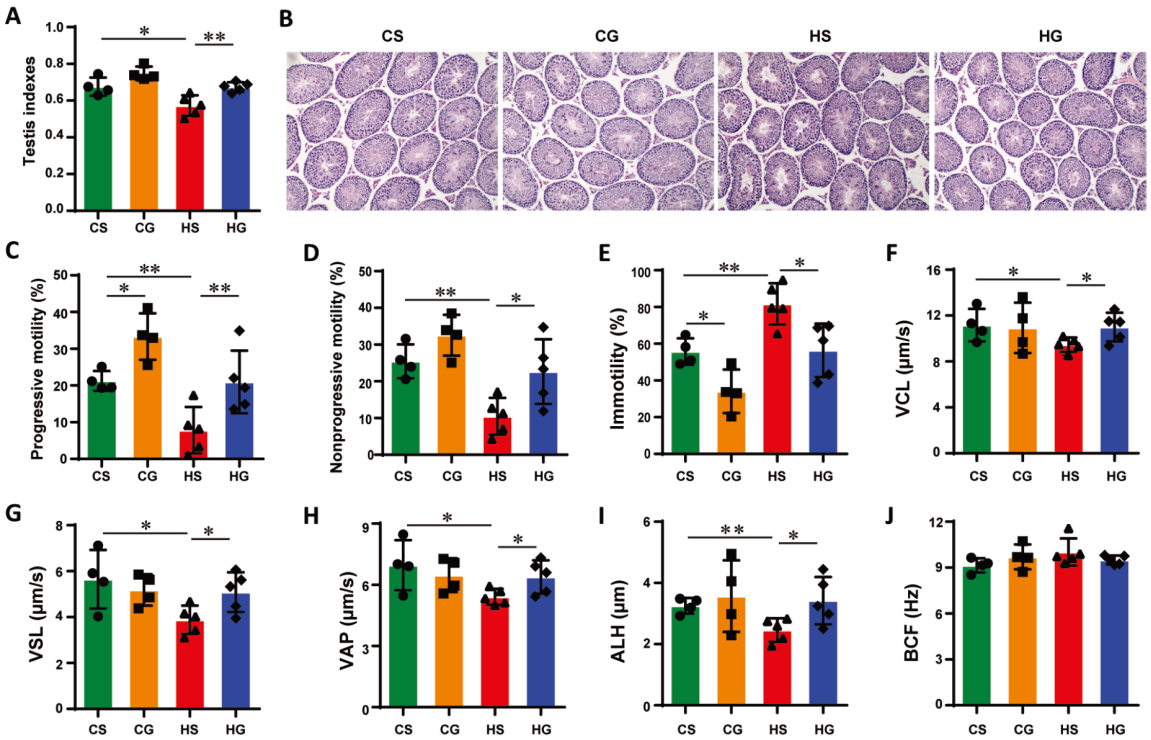

Figure 2. Genipin ameliorates HFD-induced sperm dysfunction in mice (A) Testis index of the four groups. Testis index = Testis weight/Body weight×100. (B) H&E staining of histological cross-sections of seminiferous tubules. GNP treatments recovered sperm function in HFD-fed mice, as indicated by sperm parameters (C) progressive motility, (D) nonprogressive motility, (E) immotility, (F) VCL, (G) VSL, (H) VAP, (I) ALH and (J) BCF of sperm from control or treated mice. Data are presented as the mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

[18,24,25]. Previous studies by our group and others showed that miR-132 directly targets and regulates the expression of SREBP-1c, which is a key regulator in lipid metabolism and steroidogenesis [26–28]. Thus, we assessed whether HFD and GNP treatments affect the expression of miR-132 in the mouse liver and testis. qRT-PCR revealed that hepatic miR-132 level was markedly decreased in the liver of HS mice compared to that in the liver of CS mice (Figure 4A). GNP treatment reversed the expression of miR-132 in mouse liver. Western blot analysis showed that the protein levels of the miR-132 target SREBP-1c were increased in the liver of the HS group, whereas this increase was impeded and ameliorated by GNP (Figure 4B). Using miRNA FISH, we detected miR-132 expression in the Leydig cells of the testis, which are the major cells synthesizing and secreting testosterone [29]. It was found that the miR-132 level in Leydig cells of HS mice was remarkably higher than that in the Leydig cells of CS mice, while GNP treatment inhibited the effect of HFD on miR-132 expression in Leydig cells (Figure 4C). Furthermore, we detected the expression of StAR, the key protein involved in steroidogenesis, in the different samples of testis slides by immunofluorescence microscopy. Accordingly, StAR was detected in almost all Leydig cells, similar to miR-132 (Figure 4D). In contrast, the StAR level in the testes of HFD-treated mice was much lower than that in the testes of CS mice. These data are consistent with our previous study showing that miR-132 inhibits StAR expression by

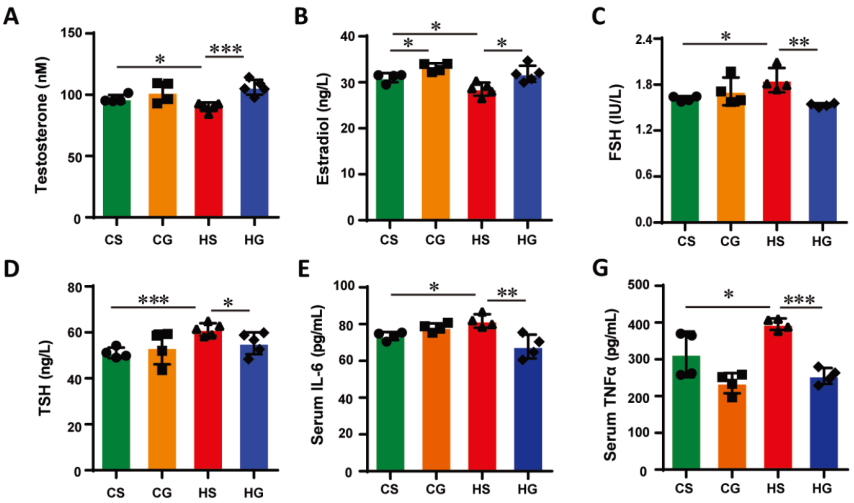

Figure 3. Hormone levels and inflammatory cytokine levels in the control and treated mice (A) Testosterone, (B) estradiol, (C) FSH and (D) TSH in the serum of control and treated mice. (E) Serum IL-6 and (F) TNFα in control and treated mice. Data are presented as the mean ± SD. $*P<0.05$, $**P<0.01$, and $***P<0.001$.

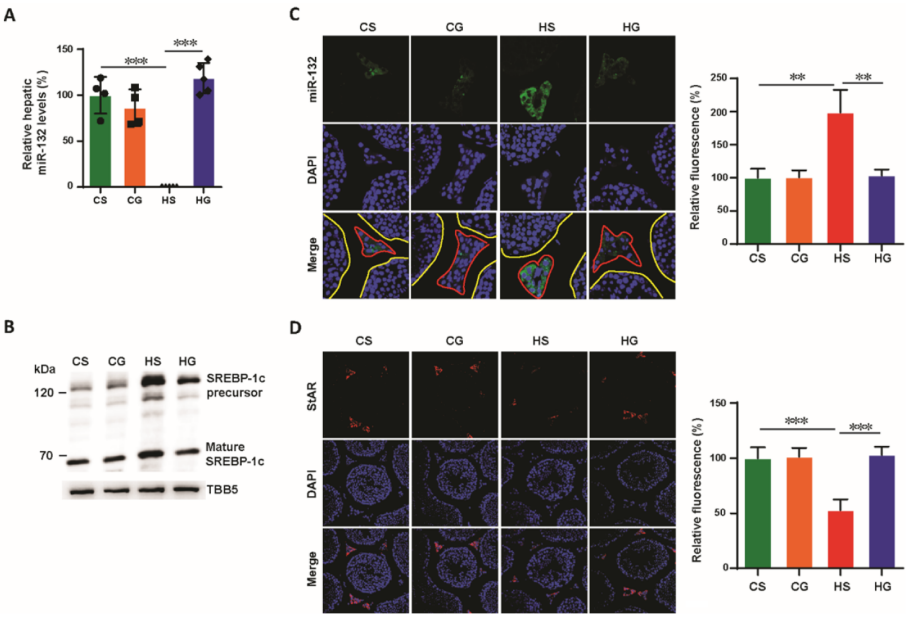

Figure 4. Tissue-specific regulation of miR-132 by HFD and GNP (A) HFD feeding inhibited miR-132 expression in the liver, which was recovered by GNP treatment. (B) The protein expression of the miR-132 target SREBP-1c in liver tissues of mice with different treatments. (C) FISH analysis showed that the miR-132 levels were higher in the Leydig cells in the testes of HFD-fed mice than in those of control mice, whereas GNP treatments recovered the miR-132 levels. (D) The protein levels of StAR in the testes of mice subject to different treatments. Data are expressed as the mean ± SD. $*P<0.05$, $**P<0.01$, and $***P<0.001$.

directly targeting the 3′ UTR of StAR mRNA [30]. GNP treatment impairs the effect of HFD on the downregulation of StAR in Leydig cells of mouse testis.

miR-132 inhibits SREBP-1c and impedes lipid accumulation

As mentioned above, HFD and GNP diversely regulate miR-132 expression in hepatic cells. Therefore, we detected whether HFD/GNP-regulated miR-132 modulates lipid metabolism in hepatic cells. HepG2 cells and primary hepatocytes were transfected with miR-132 mimic or inhibitor (Figure 5A), and TG and TC levels and lipid accumulation were determined. Compared with control cells, both HepG2 cells and hepatocytes overexpressing miR-132 had lower TG and TC levels, whereas inhibition of miR-132 increased TG and TC levels (Figure 5B,C). Overexpression of miR-132 repressed lipid accumulation in both hepatic cells, whereas the miR-132 inhibitor augmented lipid accumulation, which was detected using a fluorescent lipid probe (Figure 5D,E). Furthermore, we assessed the effect of miR-132 on the expression of the key lipogenesis factor SREBP-1c in HepG2 cells. As expected, the mRNA and protein levels of SREBP-1c in both HepG2 cells and hepatocytes were markedly impeded by miR-132 and increased by the miR-132 inhibitor (Figure 5F,G). Accordingly, qPCR analysis showed that the downstream target genes of SREBP-1c, such as *Fasn*, *Acc1*, *Scd1* and *ACLY*, were downregulated by miR-132 and upregulated by the miR-132 inhibitor in both HepG2 cells (Figure 5H) and primary

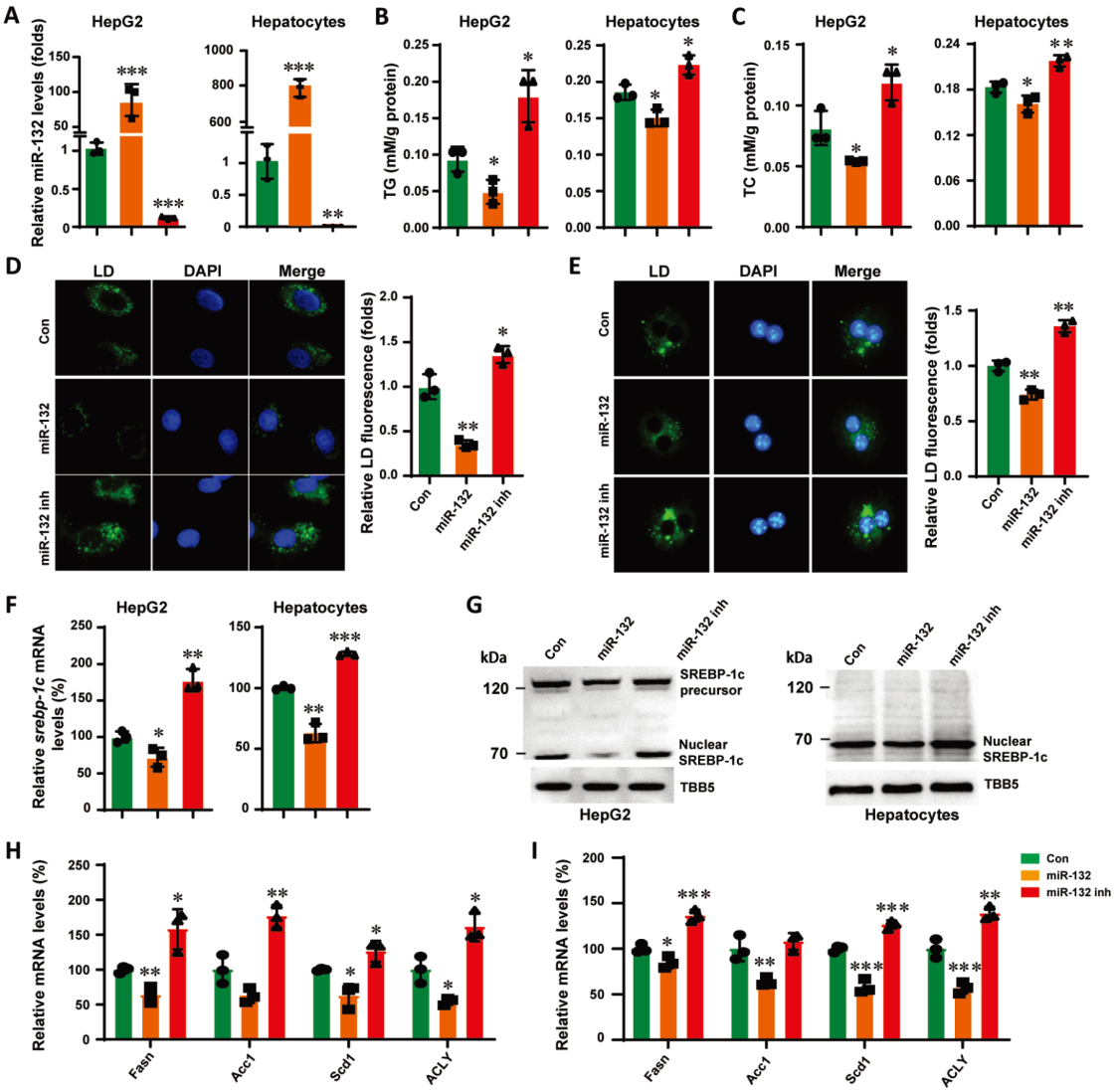

Figure 5. miR-132 regulates lipogenesis and lipid accumulation in the human liver cancer cell line HepG2 and in mouse primary hepatocytes (A) miR-132 level in cells transfected with miR-132 mimic or inhibitor. (B) TG and (C) TC levels in HepG2 cells and primary hepatocytes transfected with miR-132 mimic or inhibitor. The results showed that overexpression of miR-132 impaired TG and TC levels in cells, whereas inhibition of miR-132 upregulated TG and TC levels. $n=3$ culture plate replicates per group. (D) Representative images showing lipid accumulation in HepG2 cells with different treatments labelled with the BODIPY lipid probe. (E) Representative images showing lipid accumulation in mouse hepatocytes with different treatments. The quantification of the relative fluorescence is shown on the right. (F) The mRNA and (G) protein levels in HepG2 cells transfected with miR-132 mimic or inhibitor. (H,I) The mRNA levels of *Fasn*, *Acc1*, *Scd1* and *ACLY* were downregulated by miR-132 but upregulated by miR-132 inhibitor in (H) HepG2 cells and (I) hepatocytes, respectively. Data are expressed as the mean ± SD. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$.

hepatocytes (Figure 5I). These results indicated that miR-132 decreases lipid accumulation by targeting SREBP-1c and affects lipid metabolism.

Overexpression of miR-132 decreases SREBP-1c and StAR levels and steroidogenesis in mouse Leydig cells

We further investigated the effect of miR-132, which was also found to be regulated by HFD and GNP in mouse Leydig cells, on steroidogenesis in MLTC-1 cells, a model cell line of Leydig cells. MLTC-1 cells were transfected with miR-132 mimic (Figure 6A), and the SREBP-1c and StAR levels and progesterone levels were assessed. qRT-PCR measurements indicated that overexpression of miR-132 impeded both *SREBP-1c* and *StAR* mRNA levels in MLTC-1 cells (Figure 6B,C). Western blot analysis showed that both SREBP-1c and StAR protein levels were downregulated by miR-132 (Figure 6D). These results are consistent with our previous studies [26,30]. Furthermore, the major steroid hormone progesterone that is synthesized and secreted by MLTC-1 cells was repressed by overexpression of miR-132 (Figure 6E). These results indicated that HFD/GNP-regulated miR-132 inhibits steroidogenesis in Leydig cells, which may further affect spermatogenesis and sperm function.

Discussion

Long-term high-fat diet feeding induces obesity with a series of health problems, including metabolic syndrome and infertility [4, 19]. Dysregulated expression of various lipid metabolic genes and miRNAs contributes to this pathological process [31]. In the present study, we identified that HFD feeding induced hyperlipidemia, hepatic steatosis, serum hormone disorder and sperm dysfunction in mice, which could be recovered by GNP treatment at a dose of 20 mg/kg/day as previously described (Figures 1–3) [15]. On a molecular basis, HFD feeding regulates miR-132 expression in a tissue-specific manner, by which HFD consumption represses miR-132 in the liver but augments miR-132 expression in the testis (Figure 4). Modification of the expression of miR-132 further regulates lipid synthesis and lipid accumulation in hepatic cells and regulates hormone synthesis in steroid-producing MLTC-1 cells (Figures 5 and 6). Our study suggested a novel layer of understanding that tissue-specific regulation of miR-132 by HFD and GNP is involved in modification of lipid metabolism and sperm function through regulation of lipid metabolic genes and steroidogenetic gene expression, including *SREBP-1c* and *StAR* (Figure 6F).

Natural products and their active phytochemical constituents from plant-derived medicine combat obesity and its complications

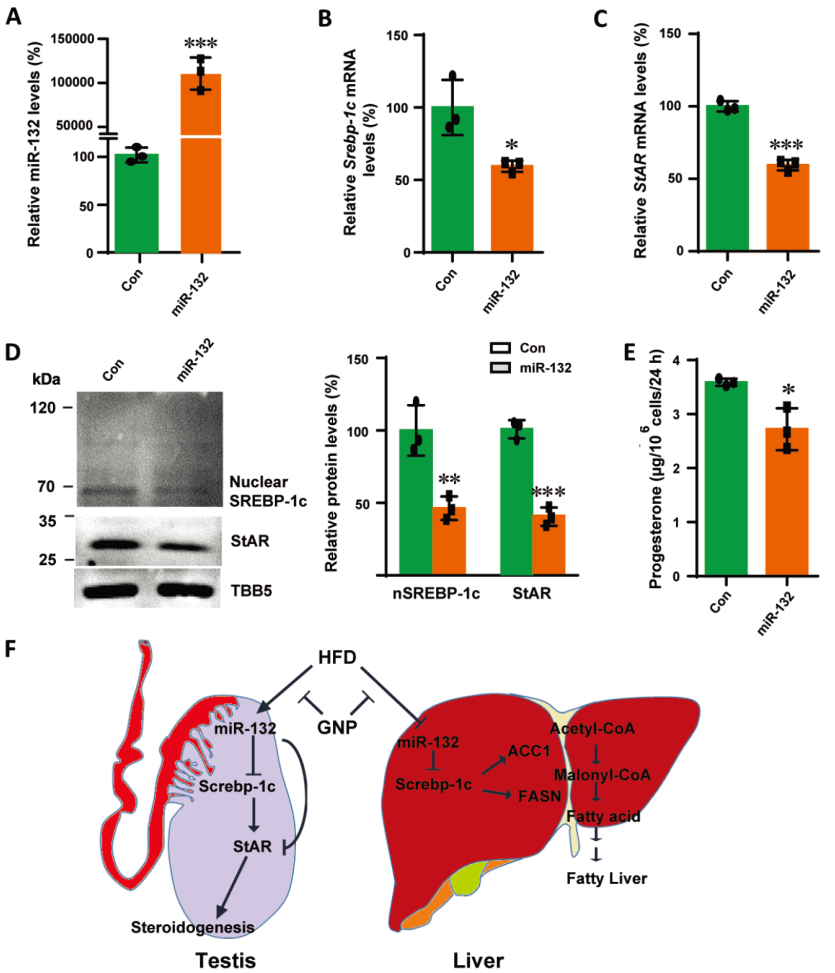

Figure 6. miR-132 represses steroidogenesis (A) Overexpression of miR-132 in MLTC-1 cells. (B,C) Overexpression of miR-132 inhibited the mRNA of *Srebp-1c* and *StAR*. (D) Overexpression of miR-132 repressed SREBP-1c and StAR protein levels. (E) miR-132 impeded progesterone synthesis in MLTC-1 cells. (F) Schematic representation of the proposed model of GNP in ameliorating HFD-induced metabolic disorder and sperm dysfunction. Data are expressed as the mean ± SD. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

by acting on multiple targets, which has been an effective strategy for the management of obesity and associated disorders [10]. GNP is the aglycone of geniposide, which is obtained from the fruit of gardenia and widely used in herbal medicine [12]. GNP has been used as an anti-inflammatory, antiangiogenic, and antioxidant and in the treatment of several inflammation-driven diseases. Our colleagues have identified the hepato-protective effect of GNP in HFD-induced obese mice [15]. Consistently, we showed that GNP treatment improved the hepatic steatosis and hyperlipidemia induced by HFD. As a chronic inflammation, obesity is accompanied by high levels of inflammatory factors, such as TNFα and IL-6 [32]. Our findings showed that the HFD-triggered TNFα and IL-6 were ameliorated by GNP. Our animal study implicated the application of GNPs in treating inflammation and metabolic disorders in humans, whereas more rigorous toxicological and pharmacological tests should be conducted for clinical trials.

Male reproduction, spermatogenesis and sperm function are controlled by sex hormones such as testosterone, E2, LH, FSH and TSH [33,34,17]. Steroid hormone synthesis occurs predominantly in the steroidogenic cells of the adrenal gland, ovary and testis, and is under the control of the hypothalamic-pituitary-gonadal (HPG) axis [26,34]. The initiation and maintenance of spermatogenesis requires the synergistic effect of FSH secreted by the pituitary gland and testosterone secreted by Leydig cells, in which testosterone plays a key role. LH binds to receptors on Leydig cells and stimulates the synthesis and secretion of testosterone by Leydig cells, providing a hormonal environment for spermatogenesis [34]. FSH acts on Sertoli cells and binds to their specific receptors to make Sertoli cells produce androgen-binding protein, which can bind with testosterone and transport it to seminiferous tubules to maintain the concentration of testosterone in the lumen [34]. Obesity and HFD feeding showed deleterious effects on hormone synthesis and further impaired spermatogenesis and male fertility [21,35]. Our current study revealed that HFD feeding for six weeks impaired sperm function and reduced serum testosterone and E2 levels. However, the serum FSH was slightly upregulated by six weeks of HFD feeding, whereas no differences were observed in LH levels among the different groups. Interestingly, our data showed an increase in serum TSH in the HFD group mice, which was reported to have inhibitory action on steroid secretion [33]. GNP treatment reversed the effect of HFD on sperm function and serum hormone levels. These results indicate that the other endogenous factors may also be involved in the regulation of testosterone under HFD feeding and GNP treatment.

miRNAs are a group of short, single-stranded, endogenously initiated noncoding RNA molecules that post-transcriptionally regulate gene expression by either suppressing its translation or enhancing its degradation [36]. Because of their effect on the regulation of the expression of various genes, miRNAs participate in key biological processes, including cell proliferation, differentiation, metabolism, carcinogenesis, immune response and other important cellular and metabolic processes [26,37]. We and others have shown that several miRNAs regulate lipid metabolism and fertility in HFD-induced obese mice [18,25,38–41]. Zhu *et al.* [15] showed that GNP alleviates HFD-induced hyperlipidemia and hepatic lipid accumulation in mice by regulating miR-142a-5p. miR-132 is a CREB-induced microRNA that is involved in several types of cancer progression, neuronal differentiation, innate immunity and obesity-related metabolic disorders [42–46]. Interestingly, miR-132 was suggested to improve insulin sensitivity because its expression in human adipose tissue is inversely related to markers of hyperglycemia and insulin resistance [47]. However, Hanin *et al.* [43] showed that transgenic mice overexpressing miR-132 had a severe fatty liver phenotype and hyperlipidemia. In this study, we found that miR-132 was inhibited in the livers of HFD-induced obese mice compared to chow diet-fed control mice. However, HFD feeding promoted miR-132 expression in Leydig cells of the testis where testosterone is synthesized. The diverse regulation of miR-132 expression in the testis and liver by HFD should be due to differential regulation of gene expression by hormones and inflammatory cytokines in these two tissues, respectively. The expression of genes in the testis is mainly regulated by hormones, while fatty liver is associated with chronic inflammation, which dominates gene expression in the liver [4,26,31]. Consistently, the serum levels of hormones and inflammatory cytokines are mediated by HFD and GNP (Figure 3). The tissue/cell-specific expression pattern of miR-132 is also consistent with previous studies showing that the expression of miR-132 is diversely regulated in different cells [45,48–50]. Overexpression of miR-132 repressed the key regulator of lipid metabolism, SREBP-1c, and impeded lipogenesis and lipid accumulation in hepatic cells, while inhibition of miR-132 expression showed the opposite results. Our previous study showed that miR-132 regulates steroidogenesis in Y1 adrenal cells by targeting StAR [30]. Consistently, we showed here that overexpression of miR-132 markedly impaired SREBP-1c and StAR expressions and repressed steroidogenesis in MLTC-1 cells. Our data indicate that tissue-specific regulation of miR-132 is involved in the regulation of metabolic disorder and sperm function in male mice by HFD and GNP. Therefore, targeting miR-132 expression as well as other miRNAs, such as miR-130a-3p and miR-26a, in certain organs by adeno-associated virus (AAV) vector or transgenic technology may contribute to the treatment of obesity-associated disease [39,40].

Collectively, the current study demonstrated a broad effect of GNP on the improvement of HFD-induced metabolic disorder and sperm dysfunction in male mice by a novel mechanism associated with tissue-specific regulation of miR-132. In addition to the reported effect of GNP on anti-inflammatory and antioxidant activity and improving pancreatic β-cell function, the function of GNP extends to ameliorating disorders in sperm function and hepatic lipid metabolism, implying that this compound is a versatile drug to treat metabolic disorders.

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Conflict of Interest

The authors declare that they have no conflict of interest

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