Advance Publication

Experimental Animals

Received: 2021.3.26

Accepted: 2021.6.17

J-STAGE Advance Published Date: 2021.7.20

1 *Original paper*

2 **AP39 ameliorates high fat diet-induced liver injury in young rats via. alleviation**
3 **of oxidative stress and mitochondrial impairment**

4

5 **Running head: AP39 ALLEVIATES HFD-CAUSED LIVER INJURY**

6

7 Yue Yu¹, Shu-Ming Ye², De-Yun Liu¹, Li-Qi Yang^{1, *}

8

9 ¹Department of Pediatrics, The Second Affiliated Hospital of Anhui Medical
10 University, Hefei, Anhui, 230601, China

11 ²Department of Orthopedics Surgery, The Second Affiliated Hospital of Anhui
12 Medical University, Hefei, Anhui, 230601, China

13

14 *Correspondence to: Li-Qi Yang, Department of Pediatrics, The Second Affiliated
15 Hospital of Anhui Medical University, 678 Furong Road, Hefei, Anhui, 230601,
16 China

17 Telephone: +86-551-65997048

18 E-mail address: yangliqi1961@163.com

19

Abstract

Non-alcoholic fatty liver disease (NAFLD) is a complication of childhood obesity and an oxidative stress-related multisystem disease. A mitochondria-targeting hydrogen sulfide (H₂S) donor AP39 has antioxidant property, while the mechanism underlying the function of AP39 on pediatric NAFLD remains undefined. Here, 3-week-old SD rats were received a high-fat diet (HFD) feeding and injected with AP39 (0.05 or 0.1 mg/kg/day) via the tail vein for up to 7 weeks. AP39 reduced weight gain of HFD rats and improved HFD-caused liver injury, as evidenced by reduced liver index, improved liver pathological damage, decreased NAFLD activity score, as well as low ALT and AST activities. AP39 also reduced serum TC, TG, LDL-C concentrations but increased HDL-C. Moreover, AP39 prevented ROS generation, reduced MDA content and increased GSH level and SOD activity. Furthermore, AP39 increased H₂S level, protected mitochondrial DNA (mtDNA), reduced mitochondrial swelling, and restored mitochondrial membrane potential (MMP) alteration. Notably, AP39 diminished HIF-1 α mRNA and protein level, possibly indicating the alleviation in mitochondrial damage. In short, AP39 protects against HFD-induced liver injury in young rats probably through attenuating lipid accumulation, oxidative stress and mitochondrial dysfunction.

Keywords: AP39, HFD, liver injury, oxidative stress, mitochondrial function

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a complication of obesity and a main cause of chronic liver disease in children [1]. Its occurrence is associated with insulin resistance, fat accumulation or steatosis, hypertension, dyslipidemia, inflammation, oxidative stress and subsequent hepatotoxicity [2-4]. In recent years, with changes in dietary structure, especially the intake ratio of fats and carbohydrates, the occurrence of NAFLD has increased dramatically, which has been regarded as one of the most prevalent public health problems [5]. There are some therapeutic drugs for NAFLD currently, but considerable side effects and poor long-term safety have been discovered [6]. Therefore, it is very necessary to explore the alternatives available for the effective treatment and prevention of pediatric NAFLD.

Hydrogen sulfide (H_2S), a gaseous signaling molecule, has a variety of biological effects in cellular signaling, including regulating inflammatory response, excessive oxidative stress and apoptosis [7-9]. It has been reported that H_2S at a non-cytotoxic level can serve as an electron donor to mitochondria for maintaining the activities of antioxidant enzymes [10]. High-fat diet (HFD) is a leading cause of NAFLD, and H_2S alleviates HFD-induced NAFLD in rats [11, 12]. Besides, H_2S donor sodium hydrosulfide (NaHS) treatment can also prevent liver injury in rats [13]. These findings indicate that H_2S and its relative compounds may have the potential to prevent liver injury.

AP39 (10oxo-10-(4-(3-thioxo-3H-1,2-dithiol-5yl)phenoxy)decyl) is a mitochondria-targeting donor of H_2S that can trigger an elevation in H_2S production

in the mitochondria [14]. AP39 has shown antioxidant and other cytoprotective effects under oxidative conditions [15]. Studies have shown that AP39 reduces the release of reactive oxygen species (ROS) and the expression of hypoxia-inducible factor (HIF)-1 α [16]. Also, AP39 has a protective role in damage of multiple organs such as the heart, lung and neuron [17-20]. However, to date, there is no research to clarify whether AP39 can prevent liver injury.

The aim of this study was to investigate the role of AP39 in liver injury in HFD-fed young rats and its underlying mechanisms. Our results demonstrated the protective effect of AP39 on obesity-related liver injury *in vivo*, providing a theoretical and scientific basis for the use of AP39 in preventing pediatric NAFLD.

Materials and methods

Animal experiments

All of our animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Anhui Medical University. Healthy male Sprague-Dawley (SD) rats were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (China). After feeding under controlled laboratory conditions for a week to acclimate to their surroundings, the three-week-old rats were randomly divided into the control group, HFD group, HFD+AP39 low-dose (HFD+L-AP39) and HFD+AP39 high-dose (HFD+H-AP39) groups (n = 6 per group).

As shown in Figure 1A, the animals (3 week old) in the control group were fed with a normal fodder (purchased from Beijing Huanyu Zhongke Biotechnology Co.,

Ltd.) for 7 weeks, while the other groups were fed a HFD (69% of basic feed, 10% of lard oil, 2% of cholesterol, 5% of sugar, 0.5% of cholate, 10% of yolk powder, 3% of yeast powder and 0.5% of decavitamin; BetterBiotechnology Co., Ltd., Nanjing, China) [21]. To investigate the impact of AP39 on HFD rats, the rats in HFD+L-AP39 and HFD+H-AP39 groups were injected daily by tail vein with AP39 (APExBIO, USA) at the dose of 0.05 mg/kg or 0.1 mg/kg, respectively [19]. The rats in control and HFD groups were received the same volume of saline. The food intake and body weight of all the rats was recorded per week for up to 7 weeks. At the end of the experiment, all of the tested rats were sacrificed by intraperitoneal injection of overdose (200 mg/kg) of pentobarbital sodium (Nembutal; Shandong XiYa Chemical Industry Co., Ltd.). Thereafter, the blood was harvested from the inferior vena cava of rats, and the livers were removed, photographed and weighed. Liver weight index was calculated based on the formula liver wet weight / body weight $\times$ 100%. Tissue samples were either fixed in 4% paraformaldehyde solution or frozen in liquid nitrogen and stored at -70 °C until use.

Histological analysis of liver

For assessment of lipid staining, the fixed tissues were dehydrated in 20% and 30% sucrose solution successively, and embedded with OCT embedding agent, and then sectioned into 10 μ m. The slices were stained with oil red O solution (Sigma, USA) and counterstained with hematoxylin (Solarbio, China). For hematoxylin and eosin (H&E) staining, the fixed and paraffin-embedded liver samples were cut into 5 μ m

104 slices and stained with hematoxylin and eosin (Sangon, China). All pathological
105 images were observed under an optical microscope at 200 × magnification. After that,
106 based on H&E staining analysis, the NAFLD activity score, a histological scoring
107 system for NAFLD, was calculated as the unweighted sum of the scores for steatosis
108 (0-3), lobular inflammation (0-3), and ballooning (0-2) [22].

109 **Biochemical parameters**

110 Serum alanine transaminase (ALT) and aspartate transaminase (AST) activities
111 were determined using the commercial ALT and AST assay kits. Total cholesterol
112 (TC), triglyceride (TG), low-density lipoprotein-cholesterol (LDL-C) and
113 high-density lipoprotein-cholesterol (HDL-C) contents in serum were measured by
114 the TC, TG, LDL-C and HDL-C assay kits, respectively. The levels of oxidative stress
115 parameters malondialdehyde (MDA), glutathione (GSH) and superoxide dismutase
116 (SOD) in liver tissues were examined by the corresponding MDA, GSH and SOD
117 assay kits. The above kits were purchased from Nanjing Jiancheng Bioengineering
118 Institute (China). In addition, hepatic H₂S level was measured using a H₂S assay kit
119 (Solarbio). All experimental procedures were performed in accordance with the
120 manufacturer's instructions.

121 **DHE staining**

122 The embedded liver tissues were sectioned into 10 μm, and incubated with the
123 fluorescent dye dihydroethidium (DHE; Beyotime, China; 1 : 100 dilution) at room
124 temperature for 30 min. The sections were examined using a 400 × fluorescence

microscope.

Quantitative real-time polymerase chain reaction (RT-qPCR)

For quantification of *HIF-1 α* , total RNA was extracted using a high-purity total RNA rapid extraction kit (BioTeke, China) and reverse-transcribed into cDNA using the M-MLV reverse transcriptase. Quantitative real-time polymerase chain reaction (RT-qPCR) was then performed on the ExicyclerTM 96 fluorescence quantifier using SYBR Green Master Mix. The relative mRNA expression of *HIF-1 α* was calculated by the $2^{-\Delta\Delta C_t}$ method and normalized to β -actin. For mitochondrial DNA (mtDNA) content determination, DNA extraction was performed using a tissue genomic DNA extraction kit (BioTeke). The copy number of mtDNA was evaluated by RT-qPCR, as described in a previous study [23]. The primers used, synthesized by GenScript (China), were listed in Table 1.

Immunoblot analysis

Western blot was performed according to Jia et al.'s description [24]. The target protein HIF-1 α was separated, quantified and blotted with anti-HIF-1 α (Affinit, China; 1 : 500 dilution). β -actin (Proteintech, China; 1 : 2000 dilution) was used for normalization.

Mitochondrial swelling assessment

Hepatic mitochondria were analyzed using the tissue mitochondrial isolation kit (Beyotime). On the basis of the previous description [25], the degree of liver

mitochondrial swelling was represented by the relative absorbance value (ΔA) of mitochondrial suspension at 520 nm for 20 min. The Larger ΔA indicates the more complete mitochondrial structure. A large ΔA represents a stronger buffering ability of calcium ions and better structural integrity in mitochondria of liver.

Detection of mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was detected using the MMP assay kit (Beyotime). The prepared mitochondria were incubated with the fluorescent cationic dye JC-1, followed by analyzing with a fluorescence microplate reader. The red/green fluorescence ratio indicated the activity of hepatic mitochondria.

Statistical analysis

Quantitative data were reported as mean $\pm$ SD. Statistical analysis was performed with GraphPad Prism 8.0 software. One-way ANOVA followed by Tukey's multiple comparisons test or the Kruskal-Wallis nonparametric analysis of variance followed by Dunn's multiple comparisons test was used for comparative analysis of multiple groups of independent samples. A *p*-value under 0.05 was considered a statistically significant difference.

Results

Effect of AP39 on body weight

As illustrated in Figure 1A, the tested young rats with a similar average weight

were received a HFD feeding and treated with AP39 for 7 weeks. We found that HFD resulted in a significant increase in the body weight of rats compared with normal diet rats. Administration of AP39 reduced HFD-induced weight gain in a dose-dependent manner ($p < 0.05$, Figure 1B). In addition, from the 3rd week to the 7th week of feeding, HFD obviously reduced the food intake of rats, while the food intake of rats with AP39 treatment showed an increasing trend, but it was not significant except for the 5th week (Figure 1C), which was consistent with Jia et al. and Ren et al.' studies [12, 24]. These findings indicate the potential of AP39 in childhood obesity.

AP39 counteracts HFD-induced liver injury

Childhood obesity is strongly associated with the occurrence of NAFLD [26]. Here, we focused on the function of AP39 in HFD-caused liver injury in young rats. At the end of the experiment, the livers of the experimental rats were photographed, as shown in Figure 2A. We observed a significant increase in liver size after HFD, with the color changing from dark red to brown yellow, which were improved by AP39 treatment. Moreover, the liver weight index of HFD rats was higher, while AP39 injection partially abolished it ($p < 0.05$, Figure 2B). Histological analysis of the liver using oil red O (Figure 2C) and H&E staining (Figure 2D) revealed the changes in hepatic steatosis in rats. We observed that there was increased lipid infiltration area and fatty vacuole area in the liver of HFD rats. However, these liver pathological damages caused by HFD were abrogated and returned to almost normal level after AP39 treatment. NAFLD activity score further confirmed that AP39

attenuated HFD-induced liver injury ($p < 0.05$, Figure 2E). These findings suggest a beneficial effect of AP39 on HFD-caused liver injury *in vivo*.

AP39 prevents serum lipid accumulation

We further verified the lipid-lowering role of AP39, as shown in Figure 3. Serum ALT and AST activities were significantly increased in HFD-induced rats, while AP39 treatment eliminated these changes ($p < 0.05$, Figure 3A). Meanwhile, the changes of serum lipid profiles TC, TG, LDL-C and HDL-C were examined. AP39 treatment could induce a reduction in TC, TG, LDL-C levels and an increase in HDL-C in HFD rats ($p < 0.05$, Figure 3B-E). Overall, these data suggest that AP39 effectively alleviates lipid accumulation in HFD-fed young rats.

Antioxidant effect of AP39 in NAFLD

To illuminate the effect of AP39 on oxidative stress, hepatic ROS and oxidative stress-related MDA, GSH, SOD were measured. As visualized by DHE staining, HFD resulted in increased ROS production, which was eliminated by AP39 administration (Figure 4A). In addition, AP39 remarkably decreased MDA content, and increased GSH level and SOD activity in HFD-fed young rats ($p < 0.05$, Figure 4B-D), indicating the antioxidant effect of AP39.

AP39 diminishes HFD-caused mitochondrial dysfunction

As expected, the H₂S level in the liver of HFD-fed young rats was lower than that of control rats, while AP39 enhanced it ($p < 0.01$, Figure 5A). We then explored

the role of AP39 in HFD-induced mitochondrial dysfunction. The mtDNA integrity, mitochondrial swelling and MMP alteration were measured to evaluate mitochondrial function. The copy number of mtDNA was increased after AP39 treatment ($p < 0.01$, Figure 5B). In addition, AP39 increased the absorbance at 520 nm, suggesting the amelioration of HFD-induced mitochondrial swelling ($p < 0.05$, Figure 5C). The loss of MMP was decreased in AP39-treated HFD rats as compared to HFD-fed rats ($p < 0.05$, Figure 5D). Noteworthy, AP39 significantly downregulated hepatic HIF-1 α expression at mRNA and protein levels ($p < 0.05$, Figure 5E). These results indicated that the adverse effects of HFD on hepatic mitochondrial dysfunction are partially reversed with AP39 treatment.

Discussion

In the present study, we demonstrate the effectiveness of AP39 in preventing HFD-induced liver injury *in vivo*. Treatment with AP39 alleviates obesity, hepatic steatosis, oxidative stress and mitochondrial dysfunction in HFD-fed young rats. These findings indicate that AP39 may be a promising therapeutic treatment for the prevention of pediatric NAFLD. Our finding is the first report to reveal the impact of AP39 on obesity-related liver injury.

Herein, three-week-old rats were fed with HFD for 7 weeks to mimic the symptoms of pediatric NAFLD. HFD-feeding significantly increased body weight and liver weight index, reduced food intake, elevated NAFLD activity score, ALT and AST activities and hepatic steatosis in young rats, which are known to aggravate liver

injury and may accelerate the progression of NAFLD [27, 28]. A growing body of evidence supports that H₂S can protect against tissue damage, such as heart, liver, lung and retina [29]. AP39, a mitochondrial-targeted H₂S donor exhibits beneficial effects on tissue injury including heart, lung and neuron [17-20]. In our study, AP39 decreased body weight gain, liver weight index and liver color almost reaching normal level, as well as decreased ALT and AST activities in HFD-fed young rats. These results clearly validate that AP39 could improve HFD-induced liver injury.

Liver is a key lipid metabolic organ and abnormal lipid metabolism is tightly related to the damage of liver function. A HFD is responsible for the imbalance between lipid acquisition and disposal in the liver, which subsequently accelerates the progression of NAFLD [30]. Our results demonstrated that AP39 decreased lipid accumulation, as manifested by reduced TC, TG, LDL-C and elevated HDL-C in serum of HFD-fed rats. Previous studies have confirmed that fat accumulation in liver can block lipid metabolic homeostasis and cause severe hepatotoxicity [31]. It seems that our findings indicate the protection of AP39 on HFD-induced liver injury by maintaining the balance of lipid metabolism, although the potential mechanism by which AP39 regulates NAFLD-related lipid metabolism is still elusive until now.

A HFD results in elevated ROS production and excessive oxidative stress in liver [32], which is in line with our finding showing that ROS level was elevated in the liver of HFD-fed rats. Interestingly, in our study, AP39 suppressed HFD-induced ROS generation. Moreover, lower oxidant MDA and more antioxidants GSH and SOD are required to attenuate and repair obesity-induced liver injury [33]. Several lines of

evidence have shown that AP39 has an antioxidant effect [16-18, 34]. Similarly, here, we found that AP39 inhibited MDA content and increased GSH level and SOD activity in HFD-fed young rats, suggesting that AP39 may ameliorate HFD-induced liver injury by inhibiting excessive oxidative stress.

Mitochondria modulate lipid metabolism and oxidative stress, and their dysfunction can lead to the disruption of lipid homeostasis, resulting in the occurrence of liver diseases including NAFLD [32, 35]. mtDNA is a major target of ROS, and HFD-feeding may damage mtDNA and aggravate liver injury [36-38]. Here, we observed a significant reduction in mtDNA quantity in the liver of HFD-fed rats, which was reversed by AP39. Besides, HFD-feeding increased the degree of mitochondrial swelling and the loss of MMP, which are similar to previous studies [25, 39]. Notably, AP39 restored hepatic mitochondrial function. Our findings were consistent with the research that AP39 protects against the loss of mtDNA and preserves mitochondrial activity [14, 18]. The above results manifest that AP39 protects mitochondrial function in HFD-induced liver injury. More importantly, mtDNA copy number is correlated with the expression of HIF-1 α in liver [40]. *HIF-1 α* , a key transcription factor, has been described as an important regulator of mitochondrial biogenesis, which can affect the development and progression of NAFLD [41, 42]. Covarrubias et al.'s study revealed that AP39 treatment reduces HIF-1 α level and improves mitochondrial function [16]. In the present study, we found that HFD-feeding upregulated HIF-1 α expression in the liver, while AP39 abolished it, which further confirmed the beneficial effect of AP39 on HFD-induced

liver injury.

The pathogenesis of NAFLD is complicated and involves glucose and lipid metabolism, oxidative stress, mitochondrial damage and inflammation. HFD can increase hepatic lipid influx and de novo lipogenesis and impair insulin signaling, thus promoting triglyceride accumulation in liver and ultimately NAFLD [43]. Lensu et al.'s study found that HFD-feeding reduces food intake and enhances body weight gain and body fat content of rats [44], which is consistent with our findings. Lipid accumulation in the liver in NAFLD may be due to an imbalance in lipid metabolism as characterized by decreased oxidative capacity or increased lipogenic activity. In addition, metabolic imbalance leads to mitochondrial functional overload, which ultimately causes liver injury. Moreover, in fat-engorged hepatocytes, several vicious cycles involving ROS, lipid peroxidation products alter respiratory chain polypeptides and mtDNA, thus partially blocking the flow of electrons in the respiratory chain. Overreduction of upstream respiratory chain complexes increases mitochondrial ROS. Oxidative stress increases the release of lipid peroxidation products and inflammatory cytokines, which together trigger the liver lesions of NAFLD [45]. Therefore, in our study, HFD-feeding increased weight gain, reduced food intake of young rats, possibly due to the enhancement of oxidative stress/mitochondrial dysfunction in NAFLD. Of note, AP39 partially ameliorates the liver damage caused by HFD, indicating the protection of AP39 on HFD-induced liver injury in young rats.

In conclusion, AP39 exerts a beneficial effect on HFD-induced liver injury in young rats possibly by modulating lipid metabolism, oxidative stress and

mitochondrial function. Current and future investigation on the effects of AP39 may provide a novel insight into the treatment of pediatric NAFLD.

Author's contributions

L.Y. designed the research study. Y.Y. and S.Y. performed the experiments. Y.Y. and D.L. analyzed the data. Y.Y. drafted the article. L.Y. revised the article. All authors read and approved the manuscript.

Data availability statement

The data used to support the findings of this study are included within the manuscript.

Disclosure statement

The authors all claim no conflicts of interest.

Funding

This work was supported by the Clinical Research Cultivation Program of The Second Affiliated Hospital of Anhui Medical university (No.2020LCYB07) and the Quality Engineering Project of Universities in Anhui Province (No. 2019jyxm1012).

References

1. Matteoni C A, Younossi Z M, Gramlich T, Boparai N, Liu Y C, McCullough A J, Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity, Gastroenterology 116(6) (1999) 1413-9.

- 310 2. Tiniakos D G, Vos M B, Brunt E M, Nonalcoholic fatty liver disease: pathology and
311 pathogenesis, *Annual review of pathology* 5 (2010) 145-71.
- 312 3. Cobbina E, Akhlaghi F, Non-alcoholic fatty liver disease (NAFLD) - pathogenesis,
313 classification, and effect on drug metabolizing enzymes and transporters, *Drug*
314 *metabolism reviews* 49(2) (2017) 197-211.
- 315 4. Xu L, Nagata N, Ota T, Impact of Glucoraphanin-Mediated Activation of Nrf2 on
316 Non-Alcoholic Fatty Liver Disease with a Focus on Mitochondrial Dysfunction,
317 *International journal of molecular sciences* 20(23) (2019).
- 318 5. Kumar S, Kaufman T, Childhood obesity, *Panminerva medica* 60(4) (2018)
319 200-212.
- 320 6. Takahashi Y, Sugimoto K, Inui H, Fukusato T, Current pharmacological therapies
321 for nonalcoholic fatty liver disease/nonalcoholic steatohepatitis, *World journal of*
322 *gastroenterology* 21(13) (2015) 3777-85.
- 323 7. Olas B, Hydrogen sulfide in signaling pathways, *Clinica chimica acta; international*
324 *journal of clinical chemistry* 439 (2015) 212-8.
- 325 8. Ge X, Sun J, Fei A, Gao C, Pan S, Wu Z, Hydrogen sulfide treatment alleviated
326 ventilator-induced lung injury through regulation of autophagy and endoplasmic
327 reticulum stress, *International journal of biological sciences* 15(13) (2019)
328 2872-2884.
- 329 9. Wang R, Physiological implications of hydrogen sulfide: a whiff exploration that
330 blossomed, *Physiological reviews* 92(2) (2012) 791-896.
- 331 10. Wen Y D, Wang H, Kho S H, Rinkiko S, Sheng X, Shen H M, et al., Hydrogen

sulfide protects HUVECs against hydrogen peroxide induced mitochondrial dysfunction and oxidative stress, PloS one 8(2) (2013) e53147.

11. Mani S, Cao W, Wu L, Wang R, Hydrogen sulfide and the liver, Nitric oxide : biology and chemistry 41 (2014) 62-71.

12. Ren R, Yang Z, Zhao A, Huang Y, Lin S, Gong J, et al., Sulfated polysaccharide from *Enteromorpha prolifera* increases hydrogen sulfide production and attenuates non-alcoholic fatty liver disease in high-fat diet rats, Food & function 9(8) (2018) 4376-4383.

13. Tan G, Pan S, Li J, Dong X, Kang K, Zhao M, et al., Hydrogen sulfide attenuates carbon tetrachloride-induced hepatotoxicity, liver cirrhosis and portal hypertension in rats, PloS one 6(10) (2011) e25943.

14. Szczesny B, Módis K, Yanagi K, Coletta C, Le Trionnaire S, Perry A, et al., AP39, a novel mitochondria-targeted hydrogen sulfide donor, stimulates cellular bioenergetics, exerts cytoprotective effects and protects against the loss of mitochondrial DNA integrity in oxidatively stressed endothelial cells in vitro, Nitric oxide : biology and chemistry 41 (2014) 120-30.

15. Ahmad A, Olah G, Szczesny B, Wood M E, Whiteman M, Szabo C, AP39, A Mitochondrially Targeted Hydrogen Sulfide Donor, Exerts Protective Effects in Renal Epithelial Cells Subjected to Oxidative Stress in Vitro and in Acute Renal Injury in Vivo, Shock (Augusta, Ga.) 45(1) (2016) 88-97.

16. Covarrubias A E, Lecarpentier E, Lo A, Salahuddin S, Gray K J, Karumanchi S A, et al., AP39, a Modulator of Mitochondrial Bioenergetics, Reduces

Antiangiogenic Response and Oxidative Stress in Hypoxia-Exposed Trophoblasts:
Relevance for Preeclampsia Pathogenesis, *The American journal of pathology*
189(1) (2019) 104-114.

17. Karwi Q G, Bornbaum J, Boengler K, Torregrossa R, Whiteman M, Wood M E, et
al., AP39, a mitochondria-targeting hydrogen sulfide (H₂S) donor, protects
against myocardial reperfusion injury independently of salvage kinase signalling,
British journal of pharmacology 174(4) (2017) 287-301.

18. Zhao F L, Fang F, Qiao P F, Yan N, Gao D, Yan Y, AP39, a Mitochondria-Targeted
Hydrogen Sulfide Donor, Supports Cellular Bioenergetics and Protects against
Alzheimer's Disease by Preserving Mitochondrial Function in APP/PS1 Mice and
Neurons, *Oxidative medicine and cellular longevity* 2016 (2016) 8360738.

19. Wepler M, Merz T, Wachter U, Vogt J, Calzia E, Scheuerle A, et al., The
Mitochondria-Targeted H₂S-Donor AP39 in a Murine Model of Combined
Hemorrhagic Shock and Blunt Chest Trauma, *Shock (Augusta, Ga.)* 52(2) (2019)
230-239.

20. Ahmad A, Szabo C, Both the H₂S biosynthesis inhibitor aminooxyacetic acid
and the mitochondrially targeted H₂S donor AP39 exert protective effects in a
mouse model of burn injury, *Pharmacological research* 113(Pt A) (2016) 348-355.

21. Shi T, Yang X, Zhou H, Xi J, Sun J, Ke Y, et al., Activated carbon
N-acetylcysteine microcapsule protects against nonalcoholic fatty liver disease in
young rats via activating telomerase and inhibiting apoptosis, *PloS one* 13(1)
(2018) e0189856.

- 376 22. Yin Z, Murphy M C, Li J, Glaser K J, Mauer A S, Mounajjed T, et al., Prediction
377 of nonalcoholic fatty liver disease (NAFLD) activity score (NAS) with
378 multiparametric hepatic magnetic resonance imaging and elastography, *European*
379 *radiology* 29(11) (2019) 5823-5831.
- 380 23. Wang W, Wang M, Ruan Y, Tan J, Wang H, Yang T, et al., Ginkgolic Acids Impair
381 Mitochondrial Function by Decreasing Mitochondrial Biogenesis and Promoting
382 FUNDC1-Dependent Mitophagy, *Journal of agricultural and food chemistry* 67(36)
383 (2019) 10097-10106.
- 384 24. Jia N, Lin X, Ma S, Ge S, Mu S, Yang C, et al., Amelioration of hepatic steatosis
385 is associated with modulation of gut microbiota and suppression of hepatic
386 miR-34a in *Gynostemma pentaphylla* (Thunb.) Makino treated mice, *Nutrition &*
387 *metabolism* 15 (2018) 86.
- 388 25. Ren T, Zhu L, Shen Y, Mou Q, Lin T, Feng H, Protection of hepatocyte
389 mitochondrial function by blueberry juice and probiotics via SIRT1 regulation in
390 non-alcoholic fatty liver disease, *Food & function* 10(3) (2019) 1540-1551.
- 391 26. Mann J P, Valenti L, Scorletti E, Byrne C D, Nobili V, Nonalcoholic Fatty Liver
392 Disease in Children, *Seminars in liver disease* 38(1) (2018) 1-13.
- 393 27. Xia H M, Wang J, Xie X J, Xu L J, Tang S Q, Green tea polyphenols attenuate
394 hepatic steatosis, and reduce insulin resistance and inflammation in high-fat
395 diet-induced rats, *International journal of molecular medicine* 44(4) (2019)
396 1523-1530.
- 397 28. Kitade H, Chen G, Ni Y, Ota T, Nonalcoholic Fatty Liver Disease and Insulin

- 398 Resistance: New Insights and Potential New Treatments, *Nutrients* 9(4) (2017).
- 399 29. Wu D, Wang J, Li H, Xue M, Ji A, Li Y, Role of Hydrogen Sulfide in
400 Ischemia-Reperfusion Injury, *Oxidative medicine and cellular longevity* 2015
401 (2015) 186908.
- 402 30. Ipsen D H, Lykkesfeldt J, Tveden-Nyborg P, Molecular mechanisms of hepatic
403 lipid accumulation in non-alcoholic fatty liver disease, *Cellular and molecular life*
404 *sciences : CMLS* 75(18) (2018) 3313-3327.
- 405 31. Miller C N, Yang J Y, Avra T, Ambati S, Della-Fera M A, Rayalam S, et al., A
406 dietary phytochemical blend prevents liver damage associated with adipose tissue
407 mobilization in ovariectomized rats, *Obesity (Silver Spring, Md.)* 23(1) (2015)
408 112-9.
- 409 32. Sumida Y, Niki E, Naito Y, Yoshikawa T, Involvement of free radicals and
410 oxidative stress in NAFLD/NASH, *Free radical research* 47(11) (2013) 869-80.
- 411 33. Zhang L, Li H X, Pan W S, Ullah Khan F, Qian C, Qi-Li F R, et al.,
412 Administration of methyl palmitate prevents non-alcoholic steatohepatitis (NASH)
413 by induction of PPAR- α , *Biomedicine & pharmacotherapy = Biomedecine &*
414 *pharmacotherapie* 111 (2019) 99-108.
- 415 34. Geró D, Torregrossa R, Perry A, Waters A, Le-Trionnaire S, Whatmore J L, et al.,
416 The novel mitochondria-targeted hydrogen sulfide (H₂S) donors AP123 and
417 AP39 protect against hyperglycemic injury in microvascular endothelial cells in
418 vitro, *Pharmacological research* 113(Pt A) (2016) 186-198.
- 419 35. Paradies G, Paradies V, Ruggiero F M, Petrosillo G, Oxidative stress, cardiolipin

and mitochondrial dysfunction in nonalcoholic fatty liver disease, World journal of gastroenterology 20(39) (2014) 14205-18.

36. Jha S K, Jha N K, Kumar D, Ambasta R K, Kumar P, Linking mitochondrial dysfunction, metabolic syndrome and stress signaling in Neurodegeneration, Biochimica et biophysica acta. Molecular basis of disease 1863(5) (2017) 1132-1146.

37. Malik A N, Czajka A, Is mitochondrial DNA content a potential biomarker of mitochondrial dysfunction?, Mitochondrion 13(5) (2013) 481-92.

38. Malik A N, Simões I C M, Rosa H S, Khan S, Karkucinska-Wieckowska A, Wieckowski M R, A Diet Induced Maladaptive Increase in Hepatic Mitochondrial DNA Precedes OXPHOS Defects and May Contribute to Non-Alcoholic Fatty Liver Disease, Cells 8(10) (2019).

39. Simões I C M, Fontes A, Pinton P, Zischka H, Wieckowski M R, Mitochondria in non-alcoholic fatty liver disease, The international journal of biochemistry & cell biology 95 (2018) 93-99.

40. Chowdhury A, Aich A, Jain G, Wozny K, Lüchtenborg C, Hartmann M, et al., Defective Mitochondrial Cardiolipin Remodeling Dampens HIF-1 α Expression in Hypoxia, Cell reports 25(3) (2018) 561-570.e6.

41. Gonzalez F J, Xie C, Jiang C, The role of hypoxia-inducible factors in metabolic diseases, Nature reviews. Endocrinology 15(1) (2018) 21-32.

42. Carabelli J, Burgueño A L, Rosselli M S, Gianotti T F, Lago N R, Pirola C J, et al., High fat diet-induced liver steatosis promotes an increase in liver mitochondrial

biogenesis in response to hypoxia, Journal of cellular and molecular medicine
15(6) (2011) 1329-38.

43. Antonucci L, Porcu C, Iannucci G, Balsano C, Barbaro B, Non-Alcoholic Fatty
Liver Disease and Nutritional Implications: Special Focus on Copper, Nutrients
9(10) (2017).

44. Lensu S, Pariyani R, Mäkinen E, Yang B, Saleem W, Munukka E, et al., Prebiotic
Xylo-Oligosaccharides Ameliorate High-Fat-Diet-Induced Hepatic Steatosis in
Rats, Nutrients 12(11) (2020).

45. Pessayre D, Role of mitochondria in non-alcoholic fatty liver disease, Journal of
gastroenterology and hepatology 22 Suppl 1 (2007) S20-7.

Table 1. Sequence of primers used in RT-qPCR.

Gene	Primers	Sequence (5'-3')
mtDNA	Forward	TGAGCCATCCCTTCACTAGG
	Reverse	TGAGCCGCAAATTTTCAGAG
nuclear DNA (β-actin)	Forward	CTGCTCTTTCCCAGATGAGG
	Reverse	CCACAGCACTGTAGGGGTTT
HIF-1α	Forward	CTACTATGTCGCTTTCTTGG
	Reverse	GTTTCTGCTGCCTTGTATGG
β-actin	Forward	GGAGATTACTGCCCTGGCTCCTAGC
	Reverse	GGCCGGACTCATCGTACTCCTGCTT

Figures and figure legends

Figure 1. Effect of AP39 on obesity. High-fat-diet (HFD)-induced NAFLD rats were intravenously injected with 0.05 or 0.1 mg/kg AP39 once a day for 7 weeks. (A) Schematic abstract of the experimental process. (B-C) Body weight and food intake of young rats was recorded once a week for 7 weeks. $^{**}p < 0.01$ versus Control; $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$ versus HFD.

Figure 2. Effect of AP39 on HFD-induced NAFLD. (A) At the end of the experiment, the liver tissues were harvested. Gross morphology of liver tissue was observed. (B) Liver weight index was calculated according to the formula liver wet weight / body weight $\times 100\%$. (C-D) The histological alternations of liver tissues were visualized with Oil red O staining ($\times 200$) and H&E staining ($\times 200$), and the representative photomicrographs were shown. (E) NAFLD activity score, a histological scoring system for NAFLD, was counted. ** $p < 0.01$ versus Control; [^] $p < 0.05$, ^{^^} $p < 0.01$

470 versus HFD.

471

472 **Figure 3.** Effect of AP39 on lipid profiles in serum of rats. (A-E) Relative levels of
 473 serum ALT, AST, TC, TG, LDL-C and HDL-C were determined using the
 474 corresponding kits. ALT, alanine transaminase; AST, aspartate transaminase; TC, total
 475 cholesterol; TG, total triglycerides; LDL-C, low-density lipoprotein cholesterol;
 476 HDL-C, high-density lipoprotein cholesterol. ** $p < 0.01$ versus Control; ^ $p < 0.05$, ^^ p
 477 < 0.01 versus HFD.

Figure 4. Effect of AP39 on HFD-induced oxidative stress. (A) DHE staining ($\times 400$) was used for ROS detection in liver. (B-D) Levels of markers of oxidative stress, MDA, GSH and SOD, were measured with commercial kits. ROS, reactive oxygen species; MDA, malondialdehyde; GSH, glutathione; SOD, superoxide dismutase. ** $p < 0.01$ versus Control; ^ $p < 0.05$, ^^ $p < 0.01$ versus HFD.

Figure 5. Effect of AP39 on H₂S level and mitochondrial function. (A) Hepatic H₂S level was detected using the H₂S determination kit. (B) mtDNA copy number in the liver was measured by RT-qPCR. (C) The degree of mitochondrial swelling was analyzed at 520 nm wavelength on a spectrophotometer. (D) Mitochondrial membrane potential (MMP) changes were detected using JC-1 probe. (E) Hypoxia-inducible factor-1α (HIF-1α) expression at mRNA and protein levels was determined by RT-qPCR and western blot, respectively. ** $p < 0.01$ versus Control; [^] $p < 0.05$, ^{^^} $p < 0.01$ versus HFD.