

Glutathione Ameliorates Mouse Steatohepatitis

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ABSTRACT

Objectives: Non-alcoholic fatty liver disease (NAFLD) is an increasing epidemic that may promote later stages of liver disease. It was reported that oxidative stress and lipid per oxidation are key pathological factors in non-alcoholic fatty Hepatitis (NASH). The objective of this study was to explore the protective effect of glutathione (GSH) on liver oxidative damage in NASH mouse model.

Methods: Male C57BL/6J mice were treated for 30 weeks by a high fat diet and intravenous injections of oxidized low density lipoproteins (oxLDL) without or with daily intra peritoneal injections of GSH for the last 10 weeks. Then the mice were sacrificed with appropriate controls. Blood and liver were harvested for analysis, including plasma and liver triacyl glycerol, total cholesterol, free fatty acids, insulin resistance, malondialdehyde (MDA), GSH, superoxide radicals, protein oxidation, inflammatory markers, and apoptosis in the liver tissue.

Results: The HFD combined with intravenous injections of oxLDL induce NASH in mice, characterized by elevated level of the lipid profiles, MDA, superoxide radical generation, protein carbonyls, and nitro tyrosine and higher expression of markers inflammatory markers, and apoptosis ($p < 0.001$). Introduction of GSH significantly mitigated these perturbations ($p < 0.001$).

Conclusions: These experimental findings suggest that intra peritoneal injections of GSH ameliorate liver oxidative damage in NASH mouse model. The results provide a new perspective for understanding NAFLD and increase therapeutic armamentarium of antioxidants.

Background

Non-alcoholic fatty liver disease (NAFLD) is one of the most common causes of liver disease in western countries and its prevalence has increased globally over the past years [1]. It is estimated that about 25% of the adults in the United States and more than 30% individuals in Japan have fatty liver in the absence of excessive ethanol consumption [2]. In China, NAFLD is becoming more and more prevalent and reported to afflict 20% of the general populations [3]. Despite the alarming rate of NAFLD, there are limited knowledge in the pathogenesis behind NAFLD and no effective medicine for NAFLD approved by governmental agencies.

Therefore, it is urgent to understand the pathogenesis of NAFLD to establish proper treatment [4]. NAFLD is characterized by lipid accumulation in the liver (steatosis) with or without inflammation (hepatitis). The progression from nonalcoholic simple fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH) is a key step in pathogenesis, as it indicates the disease process has been an accelerating stage [5,6]. At present, several mechanisms was found to account for steatohepatitis including increased adipose tissue (VAT), dyslipidemia, gut flora dysfunction, insulin resistance, oxidative stress and lipid peroxidation [7,8].

At the beginning, the increased VAT and dyslipidemia amplifies the delivery of free fatty acid (FFA) to liver, impairing the hepatic lipid content and initiating the subsequent disease process [9,10]. Higher FFA in the liver cause sustain stimulating of neutrophil and macrophage, inducing oxidative stress by the production of reactive oxygen species (ROS) [11]. Furthermore, the metabolism of the excess FFAs may enhance the lipid peroxidation, producing large amount of lipid peroxide (LPO) like Malonaldehyde (MDA) and 4-hydroxynonenal (HNE) [12,13]. Oxidative stress and lipid peroxidation are both important mechanism in the development of NASH. They may bring damage to cell structures such as membranes, proteins and DNA of cell, triggering inflammatory response, leading hepatitis [14,15].

Recent studies have revealed that oxidative stress and lipid peroxidation plays a dominant role in the progression of NAFLD to NASH [16,17]. It has been suggested that increased production of superoxide radicals from mitochondria and cytoplasm is crucial for the development of NASH [18]. OxLDL is the highly reactive form of LDL after lipid peroxidative modification [19]. Until today, a large amount of data has showed oxidized low-density lipoproteins (oxLDL) play an important role in oxidative stress and lipid peroxidation during obesity-related inflammatory disorder, such as metabolic syndrome; type 2 diabetes mellitus and atherosclerosis [20-22]. Recent evidence also suggests oxLDL as a new risk factor for steatohepatitis. OxLDL is capable of activating macrophage, enhancing transmigration of inflammatory cell [23-25] and inducing apoptosis [26]. Administration of oxLDL to HFD-fed mice can built a mouse model for NASH, displaying the entire pathology of NASH, including steatois, hepatic inflammation, oxidative stress and lipid peroxidation [27].

It has been suggested increased oxidative stress correlates with steatohepatitis progression in patients [28,29]. Since impaired antioxidant defense mechanisms can partially account for NAFLD pathogenesis, treatment strategies that affect the antioxidant enzymes might be useful for individuals suffering from NAFLD. Reduced glutathione (GSH) is an endogenous antioxidant that protects the liver from different intrinsic and extrinsic oxidants produced as a result of cellular metabolism by neutralizing the oxidants and sustaining cellular homeostasis [30,31]. GSH from GSH hepatocyte transporters is known to show scavenging activity against reactive oxygen species (ROS) generated by Kupffer cells, thereby maintaining the hepatic structural integrity [32-36]. GSH levels are depleted during severe disease caused by enhanced oxidative insult and mitochondrial GSH is believed to act as the first line of defense against damage mediated by superoxide radicals [35,37]. However, whether GSH could protect NAFLD has not been well understood. Herein, we study the viability of GSH to combat oxidative stress-mediated steatohepatitis in high fat diet supplemented mice.

Methods

This study was approved by the Institutional animal ethical committee at the Department of Infectious Diseases, Sun Yat-Sen University, China.

Experimental Animals

Four-week-old Male C57BL/6J mice purchased from vendors in China were maintained on 12:12h light and dark cycles and were free access to pellet diet and *ad libitum* water. After a week of acclimatization, the Non-alcoholic fatty Hepatitis (NASH) mouse model was developed by feeding high fat diet (HFD-60; Oriental Yeast Co. Ltd, Tokyo, Japan) and intravenous injections of oxLDL (Solarbio Co., Ltd. Pekin, China). All mice were divided into three groups. Group I (n=10) fed a normal diet for 30 weeks. For 30 weeks, with daily intravenous injections of 0.2 ml phosphate buffered saline in the last three weeks and served as the controls. Group II (n=10) received a high fat diet (HFD) along with intravenous injections of oxLDL 0.2ml on alternate days for 30 weeks. Group III (n=10) received the same experimental regimen as Group II along with daily intraperitoneal injections of GSH for the last 10 weeks (150 mg/kg body weight). The food and water intake of the mice and their body weight was recorded twice a week. During the experimental period, the body weights and organ weights of mice were measured with an electronic balance (ATY 224, Shimadzu, and Columbia, MD). At the end of the experiment, the mice were anaesthetized using diethyl ether and euthanized. Liver tissue was snap frozen by *liquid nitrogen* immediately and stored at -80°C until further use. Before termination, blood was collected from the retro-orbital sinus using a heparinized syringe. The plasma was separated by centrifugation at 8000g and stored at -80°C.

Histology

The liver tissues were fixed, paraffinized, cut to 5µm sections, deparaffinized and subjected for hematoxylin and eosin (H&E) staining. Histological changes were observed under a light microscope (*Olympus Optical Co., Lake Success, NY*).

Superoxide Radical Estimation

The generation of superoxide radicals in the liver was detected using electron spin resonance (ESR)[38]. The brief procedure was as follows:

- a) Recorded the ESR spectra on a spectrophotometer (Model UV-2602; Labomed, Inc. USA): an aqueous quartz flat cell (60 x 10 x 0.31mm, effective volume 160µl) was used.
- b) Measured optical absorption spectra: using a multi-channel photodetector. A nitron spin trap 5, 5-dimethyl-1-pyrroline N-oxide (DMPO) was used for the detection of superoxide radicals.

The incubation conditions for the reaction mixture were as follows: mitochondria 0.7-10 μ g/ μ l from Groups I, II and III, 70mM sucrose, 220mM manitol, 2mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.4), 2.5mM potassium phosphate buffer, 0.5mM EDTA, 5mM malate and 10mM glutamate. ESR was recorded for 2 min after the addition of the enzyme source and 1.5 M DMPO (20 μ l) to the reaction mixture. The ESR spectrometer (JES-FA200, JEOL, Tokyo, Japan) parameters were maintained at a magnetic field of 325Mt, power of 1.00Mw, frequency of 94,380 GHz, modulation amplitude of 2 Mt, gain of 500, scan time of 0.5 min, scan width of 10 Mt, and temperature of 25°C throughout the experiment.

TUNEL Assay

The evaluation of apoptotic cells in tissue sections was performed using TUNEL assay as described by Gavrieli et al. [39]. In brief, tissue sections were subjected to terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end labeling (TUNEL, Intergen Co., Purchase, NY) according to the manufacturer's instructions.

Western Blot Analysis

After being subjected to 12% SDS-PAGE, the samples (25 μ g of protein) were transferred onto a nitrocellulose fluoride membrane. The membranes were blocked with 1% Bovine Serum Albumin in TBST (Tris-buffered saline, 0.01% Tween 20) for 2h at room temperature (RT), incubated with the monoclonal rabbit anti-mouse caspase-3-1:500 (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), monoclonal rabbit anti-mouse PARP-1-1:500 (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), monoclonal rabbit anti-mouse TNF- α -1:500 (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), monoclonal rabbit anti-mouse β -actin-1:500 (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) antibodies for 2h at RT, washed and incubated with goat anti-rabbit HRP-1:1000. The membranes were subjected to enhanced Chem iluminescence reaction and densitometric analyses of the blots were performed using Image J-image analysis software (National Institutes of Health, Bethesda, MD)

Biochemical Analysis

Aspartateaminotransferase (AST) and Alanineaminotransferase (ALT) activity levels, as well as fasting insulin and glucose levels, were measured by commercial kits (Roche, Mannheim, Germany) following manufacturers' instructions. Insulin resistance index was assessed by calculating the homeostasis model assessment (HOMA-IR) using the following formula: [fasting plasma glucose (mmol/L) \times fasting plasma insulin (mU/L)/22.5]. Total lipid was extracted from the liver according to Sugawara and Miyazawa [40]. Total cholesterol (TC) (Cholesterol quantitation kit Sigma-Aldrich, St. Louis, MO, USA), triacylglycerol (TG) (Triglycerides Liquicolor Kit, Human, Wiesbaden, Germany), and free fatty acids (FFA) (Wako

NEFA-HR(2) Kit, Sopachem BV, Ochten, the Netherlands) were determined enzymatically using commercial kits according to the manufacturers' protocols. The protein content in the lysates was determined using the Bradford method (Sigma Chemical Co., St. Louis, MO, USA). Further, the levels of malondialdehyde (MDA), GSH, and oxidized glutathione as well as the activities of superoxide dismutase, catalase and glutathione reductase were assayed in the liver tissue of the control and experimental mice. All these assays were carried out using commercial kits according to the protocols provided by the manufacturer.

Statistical Analysis

Normally distributed data are presented as the mean \pm standard deviation (mean \pm SD), skewed data as the median (interquartile range). All data were statistically analyzed by the Statistical Package for Social Sciences (SPSS, Version 16, Chicago, IL). One-way analysis-of-variance (ANOVA) followed by Tukey's multiple comparisons test was performed to compare the results. The results were considered statistically significant when the probability was less than 0.05 ($p < 0.05$).

Results

The Influence of GSH on the Metabolic Index of NASH Mice

An increase was observed in the body weight of mice in Group II when compared to Group I and Group III mice at 10 weeks ($p < 0.05$) and at the end of the experiment ($p < 0.001$). Further, the liver weights were significantly ($p < 0.05$) increased in Group II mice (85%) compared to Group I and Group III mice. We evaluated the glucose and insulin levels in mice from Groups I, II, and III. Fasting glucose levels were significantly higher in Group II mice compared to Group I mice. Additionally, the HOMA-IR levels were increased 8.2 fold in Group II mice compared to mice from Group I ($p < 0.05$) and there was a significant decrease in HOMA-IR levels in Group III. On the other hand, the plasma glucose and insulin levels were significantly ($p < 0.001$) increased in Group II mice compared to Groups I and III. However, these perturbations were significantly mitigated in Group III mice compared to Group II mice. We did not observe any significant difference in body weight, glucose and insulin levels HOMA-IR between Groups I and III. The levels of TG, TC, and FFA in the liver tissue were significantly ($p < 0.001$) elevated in Group II mice when compared to Group I mice, whereas, post GSH treatment, these levels were significantly ($p < 0.05$) decreased. We further estimated the lipid profiles in the plasma. The Group II mice experienced a significant ($p < 0.001$) increase in their lipid profiles compared to Group I mice. In contrast, these values were significantly recovered post GSH treatment. The activities of AST and ALT were significantly ($p < 0.001$) elevated in Group II mice when compared to Group I mice. The transaminase activities were significantly lowered in mice from Group III compared to Group II mice.

Lipid Peroxidation and GSH Levels

As it was shown that MDA levels were significantly increased in Group II mice compared to Group I mice. This elevation in MDA content was significantly ($p < 0.05$) lowered by GSH treatment.

The Levels of Superoxide Radicals

ESR spectroscopy was used for the identification of free radicals. We found that the obtained ESR data revealed a rapid increase in the specific signal value in Group II when compared to Group I mice. However, this signal value was significantly decreased in the livers of Group III mice when compared to Group II mice. These findings suggest.

Inflammatory and Apoptotic Markers

In order to investigate the inflammation-mediated hepatic steatosis in high fat diet induced mice, immune blot was performed to calculate the extent of apoptosis and inflammation in liver tissue. Group II showed ($P < 0.001$ vs. Group I; $P < 0.05$ vs. Group III) pronounced expression levels of active caspase-3, N-terminal cleavage fragment of PARP-1 and TNF- α compared to Group I. The Group III mice exhibited a significant decrease in the expression of caspase-3, PARP-1, and TNF- α compared to Group II mice.

Histopathology

Histopathological results for the liver tissues from all groups. It can be seen that the livers of Group I mice have normal morphology. Microvesicular and macrovesicular hepatic steatosis around the portal area, with hepatic necrosis, inflammatory cell infiltration and fatty vacuoles. However, all these changes were recovered to normalcy in the livers of Group III mice.

Discussion

The “two-hit” hypothesis for NAFLD progression suggests that oxidative stress is an important factor in the transition from NAFL to NASH. Oxidized LDL, the lipid peroxidation products of LDL, had been demonstrated to play an important role in metabolic syndrome. But the relation between oxidized LDL and oxidative stress in NASH has not been totally clarified. Holvoet et al. reported that higher oxidized low-density lipoprotein is associated with higher incidence of the metabolic syndrome [41]. Chalasani N et al. reported that the serum level of ox-LDL was obviously higher in NASH patients than controls [42]. A paper from Veerle Bieghs et al. demonstrated that the anti-oxLDL IgM induced by immunization with pneumococci reduced the steatohepatitis induced by HFD [43]. A paper from Yimin et al. showed that administration of oxLDL to HFD-fed mice successfully found a NASH mice model, of which the clinical and pathological characteristics had not been well clarified [44].

The elevation of lipid peroxidation (MDA) levels can serve as typical markers of NASH. Lipid peroxidation is the primary mechanism through which ROS can accentuate the oxidative

damage in fatty liver disease [45,46]. The condition is responsible for inflammatory signals and thus causes cellular damage. It has been suggested that the accumulation of lipid peroxidation products including 4-hydroxynonenal and MDA causes mitochondrial dysfunction [47,48]. This suggestion is supported by Bujanda et al. [48], who reported increased MDA content following NALFD in a rodent model. Given the enormous importance of superoxide radicals in disease biology, we identified the reason for the copious production of superoxides in Group II mice. The ESR data demonstrated a rapid increment in superoxide anions in Group II mice compared to Group I mice. It has been reported that respiratory chain complexes are weakly coupled in NASH settings and serve as ROS producers in addition to increasing mitochondrial fatty acid oxidation [49,50]. Increased xanthine oxidase activity and uric acid formation via enhanced purine catabolism are essential to maintain ATP levels, and are also responsible for ROS production in the biological system.

In the current study, the administration of oxLDL to HFD-fed mice successfully induce a NASH mouse model, characterized by elevated level of the lipid profiles, MDA, superoxide radical generation, protein carbonyls, and nitro tyrosine. This result adds one more evidence to the “two-hit” hypothesis that oxidative stress exerts a strong influence on the progression from NAFL to NASH. Based on the knowledge in the contribution of oxidative stress to hepatic injury in patients with NASH. It is thought that antioxidants may be beneficial to patients with NASH [51]. Recent experimental data has implied that some antioxidants favorably influenced the lipid metabolism and steatohepatitis of NAFLD. Jung TS et al. reported that α -lipoic acid reduces hepatic steatosis and inflammatory markers in the liver of ALA-treated rats [52]. A report from Ma T et al. suggested that geniposide exerts protective effects against hepatic steatosis in rats fed with a high fat diet by its antioxidant mechanism [53]. Jamil A Haque reported that genetically GSH-deficient mice could be inducing to steatohepatitis by normal diet [54]. Oz HS et al. reported that oral administration of GSH-enhancing agent, the S-adenosyl methionine or 2(RS)-n-propylthiazolidine-4(R)-carboxylic acid (PTCA), significantly improved the abnormal enzyme activities, fatty degeneration, inflammation, and necrosis of the NAFLD mouse model induced by methionine-choline deficient (MCD) diet [55].

The present study implied intravenous injections of GSH ameliorated the steatohepatitis in mouse model induced by oxLDL, indicating the antagonistic effect of GSH on oxidative stress and lipid peroxidation of ox-LDL. It is acknowledged that TNF- α plays a vital role in the onset of fatty liver disease-induced liver injury [56,57] and circulating neutrophils affect TNF-mediated caspase-3 activation [58]. PARP-1 cleavage fragments are a hallmark of apoptotic progression [59]. All our observations suggested that the use of GSH down regulate the expression of inflammatory markers and apoptosis factors in the liver tissue, which had been elevated

by oxLDL. These findings reinforce the hypothesis that GSH may favorably influence the cellular the molecular mechanisms implicated in NAFLD and would be beneficial in the treatment of hepatic steatosis, In conclusion, we demonstrated that GSH treatment can protect mice from NASH-induced oxidative damage. GSH treatment significantly reduced cell death (apoptosis) and TNF- α expression by increasing GSH concentration and inhibiting lipid oxidation. The results suggest that GSH treatment can serve as an effective therapeutic option for NASH.

Footnotes

Authors' Contribution: Feng-Juan Chen conceived and designed the study. Qing-Xian Cai, Li-Li Wu, Hong Deng and Xiao-Man Chen performed the experiments. Qing-Xian Cai and Li-Li Wu wrote the paper, Xiao-Qiong Shao, Qiu-Min Luo reviewed and edited the manuscript. All authors read and approved the manuscript.

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