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Investigation of Thiol/Disulfide Balance in Obese Rats with Non-Alcoholic Fatty Liver Disease

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ABSTRACT

Purpose: Due to the increasing prevalence of obesity worldwide, non-alcoholic fatty liver disease (NAFLD) has reached epidemic dimensions over time. NAFLD is the most common cause of childhood chronic liver disease. There is a relationship between NAFLD and oxidative stress. This study aims to investigate the changes in thiol/disulfide homeostasis parameters to determine the oxidant/antioxidant balance in obese rats with diet-induced NAFLD and healthy rats.

Methods: Twelve Wistar albino rats were used in this study. Experimentally produced NAFLD obese rats (n=6) and healthy rats were compared. Experimental NAFLD model was created with a special fatty liver diet (Altromin® C1063, Fatty Liver Diet, Exclusivet, Lage, Germany). The biochemical and histopathological features of the groups, as well as serum thiol/disulfide homeostasis parameters, were analyzed and compared.

Results: In the experimentally induced NAFLD rat model, they gained more weight than the control group. Steatosis (at least grade 2) occurred in all rats fed with special fatty liver diet for 12 weeks. Histopathologically, no high-grade inflammation was observed in rats with experimental NAFLD after feeding a diet for 12 weeks. Results revealed that aspartate transaminase and alanine transaminase levels were high, albumin levels were low, oxidant stress parameters increased, and antioxidant thiol groups decreased.

Conclusion: Experimental NAFLD is characterized by increased oxidant stress accompanying fatty tissue in the liver. Analysis of thiol/disulfide homeostasis parameters in NAFLD can be used in further studies to develop effective treatment options.

Keywords: Fatty liver; Obesity; Oxidative stress; Sulfhydryl compounds; Disulfides; Rats

INTRODUCTION

With the increasing prevalence of childhood obesity, non-alcoholic fatty liver disease (NAFLD) has become the most common liver disease in children and adolescents in industrialized countries [1-4]. The term 'NAFLD' describes the spectrum of liver pathology ranging from

mild steatosis to non-alcoholic steatohepatitis (NASH) and even cirrhosis. The main factor in pathophysiology is the inflammatory response that may occur following insulin resistance and oxidative stress, which causes fat accumulation in hepatocytes. However, there is not enough research to clearly define the role of oxidative stress [3-6], which has remained under-researched.

The prevalence of NAFLD ranges from 7.6% in the general population to 34.2% in obese children. There is increasing evidence that associates the presence of NAFLD in children with cardiovascular and metabolic consequences, such as prediabetes, type 2 diabetes, dyslipidemia, and hypertension [4]. There are studies on the significant role of increased oxidative stress in the pathogenesis of NAFLD and its close relationship with obesity and accompanying morbidity [7-11].

Oxidative stress can be defined as the excessive increase in oxidized protein end-products that may occur as a result of various reactions in the body. Thiols are known to be the most important and necessary antioxidant buffers that interact with almost all physiological oxidants [12-14]. The glutathione-containing thiol maintains the redox state of the sulfhydryl groups of proteins required for DNA repair and expression [12,13,15].

Thiol groups of proteins, thiol groups of low molecular weight compounds, cysteine residues, and other thiol groups are oxidized by the oxidant molecules in the environment and transformed into reversible disulfide bond structures (–S–S). The disulfide bond structures can be reduced to thiol groups. Thus, the vital thiol/disulfide homeostasis is maintained. In this equilibrium, thiol groups may show antioxidant responses, while disulfide bond structures may show oxidative stress [12,13]. Until recently, only one side (thiol) of the thiol/disulfide balance could be measured, while today both sides of the equilibrium can be determined using the latest test methods and the thiol/disulfide balance can be completely evaluated [13].

Due to the role of oxidative stress in NAFLD-NASH, if the pathophysiological mechanisms can be understood completely; this may contribute to the development of non-invasive diagnosis and treatment options. Novel studies on this subject are increasing [16-18]. The present study aimed to measure the changes in thiol/disulfide balance in obese rats with diet-induced NAFLD to evaluate their relationship with histopathological and basic biochemical changes and examine the role of oxidative stress in the pathogenesis of NAFLD.

MATERIALS AND METHODS

This study was performed by Scientific and Technological Research Application, Research Center Hüseyin Aytemiz Experimental Research-Application Laboratory at Kırıkkale University, with the approval of Kırıkkale University Animal Experiments Ethics Committee (on 22.03.2018; meeting number: 18/04 decision no: 25).

Subjects

In this study, 12 male Wistar albino rats, which are 2–3 months old and weighing 250–300 g, were used. The rats were acclimatized in the animal experiments laboratory of Kırıkkale University Scientific and Technological Research and Application Center. They were kept separately in standard wire cages at 25±3°C room temperature, 55±10% humidity, and 12 hours constant light and dark cycles. Their examinations and body weight follow-ups were performed periodically.
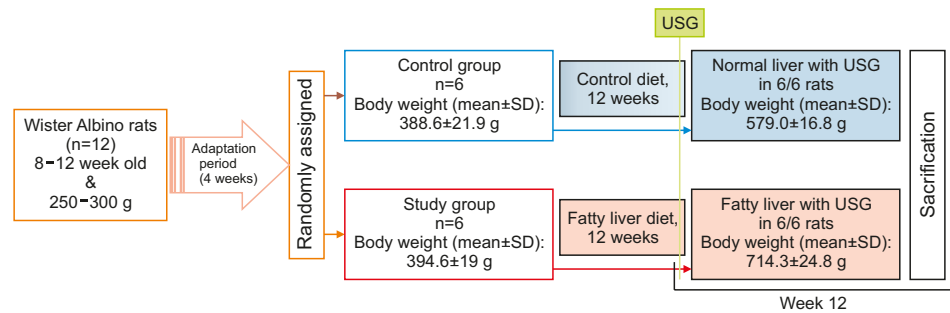
Fig. 1. The flow chart of modeling control and non-alcoholic fatty liver disease (NAFLD) rats (study design). Twelve Wistar albino rats, which are 2-3 months old and weighing 250-300 g, were used. All rats were fed with food during the 4-week acclimatization and preparation period. After the acclimatization, the rats were randomized into two groups with six rats in each group: The study group (NAFLD) (n=6) and the control group (n=6). The energy content of the fatty liver diet was 4,202 kcal/kg, and the distribution of nutrients included 29% fat, 14% protein, and 57% carbohydrate. The control diet's energy content was 3,506 kcal/kg, and the nutrient distribution was 13% fat, 20% protein, and carbohydrate 60.7%. The presence of hepatosteatosis was confirmed using liver ultrasonography in all rats in the study group at 12 weeks. At the end of the 12th week, all subjects were properly sacrificed. Blood samples were taken from all rats for the analysis. The livers were removed and submerged in 10% formaldehyde solution.
SD: standard deviation, USG: ultrasonography.

Experimental protocol

All rats were fed with food during the 4-week acclimatization and preparation period. After the acclimatization, the rats were randomized into two groups with six rats in each group: The study group (NAFLD) (n=6) and the control group (n=6) (**Fig. 1**).

A high ketoacidogenic and high-fat diet (Altromin® C1063, Fatty Liver Diet [FLD], Exclusivet®, Lage, Germany) was initiated in the study group to induce experimental hepatosteatosis. The energy content of the fatty liver diet was 4,202 kcal/kg, and the distribution of nutrients included 29% fat, 14% protein, and 57% carbohydrate. A control diet (Altromin® C1000, Control Diet, Exclusivet®) was given to the control group. The control diet's energy content was 3,506 kcal/kg, and the nutrient distribution was 13% fat, 20% protein, and carbohydrate 60.7%.

Body weights of all rats were recorded weekly using the same device. The presence of hepatosteatosis was confirmed by liver ultrasonography in all rats in the study group at 12 weeks. At the end of the 12th week, all subjects were properly sacrificed. The fasting period was set at 12 hours before the operation. The rats were placed on their back and fixed to the operating table using their four extremities. Approximately 5 mL of blood was collected through intracardiac route. Then, the rats were sacrificed.

Blood samples were taken from all rats for the analysis of thiol/disulfide homeostasis parameters, serum albumin, alanine transaminase (ALT), and aspartate transaminase (AST) levels were centrifuged at 3,500 rpm for five minutes. Separated serum samples were then transferred to Eppendorf tubes and stored at −80°C until analysis. The livers were removed and taken in 10% formaldehyde solution.

Liver ultrasonography

The abdominal areas of the rats were shaved, and liver examination was performed using a portable ultrasound system (Honda Electronics HS-2000 VET Portable; Honda Electronics, Aichi, Japan).

Biochemical analysis

ALT and AST were evaluated at the end of the 12th week in both groups. Separated serum samples were then transferred to Eppendorf tubes and stored at –80°C until analysis. The measurements were carried out at the Kırıkkale University Faculty of Medicine Biochemistry Laboratory.

Liver histopathological examination

After sacrificing the animals, their liver tissues were removed, sectioned, and fixed in 10% formaldehyde solution for 72 hours. Tissues were sampled and transferred to tissue tracking cassettes. They were dehydrated using a graded alcohol series, fixated using xylene, and finally embedded in paraffin with a tissue tracking device. After tissue processing, they were blocked (Leica TP 1020; Leica Biosystems, Seoul, Korea). These paraffin blocks were cut into 4–6 μm sections using a rotary microtome (Leica RM 2155). Then, the sections were stained using hematoxylin and eosin. Zeiss ICC 5 camera was utilized with the ZEN imaging software under the light microscope (Zeiss Axiolab.A1; Zeiss, Jena, Germany), and the images were taken microscopically using a digital camera.

Microscopic examinations were performed by an experienced pathologist who was blinded to the study information. During the microscopic examination, 10 random areas of a 200× light microscope were evaluated in each section. An average of 10 fields was taken for the degree of inflammation and steatosis. A histological scoring system developed by NASH Clinical Research Network was used to grade inflammation and steatosis [19].

Thiol/disulfide homeostasis parameters measurement

Separated serum assays were then transferred to Eppendorf tubes and stored at –80°C until analysis. Serum thiol/disulfide homeostasis tests were measured using the automated spectrophotometric method developed by Erel and Neselioğlu [13]. Serum levels of native thiol and total thiol were measured using spectrophotometry utilizing Cobas c501 (Roche Diagnostics, Indianapolis, IN, USA). In this method, the level of native thiol was measured first when the serum had interacted with 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) without any procedure. To measure the total thiol levels, dynamic disulfide bonds in the serum samples were reduced using sodium borohydride (NaBH4) resulting to the formation of free functional thiol groups.

Formaldehyde was then used to completely remove the unused NaBH4. The total thiol groups (reduced+native) were measured after reacting with DTNB. Since the reduction of a disulfide bond forms two thiol groups, the number of dynamic disulfide bonds was calculated as half the difference between the total thiol and native thiol. After determining native thiol levels, total thiol and disulfide levels, the ratios of disulfide/native thiol, disulfide/total thiol, and native thiol/total thiol were calculated.

Statistical analysis

Statistical analysis was performed using SPSS version 21.0 package program (IBM Co., Armonk, NY, USA). Descriptive statistics number, percentage, mean, and standard deviation were summarized as median (minimum-maximum) for non-normally distributed data. The conformity of the variables to the normal distribution was examined using visual (histogram and probability charts) and analytical methods (Shapiro–Wilk test). Numerical variables determined according to the state of normal distribution were compared between the two groups using the t-test in independent groups. Numerical variables that did not show normal
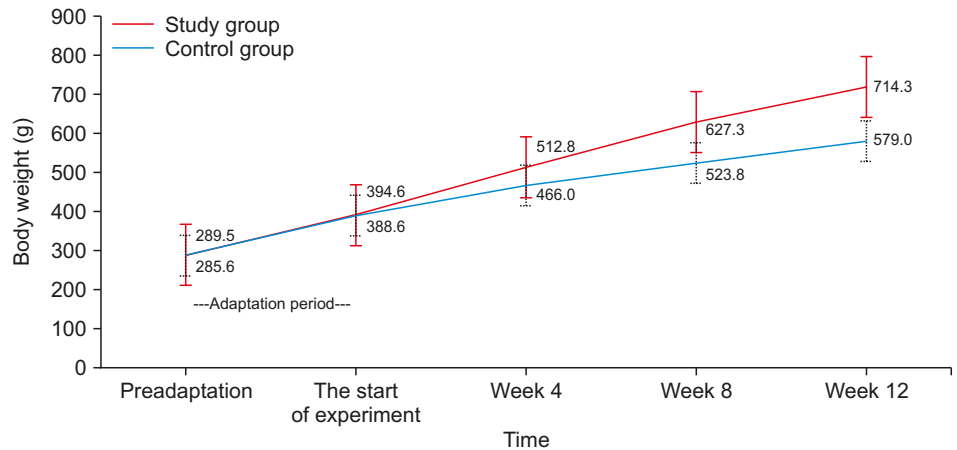
Fig. 2. The body weight course of the control group and the study (non-alcoholic fatty liver disease) group during the experimental period. Weekly body weight measurements of rats are marked as mean and error bars.

distribution were compared between the two groups using the Mann–Whitney U-test. In the statistical analysis in this study, $p < 0.05$ was considered statistically significant.

RESULTS

Body weights

After the adaptation period, the initial median body weight was 391 g (min: 361 g, max: 422 g) in the control group and 390 g (min: 367 g, max: 419 g) in the study group. The body weight changes of both control and study groups during the experimental period are graphically shown in **Fig. 2**.

While the mean body weights of the rats in both groups were similar at the beginning (p=0.873), the body weights of the rats fed with FLD were significantly higher than the control group at the end of the 4th week (p=0.01), 8th week (p=0.004), and 12th week (p=0.004) (**Table 1**).

ALT, AST, and albumin levels

When the experiment was terminated, AST and ALT levels were higher in the study group (subjects with NAFLD) than the control group. Also, albumin levels were lower in the study group (**Table 2**).

Liver ultrasonography

In the study group, the presence of hepatosteatosis was observed in all rats (6/6) in the ultrasonographic liver evaluation performed after 12 weeks of FLD. Ultrasonographic grades

Table 1. Comparison of the control and study group body weights at the beginning and at the end of the experiment

Duration of feeding	Study group	Control group	*p*-value
Initial body weight (g)	390 (367–419)	391 (361–422)	0.871
4th week body weights (g)	506 (488–565)	471 (430–495)	0.010
8th week body weights (g)	614 (602–687)	529 (478–546)	0.004
12th week body weights (g)	702.5 (695–760)	578.5 (553–597)	0.004

Values are presented as median (min–max).

Table 2. Comparison of transaminases and albumin levels of the groups at the end of the experiment

Variable	Study group	Control group	*p*-value
ALT (IU/L)	128 (77–267)	69 (59–79)	0.004
AST (IU/L)	159 (112–217)	105 (92–121)	0.015
Albumin (g/dL)	3.61 (3.36–3.86)	4 (3.73–4.03)	0.006

Values are presented as median (min–max).
ALT: alanine transaminase, AST: aspartate transaminase.

Table 3. Liver histopathological features of control and study groups

Subjects	Percentage of steatosis	Steatosis grade	Lobular inflammation score
Study (NAFLD) group			
Rat-S1	45	2	0
Rat-S2	75	3	0
Rat-S3	70	3	0
Rat-S4	45	2	1
Rat-S5	35	2	0
Rat-S6	55	2	0
Control group			
Rat-C1	0	0	0
Rat-C2	0	0	0
Rat-C3	0	0	0
Rat-C4	0	0	0
Rat-C5	0	0	0
Rat-C6	0	0	0

NAFLD: non-alcoholic fatty liver disease, S: study group, C: control group.

of steatosis in the study group were evaluated as grade 2 (in 4 rats) and grade 3 (in 2 rats). At the end of the experiment, no steatosis was observed in any rat in the control group.
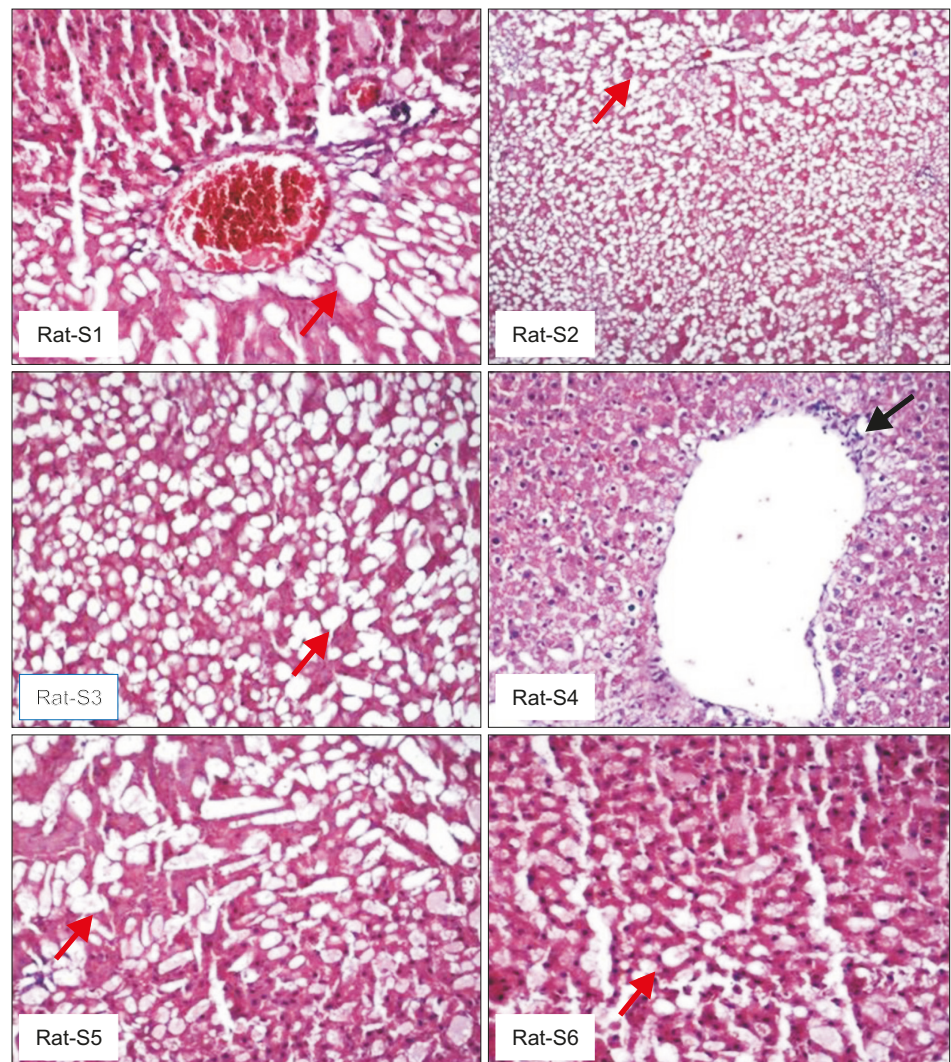
Histopathological examination of the liver

At the end of the experiment, steatosis (grade 2–3) was observed in all rats (100%) in the study group. Grade 2 steatosis was found in 4/6 rats, while grade 3 was found in 2/6 rats. Steatosis and inflammation were not observed in any of the rats in the control group at the end of the experiment. While one of the rats in the study group had grade 2 lobular inflammations (1 inflammation area/200× area) with grade 2 steatosis (steatohepatitis), there was no lobular inflammation accompanying steatosis in the other five rats. The histopathological characteristics of the rats' livers in the study and control groups are shown in **Table 3**. Images of histopathological examinations are given in **Fig. 3** (study-NAFLD group) and **Fig. 4** (control group).

Thiol/disulfide homeostasis parameters

When parameters reflecting oxidative stress of thiol homeostasis were compared using disulfide (DS), DS/Native Thiol (Index 1), and DS/Total Thiol (Index 2), they were found significantly higher in patients with NAFLD than those in the control group. Index 3 of antioxidant parameters: Native Thiol/Total Thiol was at a significantly lower level in the patient group (**Table 4**).

DISCUSSION

The present study showed that rats fed with a high-fat diet gained more weight than rats fed with a control diet. All rats fed with FLD developed obesity and fatty liver. In other studies, some examples showed that a significant weight gain (obesity) occurs in rats fed with a high-
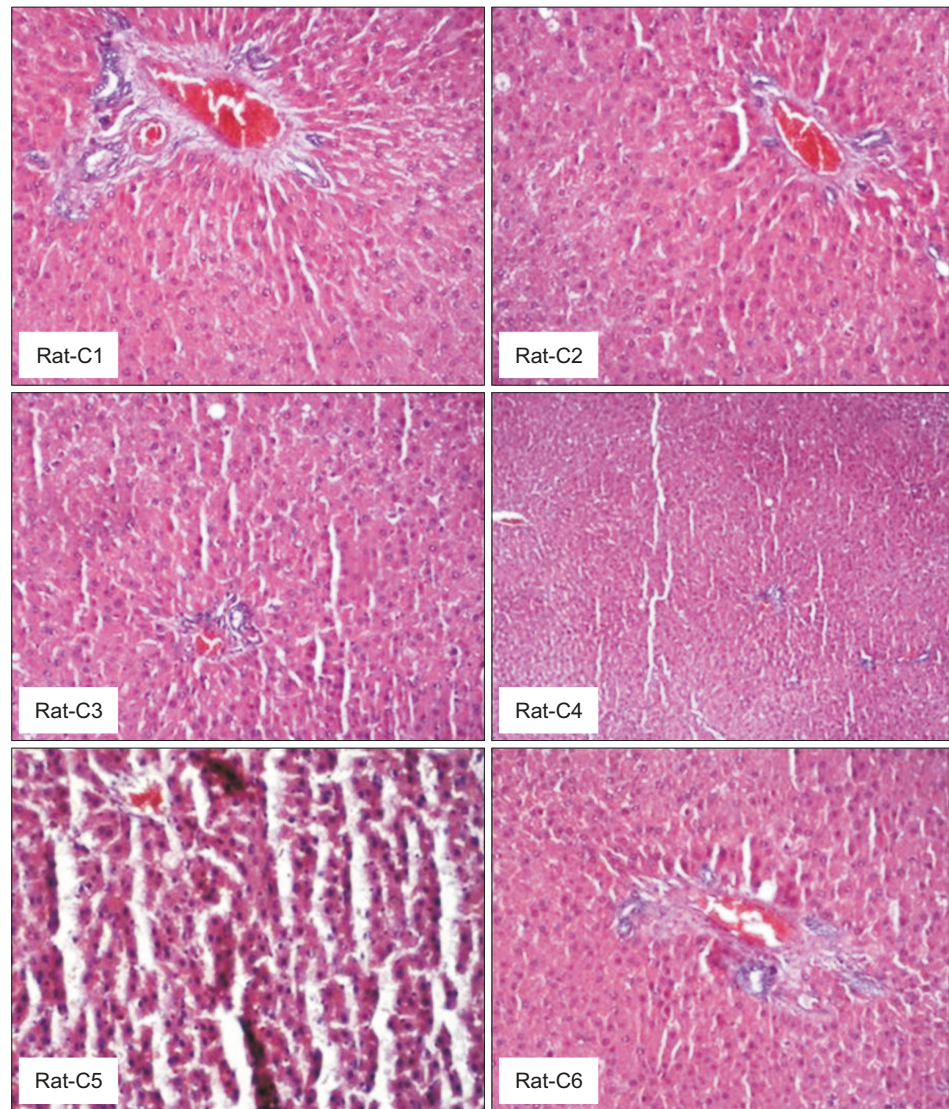
Fig. 3. Liver histopathological examination images of the rats in the study group. Grade 2 steatosis was seen in Rat-S1 (×20, H&E), Rat-S4 (×20, H&E), Rat-S5 (×20, H&E), and Rat-S6 (×20, H&E), while grade 3 steatosis was seen in Rat-S2 (×4, H&E) and Rat-S3 (×20, H&E) (red arrows). In addition, minimal lobular inflammation was observed in Rat-S4 (black arrow).
H&E: hematoxylin and eosin.

Table 4. Comparison of thiol disulfide homeostasis parameters of groups

Parameter	Study group (NAFLD)	Control group	*p*-value
Antioxidant			
Native thiol	243.2 (194.9–288)	266.6 (251.1–297)	0.310
Total thiol	292.25 (227.3–342)	305.75 (287.4–319.6)	0.631
Index 3: Native thiol/Total thiol	84.37 (79.81–86.28)	88.54 (84.09–92.93)	0.025
Oxidane stress			
Disulfide	26.53 (16.20–28.25)	17.08 (11.30–24.95)	0.025
Index 1: Disulfide/Native thiol	9.26 (7.95–12.65)	6.48 (3.81–9.46)	0.025
Index 2: Disulfide/Total thiol	7.82 (6.86–10.10)	5.73 (3.54–7.95)	0.025

Values are presented as median (min–max).
NAFLD: non-alcoholic fatty liver disease.

fat diet after four to eight weeks [20,21]. Similarly, the body weights of the rats fed with a high-fat diet were significantly higher on the 4th week than the control group.

Fig. 4. Liver histopathological examination images of the rats in the control group. Regular liver tissue was observed in the histopathological appearance of the control group. Steatosis and inflammation were not observed (Rat-C1 [×20, H&E], Rat-C2 [×20, H&E], Rat-C3 [×20, H&E], Rat-C4 [×4, H&E], Rat-C5 [×20, H&E], Rat-C6 [×20, H&E]).
H&E: hematoxylin and eosin.

In our study, a more pronounced increase in body weight was observed after the 8th week following the diet initiation. On the other hand, the increase in body weight was observed in the high-fat diet group than the control group after the 16th week in Xu et al.'s [21] study.

It is assumed that this difference may be due to the use of younger subjects with initial weights of 140–160 g in the study mentioned. Moreover, it is possible that younger rats in the rapid growth phase show a similar weight gain trend. High-fat diet periods varying between 4–16 weeks for the development of diet-induced obesity encountered in the literature suggested that the subjects selected in different studies may exhibit different weight gain rates in different growth-development periods due to their different initial ages and weights. Additionally, these facts may be related to the genetics of the rats [20-22].

Based on the laboratory reports presenting the reference range of biochemical parameters in rats, it was reported that the normal ranges may vary depending on many factors, such as the type, sex, and age of the rats. However, in male rats 8-16 weeks of age, these levels were reported as AST 105 (74–143) U/L, ALT 28 (18–45) U/L, and albumin 4 (3.4–4.8) g/dL [23]. It is a disadvantage for all studies that normal ranges cannot be determined precisely. However, to overcome this disadvantage, we have compared these values with a control group of healthy rats of the same breed and age, which were followed in the same environment. In our study, albumin levels were lower at the end of 12 weeks, while AST and ALT levels were higher in subjects with NAFLD than the control group. Similarly, Xu et al. [21] compared the biochemical and histopathological characteristics of rats given with a high-fat diet and standard control diet, and it was found that serum ALT levels increased in rats given with a high-fat diet over the 12th week. They showed that it peaked at the 16th week. Karaçor et al. [22] investigated the effects of high-fat and high-carbohydrate diets on rat liver and its role in weight gain for 16 and 20 weeks. When they compared four different rat groups, they found that ALT levels were significantly higher in the 20-week high-carbohydrate group. In the same study, it was observed that there was no significant difference between the albumin levels of the groups fed with high carbohydrate and high fat diets. In our study, higher AST and ALT levels were found in subjects with NAFLD at the end of 12 weeks than the control group. Dimitrova-Shumkovska et al. [24] found that serum albumin levels as well as total protein, urea, and creatinine levels were significantly decreased at the end of 18 weeks in rats fed with a diet with high-fat and high-cholesterol content. In our study, it was evaluated in accordance with that serum albumin levels were lower in rats fed with high-fat diet than the control group. This result was interpreted to be related to impaired protein synthesis in the liver as a result of a high-fat diet [25].

Karaçor et al. [22] reported that although there were signs of fibrosis, inflammation, and steatosis in the portal area on histological examination in all groups, no statistically significant difference was detected. In our study, less frequent and milder inflammation was attributed to the shorter diet duration. Supporting this interpretation, Xu et al. [21] reported that in rats given with high-fat diet at different periods, steatosis started at the 4th week, the steatosis degree progressed after the 8th week, and mild inflammation was observed in the 12th week. In the same study, there was no hepatic perisinusoidal fibrosis up to 24 weeks in subjects fed with a high-fat diet. They showed that the inflammation score was significantly higher at the 12th week than at the 4th, 8th, and 16th weeks. Similarly, in our study, all rats which received a high-fat diet for 12 weeks had a minimum grade 2 steatosis, while only one of six rats had grade-1 inflammatory steatosis. In Xu et al.'s [21] research, the reason for the earlier onset of lobular inflammation may be the energy content of the diet they used (4,800 kcal/kg), which was higher than the energy content of our diet (4,202 kcal/kg). In addition, Sprague-Dawley rats were used in the study mentioned [21], while Wistar rats were used in our study. Physiological differences between Sprague-Dawley rats and Wistar rats might affect the time it takes for the development of fat and inflammation in the liver [26].

It is shown with all these studies that mild steatosis can develop after being fed with a high-fat diet for 6–8 weeks, and a steatohepatitis model accompanied by inflammatory changes can develop after being fed with a high-fat diet for more than 8–12 weeks.

In our study, the findings showed that thiol/disulfide homeostasis parameters (DS, DS/Native-Thiol, DS/Total-Thiol) reflecting oxidative stress were significantly higher in obese rats developing NAFLD after feeding with a high-fat diet for 12 weeks. Native Thiol/Total

Thiol ratio, which was one of the antioxidant parameters, was significantly lower in the study group than the control group. These results showed that oxidative stress increased in obese rats with grade 2 and 3 hepatosteatosis, and no inflammation was observed except in one rat with mild lobular inflammation. Increased oxidative stress parameters without significant inflammation in these obese rats with fatty liver may be due to increased fat accumulation, which is as a substrate for lipid peroxidation and formation of free oxygen species in the liver and adipose tissues [15,27,28].

Marushchak et al. [8] analyzed glutathione reductase activity, glutathione peroxidase activity, reduced glutathione (GSH), and oxidized glutathione (GSSG) levels in erythrocytes, adipose, and liver tissues using different methods and showed an imbalance in favor of GSSG associated with increased GSH consumption in obese rats. The authors attributed this result to the increase in GSH consumption during the neutralization of free radicals caused by the activation of lipid peroxidation. This study demonstrates the inability of obese rats to maintain thiol-disulfide balance, and this seems compatible with our results about increased oxidative stress. Glutathione is known to maintain the redox state of the sulfhydryl groups of proteins required for DNA repair and expression and inhibition of the replication of mature hepatocytes in the presence of oxidative stress [12,13,15,29]. Our findings showed that even in mild hepatosteatosis without significant inflammation and no fibrosis, thiol-disulfide balance is disrupted in favor of increased oxidative stress in rats with NAFLD. Metabolic and biochemical changes or changes in oxidative stress parameters at different stages of steatosis and steatohepatitis obtained in groups with different high-fat diet content and/or duration can be compared.

Karaman et al. [30] investigated the effects of high fat diet on the thiol-disulfide metabolism of the rats. They found that the compensatory response failed in the glutathione and the impairment of redox imbalance in thiol/disulfide metabolism after 180 days alimentary. The difference with our study was that the histopathology of both liver and fatty liver were not evaluated. Also, the ratio of thiol/disulfide indexes was not calculated and discussed.

An additional result of our study is that the role of oxidative stress in the pathogenesis of NAFLD, which is a current scientific curiosity, can be investigated by choosing this new method, which is simple, cheap, fast, and non-invasive. We could not find any other study using this new method to examine thiol/disulfide homeostasis parameters in rats with diet-induced NAFLD. In our opinion, given that oxidant and antioxidant parameters can be evaluated quickly and cheaply, using this new method adds value and originality to our study.

The present study has several limitations. The sample size was small, and the rate of inflammation was low. However, there are strong sides, such as enough duration of feeding and histopathological evaluation of tissue samples.

In conclusion, this study showed that NAFLD could be induced by a high-fat diet, and thiol-disulfide homeostasis is impaired in favor of oxidative stress in obese rats with diet-induced NAFLD. This result encourages to plan and design different experimental studies in which oxidative stress parameters, such as DS, DS/Native-Thiol, and DS/Total-Thiol, can be used as markers to show the efficiency of different diagnostic or therapeutic interventions in patients with NAFLD. Analysis of thiol/disulfide homeostasis parameters in NAFLD can be used in further studies to develop effective treatment options.

ACKNOWLEDGEMENTS

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