

## **The effects of rifaximin on fructose-associated steatohepatitis: A study in a rat model**

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**ABSTRACT**

**Objective:** metabolic-dysfunction-associated-steatotic-liver-disease and its related mortality are increasing worldwide. This study evaluated the potential of rifaximin in preventing and treating steatohepatitis induced by a high fructose diet by modulating intestinal pathology.

**Material-methods:**Forty-two rats were randomly divided into six groups: one group received a normal diet, another was fed a fructose diet, two groups received rifaximin (once or three times weekly) along with a fructose diet, and the remaining two groups were given rifaximin (once or three times weekly) with a normal diet. After eight weeks, liver tissues were examined for malondialdehyde, tumor necrosis factor- $\alpha$ , nuclear factor- $\kappa$ B, and nuclear factor erythroid 2-related factor 2 using Western blot analysis, while blood samples were analyzed for uric acid, liver enzymes, triglycerides, and cholesterol; plasma tumor-necrosis-factor- $\alpha$  was measured by ELISA.

**Results:**The fructose diet group showed significant increases in body and liver weights, ballooning degeneration, lobular inflammation, and macrovesicular steatosis. Metabolic dysfunction-associated steatotic liver disease developed in 21 rats, yet steatohepatitis was observed only in the fructose-only group. Biochemical markers, including liver enzymes, triglycerides, and cholesterol, were significantly elevated in the fructose group. Moreover, plasma and tissue tumor-necrosis-factor- $\alpha$  and Nuclear-factor- $\kappa$ B levels were higher in the fructose group ( $p = 0.03$ ), while Nrf-2 levels were elevated in the rifaximin-treated groups ( $p = 0.043$ ). Additionally, MDA levels were markedly increased in the fructose-only group ( $p = 0.033$ ) and decreased dose-dependently with rifaximin treatment ( $p = 0.029$ ).

**Conclusion:**These findings suggest that rifaximin's anti-inflammatory and antioxidant effects may alleviate fructose-induced steatohepatitis, although further clinical studies are warranted.

**Keywords:** Fructose, Non-Alcoholic Fatty Liver Disease, Rifaximin

## INTRODUCTION

Metabolic dysfunction-associated steatotic liver disease (MASLD), one of the most common liver diseases in the world, can begin as simple steatosis and progress to cirrhosis [1, 2]. It has been suggested that increased dietary fructose consumption is causing a parallel increase in diabetes, obesity, and MASLD in industrialized societies [3, 4]. In daily clinical practice in Turkey, it has been observed that MAFLD has a very high prevalence [5].

Endotoxin-dependent cytokine production has been shown to play a role in the pathogenesis of MASLD. Increased bacterial overgrowth has been found in the small intestine of patients with metabolic dysfunction-associated steatohepatitis (MASH) compared to healthy controls [6]. In MASH, cytokines, and especially tumor necrosis factor (TNF)- $\alpha$ , are released from Kupffer cells in response to endotoxin released from intestinal flora, hepatocytes, and adipose tissue macrophages, and free fatty acids increase. In addition, oxidative stress increases Kupffer cell activation and free fatty acid oxidation in hepatocyte mitochondria, peroxisomes, and microsomes. Nuclear factor erythroid 2-related factor (Nrf-2), an antioxidant whose level decreases in oxidative stress. Malondialdehyde (MDA) is an essential product of membrane lipid peroxidation, [7].

Fructose is absorbed by active transport in the intestines; insulin is required to enter the cells, thus generating a glycemic response. Long-term fructose administration causes hepatic macro- and microvesicular steatosis, a 98% increase in hepatic triglyceride, and an 89% increase in hepatic cholesterol content [8]. Endotoxin levels were high in portal plasma in fructose-induced MASLD in mice. Increased endotoxin levels may activate proinflammatory cytokines, leading to pathologies ranging from simple steatosis to the development of MASH [9]. Rifaximin (RFX) is a non-absorbable antibiotic in the gastrointestinal tract and has minimal systemic effects. Rifaximin has effectively treated hepatic encephalopathy and irritable bowel syndrome in recent years. RFX acts by inhibiting bacterial translocation and through bacterial

decontamination [10]. This study aims to investigate the inhibitory role of RFX in an experimental model of fructose-induced MASLD. In our study, the effects of a fructose-rich diet on various physiological and biochemical parameters and the potential reducing effects of RFX supplementation were investigated in rats.

## **MATERIAL AND METHOD**

The study was carried out at the XXX Research Center after obtaining approval from the XXX Animal Experiments Ethics Committee, following the standard ethical rules for experimental animal studies. The study was conducted in accordance with the Declaration of Helsinki. A total of 42 male Sprague-Dawley rats (average weight 220g) were housed at  $22\pm 1^{\circ}\text{C}$  with a 12-hour light/dark cycle and fed standard pellet feed and water. The experiment lasted eight weeks. Standard pellet feed and tap water were used for feeding the animals. The body weights of the rats were monitored throughout the experiment. The experiment lasted eight weeks. Rats in rifaximin groups received 15 mg/kg, and those on a fructose diet had 50% fructose in their drinking water. The study groups (n=7) were: Control (Normal diet), Fructose (fructose diet), F+Rif1 (fructose diet+rifaximin once a week), F+Rif3 (fructose diet+rifaximin three times a week), ND+Rif1 (Normal diet+rifaximin once a week), and ND+Rif3 (Normal diet+ rifaximin three times a week).

Bottles were regularly cleaned, and solutions were refreshed to prevent pathogen development. After eight weeks, rats were fasted overnight, decapitated under anesthesia, and blood samples were taken and centrifuged. Livers were removed and weighed, and tissue samples were fixed in 10% formalin for further analysis.

### **Biochemical Analysis**

Aspartate aminotransferase(AST), alanine aminotransferase(ALT), gamma-glutamyltransferase(GGT), alkaline phosphatase (ALP), cholesterol, triglyceride, glucose, and

uric acid levels were measured in serum (Olympus AU600). Serum TNF- $\alpha$  levels were studied by Enzyme-Linked ImmunoSorbent Assay (ELISA) method using an appropriate commercial kit.

### **Measurement of Protein Expression by Western Blot Analysis**

Tissue TNF- $\alpha$  (Anti-TNF- $\alpha$  antibody, Abcam, Cambridge, UK), nuclear factor kappa-light chain enhancer of activated B cells (NF- $\kappa$ B) (Anti-NF $\kappa$ Bp65 antibody, Abcam, Cambridge, UK), Nfr-2 (Nuclear factor (erythroid-derived 2)-like Abcam Cambridge, UK), were studied by Western Blot method using kits.

Liver tissue was homogenized in a 1:10(w/v) solution of 5 $\mu$ M soy (soluble powder; Sigma, St. Luis, MO, USA) as a trypsin inhibitor and 10mM Tris-HCl (pH7.4, 0.1mM NaCl). Protein concentration was measured using the Lowry method (Protein Kit, Sigma). Proteins were transferred to nitrocellulose membranes (Schleicher and Schuell Inc, Keene, NH, USA) incubated for 1 hour. After washing, protein loading was verified using a  $\beta$ -actin monoclonal antibody (A5316; Sigma, St. Luis, MO, USA). Protein levels were analyzed densitometrically using Image J (National Institute of Health, Bethesda, USA).

### **Analysis in high-performance liquid chromatography (HPLC)**

Lipid peroxidation was measured in terms of malondialdehyde (MDA) production, using the high-performance liquid chromatography (HPLC, Shimadzu, Tokyo, Japan) method using Shimadzu UV-Vis SPD-10 AVP detector and C18-ODS-3.5 $\mu$ m, 4.6 $\times$ 250mm column.

## Histopathological Evaluation

After opening the abdomen, hepatic lobes were removed, weighed, and placed in a 10% formalin and standard saline solution. Tissue from the right lobe was stained with Hematoxylin-eosin (HE) and examined under an Olympus BX-50 light microscope. Scoring was performed for hepatic inflammation, macrovesicular steatosis, and ballooning degeneration based on images from 10 random fields.

Mallory's bodies were assessed as present/absent, and inflammation was scored as a percentage using a 4-point scale: grade 0 (no inflammation at x200), grade 1 (less than 2 foci), grade 2 (2-4 foci), grade 3 (more than 4 foci). Ballooning degeneration was scored as: 0 (none), 1 (mild), 2 (diffuse). Fibrosis was scored as follows: 0 (none), 1 (perisinusoidal or periportal fibrosis), 2 (perisinusoidal or portal or periportal), 3 (cirrhosis and severe inflammation in the portal). Steatosis as follows were evaluated: 0 (none), 1 (steatosis in 5% of hepatocytes), 2 (5-33%), 3 (33-66%), 4 (>66%). In addition, MASLD activity scores were collected, and a score of >5 was accepted as MASH [1, 11].

## Statistical analysis

Paired t-test was used to evaluate the between-group parameters of the data obtained in the study. Mann-Whitney-U test was preferred in dual evaluations and was given as mean  $\pm$  standard deviation. In addition, Pearson Spearman correlation tests were used for some parameters. Statistical evaluations were made using the SPSS 12.0 package program. A p-value of <0.05 was considered statistically significant.

## RESULTS

In this study, MASLD developed in 21 rats in 3 groups, 7 rats in each group, fed with fructose diet: Fructose (fructose diet), F+Rif1 (fructose diet+rifaximin once a week) and F+Rif3 (fructose diet+rifaximin three times a week). However, steatohepatitis findings were evident

only in the group receiving the fructose diet. Significant weight gain and liver weight gain were found in the fructose diet group compared to the control group ( $p < 0.05$ ). No significant difference was found in weight gain and liver weight between the fructose diet group and the group receiving different doses of RFX in addition to the fructose diet ( $p > 0.05$ ). Again, no significant difference was found between the groups receiving RFX in addition to the normal diet and the control group in terms of weight measurements and liver weights (Table 1).

There was a significant increase in uric acid, ALT, ALP, GGT, triglyceride, and cholesterol levels in rats fed with fructose compared to the control group ( $p < 0.05$ ). There was a decrease in the biochemical parameters measured in the groups' blood given different doses of RFX ( $p < 0.05$ ). No significant difference was observed when the fasting glucose level measurements were examined. Glucose measurements between groups were analyzed, and similar measures were found (Table 2).

Plasma TNF- $\alpha$  levels were significantly highest in rats on a fructose diet compared to the control group ( $p = 0.03$ ). However, RFX treatment significantly decreased TNF- $\alpha$  levels compared to the non-rifaximin group ( $p = 0.04$ ,  $p = 0.02$ ) with no significant difference between different RFX doses ( $p = 0.06$ ). In our study, tissue TNF- $\alpha$  levels were highest in the fructose group ( $p = 0.037$ ). TNF- $\alpha$  levels were also lower in the two groups given different doses of RFX with the fructose diet. But the results of TNF- $\alpha$  levels were the same for groups of rifaximin different amounts ( $p = 0.07$ ). In other words, TNF- $\alpha$  levels measured in plasma and TNF- $\alpha$  levels measured in tissue resulted in parallel. RFX given for therapeutic purposes confirmed our hypothesis; that is, levels were found to be low in the treatment group (Figure 1).

NF- $\kappa$ B levels were significantly higher in the fructose diet group compared to the control group ( $p < 0.001$ ). It was found to be significantly lower in the groups receiving RFX in addition to the fructose diet compared to those receiving only fructose ( $p = 0.026$ ). NF- $\kappa$ B value was found to



be lower in the RFX in addition to the normal diet and control groups ( $p < 0.05$ ), but this result was not statistically significant ( $p = 0.088$ ) (Figure 2).

Nfr-2 was lower in the fructose alone group ( $p < 0.001$ ). A significant difference was found in the groups receiving RFX in addition to the fructose diet compared to the group receiving only fructose ( $p = 0.043$ ). However, the protective role of RFX was independent of the dose. When the fructose diet group was compared with the ND+Rif1, ND+Rif3 groups, Nrf-2 values were found to be higher in the ND+Rif1, ND+Rif3 groups ( $p = 0.022$ ) (Figure 3).

MDA was significantly higher in the group using only fructose compared to the other groups ( $p = 0.033$ ). At the same time, there was a statistically significant decrease in MDA levels in all groups receiving different doses of RFX compared to the control group ( $p = 0.029$ ). While the level of MDA, one of the inflammation markers, was low in all groups receiving rif and the control group, the level of MDA was high in the group receiving fructose (Figure 4). Accordingly, it was interpreted that fructose exposure increases inflammation, while rifaximin reduces inflammation.

In the histopathological examination, steatohepatitis developed with a fructose diet. Ballooning degeneration, lobular inflammation, and macrovesicular adiposity were significantly observed in the fructose diet group. However, it was observed that steatohepatitis was decreased in the only RFX-given groups when compared to the group given RFX at different doses in addition to the fructose diet. Steatohepatitis findings were similar in the control and normal diet groups. While fibrosis was not observed in any group, other histopathological findings are shown in Table 3 and Figure 5.

## DISCUSSION

In this study, we investigated the effects of a fructose-rich diet on physiological and biochemical parameters in rats, as well as the potential mitigating effects of rifaximin supplementation. Our findings revealed significant weight gain in the fructose diet group compared to the control group, with no substantial difference in weight gain between the fructose diet group and those that received varying doses of rifaximin. Liver weights were also significantly elevated in the fructose group; however, no significant differences were found between the fructose diet group and those receiving rifaximin. Biochemically, we noted increased levels of uric acid, ALT, ALP, GGT, triglycerides, and cholesterol in the fructose-fed rats, while rifaximin supplementation resulted in a significant decrease in these parameters. Moreover, TNF- $\alpha$  levels were significantly higher in the fructose group and were notably lower in the rifaximin-treated groups, indicating rifaximin's protective potential against steatohepatitis. The study also documented significantly elevated NF- $\kappa$ B levels in the fructose group and a marked reduction in both NF- $\kappa$ B and Nrf-2 levels in the rifaximin groups, suggesting that rifaximin may inhibit the inflammatory response caused by a fructose-rich diet. Histopathological assessments confirmed the development of steatohepatitis associated with the fructose diet and showed improvement in histological findings in rifaximin-treated groups. Overall, these results highlight the detrimental effects of a fructose-rich diet on metabolic health and the potential therapeutic benefits of rifaximin in mitigating steatohepatitis and associated inflammatory responses.

Markers such as TNF and NFKB, which are indicators of oxidative stress in the blood, were elevated, and pathological indicators of steatohepatitis such as an increase in inflammatory cells, ballooning degeneration and fibrosis were increased in the liver tissues evaluated in histopathological examination.

In animal and human studies, it has been suggested that endotoxins secreted from the intestine play a critical role in the development of MASLD. The liver is in constant communication with products derived from the intestine. Many studies have observed and proven that the intestinal microflora LPS-TLR4 signaling pathway may play a critical role in the pathogenesis of MASLD [12, 13]. RFX is part of most treatments in the gastroenterological field. It is a gut-selective, oral antimicrobial agent that specifically reduces the recurrence of hepatic encephalopathy (HE) [14, 15].

Most animal studies have concluded that chronic fructose intake induces high Reactive Oxygen Species (ROS) formation in the liver of rodents [11, 12]. Bergheim et al. reported that feeding 30% fructose solution to mice for eight weeks significantly increased their markers for ROS formation [16]. In our study, we found that rats fed 50% fructose solution for eight weeks developed steatohepatitis. We found a significant reduction in histopathological findings of steatohepatitis, such as steatosis, inflammation, and ballooning degeneration, with RFX treatment. Our study is a rare study showing that different doses of RFX prevent early signs of fructose-induced steatohepatitis in rats. In one study, increased portal endotoxin levels and ROS formation were associated with induction of intrahepatic TNF- $\alpha$  expression [17]. Sapp et al. observed that rapamycin reduced fructose in a zebrafish MASLD model [18]. In mice fed a diet rich in fructose and cholesterol, endotoxin levels and lipid peroxidation increased TNF- $\alpha$  expression in portal blood. They noted a decrease in inflammatory markers when polymyxin B and neomycin were used simultaneously [16]. RFX has been shown to exert an anti-inflammatory effect through NFkB. In steatohepatitis models using RFX, intestinal permeability increases and circulating NFkB decreases [19, 20]. Our data were in line with other studies. NF-kB, a proinflammatory cytokine, was similar between the control group and the groups receiving RFX in addition to the normal diet, suggesting that RFX is hepatotoxic or protective against steatohepatitis.

Treatment of rifaxime is known to lead to a decrease in lipid peroxidation, thus reducing the levels of ROS and MDA [21]. In studies in patients with non-alcoholic steatohepatitis proven by liver biopsy, improvements in liver enzymes (ALT, AST, GGT), circulating endotoxins, TNF- $\alpha$  levels and metabolic homeostasis were observed when 6 months of RFX was applied [19]. It has been proven in the literature that rifaximin regresses liver inflammation markers involved in the pathogenesis of steatohepatitis. As seen in the study by Longo et al., in our study, it was determined histopathologically and biochemically that steatohepatitis regressed in

rat liver samples in the rifaximin group [22]. In our study, tissue MDA and Nrf-2, markers of oxidative stress, were analyzed. We found that these two markers increased in rats fed a high-fructose diet and decreased with RFX. In addition, ALT level, cholesterol, triglyceride levels and other basic biochemical and metabolic features of steatohepatitis increased in rats fed a high-fructose diet and decreased with RFX.

## LIMITATIONS

In our study, we detected early signs of steatohepatitis histopathologically. However, we did not observe fibrosis development in any of the rats. Therefore, we thought that the rats should be fed a high-fructose diet for a longer period of time. In addition, this situation was identified as a limitation of the study.

## CONCLUSION

In conclusion, our study shows that RFX shows early signs of fructose-induced steatohepatitis, drawing attention to rfx treatment in alleviating NAFLD/MASLD. The modulation of oxidative stress markers and improvement in histopathological findings highlight the need for further research to elucidate the mechanisms underlying the gut-liver axis in MASLD and to investigate the long-term effects of interventions such as RFX.

## Competing interests

The authors declare that they have no competing interests.

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**Table 1.** Rat and liver weights in the groups

Groups	Basal weight	Final weight	Liver weight
Group 1 (control)	219±20.4 <sup>a</sup>	280±13.2 <sup>b</sup>	8.84±1.19 <sup>b</sup>
Group 2 (fructose)	219±44.5 <sup>a</sup>	355±6.8 <sup>a</sup>	12.23±1.30 <sup>a</sup>
Group 3 (fructose+R1)	220±20.1 <sup>a</sup>	352.14±13.1 <sup>a</sup>	10.7±1.40 <sup>a</sup>
Group 4 (fructose+R3)	220±30.1 <sup>a</sup>	350.86±6.81 <sup>a</sup>	10.4±1.49 <sup>a</sup>
Group 5 (Normal diet+ R1)	220±26.0 <sup>a</sup>	265±6.8 <sup>b</sup>	9.1±1.09 <sup>b</sup>
Group 6 (Normal diet+ R3)	220±30.5 <sup>a</sup>	273±6.8 <sup>b</sup>	8.88±1.01 <sup>b</sup>

\* The data are presented as mean ± Standard error

a–d: The difference between the groups with different letters in the same line is statistically significant (  $p < 0.05$  )

all results are given as mean ± SD.

**Table 2.**Biochemical parameter results of the groups

Glucose	Uric acid	ALT	ALP	GGT	Triglyceride	Cholest
110.43±3.7 <sup>a</sup>	1.29±0.54 <sup>c</sup>	60±6.8 <sup>b</sup>	306.7±6.4 <sup>b</sup>	0.43±0.1 <sup>c</sup>	120±6.8 <sup>c</sup>	53±2.9 <sup>b</sup>
109.86±2.4 <sup>a</sup>	4.54±1.59 <sup>a</sup>	75.2±5.0 <sup>a</sup>	463.1±60.9 <sup>a</sup>	1.5±0.2 <sup>a</sup>	180±41 <sup>a</sup>	68.4±3.1
112.29±10.3 <sup>a</sup>	3.07±0.30 <sup>b</sup>	66.7±1.6 <sup>b</sup>	395.5±77.3 <sup>b</sup>	1.0±0.2 <sup>b</sup>	150±40.8 <sup>b</sup>	58±7.3 <sup>b</sup>
112.57±2.7 <sup>a</sup>	2.21±0.62 <sup>b</sup>	61±10.6 <sup>b</sup>	350.8±49.1 <sup>b</sup>	0.74±0.22 <sup>b</sup>	130±26.7 <sup>c</sup>	55.7±10
111.43±6.29 <sup>a</sup>	1.36±0.34 <sup>c</sup>	62.8±2.19 <sup>b</sup>	314.4±25.7 <sup>b</sup>	0.46±0.21 <sup>c</sup>	125±68.8 <sup>c</sup>	54.7±3.1
110.14±8.1 <sup>a</sup>	1.50±0.25 <sup>c</sup>	62.7±6.6 <sup>b</sup>	310.8±20.8 <sup>b</sup>	0.44±0.17 <sup>c</sup>	125±35.5 <sup>c</sup>	56.5±4.9

\* The data are presented as mean ± Standard error.

a–d: The difference between the groups with different letters in the same line is statistically significant.  $p < 0.05$

all results are given as mean ± SD.

**Table 3.** Histopathological findings

	Control	Fructose	F+Rif 1	F+Rif 3	ND+Rif 1	ND+Rif 3	p*
<b>Inflammatory focus</b>	0.14±0.378	1.29±0.488	0.43±0.535	0.43±0.535	0.14±0.378	0.14±0.378	0.003
<b>Macrovesicular adiposity</b>	0.00±0.00	1±0.00	0.575±0.535	0.57±0.535	0.00±0.00	0.00±0.00	<0.001
<b>Ballooning degeneration</b>	0.14±0.378	2.71±0.488	2±0.577	1.86±0.690	0.14±0.378	0.29±0.488	<0.001
<b>Pericellular fibrosis</b>	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	>0.005

\*Kruskal-Wallis test value, all results are given as mean ± SD.

**Figure 1:** Hepatic tissue TNF- $\alpha$  levels

**Figure 2:** Hepatic tissue NF- $\kappa$ B levels

**Figure 3:** MDA levels of the groups

**Figure 4:** Hepatic tissue Nrf-2 levels

**Figure 5a:** Histopathological appearance of the hepatic tissue in the rats in the control group (H&E, x200).

**Figure 5b:** Severe ballooning degeneration and sporadic hepatic inflammation were found in the livers of the rats who were fed with 50% fructose solution. In this group, steatohepatitis findings were observed most extensively (H&E, x200).

**Figure 5c:** Regression in ballooning degeneration was found in the group who received RFX once a week in addition to 50% fructose solution compared to the group who received a fructose diet alone (H&E, x200).

**Figure 5d:** Macrovesicular adiposity, severe ballooning degeneration, or pericellular fibrosis were not found in the livers of the rats who were given RFX three times a week in addition to 50% fructose solution. The mildest findings were observed in this group compared to those who received a fructose diet (H&E, x200).