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Original Research

Effect of Bile on Fatty Liver and Metabolism in Rats

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Abstract

Objectives: Non-alcoholic fatty liver disease (NAFLD) is a range of liver disorders ranging from accumulation of fat in the liver (steatosis) to non-alcoholic steatohepatitis (necrosis and inflammation), eventually progressing in some individuals to fibrosis, cirrhosis, and liver failure, respectively.

Methods: The bile taken from the gallbladder, which was removed as a specimen from donor hepatectomy and living donor liver transplantation (LDLT) performed at Liver Transplantation Institute, was delivered daily to İnönü University Experimental Animal Production and Research Center under suitable conditions for use in the project. Monthly weights of all rats included in the study and the amount of bile consumed by rats in separate cages in the bile group were recorded.

Results: When the change in rat weights according to months was analyzed in the three groups 1st month, 2nd month, and 3rd month are made separately, the comparisons between the groups. Statistically significant differences were detected in the first month ($p<0.001$) and in the 3rd month ($p=0.001$).

Conclusion: This study aimed to determine whether bile contributes to the reduction of fatty liver. It was aimed to observe the effect of human bile fluid by giving it to study animals. In this study, by comparing the rats to which we gave bile by creating fatty liver, with the group with fatty liver damage and the control group, it was shown that bile improved fatty liver. In addition, it has been determined that bile has significant effects on routinely used laboratory tests such as ALT, ALP, cholesterol, triglyceride, HDL, and VLDL. When the results are evaluated, they make important contributions to the question of whether bile can be used by exogenous administration in the human body.

Keywords: Bile, bile acid, fatty liver

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Hepatitis Non-alcoholic fatty liver disease (NAFLD) is a group of liver diseases that includes steatosis, or the buildup of fat in the liver, and non-alcoholic steatohepatitis, or the inflammation and necrosis of the liver. In certain cases, NAFLD eventually progresses to cirrhosis, fibrosis, and liver failure.[1] A significant contributor to liver-related morbidity and death, nonalcoholic fatty liver disease (NAFLD) is being recognized as a hepatic manifestation of

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metabolic syndrome due to its prevalence linked to obesity, diabetes, and insulin resistance.[2,3]

Cirrhosis, cancer, and liver failure are all related to non-alcoholic fatty liver disease (NAFLD).[4] The prevalence of fatty liver disease is rising as a result of industrial food consumption, malnourishment, and alcohol usage. According to recent research, between 80 and 100 million Americans, or 30 to 40% of the country's population, suffer from NAFLD.[5] NAFLD is the primary cause of liver transplants globally, comes at a high financial cost, and lowers quality of life.[6] Because of this, research using animal experimental models to treat and prevent fatty liver has started to rise.[7,8]

Lipid droplets build up in the cytoplasm of hepatocytes, which results in fatty liver. Fatty liver is defined as having more than 5% of its weight in lipids and more than 2% of triglycerides (TG).[9]

Hepatosteatosis is categorized as mild, moderate, or severe based on factors such as lobular and portal inflammation, hepatocyte ballooning, and steatosis. A liver biopsy is the most accurate way to grade, diagnose, and determine the prognosis of steatohepatitis; thus, those who are at risk of liver disease should have a liver biopsy.

The liver produces bile, which is involved in the breakdown and assimilation of lipids. With an osmolality of 300 mOsm/kg, it is the physiological secretion of the liver that is comparable to plasma. Because it comprises bilirubin, salts attached to bile acids, phospholipids, cholesterol, proteins, electrolytes, bile dye, water, and several metabolites, it is the physiological secretion of the liver. The removal of metabolites—such as cholesterol and bilirubin—from the body that is produced as a result of the breakdown of blood products is another purpose of bile. Along with penicillin, sulfate, glucuronide, glutathione molecules, metals including copper and zinc, prostaglandins, fat-soluble vitamins, adrenal cortical hormones, and other steroid hormones are also expelled with bile.[10,11]

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The gut is affected by bile salts in two significant ways. It first influences the separation of fat globules into minute pieces by lowering the surface tension on the oil particles. Second, bile salts combine with micelles, which are tiny lipid complexes. They return to the intestinal content after aiding in the transfer of lipids. Bile salts are critical for the absorption of fat-soluble vitamins because of their impact on fat absorption. By stool, 40% of lipids are lost in the absence of bile salts.[13,14]

Hepatic cholesterol metabolism produces bile acid amphipathic (i.e., containing both hydrophobic and hydrophilic) steroids, which are crucial for preserving energy balance. Despite their historical use as detergents in lipid emulsification, digestion, and absorption, fatty acids were discovered at the start of the twenty-first century to be ligands for the nuclear farnesoid X receptor (FXR), which regulates their synthesis.[15]

The body needs certain metals, including iron, zinc, copper, and selenium, for various metabolic processes. In different disorders, the number of atoms in the liver increases and decreases. Exposure to hazardous heavy metals like lead, mercury, manganese, zinc, nickel, chromium, and zinc rises with industrialization.[16]

An average of 250–300 living donor liver transplants are carried out each year at the İnönü University Liver Transplantation Institute. This high-volume transplant clinic provides bile. By giving the bile from the gallbladder specimen removed from donor hepatectomy and living donor liver transplantation performed in our center for living donor liver transplantation to rats that develop fatty liver, the study aims to determine the effects of the bile on fatty liver and metabolism. It was looked into whether bile could slow the progression of nonalcoholic fatty liver disease (NAFLD) by enhancing hepatic lipid metabolism and decreasing fat buildup. Rat liver biopsies were performed to assess the extent of hepatosteatosis. Additionally, blood analyses were used in the laboratory to study the follow-up parameters of hepatosteatosis.

Methods

The İnönü University Faculty of Medicine Animal Studies Local Ethics Committee was contacted before the commencement of the studies, and clearance was obtained (approved number: 2021/8-9/11732). The study was carried out in January 2023 and February 2022. The İnönü University Scientific Research Projects Coordination Unit provided financial assistance for the study (project approval number: TSA-2022-2734).

While the power analysis of the study was 0.05, the power of the test (1-beta) was 0.80, the effect size was 0.90 and the

alternative hypothesis (H1) was two-sided, the minimum sample size required to find a significant difference using this test was 25 in each group. It was calculated that there should be 75 rats in total.

75 Wistar albino female rats, three months old and weighing 200–300 g, were bred by İnönü University Experimental Animal Production and Research Center and utilized in the research. 75 Wistar albino female rats were randomly assigned to three groups (n=25) as part of the project's scope.

The same number of rats that developed hepatosteatosis after being fed a high-fat diet (ARD-24, Diets D12451, Ankara, Turkey) with 45% of the food content being fat after 12 weeks were produced as the harm group and 25 rats fed a normal diet were established as the control group.

By including 20 milliliters of bile fluid per day into the dietary regimen, a bile group was established to assess the impact of bile on hepatosteatosis and metabolism.

Rats were kept in living quarters with regulated temperature (22±1°C) and humidity (55±5%) with a 12:12 h light/dark cycle. The rats were fed normal pellet feed measuring 8 mm, and they had unlimited access to water. The study groups are summarized in Table 1.

Under appropriate conditions, the gallbladder's bile—which was removed as a specimen from the Liver Transplantation Institute's donor hepatectomy and living donor liver transplantation (LDLT)—was sent every day to the İnönü University Experimental Animal Production and Research Center for use in the project. All of the study's rats' monthly weights were noted, as was the quantity of bile eaten by the rats in the bile group who were housed in different cages.

Measurements were made of the following: high-density lipoprotein (HDL) from intracardiac blood samples taken at the end of the third month; triglyceride; aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Lactate Dehydrogenase (LDH), cholesterol, total bilirubin, Alkaline Phosphatase (ALP), Gamma Glutamyl Transferase (GGT), and very low-density lipoprotein (VLDL). In the biochemistry lab of İnönü University Turgut Özal Medical Center, measurements were taken on several instruments.

Table 1. Summary of Study Groups

Study groups	Group Name	Explanation
1. Group	Control group (n=25)	8 mm standard pellet feed was used for 12 weeks and access to drinking water was provided *ad libitum*.
2. Group	Damage group (n=25)	Rats were fed with 45% high-fat feed for 12 weeks and had access to drinking water *ad libitum*.
3. Group	Bile group (n=25)	Rats were fed with 45% high-fat feed for 12 weeks, and access to bile fluid was provided *ad libitum*. The bile fluids of the cages were renewed every other day.

As part of the experiment, rats were put to death intraperitoneally with 300 mg/kg of ketamine and 25 mg/kg of xylazine to identify fatty liver. The pathology department then assessed the level of hepatosteatosis in the liver tissues that were removed.

The livers of the rats were fixed in 10% formaldehyde solution, embedded in paraffin, sectioned at 4-5 µm thickness by a microtome and stained with routine hematoxylin-eosin (HE) for microscopic examination. The morphological evaluation was made under a light microscope (Olympus BX51).

The percentage of hepatocytes (0% to 100%) with macro- or micro-vesicular fat was used to semi-quantitatively quantify the liver steatosis. The proportion of fat in the hepatocytes was expressed as a score between 0 and 4 (0): less than 5%, 1: 5–15%, 2: 16–25, 3: 26–35, and 4: more than 35% steatosis.

The following elements are present in liver tissue: sodium (Na23), potassium (K39), vanadium (V51), copper (Cu65), zinc (Zn66), rubidium (Rb85), and molybdenum (Mo98), lithium (Li7), magnesium (Mg24), and aluminum. Atomic weights of iron (Fe57), nickel (Ni60), calcium (Ca44), chromium (Cr52), manganese (Mn55), selenium (Se78), and aluminum (Al27) were noted. Measurements were conducted at the İnönü University Faculty of Engineering's Chemical Engineering laboratory.

The Shapiro-Wilk test was used to determine if the quantitative data in the research were appropriate for a normal distribution. The summary of non-normally distributed data was represented by the median (minimum-maximum), whereas the summary of normally distributed data was mean±standard deviation. The Kruskal Wallis test, One-Way Analysis of Variance, and One-Way Analysis of Variance in Repeated Measurements were employed in statistical studies when needed. The Kruskal Wallis test in multiple comparisons was conducted using the Mann-Whitney U test with Bonferroni correction, the One-Way Analysis of Variance in Repeated Measurements was conducted using the dependent sample t-test with Bonferroni correction, and the Tukey test was utilized for the one-way analysis of variance. A p-value of less than 0.05 was deemed statistically significant in the statistical analysis used. Every analysis was carried out with IBM.

Results

When the change in rat weights according to months was analyzed in the three groups 1st month, 2nd month, and 3rd month are made separately, the comparisons between the groups. Statistically significant differences were detected in the first month (p<0.001) and in the 3rd month (p=0.001) (Table 2).

The total amount of bile consumed in 24 hours by 5 rats in each cage in the Bile group fed with bile is given in Table 3. When the control group fed normally, the damage group fed a high-fat diet, and the group given bile along with a high-fat diet were compared in terms of the percentage of fatty liver, a statistically significant difference was detected (p<0.001) (Table 4).

Upon evaluating the laboratory tests, statistically significant changes were found in the levels of ALT, cholesterol, ALP, and HDL between the groups that were fed a normal control, the damage group that was fed a high-fat diet, and the group that received bile fluid in addition to a high-fat diet (p<0.001). Triglycerides, VLDL levels, and a high-fat meal were supplied to the harm group, the control group, and the group that also received bile.

Statistically significant differences were found (p=0.015) when the values were compared. There was no statistically significant difference seen (p=0.504) between the direct bilirubin values of the control group, which was fed normally, the harm group, which was fed a high-fat diet, and the group that received bile in addition to a high-fat diet. Upon comparing the AST readings of the normal control group, the high-fat diet-eating damage group, and the group that consumed bile in addition to a high-fat diet, no statistically significant difference was seen (p=0.113). Upon comparing the LDH levels of the normal control group, the high-fat diet group that underwent damage, and the group that combined a high-fat diet with bile, no statistically significant difference was seen (p=0.928).

There was no discernible statistical difference in the total bilirubin values of the groups that were fed normally as the control, the harm group that followed a high-fat diet, and the group that took bile in addition to the high-fat diet (p=0.227). Upon comparing the GGT values of the control group, the high-fat diet group that received damage, and the group that received bile in addition to a high-fat diet, no statistically significant variation was seen (p=0.354) (Table 5).

Table 2. Analysis table of rat weights according to months in terms of groups

Variable **	Group*			p***
	Control	Damage	Bile	
Beginning	188.92[a,b]±7.182	163.16[b]±11.123	173.8±9.161	<0.001
1. Month	252.24[a,b]±12.451	225.16±21.005	228.708±19.767	<0.001
2. Month	275.44[a,b]±15.387	250.8±24.157	251.583c±23.063	<0.001
3. Month	282.087[a,b]±15.951	255.391±27.696	262.545±24.114	0.001

*: [a]: Different according to damage group, [b]: Different according to bile group; **: Variables are summarized as 'mean±standard deviation'; ***: One-Way Analysis of Variance.

Table 3. Amount of bile fluid drunk in cages in the bile group

Variable*	Group**					p***
	1. Cage	2. Cage	3. Cage	4. Cage	5. Cage	
Amount of Bile Fluid Consumed (mL)	132 (50-199)	133d (40-261)	134d (45-226)	137d (47-221)	127 (37-195)	0.00674

*: Variables are summarized as 'median (min.-max.)'; **: d: It differs depending on the cage 5; ***: Kruskal Wallis test.

Table 4. Analysis table regarding the percentage values of fatty liver in terms of groups

Variable*	Group**			p***
	Control	Damage	Bile	
Percentage	0[a,b] (0-0)	10[b] (0-35)	0 (0-20)	<0.001

*: Variables are summarized as 'median (min.-max.)'; **: a: It differs according to the damage group, b: It differs according to the bile group; ***: Kruskal Wallis test.

Table 5. Serum biochemistry parameters

Variables	Groups						
	Control		Damage		Bile		
	Mean±SD	Median±Min.-Max.	Mean±SD	Median±Min.-Max.	Mean±SD	Median±Min.-Max.	p
Direct Bilirubin (mg/dL)	0.03±0.02	-	0.04±0.01	-	0.04±0.02	-	0.504**
AST (U/L)	149.57±37.88	-	140.53±47.4	-	124.06±25.74	-	0.113**
ALT (U/L)	54.35[a,b]±15.7	-	33.53±6.44	-	29.78±7.05	-	<0.001**
LDH (U/L)	957.61±407.59	-	974.95±330.27	-	1000.61±301.8	-	0.928**
Cholesterol (mg/dL)	62.96[a,b]±13.65	-	74.47±7.98	-	80.78±10.97	-	<0.001**
Total Bilirubin (mg/dL)	-	0.2 (0.1-0.2)	-	0.1 (0.1-0.2)	-	0.2 (0.01-0.2)	0.227***
ALP (U/L)	-	90[a,b] (33-143)	-	136 (102-259)	-	124.5 (77-237)	<0.001***
GGT (IU/L)	-	0 (0-5)	-	0 (0-5)	-	0.5 (0-5)	0.354***
HDL (mg/dL)	-	40[a,b] (28-69)	-	52 (40-63)	-	54.5 (45-67)	<0.001***
Triglyceride (mg/dL)	-	91[a,b] (60-249)	-	113 (83-253)	-	134.5 (17-201)	0.015***
VLDL (mg/dL)	-	18.2[a,b] (12-49.8)	-	22.6 (16.6-50.6)	-	26.9 (3.4-40.2)	0.015***

AST: Aspartate Aminotransferase; ALT: Alanine aminotransferase; LDH: Lactate Dehydrogenase; ALP: Alkaline Phosphatase; GGT: Gamma-glutamyl transferase; HDL: High density lipoprotein; VLDL: Very low density lipoprotein; **: One-way analysis of variance; ***:Kruskal Wallis test.

When the levels of atoms in the liver tissue are examined across the groups, the concentrations of molybdenum (Mo98), copper (Cu65), zinc (Zn66), sodium (Na23), potassium (K39), vanadium (V51), and rubidium (Rb85) differ statistically significantly. (p is less than 0.0550). In terms of ppb atoms, there is statistical significance between groups for lithium (Li7), magnesium (Mg24), aluminum (Al27), calcium (Ca44), chromium (Cr52), manganese (Mn55), iron (Fe57), nickel (Ni60), and selenium (Se78). No discernible change has occurred. (p>0.05) (Table 6).

Discussion

This study aimed to investigate the potential role of bile in the decrease of fatty liver. The purpose of administering human bile fluid to study animals was to see what effects it might have.

The liver secretes bile, which travels via the bile duct system and into the duodenum. Following cholecystokinin activation, some bile enters the gastrointestinal system where it is concentrated before being distributed during digestion. In particular, bile plays a role in the digestion of fat. Additionally, it is a crucial and exclusive method for excreting copper, bilirubin, and cholesterol. enters the intestine: bile, immunoglobulins, bile acids, etc.[17]

When the groups in our study were compared, total bilirubin readings did not change, despite differences in the levels of copper in the liver, cholesterol, HDL, triglyceride, and VLDL.

Up to 90% of NAFLD cases show an asymptomatic rise of ALT and AST values when other liver disease causes are ruled out, according to research by Pouwels et al.[18] In NAFLD patients, ALT rises to occur more frequently than AST elevations. Compared to ordinary steatosis, NASH typically has higher ALT levels. Levels of albumin, bilirubin, and ALP may also be raised. No change in AST levels was identified, even though ALT levels were shown to be different in our investigation, which is consistent with the conclusion in this review.

The distribution of components in the liver tissue for each of the three groups—control, injury, and bile—displays variations when Table 5 is studied. In all three groups, the elements Li, Na, Mg, Al, K, Ca, Mn, Fe, and Zn have a very homogenous distribution. Except for Al and Li, these elements are crucial to human existence. Nonetheless, it is noted that the damage group and bile group exhibit noticeably higher accumulations of metals such as nickel, copper, chromium, selenium, vanadium, rubidium, and molybdenum in comparison to the control group. This is to be expected given the significance of liver tissue to the body.

Lithium, salt, iron, and nickel levels in liver tissue drop in the

Table 6. Analysis table regarding the amounts of atoms in liver tissue in terms of groups

Variable*	Group**			p***
	Damage group	Control groub	Bile groub	
Lİ7 ppb	34.375 (3.85-71)	58.775 (34.375-89.3)	22.15 (3.85-77.1)	0.21793
NA23 ppm	512.619 (463.918-564.87)	537.213[b] (489.264-555.724)	456.601 (413.521-495.886)	0.04372
MG24 ppm	218.779 (200.157-226.721)	226.05 (193.015-228492)	228.043 (223.307-231.825)	0.08982
AL27 ppb	409.175 (225.1-648.275)	468.95 (155.775-1231.6)	538.3 (117.5-1289)	0.81058
K39 ppm	2199.806[a,b] (1977.853-2240.771)	2546.138 (2198.558-2569.618)	2349.414 (2299.303-2459.962)	0.02065
CA44 ppm	39.956 (32.282-61.829)	40.862 (39.835-47.267)	44.186 (36.149-57.056)	0.78432
V51 ppb	140.95[a] (84.75-229.025)	69.575[b] (44.7-112.575)	173.55 (116.4-219)	0.01674
CR52 ppb	279.35 (191.85-448.925)	354.1 (297.6-507.25)	390.575 (310.35-428.875)	0.20488
MN55 ppb	2375.225 (1949.025-2769.05)	2427.575 (2205.75-2727.925)	2754.1 (2609.525-3288.75)	0.06654
FE57 ppm	287.226 (129.296-300.063)	288.599 (247.101-384.551)	207.335 (182.735-224.054)	0.07502
Nİ60 ppb	33.275 (12.3-40.25)	5.3 (0-40.25)	19.3 (0-68.225)	0.41066
CU65 ppb	2907.825[ab] (2526.325-3282.275)	4031.15 (3020.875-4299.625)	3833.35 (3211.625-3925.175)	0.02086
ZN66 ppm	21.259[ab] (19.675-22.127)	24.661 (20.326-25.228)	23.827 (22.427-25.461)	0.03579
SE78 ppb	216.2 (0-684.65)	937.9 (399.375-1679.675)	496.975 (0-1022.675)	0.10791
RB85 ppb	1657.75[ab] (1538.675-1834.7)	10957.33[b] (10509.95-11504.85)	2061.725 (1716.725-2121.8)	0.00306
MO98 ppb	566.7[ab] (408.725-573.725)	829.6[b] (647.45-935.3)	668.5 (636.525-766.8)	0.00443

*: Variables are summarized as 'median (min.-max.)', **: [a]: Different from the control group, [b]: Different from the bile group., ***: Kruskal Wallis test.

bile-fed group while other elements rise. The body's requirements, intake, and tissue buildup can be used to explain these findings. That is, if it is required in other bodily areas, it is used up and does not build up; if it is not required, it builds up in the liver. This perspective is supported by the elevated concentrations of all harmful components in the liver.

It is possible to say that bile plays a role in the liver's buildup of inorganic substances from nutrient-rich food, particularly harmful substances.[19] According to research by Ashley et al., there is a reciprocal association between hepatic lipid accumulation and fibrosis. In this study, there was a substantial difference in the end-of-study weights of the groups fed a high-fat diet and those fed a regular diet. Despite having a shorter research time, there was a noticeable difference between the three groups' monthly weights, particularly in the third month, when the comparison was made.

According to Donkers et al.,[20] bile acid intake may be a novel strategy for treating hepatosteatosis and obesity by lowering intestinal fat absorption and boosting energy at the same time. Fatty acid absorption and de novo lipogenesis both influence the lipid buildup in the liver that is brought on by a high-fat diet. Hyperglycemia and insulin resistance are linked to obesity and high-fat diets.[19] There is compelling evidence that the pathophysiology of extreme obesity, insulin resistance, non-alcoholic fatty liver disease (NAFLD), dyslipidemia, and type 2 diabetes is shared. This is due to aberrant fatty acid metabolism and signaling.[16]

Metabolic implications of fatty liver are a global health concern. Bile acids are involved in the metabolism of fat and energy. The liver produces primary bile acids, which the intestinal bacteria transforms into secondary bile acids. Both animal models of non-alcoholic steatohepatosis (NASH) and fatty liver disease have been the subject of substantial research on bile acid receptors and their agonists. Bile acid receptor agonists have been shown in clinical studies to offer promise in the treatment of NASH.[21] At the moment, research on the benefits of natural products and herbal extracts on non-alcoholic fatty liver disease (NAFLD) is becoming more and more popular. Several of these studies have been done on a variety of herbal items that have strong anti-NAFLD properties.[22,23]

With a high incidence and prevalence across all age and gender categories, nonalcoholic fatty liver disease has emerged as the leading cause of chronic liver disease in both industrialized and developing nations. NAFLD impacts several organ systems outside of the liver in addition to being linked to mortality or morbidity related to the liver. The most prevalent conditions are type 2 diabetes, chronic renal disease, and cardiovascular illnesses.[24]

Cachexia can be affected by exogenous administration of artificial bile acids (ursodeoxycholic acid).[25] A medication called ursodeoxycholic acid is used to treat and control cholestatic liver disease. It affects the liver by several intricate and synergistic processes, including modifications to the bile acid pool, cytoprotection, immunomodulation,

and choleretic action.[26] Yet, there isn't presently a nonalcoholic steatohepatitis (NAFLD) medication that has been licensed by the Food and Drug Administration (FDA).[27]

Despite the identification of several chemical processes, routes, and histological mechanisms associated with fatty liver, no effective therapeutic approach is now in common use. Strategies for weight loss and dietary cholesterol reduction are still employed in the battle against fatty liver. We believe that larger-scale animal studies and later human studies will provide additional insight into the advantages of actual bile fluid, given the areas in which physicians employ fake bile acids.

In this study, it was demonstrated that bile improved fatty liver by comparing the rats to which we provided bile by inducing fatty liver, with the group with fatty liver damage and the control group. Additionally, it has been found that bile significantly affects commonly used laboratory tests including HDL, VLDL, ALT, ALP, cholesterol, and triglycerides. When the data are analyzed, they significantly advance the debate over whether the human body can utilize bile that has been administered externally.

Conclusions

In our investigation, bile acid generated by the human body was utilized in place of synthetic bile acid. The results of this investigation will significantly advance our knowledge of bile's impacts.

Our research revealed that giving bile to rats with high-fat diet-induced NAFLD enhanced liver function, raised hepatic antioxidant activity, and decreased blood levels of lipid profiles. In rats with nonalcoholic fatty liver disease (NAFLD), bile therapy reduced lobular inflammation, liver steatosis, and ballooning, according to pathological examination.

Important findings from this study suggest that bile may be a novel therapeutic agent for lowering body weight, liver fat mass, and body fat percentage. To fully understand the impact of bile on human metabolism, more research is required.

Disclosures

Ethics Committee Approval: The İnönü University Faculty of Medicine Animal Studies Local Ethics Committee was contacted before the commencement of the studies, and clearance was obtained (approved number: 2021/8-9/11732). The study was carried out in January 2023 and February 2022. The İnönü University Scientific Research Projects Coordination Unit provided financial assistance for the study (project approval number: TSA-2022-2734).

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