Clinical and Experimental Health Sciences

Investigation of Changes in Liver Microanatomy in the Steatosis Model Created by Permanent Canula in Rats

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ABSTRACT

Objective: The knowledge of nonalcoholic fatty liver disease (NAFLD) and Nonalcoholic Steatohepatitis (NASH) is limited to the findings from available suitable models for this disease. A number of rodent models have been described in which relevant liver pathology develops in an appropriate metabolic context. In this experimental study, it was aimed to create a new liver fat model by giving fat from the portal vein of rats and to visualize the changes in the liver with advanced microscopic techniques.

Methods: 28 female rats were used in the study. Permanent intraabdominal cannulas were inserted into the portal vein of the rats. Rats were randomly divided four group. Intralipid 20% substance was injected through cannula to the experimental groups during the test period. Control group received saline at the same rate. At the end of the experiment, the animals were visualized with a laser speckle microscope and livers were divided into sections according to the stereological method. The sections were painted with Hematoxylin-Eosin, Oil red o, Masson trichoma, Bodipy, Nile red. Sections were evaluated under a microscope.

Results: Ballooning, inflammation and fibrosis were observed in the 2 week intralipid group. In the 1 week intralipid group, the rate of parenchyma decreased while the sinusoid rate increased, and sinusoid rate increased significantly in the 2 week intralipid ($p<0.05$).

Conclusion: According to the findings, steatohepatitis was detected in the 2 week intralipid, whereas only steatosis was observed in the 1 week intralipid. Thus, it was concluded that the newly formed rat model causes steatosis.

Keywords: NAFLD, Steatosis, Portal vein, Confocal microscope, Laser speckle.

1. INTRODUCTION

The prevalence of nonalcoholic fatty liver disease (NAFLD) is between 4% and 47% in different populations (1,2).

Alcoholic and nonalcoholic fatty liver is a disease characterized by the accumulation of triglycerides in hepatocytes. In nonalcoholic steatosis, not only fat, but also intralobular inflammation, hepatocellular ballooning, and advanced fibrosis are seen (3). Nonalcoholic steatosis is the most common chronic liver disease in the world (4), a metabolic disease that occurs in 10-35% of the world population and more than 50% of obese people (5).

Animal studies for the nonalcoholic steatosis model not only explain the pathogenesis of steatosis, but also provide important information for testing the therapeutic effects of various agents. However, these animal models need to coincide with both the pathophysiology and histopathology of human nonalcoholic steatosis (6,7). Currently, there are many animal models for nonalcoholic steatosis. None of these models demonstrates the entire process of human steatosis. Therefore, new models are needed to provide information about the pathogenesis and treatment of nonalcoholic steatosis (4).

The aim of this study was to create a non-alcoholic steatosis test model in rat liver by injecting fat from the portal vein using a permanent cannula to create a similar model of human fatty liver. In addition, it was aimed to detect the changes in liver tissue in more detail by making necessary stains after steatosis. At the same time, it is aimed to show uptake of fatty acid and possible accumulation of fat in intracellular fluid with fluorescent dyes to be applied and to detect all structural changes in liver tissue quantitatively by the latest stereological methods.

2. METHODS

The ethical approval of the study was obtained from Istanbul Medipol University Animal Experiments Local Ethics

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Committee (date: 14.04.2014 and decision number: 17). A total of 28 female Sprague-Dawley rats weighing 250-300 g were used in the study. The animals were obtained from Istanbul Medipol University Medical Research Center (MEDITAM). All experiments were done in Regenerative and Restorative Medicine Research Center (REMER). The animals were maintained at the controlled temperature of 23 ± 1°C, humidity of 55 ± 5%, in a 14-hour light/10-hour dark cycle. Throughout the study, the animals were provided with soy-free, in-house-prepared pelleted feed and filtered drinking water, ad libitum. Experimental groups (n=14) and control groups (n=14) were divided into 1-week intralipid (n=7), 1-week saline (S) (n=7) and 2-week intralipid (n=7), 2-week saline (n=7).

One-week experiment group was injected intralipid 20% substance + saline through the portal cannula for one week (1w/intralipid). One-week control group received saline in the portal cannula (1w/saline). Two-weeks experiment group was injected intralipid 20% + saline from the portal cannula for two weeks (2w/intralipid), while the control group was injected with saline for two weeks (2w/saline).

2.1. Surgical Procedures

Before surgery, animals were anesthetized. For surgical operations, the Ritsma protocol was used (8) (Fig. 1 A, B, C.). The edges of the cannula were adhered to the vessel with tissue adhesive (9). The reservoir with the cannula was placed in the pocket we created (Fig. 1 D, E).

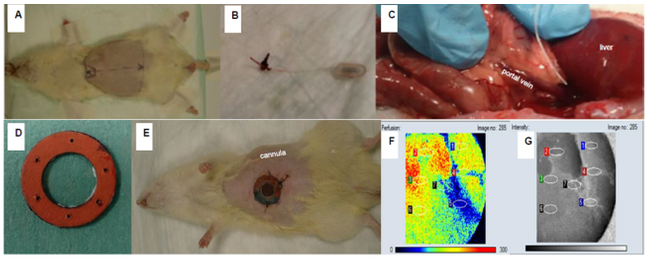

Figure 1. *Preparation of the rats for surgical operation and insert the cannula into the portal vein and blood flow image.*

A. Shaving and incision. **B.** Catheter preparation. **C.** Inserting the cannula into the portal vein. Abdominal imaging window developed by the materials in our laboratory and **D.** placement of the rat in the portal vein with cannula. **E.** Laser speckle microscopy shows the imaging process of the liver. **F.** amount of perfusion (blue: low flux, green: medium flux, red: high flux). **G.** measured areas in liver.

2.1.2. Intralipid injection:

After surgical procedures, intralipid ® 20% substance from portal vein was given to the experimental groups in the form of 2.4 g fat / kg + saline every day during the experiment period, while steatosis was aimed to occur in the control groups 0.9% saline administered daily during the same period.

2.1.3.Live microscopic examination of steatosis model developed rats:

Blood flow in the liver was evaluated with laser speckle microscopy while the rats were under anesthesia. The distance between the rat and the microscope objective was adjusted and the measurement started (Fig. 1 G, H). For this purpose, the same areas were used to measure on the same lobe of each rat. All rats were imaged between the 5th and 10th minutes and the values were recorded.

2.1.4.Examination of steatosis model rats by biochemical, histological and stereological methods:

At the end of the experiment, all rats were anesthetized, blood was taken from the heart and euthanized than liver tissue was collected (10,11).

2.1.5. Biochemical Analyses:

Aspartate aminotransaminase (AST), Alanine aminotransferase (ALT) serum levels were determined. Rat Eliza kits (Sigma) were used for the samples. The solutions were prepared following the protocols in the assay kits.

2.1.6. Fluorescent Staining:

Nile red staining and Bodipy staining; The stock solution was prepared before staining and then imaged on a confocal microscope (Zeiss LSM 780 NLO Multi Photon and Confocal Microscope; Zeiss Axio Observer Z1, fully motorized inverted microscope).

2.1.7. Histological investigation

For histological investigation, 8 µm sections were taken from frozen liver tissues in cryostat. Images were taken with axio zoom microscope.

2.1.8. Oil Red O staining (ORO)

With this dye, the presence of lubrication under fluorescence microscopy was evaluated.

2.1.9. Hemotoxylin-Eosin staining:

Hemotoxylin and eosin staining were performed on sections taken at 5 µm size. It was evaluated whether there was ballooning and whether the lubrication was macrovesicular or microvesicular.

2.1.10. Masson trichoma staining:

For staining, 8 µm sections were taken from the tissues with cryostat. Fibrosis in liver tissue was evaluated with stained sections.

2.2. Morphometric Analyses

In our study, morphometric evaluations were performed to obtain quantitative results. For this purpose, after the blood collection from rats, removed liver lobes were divided into 6-8 sections and tissue fragments were taken according to systematic random sampling method (12). A piece of tissue of 1 cm^3 was taken for each animal. Subsequently the fractions were frozen at – 80 ° C. Sections of frozen tissue were then taken in 5 µm sections on the cryostat. Sections were selected by systematic random sampling method

(one out of every 16 sections). All sections were stained with Hematoxylin-Eosin. Mean diameter of central veins in liver and liver sinusoids to liver parenchyma ratio (Vv) were calculated on these sections. To estimate the mean diameter of central veins, major (a) and minor (b) diameters of central veins were measured and the formula $(D = \sqrt{a.b})$ was used However, sinusoids to parenchyma ratio was determined by point counting method (13).

2.3. Statistical Analyses

Mean, standard deviation, median lowest, highest, frequency and ratio values were used in descriptive statistics of the data. Distribution of variables was measured by Kolmogorov Simirnov Test. Mann-Whitney u test was used for the analysis of quantitative independent data. The chi-square test was used for the analysis of qualitative independent data, and the Fischer test was used when the chi-square test conditions were not met. SPSS 22.0 program in analysis.

3. RESULTS

In the 1w/intralipid group, steatosis, including mostly microvesicular-weighted macrovesicles, was observed. Hepatocellular ballooning was not observed. There were lobular inflammations. In the 2w/intralipid group; steatosis was predominantly macrovesicular. There was lobular inflammation. Hepatocellular ballooning was observed. Perisinusoidal fibrosis and portal fibrosis were also present. Lipogranulomas were large in size. Steatosis ballooning and lobular inflammation were not seen in the control group (Fig.3) (Table 1).

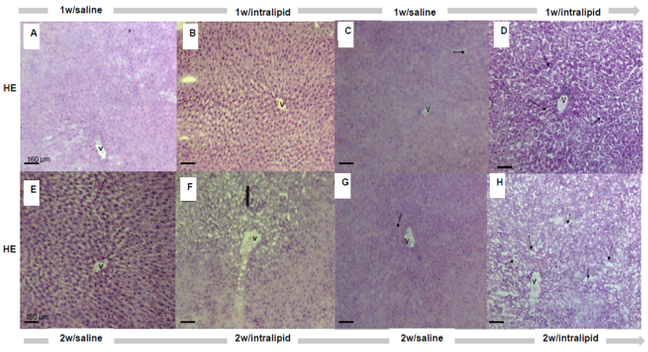

Figure 2. *Histological investigation of central vein and sinusoid/ parencyma.*

A. 1w/saline central vein. **B.** 1w/intralipid central vein **C.** 1w/saline sinosoid/ parencyma. **D.** 1w/intralipid sinusoid/parencyma. **E.** 2w/saline central vein. F. 2w/intralipid central vein. **G.** 2w/saline sinosoid/parencyma. **H.** 2w/intralipid sinusoid/parencyma. V: central vein, narrow: sinusoid (HE staining).

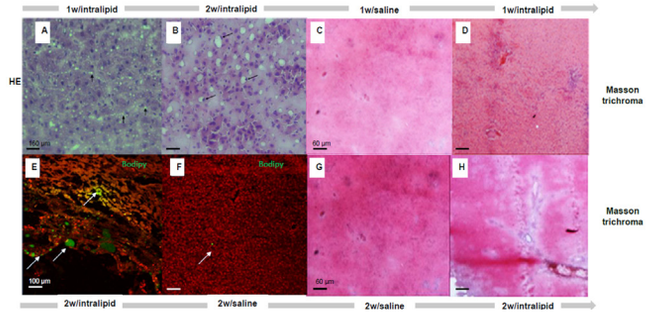

Figure 3. *Ballooning, fibrosis and fat accumulation images.*

A. 1w/intralipid. **B.** 2w/intralipid (short black arrows show microvesicular ballooning, long black arrows show macrovesicular ballooning HE). **C.** 1w/ saline. **D.** 1w/intralipid (less periportal fibrosis). **G.** 2w/saline. **H.** 2w/intralipid (pericinusoidal and periportal fibrosis) Masson trichroma. **E.** 2w/intralipid. **F.** 1w/intralipid; the green spots are the foci where the bodipy fluorescent dye radiates. Shows intracellular fat accumulation (Bodipy staining).

Table 1. *Summary of Results.*

	1w/saline	1w/intralipid	2w/saline	2w/intralipid	p
Mean central vein diameters (μ) Med±s.d.	5.8 ± 0.7	8.1 ± 2.6	5.8 ± 0.7	10.7 ± 2.8	**0.00**[m]
Sinusoids / parenchyma (Vv) Med±s.d	4.5 ± 2.8	14.4 ± 5.5	4.4 ± 1.3	22.4 ± 3.9	**0.00**[m]
Ballonning (n=42 sample each group)	(+) n-(%) 0; 0.0%	(+) n-(%) 0; 0.0%	(+) n-(%) 0; 0.0%	(+) n-(%) 8; 19.0%	**0.000**[X²]
Fibrosis (n=42 sample each group)	Perisinusoidal; 0.0%	Perisinusoidal; 0.0%	Perisinusoidal; 0.0%	Perisinusoidal; 11.9%	**0.000**[X²]
	Periportal; 0.0%	Periportal; 14.3%	Periportal; 0.0%	Periportal; 88.1%	
Fat granule rate (n=42 sample each group)	%5; 0.0%	%5; 33.3%	%5; 0.0%	%5; 28.6%	**0.000**[X²]
	%15; 0.0%	%15; 0.0%	%15; 0.0%	%15; 14.3%	
	> %50; 0.0%	> %50; 0.0%	> %50; 0.0%	> %50; 57.1%	
Fatty acid release (n=42 sample each group)	(+) n-(%) 0; 0.0%	(+) n-(%) 32; 76.2%	(+) n-(%) 0; 0.0%	(+) n-(%) 37; 88.1%	**0.000**[X²]
Intracellular fat accumulation	(+) n-(%) 0; 0.0%	(+) n-(%) 0; 0.0%	(+) n-(%) 0; 0.0%	(+) n-(%) 42; 100%	**0.000**[X²]
ALT(U/L) Med±s.d.	96.9 ± 42.4	81.6 ± 32.9	83.6 ± 34.7	117.5 ± 58.8	0.25[m]
AST(U/L) Med±s.d.	188.3 ± 95.4	217.0 ± 97.5	162.6 ± 38.5	343.4 ± 194.3	**0.05**[m]
Blood flow (mm/min) Med±s.d.	162.1±22.4	109.6 ± 48.3	160.5 ± 44.1	120.1 ± 10.5	0.089[k]

Liver histopathological and biochemical parameters in control and experimental groups. [m] Mann-Whitney U test. [X²] Chi-square test. [K] Kruskal-Wallis (Mann-Whitney U test). (p <0.05)

3.1. Histological and Fluorescent Staining Findings

Ballooning / fibrosis results (Fig. 1, 3) and Oil red o, Nile red and Bodipy staining results are shown in Table 1 (Fig. 3, 4).

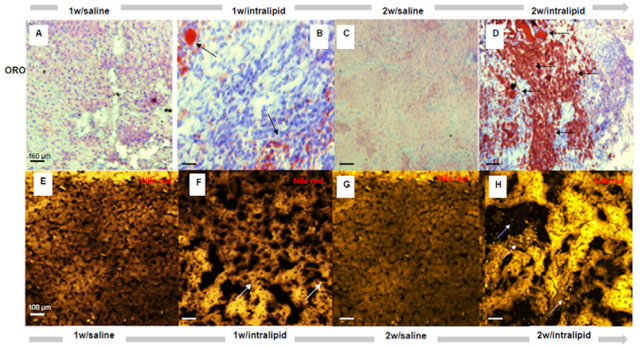

Figure 4. *Lipid droplet and fatty acids images.*

A. 1w/saline. **B.** 1w/intralipid. **C.** 2w/saline. **D.** 2w/intralipid. **G.** 2w/saline (short black arrows shows oil granules, ORO). **E.** 1w/saline. **F.** 2w/intralipid. **G.** 2w/saline.**H.** 2w/intralipid, white arrows show fatty acids, the golden yellow spots are the foci where the nile red dye radiates. (Nile red staining).

3.2. Morphometric, Biochemical and Liver Perfusuion (Blod flow) Findings

Results are shown in the Table 1 (Fig. 2, 3).

4. DISCUSSION

The knowledge of NAFLD and NASH is limited by the suitable models for this disease at the end of previous studies. A number of rodent models have been described in which relevant liver pathology develops in an appropriate metabolic context. These models play a key role in research on NASH. To date, not a single rodent model has been able to explain the progression of this disease in humans, but these models can mimic the characteristics of the disease in humans. Therefore, it is important that researchers select appropriate rodent models (14).

When rats were evaluated macroscopically, large or small lipogranuloma was observed in all of the 2w/intralipid (Fig. 4 A-D). Lipogranulomas are not necessary for the diagnosis of NAFLD, but since it is a frequently observed finding in NASH (15), it has been valuable data indicating that our model may have positive results macroscopically.

Morphometric examinations in our study: mean central vein diameter was found to be larger in both 1w/intralipid and 2w/intralipid than control group rats. When the 2w/intralipid and 1w/intralipid were compared (Fig. 2 A-D), the mean central vein diameter was larger in the 2w/intralipid compared to the other group, indicating that the mean central vein diameter increased as fat was fed from the portal vein. However, it was observed that the vessel diameters in the portal area increased more in the 2w/intralipid compared to the 1w/intralipid. There was no such study on the diameter of the central vein in parallel with our study. Shih et al. 2016 (16), stated that the change in central vein diameter did not correlate with blood flow in their transplantation study.

When the sinusoid / parenchyma ratio was calculated morphologically; The rats in the 1w/intralipid group were found to have increased sinusoidal space compared to the control group, but decreased liver parenchyma. In the 2w/intralipid group, it was observed that the sinusoidal space increased and liver parenchyma decreased more than the 1w/intralipid group. It is noteworthy that the parenchyma decreases and the sinusoidal space increases as the duration of the experiment increases. Noorafhsan et al. 2005 (17), in their study of diabetic rats reported that changes in the volume of sinusoid volume to changes in hepatocyte volume. Altunkaynak and Ozbek 2009 (12), female rats in their study of high fat content in the increase in sinusoidal volume due to necrosis parenchyma loss and decreased the density of hepatocytes were connected. Again, Yahyazadeh et al. 2017 (18), in their high-fat diet study, stated that feeding with fatty diet causes vascular dilatation in the liver and this may be due to inflammatory changes. Brancatelli et al. (19) attributed the cause of sinusoidal dilatation to increased venous pressure.

Ballooning and Mallory-Equivalent bodies represent liver cell damage and are critical in making the diagnosis of NASH (20). In our study, ballooning was observed only in the 2w/intralipid group (Fig. 3), whereas no ballooning was observed in the one-week experimental group. This indicates the presence of steatohepatitis in the 2w/intralipid group.

NASH is part of the NAFLD spectrum, characterized by lobular inflammation and progressive perisinusoidal fibrosis (21). Liver fibrosis is a liver disease that can result in the accumulation of extracellular matrix proteins, mainly collagen, and subsequent cirrhosis, portal hypertension, hepatic failure and HCC due to chronic liver damage (22).

In our study, inflammation was detected in 2w/intralipid groups, but not in the majority of the 1w/intralipid groups. This inflammation in the 2w/intralipid groups is mainly caused by lobular infiltration. This is an important symptom of steatohepatitis (23), indicating that steatohepatitis occurs in the 2w/intralipid group. When sections were stained with Masson trichoma stain used to show fibrosis, fibrosis was observed in some of the 1w/intralipid groups. Both periportal and perisinusoidal fibrosis were present in the two-week experimental group. In the 1w/intralipid group, periportal fibrosis was seen in some regions alone. However, the presence of both periportal and perisinusoidal fibrosis in the 2w/intralipid group is an indication that the model we developed was going to steatohepatitis.

When oil red o stained sections were evaluated, oil droplets were observed in experimental groups compared to control groups in our study. When the 1w/intralipid group and the 2w/intralipid group were compared, the percentage of fat droplets on the cross-section in the 2w/intralipid group was higher than the 1w/intralipid group. In addition, when comparing the size of the oil droplets in the hematoxylin-eosin stained sections, an image was obtained with microvesicular oil drops, mostly on the basis of macrovesicular weight in the 2w/intralipid group. In the 1w/intralipid group, a few rats in the group had less macrovesicular weighted fat droplets than the 2w/intralipid group, while the other

rats had microvesicular weighted fat droplets, including macrovesicles. Although microvesicular fat droplets have a poor prognosis in the long term, they are known to carry a great risk for the development of steatohepatitis and liver damage (24). In our study, we can say that simple steatosis occurs in the animals in the 1w/intralipid group and the risk of steatohepatitis is high. The presence of severe steatosis in rats in the 2w/intralipid group was determined by staining.

When all these histological findings were evaluated together; In the 1w/intralipid group, non-alcoholic fatty liver was evaluated; mild steatosis (> 5%) as well as periportal inflammation and no ballooning. In 2w/intralipid group, severe steatosis (33% – 66%), lobular inflammation was observed as 2-4 foci per foci, with several ballooned hepatocytes in sections. Given these data, it is possible to mention the presence of NAFLD in both experimental groups. When non-alcoholic steatohepatitis is graded, it is possible to mention only mild steatosis in the 1w/intralipid group and panacinar steatosis in the 2w/intralipid group. Since fibrosis was not observed in the 1w/intralipid group except portal fibrosis, staging could not be performed, whereas the 2w/intralipid group showed stage 2 characteristics. In the light of these data, steatosis has occurred in our 1w/intralipid group, but it can be said that the 2w/intralipid group has gone to steatohepatitis.

Abnormal intrahepatic fat accumulation (steatosis) in the form of cytoplasmic lipid droplets is an early pathophysiological feature of alteration of liver metabolism. For this reason, when we evaluated the fatty acid intake by Bodipy staining to detect the fat droplets, the foci of radiation were determined in certain areas in 2w/intralipid groups. This radiation showed us that fatty acid intake and accumulation were present in the 2w/intralipid group.

In order to detect intracellular fat accumulation by Nile red staining, fluorescence radiation from the stained sections was evaluated under a microscope. The abundance and scarcity of the focal points were examined from the sections where there was radiation. According to the results, more radiant focal points were detected in some rats in the 1w/intralipid group, and less in some rats in the 2w/intralipid group. Thus, more intracellular fat accumulation was observed in the 2w/intralipid group than in the 1w/intralipid group. Foci with radiation are often more frequent in or near the portal area, especially where cell infiltration occurs.

The accumulation of end products of enzymatic reactions in the liver may contribute to liver microvascular damage and NAFLD progression by further enhancing liver dysfunction. Since the end products of the enzymatic reaction are essentially advanced glycation end products, it has been disclosed that by stimulating the receptor, it exacerbates liver damage, fibrosis and inflammation, which in turn activates oxidative and inflammatory pathways (24).

In the studies to date, microcirculation change of NAFLD has been mentioned (25, 26). For this purpose, in our study, blood flow was evaluated by laser speckle for microcirculation of liver blood flow. Although no significant difference was found between the one week groups, it was observed that blood flow slowed down in some parts of the liver lobe compared to other groups in the 1w/intralipid group. Seifalian et al. 1999 (26) , in their study found that decreased microcirculation in the steatosis group and as a result of hepatic fat infiltration of the liver blood flow and parenchymal microcirculation were interpreted as slowed. McCuskey et al. 2004 (27), in their study of steatohepatitis with dietary fat in the flow of fat to go down to the direction of fibrosis interpreted their studies. In a NAFLD study performed by Pereira et al. 2017 (28), with a high-fat diet, it was found that liver blood flow decreased by up to 47%. This situation is similar to our study. When the 2w/intralipid groups were compared with the saline groups, it was observed that blood flow decreased in the portal vein fat treated groups compared to the saline groups and a significant difference was observed. Our results coincide with other studies (26,28).

As a result of the ALT evaluation of the blood samples of the experimental groups, no statistically significant difference was observed between the groups. However, according to the AST results, there was no difference between the 1w/intralipid group and the control group, whereas the AST values of the 2w/intralipid group were higher than the other groups. When the results were examined, the AST value of the 2w/intralipid group was found to be significant compared to the saline group. AST / ALT ratio of 1.33 and above is indicative of steatosis (29). In fact, the increase in AST is directly proportional to fibrosis (30). When we look at all of our groups, the ratio is over 1.33. Surprisingly, this rate was high in our control groups. This model, which we developed because we observed fibrosis formation due to the fact that AST was higher in the 2w/intralipid group compared to other groups, shows that this model is similar to other steatohepatite models (16). At the same time, as the increase in AST is a determinant factor in the blood after liver tissue damage, this result confirms the histological data of our study.

When we compare the data obtained in our study with other models, we can say that we created a new model for NAFLD studies by showing the presence of fatty acids in hepatocytes in addition to ballooning, steatohepatitis and fibrosis in our model.

The formation mechanism of NAFLD has been discussed in all studies until today and it has been stated that not all models made reflect the same spectrum in humans. Therefore, we set out to create a new model closest to the human NAFLD. As a matter of fact, in our study, when we look at the general parameters in this model, we observed that NAFLD was formed by administering oil directly from the portal vein as well as parenteral models. With this new model, we think that may be opened a new door in NAFLD formation mechanism studies.

We recommend that researchers focus on the development of liver carcinoma, the last step of NAFLD. Since cardiovascular diseases are seen in people with NAFLD, it is necessary to determine the presence of cardiovascular diseases in the lubrication performed with this model. At the same time, other studies in which our model will be used can be obtained from different parameters and molecular evaluation of NAFLD to provide new information

about the mechanism of this disease. With this information can help in the development of drug therapy.

Conflicts of interest: None

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