Histopathological changes and onset of severe hepatic steatosis in rats fed a choline-free diet

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Abstract. Hepatic steatosis significantly increases morbidity and mortality associated with major liver surgery. Several rodent models of hepatic steatosis have been previously reported, which aimed to investigate the effect of various pharmaceutical agents and interventional procedures on the pathophysiology of steatotic liver. The aim of the present study was to investigate the time frame of severe hepatic steatosis in rats after they were fed a choline-free diet and any associated histopathological changes. The duration of feeding with a choline-free diet required to develop severe hepatic steatosis was investigated in Wistar rats. The severity of hepatic steatosis in liver specimens was evaluated at 8, 10, 12 and 14 weeks following the onset of the choline-free diet. Comparisons were made with rats receiving standardized laboratory food. Feeding rats for 12-13 weeks with a choline-free diet led to 66% fatty liver infiltration, which exceeded 68% after 14 weeks. Prior to 8 weeks, the fatty infiltration reached 43%, with a gradual increase revealing a stronger rate from 8-12 weeks and a gradual decline after 14 weeks. At 12-13 weeks the fatty infiltration was considered representative of severe hepatic steatosis. Macrovesicular fatty infiltration revealed a significant increase at a steady rate between 8 and 14 weeks, with evidence of the onset of lobular inflammation and steatohepatitis after 14 weeks of feeding with the choline-free diet. Microvesicular fatty infiltration demonstrated a lower growth rate between 8 and 12 weeks while maintaining a steady rate between 12 and 14 weeks. Mixed fatty infiltration maintained its steady rate of hepatic parenchyma from 8.8-9.5%. Rats fed with the standard laboratory diet did not demonstrate fatty infiltration >4.5%, so they did not develop hepatic steatosis. Developing an ideal model of hepatic steatosis is a particular challenge. The findings of the present study indicate that severe hepatic steatosis in rodents may lead to the development of steatohepatitis after feeding with a choline-free diet for at least 14 weeks. This model is of particular interest in experimental liver surgery and associated surgical maneuvers, and is easily reproducible.

Introduction

Hepatic steatosis [fatty liver disease (FLD)] significantly affects morbidity and mortality of patients undergoing major liver surgery (1-7). The prevalence is increased in the developed world (20-30% of the general population), with obese patients showing frequencies above 95% with increased markers of incidence of steatohepatitis (2,7-13). The etiology of hepatic steatosis has not been fully elucidated and etiological factors include dietary habits, obesity, dyslipidemia, alcoholism, hepatic and metabolic disorders (2,14-18).

In recent years, liver surgery presents excellent growth by reducing morbidity and postoperative mortality (14,18,19). However, postoperative mortality in surgical patients with hepatic steatosis remains in high rates (>14%) (14,19-23). The surgical technique in these procedures involves manipulations of prolonged hepatic ischemia followed by reperfusion [ischemia/reperfusion (IR)], which cause a significant degree of inflammation and ischemic damage by greatly aggravating survival (14,20,24-27).

Various models of hepatic steatosis in rodents are presented in the literature aiming to investigate both pathophysiology and the study of the effect of various pharmaceutical agents and/or interventional procedures in livers with severe fatty infiltration. The aim of the present study is to investigate the time frame for the development of a severe hepatic steatosis model in rats after choline-free diet and the assessment of histopathological changes of the hepatic parenchyma, such as hepatic steatosis, microvesicular, macrovesicular and mixed steatosis, necrosis, ischemia and inflammation, compared to normal rats (1-27).

Materials and methods

Animals. Male Wistar rats (n=96) aged 12-14 weeks and weighing 250-300 g were randomly divided into 2 groups. The
first group of 48 rats was characterized as control group C and freely received the standard laboratory diet (concentrated feed with a protein concentration of 20-27%), while the remaining 48 rats were group A and received choline-free diet (Mucedola, PF1877) for 14 weeks. Rats had free access to food and water (daily water consumption was about 10 ml/100 g of weight) while living conditions included constant ambient temperature (22°C and 60% humidity) and a 12-h light-dark cycle. This experimental study was implemented at the Experimental Research Center of ELPEN (Athens, Greece) and was approved by Veterinary Authority of East Attika Prefecture (Protocol ref. no. 1633 directive 609/1986) and performed complying with the rules of experimentation and 3Rs (Replace, Reduce and Refine).

Experimental design. In the pilot phase of the experiment the type of food and the induction time of severe hepatic steatosis (>66%) were investigated. After reviewing the literature on liver steatosis models the choline-free diet was selected (2). From the existing data, 8-10 week feeding with choline-free diet leads to moderate hepatic steatosis, a 12-14 week feeding achieves a steatosis rate of >66%, whereas after this time, rats significantly reduce food intake and show a severe steatohepatitis and cirrhosis over the next 3-4 weeks (2). From this test feeding of rat animals, a significant steatosis development occurred after 8 weeks during the pilot phase. Therefore, in order to confirm the existing literature, it was decided to feed the rats with choline-free diet (group A) and to randomly divide them into subgroups of 12 rats named A1, A2, A3, and A4. Every two weeks after the 8th week to the 14th (8th, 10th, 12th, 14th week) euthanasia was performed in each subgroup, in order to determine the time and the degree of hepatic steatosis reaching a rate >66% of the hepatocytes. Similarly, to compare with control group C rats fed with a standard laboratory diet were randomly divided into 4 subgroups of 12 rats and named C1, C2, C3, and C4. Euthanasia was performed in each control subgroup every 2 weeks from 8th week to week 14 (8th, 10th, 12th, 14th week) to study possible differences with the corresponding subgroups receiving choline-free diet for hepatic steatosis. Specifically, the experimental groups were: Group A, 48 rats received choline-free diet for the induction of hepatic steatosis and were divided into subgroups: A1, (n=12) were subjected to euthanasia at 8 week after the start of feeding; A2, (n=12) were subjected to euthanasia at 10 week after the start of feeding; A3, (n=12) were subjected to euthanasia at 12 week after the start of feeding; A4, (n=12) were subjected to euthanasia at 14 week after the start of feeding. Control group C, with 48 rats that received a standard laboratory diet after their birth and at the age of 12-14 weeks (experimental start time) were selected to continue to receive the same diet and to be studied comparatively after 8 to 14 weeks with rats of group A. In particular, the subgroups of control group C were: C1, (n=12) were subjected to euthanasia at 8 week after the start of the experiment; C2, (n=12) were subjected to euthanasia at 10 week after the start of the experiment; C3, (n=12) were subjected to euthanasia at 12 week from the start of the experiment; C4, (n=12) were subjected to euthanasia at 14 week after the start of the experiment.

Hepatic steatosis. The duration of steatosis for male rats (initial body weight 250-300 g) was ultimately determined by the control group at 12-14 weeks using the recombined Mucedola-PF1877 choline-free diet. The mean consumption of choline-free diet is amounted to 20.41 g/animal with an average gain of body weight (BWt) daily of 3.49 g/24 h. For example, the corresponding average daily standard consumption of typical laboratory food in normal rats was about 16.78 g/animal, with an average daily increase of 1.18 g/24 h. The daily BWt increase for rats with hepatic steatosis ranged from 4.09 g/24 h to 1.714 g/24 h (in the last week), with a final weight ranging from 528 to 636 g, in contrast with the age-matched animals without hepatic steatosis, whose weight ranged from 340 to 424 g. The percentage of liver weight in rats with hepatic steatosis after 12-14 week feeding with choline-free diet corresponded to 4.61±0.43% of total body weight. On the contrary, the weight percentage of normal liver in the control group was only 3.8±0.25% of the total BWt of rats of the same age without hepatic steatosis. After 12-14 weeks, fatty infiltration of hepatic parenchyma >66% was achieved, causing moderate to severe FLD (2.28-30).

Histopathological examination. The liver specimens to be studied for histopathological changes were dipped in a 10% Formol solution (10 ml of formol 100% in 90 ml of ddH2O). During their processing, tissue sections of 10 μm were taken, stained with the combination of hematoxylin and eosin and then at x200 visual magnification were studied. The main features of the confirmation of the extent of hepatic fatty filtration are mentioned, as well as the ratio between the microvesicular (microlacunar) and the macrovesicular (macrolacunar) form of hepatic steatosis in the experimental groups.

Statistical analysis. Continuous variables were described using the average levels, standard deviations and medians, while the categorical ones were described using the frequencies and the corresponding rates. The control of the regularity of the measurements distribution was done using the Kolmogorov-Smirnov test and normal probability plot. Two-way analysis of variance (ANOVA) without repetitive measurements was used to study factors, including inflammation, necrosis, ischemia, body weight, time, steatosis (microvesicular, macrovesicular and mixed), and their interaction. Multiple comparisons were made with the post hoc Bonferroni test. In statistically significant interaction one-way ANOVA model was used so that multiple comparisons are made with Bonferroni test. The non-parametric analysis, was made using the Kruskal-Wallis test and Mann-Whitney test. All statistical analyses were performed with the SPSS statistical package, version 17.00 (SPSS, Inc., Chicago, IL, USA). All tests are two-sided. The P-value <0.05 was established as a statistically significant difference level.

Results

From the parametric and non-parametric study of the percentage of fatty infiltration between groups A and C was confirmed a strong statistically significant difference (P<0.0005). In control group C lean rats in which the liver parenchymal fatty infiltration rate ranged from 0 to 4.5% for all four subgroups (C1-4), while in rats with hepatic steatosis in group A ranged from 43.3% (subgroup A1), up to 68.4%
(subgroup A₄) with maximum values observed after free choline feeding for 14 weeks for the A₄. The percentage of macrovesicular fatty infiltration in subgroup A₄ reached 54.7% of total fatty infiltration (corresponding to 37.4% of total liver parenchyma), the equivalent of microvesicular fatty infiltration averaged 35.8% (corresponding to 24.5% of total hepatic parenchyma), while mixed fatty infiltration was 9.5% (corresponding to 6.5% of total hepatic parenchyma).

Histopathological findings. The control group C, fed with the standard laboratory diet, did not show fatty infiltration greater than 4.5% in any of the C₁-₄ subgroups (Fig. 1). Therefore, hepatic steatosis cannot be considered. This percentage is considered to be normal, especially when it comes to the form of microvesicular (microlacunar) fatty infiltration, which was the most common form of fatty liver degeneration in all subgroups of the control group. No necrosis, ischemia or inflammation was developed in any subgroup of the control group. The steatosis did not develop in any subgroup greater than 4.5%. Microvesicular (microlacunar) hepatic fatty infiltration was developed in a percentage of 68.9‑70.2%, macrovesicular in a percentage of 1.7 to 2.5% and the mixed one from 27.3‑29.4%. Under no circumstances was observed a statistically significant difference between the control group subgroups for any of the histological parameters (Fig. 1).

The subgroups of group A (A₁-₄) showed no necrosis or ischemia. Therefore, they showed no difference from the control group. All subgroups of group A showed mild inflammation (rarely one leukocyte infiltration focus in 20‑30% of the studied optical fields). There was no statistically significant difference among subgroups of A, while a statistically significant difference (P<0.01) was observed in the corresponding subgroups of control group C.

In particular, subgroup A₁ (Fig. 2) of rats receiving choline-free diet for 8 weeks (from the age of 12‑14 weeks) developed fatty liver infiltration of 43.3%, macrovesicular to 21.9%, microvesicular at 17.6%, and mixed at 8.7% (Fig. 3). In each case, compared to the control subgroup C₁, there was a strong statistically significant difference (P<0.0001) for the fatty infiltration parameters (macrovesicular, microvesicular and mixed), with subgroup A₁ showing the highest values.

The A₂ subgroup of rats (Fig. 4) which received choline-free diet for 10 weeks (from the age of 12-14) developed 51.7% fatty liver infiltration, 26.5% macrovesicular, 20.8% microvesicular, and mixed in the 9.3% (Fig. 5). In each case, compared to the control subgroup C₂, there was a strong statistically significant difference (P<0.0001) for the parameters of macrovesicular, microvesicular and mixed fatty infiltration, with subgroup A₂ showing the highest values.

The A₃ subgroup of rats (Fig. 6) which received choline-free rats diet for 12 weeks (from the age of 12-14 weeks) developed...
fatty liver infiltration 62.9%, with macrovesicular corresponding to 32.3%, microvesicular corresponding to 24.7%, and mixed to 9.4% (Fig. 7). In each case, compared to the control subgroup C, there was a strong statistically significant difference (P<0.0001) for the parameters of macrovesicular, microvesicular and mixed fatty infiltration, with subgroup A showing the highest values.

The A subgroup of rats (Fig. 8) which received choline-free diet for 14 weeks (from the age of 12-14 weeks) developed fatty liver infiltration of 68.4%, macrovesicular corresponding to 37.4%, microvesicular corresponding to 24.5%, and mixed to 9.5% (Fig. 9). In each case, compared to the control subgroup C, there was a strong statistically significant difference (P<0.0001) for the parameters of macrovesicular, microvesicular and mixed fatty infiltration, with subgroup A showing the highest values.

From the statistical analysis of the studied parameters of the model studying the optimal feeding time with a choline-free diet (Fig. 10), for the development of hepatic steatosis, for the key parameter of hepatic fatty infiltration a strong statistically significant (P<0.0001) difference of all subgroups of group A occurred compared to the corresponding subgroups of control group C, at each of the studied time periods (8th, 10th, 12th and 14th week), with subgroups of group A, showing very high levels of fatty liver infiltration compared to the subgroups of group C, which was in each case <4.5%. Group A showed the highest average infiltration rate (68.4%), indicating a high degree of hepatic steatosis. High percentages were also shown by the remaining subgroups of group A, but below the 66% limit, in order to consider serious steatosis. In particular, subgroup A1 (8-week diet with choline-free diet) showed a percentage of 43.3%, A2 (10-week diet with choline-free diet) 51.7% and A3 (12-week diet with choline-free diet) with a percentage of 62.9%. Subgroups A1 and A2 showed a statistically significant (p <0.0001) difference compared to subgroup A4, while subgroup A3 showed only statistically significant difference (P<0.01) compared to A4.

Regarding the parameter of macrovesicular steatosis (Fig. 10), again subgroup A4 showed the highest percentage (37.4%) which is statistically significant (P<0.01) higher than A3 (32.3%) and statistically much higher than subgroups A2 (26.1%) and A (21.7%). All A1-4 subgroups showed statistically significant (P<0.0001) much higher values for macrovesicular steatosis than the corresponding subgroups of the control group C which showed rates <1% in each case. Regarding the parameter of macrovesicular steatosis, subgroup A4 with a percentage of (24.5%) and A3 with a similar percentage (24.7%) showed strong statistically significant (P<0.01) higher values than the subgroups A2 (20.8%) and A (17.6%).
subgroups showed statistically significant (P<0.0001) much higher values for macrovesicular steatosis than the corresponding subgroups of the control group C, which showed rates <0.05% in each case. Regarding the parameter of mixed steatosis, all subgroups of group A showed a similar variation of the rate from 8.7 to 9.5%, without any statistically significant difference. On the contrary, all A<sub>1-4</sub> subgroups showed statistically significantly (P<0.0001) much higher values for mixed steatosis than the corresponding subgroups of the control group C, which showed rates <0.5% in each case.

From the statistical analysis of the studied parameters of the model studying the optimal feeding time with a choline-free diet, for the development of hepatic steatosis, no data on the necrosis and ischemia of the hepatic parenchyma were obtained since they did not develop at any stage of the study (Fig. 10). As for the inflammation parameter, all subgroups of group A exhibited mild inflammatory symptoms (periportal polymorphonuclear infiltration) without any statistically significant difference. On the contrary, all A<sub>1-4</sub> subgroups showed statistically significant (P<0.0001) higher values for the inflammation parameter than the corresponding subgroups of the control group C, where almost no outbreaks of inflammation (extremely rare mononuclear random infiltrations) were observed.

Finally, from the study of weight changes between the subgroups of group A and the control group C (Fig. 11), there was a strong statistically significant (P<0.0001) correlation, with subgroups of group A (which received a choline-free diet) developing a higher body weight compared to the corresponding subgroups of control groups (which received standardized laboratory diet) for the corresponding time intervals. By comparing subgroups of group A, only A<sub>4</sub> showed a statistically significant increase in weight (535.6 g) compared with only A<sub>1</sub> (450 g) and A<sub>2</sub> (483.7 g).

**Discussion**

In the present study, the choline-free diet led to 66% fatty liver infiltration at 12-13 weeks, and after 14 weeks the rate of infiltration exceeded 68%. Before 8 weeks, the fatty infiltration rate reached 43%, with a gradual increase, showing a stronger rate from 8 to 12 weeks and a gradual decline in the rate until 14 weeks. After 12 weeks, and in particular after the 13th week, the percentage of fatty infiltration satisfies the evidence of severe hepatic steatosis. Macrovesicular fatty infiltration showed a significant increase at a steady rate between the 8th and 14th week. Microvesicular fatty infiltration showed a lower growth rate between the 8th and 12th week while maintaining a steady rate between 12th and 14th week. Mixed fatty infiltration maintained its steady-state percentage of hepatic parenchyma from 8.8-9.5%. The highest incidence of macrovesicular fatty infiltration, along with the presence of mild neutrophilic inflammatory infiltration, confirms the gradual progression of severe hepatic steatosis to steatohepatitis, after 14 weeks, in the choline-free diet group. In terms of body weight, obesity has been developed in rats with choline-free diet (subgroup A<sub>1</sub>) as early as 8 weeks and at 14 weeks obesity is severely impaired (subgroup A<sub>4</sub>) compared to both the control group and the other subgroups of group A (A<sub>1-3</sub>).

The development of experimental models mimicking non-alcoholic fatty liver disease (NAFLD) is the key objective for investigating the etiopathology of the disease and it is treatment with therapeutic interventions (2,28-30). Unfortunately, to date no ideal model of hepatic steatosis has been developed simulating the same histopathological lesions with humans and with the same timing of progression of the lesions (2). Existing experimental models mimic different stages and pathologoanatomical lesions with different clinical signs. Key problems of the existing models are the inability to develop a sufficient extent of macrovesicular fatty liver infiltration, the failure to develop a sufficient extent of inflammation of the hepatic parenchyma and its progression to a sufficient degree of fibrosis (2,28-30). Also in several cases, the inability of development of metabolic disorders is observed (obesity, insulin resistance, hyperglycemia, hyperlipidemia, metabolic syndrome), despite the moderate or severe development of hepatic parenchymal steatosis (2,28-30).

They have been used as animal models, mice and rats in genetic, nutritional and mixed models for the development of NAFLD (2,29-32). NAFLD developmental dietary models are rather more reliable research tools. Nutrition with data leading to gradual development of hepatic steatosis due to complicated metabolic pathogenetic disorders and its progression to steatohepatitis is a basic feature of all dietary models (2,31). Clearly, the differences in gene background, physiology, nutrition, metabolism, endocrine system, habits and lifestyle, exercise and medication, change the organic reaction, the development of hepatic steatosis and its effect between humans and different animal models. In humans, the various pathogenic agents act spontaneously over many years, while in animal models, they have a violent effect over a short period of time, irrespective of their small biological life cycle. Only these differences significantly reduce the credibility of existing experimental models (2,30). NAFLD’s genetic models usually fail to simulate the polygenic pathogenic disorder that causes the disease. Activation of all multiple gene disorders usually fails, while animal models are extremely difficult to replicate and are costly to research (2,28-34).

In particular, Zucker rats (fa/fa rats) which develop obesity, hyperglycemia, hyperinsulinemia and hyperlipidemia have been used. Their disadvantage is that they develop mild macrolacunar and mild to moderate microlacunar liver hepatic steatosis but are unable to develop into steatohepatitis. In addition, in cases of extensive hepatectomy (>60-70%),
this model presents severe disorders in liver regeneration and survival (2,30). Transgenic models overexpressing the SREBP-1a gene lead to the development of metabolic syndrome and mild hepatic steatosis (2,30). Otsuka Long-Evans Tokushima obese rats show between 28-38 weeks an automatic development of microvesicular and macrovesicular hepatic steatosis, along with metabolic syndrome and type II diabetes. They do not have a similar cause pathogenesis of NAFLD, such as in humans, and should receive a special diet in order to develop hepatic steatosis and steatohepatitis (2,30,35,36).

In most cases, it is necessary to feed genetically modified models with special nutrition in order to achieve the development of severe hepatic steatosis and its progression to steatohepatitis, which is not always successful. These models are designated as mixed (composite). In particular, mixed models using ZFR genetically modified animal models require the addition of a diet rich in fats or with excess of disaccharides and a reduction in the percentage of lipopolysaccharides, or free of choline and methionine (2,28,30,37,38). The transgenic animal models overexpressing SREBP-1a in order to develop similar pathogenetic mechanisms with humans to induce NAFLD should be fed a diet rich in fructose for about 16 months (2,35). Otsuka Long-Evans Tokushima obese rats need to receive a high-fat or choline-methionine-free diet in order to develop severe hepatic steatosis, with lobular inflammation and fibrosis (2,35,39-41). Finally, Wistar rats with hereditary hypercholesterolemia (Prague hereditary hypercholesterolaemic rats) in addition to the gene disorder, require a high cholesterol (>5%) diet to develop mild to moderate hepatic fatty filtration (2).

Pure nutritional models of hepatic steatosis have been used in a variety of studies, yielding satisfactory results with relatively low development costs. Existing studies with fat-rich diets for the induction of hepatic steatosis in rats have failed to show a progression of simple hepatic steatosis in steatohepatitis and also have different effects on the development of human-like metabolic pathophysiology (insulin resistance, hyperglycemia and dyslipidemia). The right choice of the ratio of monounsaturated and polyunsaturated fatty acids (ω-3 to ω-6 fatty acids) is of great importance as well as the ratio of proteins and carbohydrates. In many cases, administration should be done by placing a gastric catheter in order to administer the required amount of food (2,28,30,42-45). Also, a high carbohydrate (fructose and sucrose) diet lasting >15-16 weeks, which may cause panlobular microvesicular hepatic steatosis has been used (2,30).

The most frequently used experimental models of NAFLD concern the choline or choline/methionine-free diet (2,30,46). The absence of choline disrupts the synthesis of phosphatidylcholine in the liver, leading to accumulation of triglycerides in the hepatocyte and development of hepatic steatosis (2,14,29,30,46). Feeding with a choline-free diet causes severe chronic-dependent hepatic steatosis, characterized by inflammation, with or without mild fibrosis, while prolonged feeding can cause even neoplasia (2,14,28-30,46).

In the choline-free diet there are changes that resemble metabolic syndrome, the development of obesity, dyslipidemia and insulin resistance after 7-12 weeks of feeding. The absence of insulin resistance is an important difference observed in this experimental model of steatosis with choline and methionine-free diet in rats with respect to humans (2,14,20-30,39,46,47). The free choline and choline/methionine diet in Wistar rats causes in a week microvesicular liver steatosis, mainly in zone III, and after 3-5 weeks there is progressive development of inflammation. After the 7th week, there is an intense macrovesicular hepatic steatosis (2,39,46,47).

Mitochondrial function is maintained at reduced but satisfactory levels despite the oxidative phosphorylation of reduced performance whereas liver regeneration after heptectomy is not significantly affected in Wistar rats. These models achieve the development of hepatic steatosis and its progression to steatohepatitis depending on the duration of feeding. However, the choline/ methionine-free diet does not cause a corresponding metabolic burden (hyperglycemia, hyperlipidemia, insulin resistance), such as the choline-free diet. The latter, ultimately, is considered the most appropriate for the development of reliable models for the study of the effects of hepatic steatosis, inflammation and oxidative stress in the liver, in combination with metabolic syndrome and FLD disorders (2,30).

In this rat model, the development of macrovesicular severe hepatic steatosis with evidence of onset of lobular inflammation and onset of steatohepatitis, was demonstrated after a 14-week feeding with choline-free diet. Although in the literature there is reference of hepatic steatosis development after the 7th-8th week, however severe hepatic steatosis (fatty infiltration >66% with predominantly macrovesicular form) develops after the 14th week. No fibrosis elements were observed during this time, as confirmed by the literature (2,28-30,46). The particular interest is in performing surgical manipulations and study their effect on liver physiology with steatosis without the synergistic effect, together with metabolic pathophysiological mechanisms.

Developing an ideal model of NAFLD is a particular challenge. The histopathological changes that occurred in animals with hepatic steatosis are in many cases unable to mimic the corresponding changes in humans. The results of this study indicated that severe hepatic steatosis in rodents may lead to the development of steatohepatitis after feeding with a choline-free diet for at least 14 weeks. This model may be of interest to researchers of experimental liver surgery and related surgical maneuvers, as it is easily reproducible. Further studies should focus on the search for longer periods of feeding with a choline-free diet, beyond the 14th week and should further investigate on the progression of steatohepatitis in order to control possible process of hepatic fibrosis and cirrhosis.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.
Authors' contributions

TK and NS conceived and designed the study, and drafted the manuscript. DM and AP performed the analysis and interpretation of data. KA, LM conducted the experiments. GK reviewed and edited the manuscript critically for important intellectual content. All authors read and approved the manuscript.

Ethics approval and consent to participate

This study was approved by the Veterinary Authority of East Attika Prefecture (approval no. 1633 directive 609/1986).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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