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A Simple and Low-cost Experimental Mouse Model for the Simultaneous Study of Steatohepatitis and Preclinical Atherosclerosis

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ABSTRACT

Recent studies have demonstrated a clinical relation between steatohepatitis and atherosclerosis. Both pathologies are of much interest due to the great morbidity and mortality associated with them and studies in animal models are necessary in order to find new therapies. The aim of the present study was to establish a new model for the simultaneous study of preclinical atherosclerosis and non-alcoholic steatohepatitis, in which BALB/c mice were fed a high-fat canine puppy diet. An experimental study was carried out on 3 BALB/c mice groups that were fed a standard diet (SDiet), a commercial atherogenic diet (AtheroDiet), or a canine puppy diet (CanDiet), for 6 months. A biochemical serum test and an intraperitoneal glucose tolerance test were carried out. The animals were euthanized at the sixth month and histopathologic slices of the liver and the thoracic and abdominal aorta were stained with hematoxylin and eosin or Masson's Trichrome. Steatohepatitis was evaluated and the thoracic and abdominal aortic intima-media thickness was measured. The ANOVA test was used for the group comparison. Steatosis and inflammation of the liver and thoracic and abdominal aortic intima-media thickness were all significantly increased in the AtheroDiet and CanDiet groups, compared with the SDiet group ($p = 0.000$). There was a positive correlation between steatohepatitis and the aortic intima-media thickness. No significant differences were found among the groups in relation to serum cholesterol or triglyceride levels; however, there was an alteration in the glucose tolerance curve in the AtheroDiet and CanDiet groups with respect to the SDiet group. There was no evidence of hepatic fibrosis in any of the groups. In conclusion, we were able to create a low-cost and accessible murine model for the simultaneous study of steatohepatitis and preclinical atherosclerosis.

Key words: Non-alcoholic fatty liver disease, atherosclerosis, high-fat diet

INTRODUCTION

Non-alcoholic Fatty Liver Disease (NAFLD) is the most common liver pathology in the Western world (Krawczyk *et al*., 2010). Histologically it encompasses simple steatosis (fat accumulation in hepatocytes) and extends to non-alcoholic steatohepatitis (NASH) (Kneeman *et al*., 2012). The latter is characterized by hepatocyte damage, inflammation and varying grades of hepatic fibrosis and it can progress into cirrhosis and liver failure (Angulo, 2010). An association between NAFLD, metabolic syndrome and cardiovascular disease (CVD) has recently been suggested. Indeed, different clinical studies have demonstrated that NAFLD patients present with increased preclinical atherosclerosis compared with non-steatosic individuals; they are supported by follow-up studies that have revealed that atherosclerotic CVD is the second most common cause of death in NAFLD patients (Lizardi-Cervera and Aguilar-Zapata, 2009).

Atherosclerosis, whether combined or not with NAFLD, is an important public health problem that is characterized by vascular site-specific chronic inflammation initiated in response to retained and modified lipids within the arterial wall. Preclinical atherosclerosis, clinically detected by the thickening of the intima-media layers of the carotid artery or the aorta, is a generalized atherosclerosis indicator, as well as one of coronary artery disease and it is also related to metabolic control (Harrington *et al*., 2010; Iwakiri *et al*., 2012; Meenakshisundaram *et al*., 2011).

Experimental studies on NAFLD or atherosclerosis are generally performed independently and therapies for these pathologies are tested using different animal models in separate experiments. Murine models have been the most commonly employed models for atherosclerosis (Daugherty, 2002). With the advent of genetic engineering, knockout and transgenic mouse models of atherosclerosis have supplemented the traditional dietary cholesterol-induced disease models (Nofer, 2012). Apolipoprotein E-deficient mice (ApoE -/- mice) and LDL receptor-deficient mice (LDLr -/- mice) are among the most widely used mouse models. In the ApoE -/- mice, there is a targeted deletion of the apoE gene which leads to severe hypercholesterolemia and spontaneous atherosclerosis and the LDLr -/- mice develop atherosclerosis, especially when fed a lipid-rich diet (Zadelaar *et al*., 2007). A review of 100 articles from the past 5 years describing experiments on atherosclerosis using murine models showed that 96% of them utilized genetically modified mice to induce atherosclerosis (64% ApoE(-/-), 22% LDLR (-/-), 7% ApoE(-/-), LDLR(-/-) and 3% others). A total of 4% did not use transgenic mice-only an atherogenic diet.

On the other hand, many animal models of NASH have been developed to date. These animal models do not replicate the full spectrum of the disease in humans. Likewise, for the study of atherosclerosis, there are transgenic mouse models and supplemented dietary-induced disease models. In many of the genetic models of NASH (e.g., sterol regulatory element binding protein (SREBP)-1c transgenic mice and phosphatase and tensin homologue deleted on chromosome 10 (PTEN) null mice], hepatic steatosis occurs first and steatohepatitis develops later. Ob/ob, db/db and KK-A mice do not spontaneously progress to steatohepatitis. On the other hand, in a methionine and choline deficient dietary model, steatohepatitis is induced very quickly. To a greater or lesser degree, all the models have a pathophysiologic or histologic difference from humans; however, they can be used in verifying hypotheses on the pathogenesis of NASH and in performing interventional studies (Takahashi *et al*., 2012).

An important aspect of some of these animal models is the fact that the use of mutant mice and some types of commercial diets is very expensive and not available worldwide, in addition to the special management that may be required. This could complicate the study of these pathologies in certain geographic regions.

Atherosclerosis and NAFLD are associated pathologies in humans and multiple therapies could benefit both diseases by affecting common pathophysiologic processes. Therefore, a model for the simultaneous study of steatohepatitis and preclinical atherosclerosis would be appropriate. The aim of the present study was to create a low-cost and accessible common model for these pathologies using BALB/c mice fed with a high-fat canine diet.

METHODS

Animals and diet: Thirty-seven male BALB/c mice (Harlan® Mexico), between 4 and 6 weeks old and with an initial weight of 22 to 25 g, were employed in the present study. Thirteen of these mice were fed a commercial atherogenic diet (AtheroDiet group) that was made up of 46.9% carbohydrates, 17.3% protein, 21.2% net fat, 1.25% cholesterol and 0.5% cholic acid (TD.02028 Atherogenic Rodent Diet, Harlan®, USA). Eight mice were fed a canine puppy diet (CanDiet group) consisting of 30% proteins, 4% fiber and 18% net fat (Perfect Fit Puppy, Waltham®, Mexico). The remaining 16 mice were fed a standard rodent diet (SDiet group) made up of 44.2% carbohydrates, 18.6% protein, 3.5% fiber and 6.2% net fat (2018S Tekland Global 18% Protein Rodent Diet, Harlan®, USA).

The mice were kept in cages in groups of a maximum of 5 mice under controlled conditions of light and temperature and they were given food and water ad libitum. After a period of 6 months of feeding, a glucose tolerance curve was made and the mice were decapitated. Blood samples were obtained for biochemical analysis and the aorta and liver were extracted and processed for histopathologic analysis. The animals were manipulated according to institutional guidelines and the Mexican Official Norm regulating laboratory animal use (NOM-062-ZOO-1999). The study was approved by the institutional Bioethics Committee of the University of Colima School of Medicine.

Glucose tolerance curve and biochemical analysis: An intraperitoneal glucose tolerance test was carried out at the 6th month. Glucose was injected intraperitoneally (2 mg kg^{-1} b.wt.) and blood glucose was determined at 0, 30, 60, 90 and 120 min after administration. Blood was collected from the tail vein of each mouse (Horio *et al*., 2005). Before the mice were euthanized, a blood sample was obtained for measuring the serum lipids (triglycerides, total cholesterol) and liver enzymes (ALT, AST), using an automatic biochemical analyzer (Cobas c111, Roche®, Mexico). The commercial ELISA kit was used for insulin detection (EZRMI-13K, Millipore).

Histologic studies: The livers were weighed and fixed in 10% formaldehyde. Three cross-sectional liver slices, two from the right lobe (central and external third region) and one slice from the left lobe, 2-3 mm thick, were dehydrated in ethanol, embedded in paraffin wax, sectioned (5 μm thick) and stained with hematoxylin and eosin. A pathologist carried out a blinded evaluation of hepatic steatosis by analyzing the percentage of liver tissue that presented with fat accumulation. This variable was classified as grade 0 (absent), grade 1 (up to 33%), grade 2 (between 33 and 66%) and grade 3 (more than 66%) and was also identified as mild, moderate and severe (Haddad *et al*., 2011).

Only the tissue with steatosis was taken into account. It was classified as having a micro or macrovesicular pattern and the percentage was quantified (1-100% of the tissue with steatosis). According to the previously described methodology and classification, inflammation was evaluated by functioning histologic zones depending on the oxygen supply: Zone 1 encircles the portal tracts where the oxygenated blood from the hepatic arteries enters; Zone 3 is located around the central veins, where oxygenation is poor; Zone 2 is located between zones 1 and 3. There were four

categories in relation to the percentage of tissue presenting with inflammatory infiltrate: none, mild, moderate and severe (0%, up to 33, 33-66% and more than 66%, respectively) (Haddad *et al*., 2011). Fibrosis was evaluated using the Masson trichrome stain.

For its study, the aorta was dissected from the sinuses of Valsalva up to the iliac bifurcation and fixed in 10% formaldehyde. Cross-sectional slices (1-2 mm thick) were cut in six different portions of the aorta: Three at the level of the thoracic aorta (ascending aorta, aortic arch and descending aorta) and three portions of the abdominal aorta (the proximal, middle and distal third). These were embedded in paraffin in order to obtain 5 μm thick histologic slices and were stained with hematoxylin and eosin for their evaluation.

Atherosclerosis of the thoracic and of the abdominal aorta was evaluated separately. Only the slice qualitatively showing the lesion with the highest grade of disease according to the Stary classification (grades I-VI) (Stary, 2000) was selected and a blinded analysis was carried out by a pathologist. The same slice was quantitatively evaluated by measuring the intima-media thickness (from the interior edge of the endothelium to the exterior edge of the middle layer) (Ku *et al*., 2006). This was done at eight equidistant sites per section, selected through systematic uniform random sampling, regardless of the presence or absence of atherosclerotic lesions at the measuring site.

The evaluations of the slices of the aorta and liver were carried out through images taken with an Axiocam MRC-5 model digital camera (Zeiss®, Germany) attached to an AxioPlan 2 M model bright field optical microscope (Zeiss®, Germany) with a motorized stage and A-plan X5 and X20 objective (total magnification X50 for the aorta and X200 for the livers). All the shots were taken under the same conditions of light and exposure.

Statistical analysis: The ANOVA test was used to compare the groups and the Bonferroni test was used for the post hoc analysis. Correlation analyses with the Spearman test were done to evaluate the correlation between the variables and the quantitative data, employing an ordinal scale. A 95% Confidence Interval (CI) was used in all tests and statistical significance was considered if $p<0.05$. The IBM SPSS Statistics 20 software was employed.

RESULTS

Morphometric and biochemical analyses: There were no significant differences among the groups in relation to the weight of the mice at the beginning and end of the experiment. Table 1 shows diverse parameters at the end of the study. Of these, the weight of the liver, the AST and

Table 1: Phenotype and serum biochemical profile at the end of the experiment

Parameter[a]	S diet	Athero diet	Can diet	p[b]
Weight (g)	31.3±1.5	32.9±2.2	31.7±1.5	0.370
L/B WR[c]	0.046±0.006	0.054±0.006	0.054±0.004	0.002
Total cholesterol (mg dL^{-1})	138±21	156±51	158±34	0.380
Triglycerides (mg dL^{-1})	224±76	182±49	175±53	0.170
ALT (U L^{-1})	144±21	190±58	226±222	0.280
AST (U L^{-1})	1001±166	1049±354	585±247	0.003
Glucose (mg dL^{-1})	130.4±11	119.3±11	124.5±20	0.270
Insulin (mU L^{-1})	46.5±31.7	79.3±53	82.5±53	0.130
Glu/Ins ratio[d]	4.1±1.9	2.1±1.7	1.8±1.2	0.020

Values of biochemical parameters are expressed as Means±SD, [b]ANOVA. [c]Liver/body weight ratio, [d]Glucose/insulin ratio

Table 2: Mean values of the glucose tolerance test

	Glucose (mg dL^{-1})				
	Time (min)				
	0	30	60	90	120
S diet	130.4	179.8	138.7	123.7	112.6
Athero diet	119.3	205.2*	139.9	119.3	104.2
Can diet	124.5	213.5*	139.5	118.6	126.3*

*Significant difference with respect to the standard diet (S diet)

Table 3: Effect of atherogenic and canine diet on aortic intima-media thickness and liver steatosis and inflammation

Parameter[a]	S diet	Athero diet	Can diet	p[a]
Thoracic aortic intima-media thickness (μm)	63.40±15.0	73.00±17.0	80.19±20	0.000
Abdominal aortic intima-media thickness (μm)	62.90±11.0	76.30±18.0	79.50±17	0.000
Liver steatosis	0.37±0.5	2.23±0.4	2.50±0.5	0.000
Microvesicular (%)	95.80±10.2	43.70±10.3	55.70±7.1	0.000
Macrovesicular (%)	4.10±10.2	56.20±10.3	44.20±7.1	0.000
Liver inflammation	0.37±0.6	1.53±0.5	1.50±0.5	0.000[a]

Values are expressed as Means±SD. Liver steatosis and inflammation scores are the result of assigning the numerical values of 0, 1, 2 and 3 to the grades of histologic alteration: Absent, mild, moderate, or severe, respectively (as has previously been reported). [a]ANOVA.

the fasting glucose/insulin ratio were different among the groups. The post hoc analysis done on these parameters revealed the following: the livers of the AtheroDiet and CanDiet groups were heavier than those of the Sdiet group. The AST values were significantly lower in the mice of the CanDiet group with respect to the other groups ($p = 0.007$). Compared with the Sdiet group, insulin values were high in the AtheroDiet and CanDiet groups but only the latter group reached a significant difference ($p = 0.04$). The fasting glucose/insulin ratio, whose low values correlated with a higher degree of insulin resistance, was significantly low in the AtheroDiet ($p = 0.03$) and CanDiet ($p = 0.02$) groups, compared with the mice that were fed the standard diet.

The values obtained on the glucose tolerance curve are shown in Table 2. Compared with the Sdiet group, there was a significant rise at minutes 30 and 120 in the CanDiet group and at minute 30 in the AtheroDiet group.

Histologic findings in liver: There were significant differences in the steatosis and inflammation grades among the groups (Table 3). The post-hoc analyses showed that the two groups with a high-fat diet (AtheroDiet and CanDiet) had a higher grade of steatosis and inflammation, compared with the SDiet group ($p = 0.000$) (Table 3). Regarding the steatosis pattern, when there was fatty change in some of the mice of the Sdiet group, 95% had a microvesicular pattern, whereas close to half of the abundant tissue with steatosis in the AtheroDiet and CanDiet groups had a macrovesicular pattern (Fig. 1). Hepatocellular ballooning which has been difficult to recreate in animal models of NAFLD/NASH, was a pronounced histologic feature in the AtheroDiet and CanDiet groups but it was absent in the SDiet group. With respect to the presence of hepatic fibrosis, only the AtheroDiet and CanDiet groups developed very mild and not statistically significant perisinusoidal fibrosis.

Histologic findings in aorta: To determine the degree of progression of atherosclerosis, cross-sectional slices of the aorta were used, in which the intima-media thickness and the grade of damage according to the stary classification were determined. The AtheroDiet and CanDiet groups

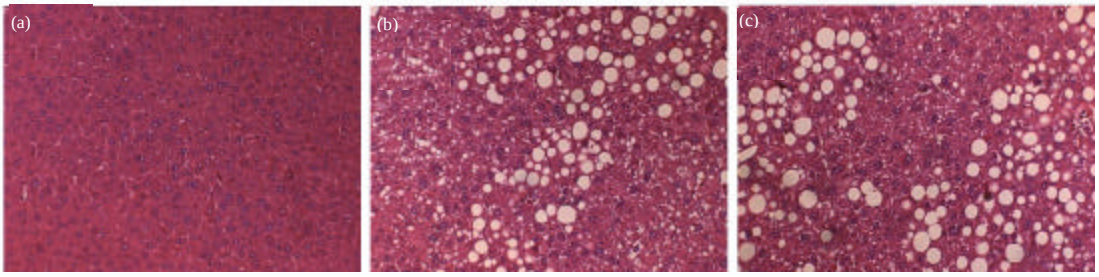

Fig. 1(a-c): Histologic images representative of livers of each group (H and E, X100), (a) Micrographs of the S diet group showing normal liver histology, (b) Can diet group showing severe steatohepatitis with a slight predominance of microvesicular steatosis and (c) Athero diet group showing severe steatohepatitis with a slight predominance of macrovesicular steatosis

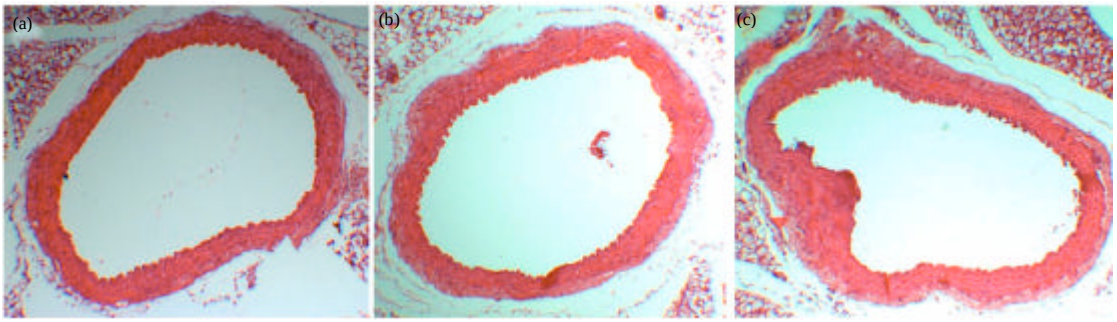

Fig. 2(a-c): Histologic images representative of aortas of each group (H and E, X50), (a) Micrographs of the S diet group showing a normal aorta, (b) Can diet group showing a stary type II lesion and (c) Athero diet group showing a stary type III lesion

showed a significant increase in the intima-media thickness of the thoracic and abdominal aorta, compared with the Sdiet group (Table 3) ($p = 0.000$). Moreover, in the thoracic aorta this thickening was significantly greater in the CanDiet group than in the AtheroDiet group ($p = 0.04$). No significant differences were found for the atherosclerotic lesions using the Stary classification, the majority of which were grade 2; there were only a few grade 1 and 3 lesions (Fig. 2).

Relation between steatohepatitis and preclinical atherosclerosis: The thickening of the aortic wall is a sign of preclinical atherosclerosis and has been associated with NAFLD. To determine the Correlation Coefficients (CC) two data series were created, one with the SDiet+AtheroDiet groups and the other with the SDiet+CanDiet groups (Table 4). As was expected, the grade of steatosis was importantly correlated with the grade of inflammation. There was also a significant relation between hepatic steatosis and the thickness of the aorta. In turn, the grade of hepatic inflammation was also correlated with the thickness of the thoracic aorta (SDiet+AtheroDiet: $CC = 0.37$, $p = 0.02$; SDiet+CanDiet: $CC = 0.43$, $p = 0.01$) and the abdominal aorta (SDiet+AtheroDiet: $CC = 0.34$, $p = 0.03$; SDiet+CanDiet: $CC = 0.45$, $p = 0.01$). The above mentioned CCs were always greater in the analyses that included the CanDiet group with the

Table 4: Correlation coefficients between the grade of hepatic steatosis and the other variables

Variables	S diet+Athero diet correlation (p)*	Sdiet+CanDiet correlation (p)*
Inflammation	0.87 (0.000)	0.86 (0.000)
ALT/AST ratio	0.56 (0.003)	0.36 (0.057)
Thickness: Thoracic aorta	0.45 (0.007)	0.60 (0.001)
Abdominal aorta	0.51 (0.002)	0.64 (0.000)

*Spearman correlation coefficient

exception of the ALT/AST index which was not significant in this data series (Table 3). Because there was a correlation between the hepatic steatosis and inflammation and the thickness of the aortic wall it can be said that the model reflected the existing relation between steatohepatitis and preclinical atherosclerosis.

DISCUSSION

It was demonstrated that mice that were fed a high-fat canine diet (a canine puppy diet) developed steatohepatitis and preclinical atherosclerosis. Both pathologies have been associated in different clinical trials and their simultaneous study should be fomented, given that cardiovascular diseases (secondary to atherosclerosis) are the main causes of death in patients with NAFLD/NASH (Brea and Puzo, 2013). Fat deposit and inflammation, among others, are common pathophysiologic factors in atherosclerosis and NAFLD. Therefore it would not be surprising that different treatments could be beneficial for both pathologies and the model presented herein enables their simultaneous evaluation.

Of the models analyzed (AtheroDiet and CanDiet), the canine diet showed the greatest correlation between steatohepatitis and atherosclerosis. An ideal animal model of NAFLD/NASH should reflect the hepatic histopathology and pathophysiology of human NAFLD/NASH. Accordingly, the liver of the animal model of NASH should show steatosis, intralobular inflammation and hepatocellular ballooning (Takahashi *et al*., 2012). All these aspects were covered in the CanDiet model. Ideally, the animal model should also create perisinusoidal fibrosis and susceptibility to liver tumors. The model proposed herein only developed very mild and insignificant perisinusoidal fibrosis and thus would not be adequate for evaluating this parameter. However it is likely that if the experiment were to last a longer period of time, this disorder could develop.

Furthermore, the ideal animal model should show metabolic abnormalities such as obesity, insulin resistance, glucose metabolism alterations, dyslipidemia and an altered adipokine profile. In the CanDiet model, significant elevations in the glucose tolerance curve and higher insulin levels were created and a significant reduction of the fasting glucose/insulin ratio (related to insulin resistance) was seen, all of which are changes suggestive of metabolic syndrome. Most likely longer experimentation duration or a larger sample in future experiments would produce even higher values of the abovementioned parameters.

The canine diet does not produce significant weight increase but neither does it cause loss, as occurs in certain methionine and choline deficient diet or atherogenic diet models (Rizki *et al*., 2006; Takahashi *et al*., 2012). There were also no significant changes in the lipid profile. The liver enzymes, ASLT and AST, were not elevated and the ALT/AST ratio did not act as a steatohepatitis indicator, as has been proposed in humans. Nevertheless it has also been reported that a large percentage of patients with NAFLD do not present with liver enzyme alterations (Clark *et al*., 2002). These are aspects to take into consideration when contemplating the use of the CanDiet model in future studies.

It is important to mention that the simultaneous study of NAFLD with preclinical atherosclerosis is an advantage that was not contemplated in previous animal models. In regard to animal models of atherosclerosis, some develop this pathology very quickly (in a few weeks) (Jawien *et al*., 2004), making them unadvisable for evaluating long-term or preventive treatments for this pathology. The proposed CanDiet model is adequate for evaluating aspects that intervene in a slow development of atherosclerosis and in the initial stages (preclinical atherosclerosis), whereas it is not apt for evaluating advanced atherosclerotic lesions. It should be pointed out that the intima-media thickness obtained from the mice fed the canine diet was superior even to that caused by the commercial atherogenic diet. However, a limitation of the present study with respect to atherosclerosis is that it does not cause significant differences if the lesion grades are evaluated only with the qualitative Stary scale, since it is a model for preclinical atherosclerosis; this disease is evaluated in humans through the thickness of the arteries, especially the carotid artery.

The atherogenic diet has been used in multiple models to develop atherosclerosis or steatohepatitis, whereas the canine diet has not been used to induce these pathologies. The canine puppy diet could reduce feed cost by more than 90%, compared with a high-fat or commercial atherogenic diet. Furthermore, the latter two require refrigeration, they stay fresh for a short period of time and their delivery can take months in countries where they are not habitually sold. In addition, using BALB/c mice instead of knockout or transgenic mice reduces the cost per experimental animal by more than 70%. Other mice, such as the C57BL/6 strain, have been used as steatohepatitis or atherosclerosis models but their availability is limited in laboratory animal facilities throughout the world when compared with the widely distributed BALB/c mice. The topics of cost and/or availability of specialized feed and mouse strains may seem insignificant to some laboratories but they can be the defining aspect as to whether a study is carried out or not in certain regions or countries of the world.

C57BL/6 mice have demonstrated a great capacity for producing atherosclerosis and steatohepatitis with fibrosis (DeLeve *et al*., 2008; Whitman, 2004) and thus their being fed a canine diet could create a study model with the most advanced stages of these pathologies. It is also worth mentioning that a canine puppy diet with a high fat proportion (18%) was used in the present study. It is likely that the commercial brand of this canine diet is not available on a worldwide basis but it is feasible to replicate this animal model with similar canine diets that contain at less 18% fat. However, these aspects need to be confirmed in future studies.

In conclusion it was possible to create an accessible and low-cost murine model for the simultaneous study of non-alcoholic steatohepatitis and atherosclerosis. Both pathologies were produced in non-advanced stages (preclinical atherosclerosis and steatohepatitis without significant fibrosis). Future experiments must evaluate its usefulness in accordance with the aims of each study.

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