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ORIGINAL ARTICLE

Fast Morphological Gallbladder Changes Triggered by a Hypercholesterolemic Diet

Alberto López-Reyes,* Denise Clavijo-Cornejo,* Javier Fernández-Torres,* Daniel Medina-Luna,* Erendida G. Estrada-Villaseñor,† Luis E. Gómez-Quiroz,‡ Marwin Gutiérrez,* Julio Granados,§ Gilberto Vargas-Alarcón,‖ Carlos Pineda,* Hiram García,* Luis A Morales-Garza,¶ María C. Gutiérrez-Ruiz,‡ Karina Martínez-Flores*

* Rheumatic and Musculoskeletal Diseases Division (Synovial Fluid Laboratory). National Institute of Rehabilitation "Luis Guillermo Ibarra Ibarra", Ministry of Health. Mexico City, Mexico.
† Pathology Service. National Institute of Rehabilitation "Luis Guillermo Ibarra Ibarra", Ministry of Health. Mexico City, Mexico.
‡ Department of Health Sciences. Autonomous Metropolitan University, Mexico City, Mexico.
§ Transplantation Department. National Institute of Medical Sciences and Nutrition Salvador Zubirán, Ministry of Health. Mexico City, Mexico.
‖ Molecular Biology Department. National Institute of Cardiology "Ignacio Chávez", Ministry of Health. Mexico City, Mexico.
¶ School of Medicine. Tecnológico de Monterrey, Monterrey, Mexico.

ABSTRACT

Introduction and aim. Obesity is a worldwide epidemic problem, described as a risk factor for hepatic diseases, such as non-alcoholic fatty liver disease and other pathologies related to development of cholesterol crystals and cholesterol gallbladder stones. It has been reported that cholesterol overload may cause hepatic damage; however, little is known about the effects of an acute hypercholesterolemic diet on the gallbladder. The aim of this manuscript was to evaluate the impact of a cholesterol-rich diet on the gallbladder. **Material and methods.** The study included ten eight-week-old C57BL6 male mice, which were divided into two study groups and fed different diets for 48 h: a hypercholesterolemic diet and a balanced Chow diet. After 48 h, the mice were analyzed by US with a Siemens Acuson Antares equipment. Mice were subsequently sacrificed to carry out a cholesterol analysis with a Refloton System (Roche), a crystal analysis with a Carl Zeiss microscope with polarized light, and a histological analysis with Hematoxylin-eosin staining. **Results.** The hypercholesterolemic diet induced an increase in gallbladder size and total cholesterol content in the bile, along with important histological changes. **Conclusion.** Cholesterol overloads not only trigger hepatic damage, but also affect the gallbladder significantly.

Key words. Histopathology. Cholesterol. Ultrasound. Lithiasis. Bile.

INTRODUCTION

Nowadays, overweight and obesity represent a major public health problem worldwide. These conditions are associated with metabolic syndrome, dyslipidemia, hyperglycemia and diabetes, which are considered pandemic disorders in developing countries.[1] Obesity may lead to fatty infiltration of multiple internal organs, including the liver, heart, kidneys, and pancreas, causing organ dysfunctions. Fatty infiltration leads to chronic inflammation and tissue damage. Cholesterol infiltration in the liver may result in non-alcoholic fatty liver disease (NALFD), which is defined as evidence of hepatic steatosis and considered the hepatic manifestation of metabolic syndrome.[2-5] NALFD affects up to a third of the population worldwide.[6] In the Middle East, the prevalence of this condition is 31.8%, whereas in North America, the prevalence is 23.6%.[7] A consequence of this liver condition is the formation of cholesterol crystals within the biliary tract.[8-10] Although it is assumed that fatty liver and fatty gallbladder result from lipid accumulation, the toxic potential of specific lipids, such as cholesterol, has been studied.[11] There are three main types of gallstones, and about 80% of them are composed of cholesterol monohydrate crystals, potentially contributing to human gallstone disease, which is a frequent health problem worldwide and accounts for approximately 95% of the biliary tract disorders.[12] Hepatobiliary ultrasound (HBUS) studies suggest the prevalence

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rate mean to be around 10%-15% in the United States, and the occurrence rates range from 5% for non-Hispanic black men to 27% for Mexican-American women.[13] In patients with cholelithiasis, the pigment stones are predominantly composed of bilirubin, calcium, salts or mixed stones. Cholesterol stones are usually yellow-green colored, whereas pigment stones are small dark stones made of bilirubin.[12,14] The presence of a gallstone can be confirmed through an ultrasonography in the fasting state, with a correct diagnosis in > 90% of the cases.[15] Modern ultrasound (US) technologies are capable of detecting stones as small as 2 mm in diameter routinely. Smaller stones may be missed or confused with biliary sludge (layering echogenic material that does not cast an acoustic shadow).[15]

In an animal model study where mice are fed a lithogenic diet (composed of 15% fat, 1% cholesterol, 0.5% cholic acid), relevant factors can be explored, such as mucin accumulation. Prior to the formation of solid crystals, liquid crystals and cholesterol crystals grow within the mucin gel accumulated in the gallbladder. Furthermore, in a fasting gallbladder, hepatic bile is concentrated 4- to 5-fold due to water absorption, enhancing cholesterol crystallization; on the seventh day on a lithogenic diet, gallbladder bile becomes supersaturated with cholesterol.[16,17] In this study, we used ultrasonographic, macroscopic, and histopathologic methods to show the changes that occur in the gallbladder during the first two days on a high cholesterol diet.

MATERIAL AND METHODS

Study subjects

A total of ten eight-week old C57BL6 male mice were acclimatized to the animal facility environment during 1 week before the study. The mice were put on a standard diet, and their circadian rhythm was synchronized with a 12-h:12-h light-dark cycle. The local Institutional Review Board approved the study protocol, and experiments were performed in accordance with current ethics guidelines and the provisions of the Institutional Animal Care and Use Committee. [18]

Experimental design

The mice were assessed, divided into two groups and fed different diets for 48 h. The first group was fed a hypercholesterolemic (HC) diet, composed of standard chow supplemented with 2% cholesterol and 0.5% sodium cholate;[19-21] the second group (controls) only received a standard balanced chow diet (Chow).[16] After 48 h, HBUS assessment was performed and the mice were subsequently sacrificed in order to perform the histological and routine laboratory analyses.

Ultrasound assessment

An experienced sonographer conducted all HBUS examinations, which were blind to the type of diet and randomized.

Specimens were sedated with avertin and shaved with a shaving machine. After cleaning the area with a dry paper towel, ultrasound gel was applied. The best images were selected and recorded. The animals were examined in a dorsal position and in left and right lateral decubitus positions. Images of the liver, gallbladder, and bile ducts were taken in longitudinal, transversal, and oblique planes.

All US examinations were performed using a Siemens Acuson Antares device, equipped with a 5-13-MHz hockey stick linear transducer. The international guidelines for gallbladder were adopted for both the scanning technique and the interpretation.

Bile cholesterol assay

Gallbladder bile was aspirated with a sterile syringe and the total cholesterol (TC) content was determined using an automated method (Reflotron system, Roche, Inc).

Crystal assay with polarized light microscopy

Upon exposing the peritoneal cavity, the bile was aspirated and further analyzed with polarized light microscopy (Carl Zeiss, Inc) in order to identify cholesterol crystals.

Histology

Gallbladders were sectioned and fixed with 10% neutral formaldehyde (FA), and the tissues were embedded in paraffin for histologic studies. 7 μm cuts of paraffin-embedded tissues were obtained and routine hematoxylin and eosin staining was conducted. The slides were studied and photographed using a digital photographic microscope (Carl Zeiss, Inc).

Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical differences between experimental groups were assessed using the Student T test adjusted by unequal variances when appropriate. All P-values below 0.05 were considered statistically significant. Prism v6.01 (Graph Pad Software Inc, California, USA).

RESULTS

A non-invasive US imaging technique was used to examine the gallbladder in living animals. The images revealed an increased organ area (mean: 8.8 mm^2) in the HC diet mice, compared to the normal gallbladder in the Chow mice (mean: 0.4 mm^2); additionally, hyperechoic spots were identified within the gallbladder (Figure 1A) of the HC diet mice. Then, we removed the gallbladder to confirm the increase in volume and we even found some changes in the bile color (Figure 1 B).

We were interested in analyzing the bile at the microscopic level, and interestingly, we found an increase of birefringent crystals characterized by their square shape and notches on the borders under polarized light in the HC mice, suggesting the presence of cholesterol crystals (Figure 1C).

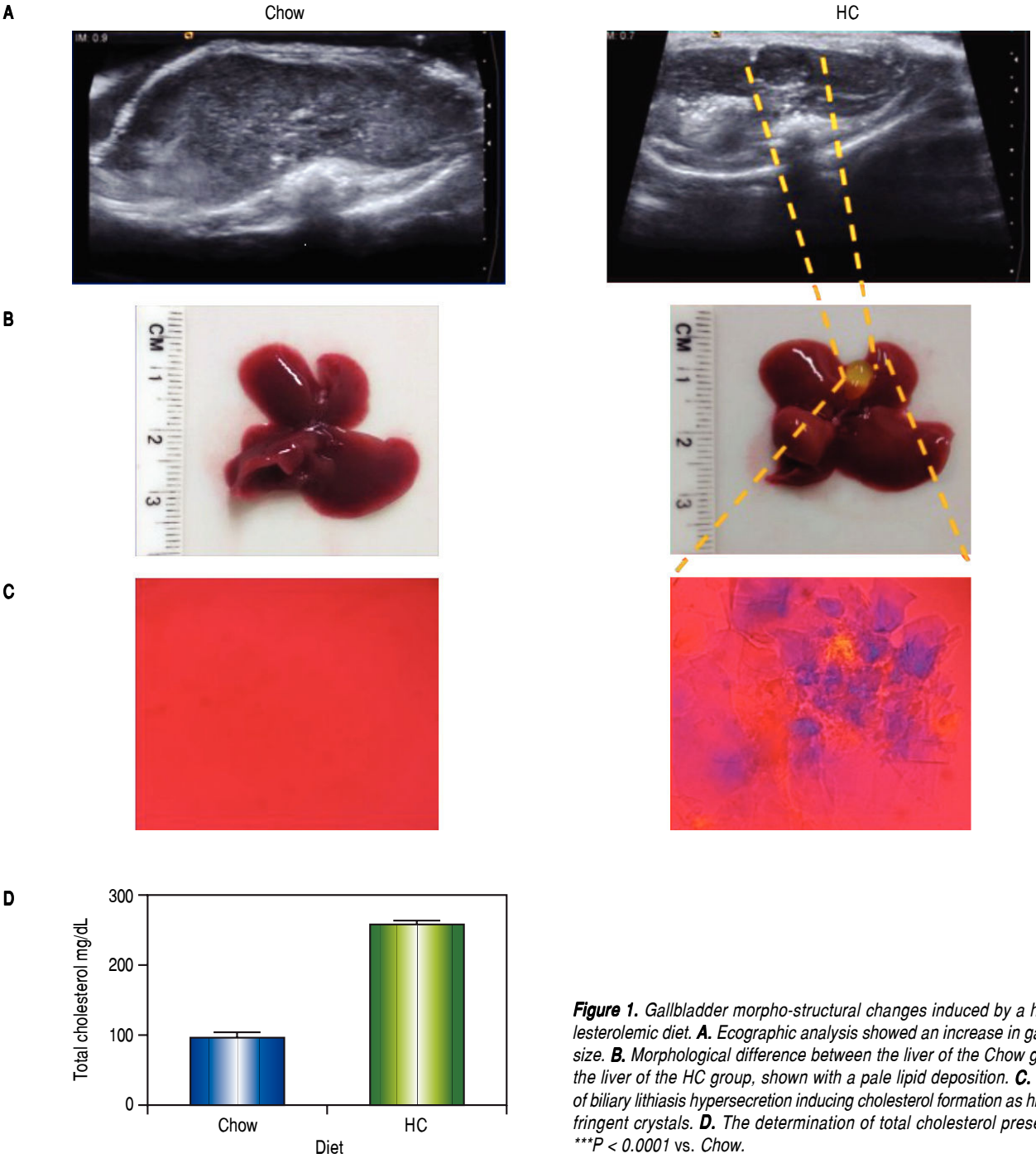

Figure 1. *Gallbladder morpho-structural changes induced by a hypercholesterolemic diet.* ***A.*** *Ecographic analysis showed an increase in gallbladder size.* ***B.*** *Morphological difference between the liver of the Chow group and the liver of the HC group, shown with a pale lipid deposition.* ***C.*** *Evidence of biliary lithiasis hypersecretion inducing cholesterol formation as highly birefringent crystals.* ***D.*** *The determination of total cholesterol presence was* ****P < 0.0001* vs. *Chow.*

We measured total cholesterol through a colorimetric reaction assay. Data show that total cholesterol content in the HC mice gallbladder increased 1.5 fold, compared to the Chow group (Figure 1D). Finally, at the histological level in the HC mice we, observed a pseudo-stratified (green arrow) epithelium, with subepithelial isolated lipid vacuoles (black arrow), increased secretion coming from the epithelial cells to the gallbladder lumen (blue arrow), and a moderate infiltrate mainly mediated by neutrophils cells (red arrow) (Figure 2A). On the other hand, the epithelium of the Chow mice showed a simple columnar structure; increased secretion, lipid vacuoles and neutrophils were not observed (Figure 2B-D).

When analyzed the features of the Chow group tissue, and took them as normal characteristics. The epithelial was 7.76 μm thick and the wall was 108.4 μm thick. The epithelium showed a simple columnar structure and the nucleus size of the epithelial cells was in average 5.11 μm. In comparison with the Chow gallbladder tissue, the HC group showed an increased epithelial thickness, reaching on average 15.3 µm (p=0.0004). The wall thickness increased significantly from 108.4 to 296.6 µm (p = 0.0204), and we saw an increase in nucleus size (p = 0.0196).

DISCUSSION

Lipid-rich diets can constitute one of the main risk factors for developing non-alcoholic fatty liver disease (NAFLD). López-Reyes, *et al*. and Domínguez-Pérez M, *et al.* have demonstrated that a cholesterol-rich diet can induce

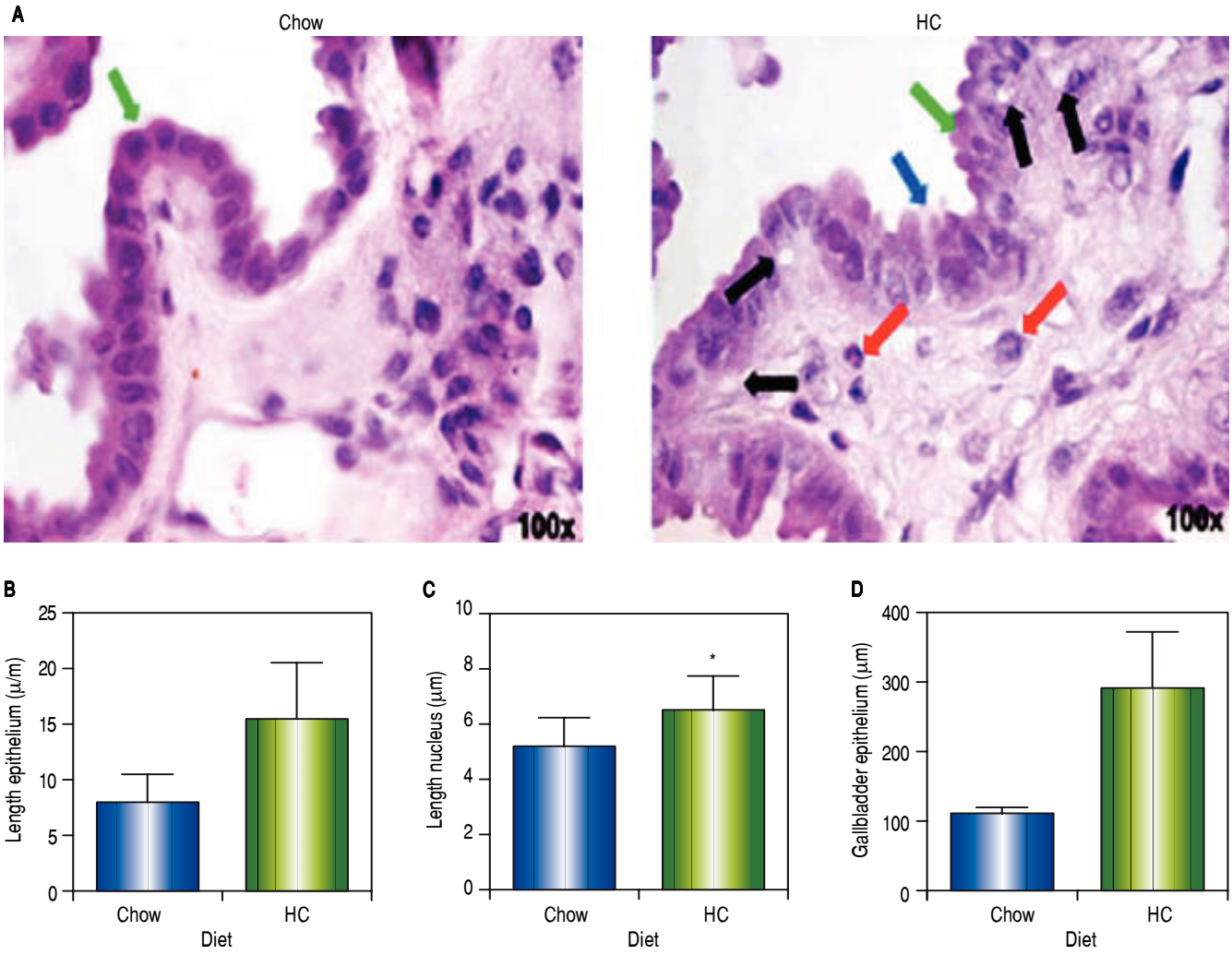

Figure 2. *Histological analysis of the gallbladder of HC mice.* ***A.*** *The gallbladders of the animals that were fed a balanced Chow diet or a hypercholesterolemic (HC) diet were stained with hematoxilin and eosin. Green arrows indicate the change from a simple columnar epithelium (Chow diet) to a pseudo-stratified structure (HC diet), blue arrows show the presence of secretion, black arrows indicate the presence of lipid droplets, and red arrows indicate inflammatory infiltrate.* ***B.*** *The graph shows the significant difference in epithelium length between the Chow diet and the HC diet (P < 0.001).* ***C.*** *Comparison of the epithelium nucleus of the gallbladder between Chow diet and HC diet (P < 0.05).* ***D.*** *Difference in gallbladder wall thickness depending on the diet (P < 0.05). Original zoom 100x.*

liver damage. In the gallbladder, a diet with high-lipid content is a major risk factor for the formation of cholesterol stones, and according to several studies, high-cholesterol secretion from the liver is the main cause of bile saturation in obesity.[22-24] Furthermore, this effect could be associated with cholesterol-metabolic disorders in the hepatic environment, also related to a decrease in the metabolism of cholesterol into bile acids.[25] Thus, the objective of this work was to use a hypercholesterolemic model to determine how being on a diet high in cholesterol for two days could lead to a microlithiasis state. Our ultrasound results show an increase in the gallbladder area, and according to Hussaini et al., an increased lipid concentration triggers cholesterol formation, leading to an increase in the vesicular area, as well as an echogenic appearance due to the presence of cholesterol crystals.[26] According to Treviño-García, *et al.*, administering a parenteral diet to intensive care patients showed an echogenic appearance after the first 3 days due to the formation of cholesterol crystals in the bile at the gallbladder level.[27] According to Handbige, US is the first approach for acute pathological assessment of the gallbladder that allows to measure the diameter and wall thickness with a sensitivity of 83% and a specificity of 95%.[28] The diagnosis of gallstones relies on detecting echogenic objects within the gallbladder lumen that produce an acoustic shadow, as it was observed in the HC group due to the presence of cholesterol micro-crystals. In support to this, it is worth pointing out that echogenic material in the gallbladder has been reported to be caused by the presence of cholesterol crystals, calcium bilirubinate pigment and other calcium salts in the mucus.[12,29] Additionally, it has been reported that the presence of these crystals suggests that the bile is saturated due to an excess in cholesterol content.[30,31] At a microscopic level, the evidence of cholesterol crystals is defined as microlithiasis, and it is considered to contribute to the formation of additional stones in the gallbladder.[12] Our findings, discovered using polarized microscopy, show cholesterol crystals featuring a square structure with indentations on the edges and birefringence. However, this is not the only structure possible for cholesterol crystals, as they can take a flat, stringy, helical, arch-like, or tubular structure.[12,32] These structures have been mainly observed in patients diagnosed with calculous gallbladder disease.[33] On the other hand, not only did we demonstrate early formation of cholesterol micro-crystals, but we also found a significant increase in total cholesterol levels in the bile. This finding confirms that cholesterol crystals presence is due to an increase of this lipid. Van Erpecum et al. found an increase in the cholesterol saturation index on the 14th day on a lithogenic diet, which triggers crystal formation.[34] Chang and Shuch used a murine model where mice were fed a diet supplemented with 1% of cholesterol and 0.5% of cholic acid during 2 days and following periods of 1, 2, 4, 8, 12, 24, and 40 weeks, to discover there was a prelithiasic phase characterized by a distention of 7.2 mm in average and targeted hyperplasia.[35] However, they did not show epithelial changes until the second week, when they found an important pseudo-stratification and mucosae with higher elongation and proliferation. This contrasts with our results, which show a change in epithelium structure as soon as the second day on a hypercholesterolemic diet, as well as formation of mucus, suggesting abnormal gallbladder function, since it is known that this secretion triggers formation of cholesterol crystals or stones. Additionally,

Van Erpecum, *et al*. reported that, in a model in which C57LBL6 strain mice were fed a lithogenic diet with 1% cholesterol during 14, 28, and 56 days, the mice featured a clear thickening on the edges and a moderate neutrophil inflammatory infiltrate. A diet containing 1 g of cholesterol, 0.5 g of cholic acid, 2 g of corn oil, 50 g of sucrose, and 20 g of casein, during 14, 28, and 56 days, showed a clear increase in thickness on the gallbladder edge and a moderate inflammatory infiltrate of neutrophil-granulocytes at the stroma and some inter-epithelial granulocytes.[35] Our findings revealed a change in the cubic pseudo-stratified epithelium, which could be caused by the HC diet composed of a higher cholesterol (2%) concentration, reinforcing the fact that even on the second day, a diet higher in cholesterol could generate faster gallbladder changes, demonstrating that a high-lipid diet not only affects the hepatic environment, as Gutiérrez-Ruiz, *et al*. have demonstrated in recent years.[5]

CONCLUSION

Our steatosis model caused by cholesterol overload showed the effect of a high-lipid diet on the gallbladder. Furthermore, the case underlines the relevance of integrated imaging to depict all these fast changes, as well as the histological changes. Finally, this model shows that high dosages of cholesterol could induce damage not only to the liver, but to the gallbladder in a short period of time, which could lead to a fast progression of cholesterol stone formation, further affecting the gallbladder and the liver.

AUTHOR CONTRIBUTIONS

Alberto López-Reyes: Designed the study and the experimental component, analyzed and interpreted the results to create the manuscript, and gave his approval to the final revision of the manuscript. Denise Clavijo-Cornejo: Provided support with bile samples and their classification into groups for cholesterol crystal identification, interpreted the results, and took part in the manuscript

creation. Javier Fernández-Torres: Contributed to cholesterol determination and development of a database with the results obtained, and took part in the manuscript creation. Daniel Medina-Luna: Carried out histological sections and staining for subsequent analysis, and took part in the discussion of the results. Erendida G. Estrada-Villaseñor: Contributed to analysis of histological sections to identify changes in the epithelium and gallbladder and development of a database, and took part in the manuscript creation.

Luis E. Gómez-Quiroz: Provided support with biological interpretation of the cholesterol results and statistical analysis for the manuscript discussion. Marwin Gutierrez: Conducted ultrasound studies and interpreted the results, and took part in the manuscript creation. Julio Granados: Contributed to microscopic analysis of cholesterol crystals, analysis of histological sections, and review of the manuscript. Gilberto Vargas-Alarcón: Provided support with development of a database of all the results, statistical analysis, and review of the manuscript. Carlos Pineda: Identified cholesterol crystals, interpreted ultrasound results, and took part in the manuscript creation. Hiram García: Administered the diet, dissected the mice, analyzed the results, and took part in the discussion of the manuscript. Luis A. Morales-Garza: Looked up bibliography related to the ultrasound findings and analysis of the ultrasound results, and took part in the discussion of the manuscript. María C. Gutiérrez-Ruíz: Provided support with interpretation of the cholesterol and histological results, and bibliography search for the manuscript discussion, and took part in the manuscript draft. Karina Martínez-Flores: Contributed to study design, experiment oversight, statistical analysis for interpretation of the results, manuscript preparation and approval of the final revision of the manuscript.

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This manuscript was developed using an animal model, and the experiments were carried out in accordance with the current ethical guidelines and provisions of the Institutional Laboratory Animal Care and Use Committee prepared by the National Academy of Sciences and published by the National Institutes of Health.

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Correspondence and reprint request:
Karina Martínez-Flores, B.Sc, M.Sc, Ph.D.
Rheumatic and Musculoskeletal Diseases Division
(Synovial Fluid Laboratory) Instituto Nacional de Rehabilitación "Luis Guillermo Ibarra".
Calz. Mexico-Xochimilco Núm. 289, C.P. 14389, Mexico City, Mexico. Tel.: +52 55 59991000, Ext.: 19501.
E-mail: karinabiologist@hotmail.com