Modulating effects of the probiotic *Lactococcus lactis* on the hepatic fibrotic process induced by CCl4 in Wistar rats

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Abstract. Hepatic cirrhosis is a chronic disease that affects one fifth of the World’s population and is the third leading cause of death in Mexico. Attempts have been made to develop treatments for this hepatic cirrhosis, which include manipulating the intestinal microbiota and thus decreasing the early inflammatory response. The microbiota is reportedly altered in patients with cirrhosis. Due to its immunomodulatory properties and its ability to survive in the gastrointestinal tract, *Lactococcus lactis* (L. lactis) has been used as a therapeutic measure in inflammatory disorders of the colon. The objective of the present study was to evaluate the efficacy of the *L. lactis* probiotic NZ9000 in preventing tetrachloromethane (CCl4)-induced experimental hepatic fibrosis. The following 4 groups were included in the experimental stage (n=5): i) Control group; ii) *L. lactis* group; iii) CCl4 group; and iv) *L. lactis*-CCl4 group. For the first 2 weeks, *L. lactis* was orally administered to the *L. lactis* and *L. lactis*-CCl4 groups; CCl4 was then peritoneally administered to the *lactis*-CCl4 group for a further 4 weeks (in addition to the probiotic), while the *L. lactis* group received the probiotic only. For the CCl4 group, CCl4 was administered for 4 weeks. The experimental groups were all compared with the control group and the *L. lactis* + CCl4 group. Tissue samples were analyzed histologically and biochemically, and the gene expression levels of interleukin (IL)-1, IL-10 and forkhead box protein P3 (FoxP3) were determined. *L. lactis* decreased hepatic cirrhosis by preventing steatosis and fibrosis, and by reducing the levels of AST and ALT. Subchronic CCl4 injury induced upregulation of the IL-1β gene in the liver, which was decreased by *L. lactis*. It was also found that the group treated with *L. lactis* showed increased expression of Foxp3 in the liver and IL-10 in the gut. These results suggested that oral administration of *L. lactis* may be a potential probiotic to prevent or protect against CCl4-induced liver injury.

Introduction

Fibrosis is defined as an excessive component deposition of the extracellular matrix (ECM), collagen and peptidoglycans in organs and tissues as a result of the proliferation and activation of fibroblasts, stellate cells and myofibroblasts (1-3). Inflammatory reactions of both the innate and adaptive immune system contribute to the development of fibrosis; in the early stages of fibrosis, neutrophils, macrophages, natural killer (NK) cells and T lymphocytes promote pro-fibrotic processes, including hepatic stellate cell activation, increased

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Abbreviations: ECM, extracellular matrix; TGF-β, transforming growth factor β; MMP, matrix metalloproteinase; SMAD, small worm phenotype and mothers against decapentaplegic homologies; NF-κB, nuclear factor κ light-chain enhancer of activated B cells; FoxP3, forkhead box P3; IL-1β, interleukin 1β; IL-10, interleukin 10; AST, aspartate aminotransferase; ALT, alanine aminotransferase; NK, natural killer; NKT, natural killer T cell; TNF-α, tumor necrosis factor α; IFN-γ, interferon γ; GALT, Gut-associated lymphoid tissue

Key words: *Lactococcus lactis*, interleukin 10, interleukin 1β, fibrosis, microbiota, probiotic

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transforming growth factor (TGF)-β, platelet-derived growth factor, fibroblast growth factor, matrix metalloproteinase (MMP) 9 and metalloproteinase inhibitor-1/-2 expression, and a decrease in MMP13 expression (1-4). Activated macrophages produce tumor necrosis factor (TNF)-α and interleukin (IL)-1, which in turn activate hepatic stellate cells and fibroblasts to induce ECM overproduction. The signal transduction triggered by TNF-α leads to the expression of fibrogenic cytokines, primarily via the NF-κB and SMAD pathways (1,3). By contrast, interferon (IFN)-γ produced by activated NK cells (and a subsequent increase in IL-10) exerts antifibrotic effects (3). During cirrhosis, collagen types I and III are deposited in the hepatic stroma, creating fine or wide fibrous septa. Subsequently, new vascular channels are formed that facilitate communication between the portal region (hepatic arteries and portal veins) and the centrilobular veins, establishing an alternative circuit through which blood can bypass the sinusoids due to the increase of collagen fibers in the Dissé space (4,5).

Continuous collagen deposition in the Dissé space of the parenchyma is associated with the loss of sinusoidal endothelial cell fenestrae, in this process, the sinusoidal space takes on a capillary-like structure rather than a channel for the exchange of solutes between hepatocytes and the plasma (4). Collectively, this alters the secretion of hepatocellular proteins such as albumin, coagulation factors and lipoproteins (1,5). A number of therapeutic strategies have been developed to prevent this process and to subsequently decrease or reverse fibrosis/cirrhosis-associated liver damage (6); for example, the use of antioxidants (7), adrenoblockers (8), anti-inflammatory cytokines (9,10) and probiotics (11) has been suggested, but a complete cure for the disease has yet to be identified.

The pharmacological basis of a number of fibrosis treatments is the interaction between the intestinal microbiome and the host, which helps to maintain homeostasis (12-14). Haller et al (15) demonstrated that Lactobacillus (L.) johnsonii of an intestinal origin did not induce TNF-α or IL-1β release, but promoted that of TGF-β, presenting a global anti-inflammatory profile in a colitis model. In addition to in vitro experiments, experimental animal models of colitis have demonstrated the usefulness of probiotics in the control of intestinal inflammation. In an acetic acid-induced rat colitis model, administration of L. reuteri R2LC immediately after induction prevented the development of colitis (16). Similarly, L. plantarum administration to rats decreased the severity of colitis in an intraperitoneal methotrexate-induced enterocolitis model (17,18).

L. lactis is a gram-positive, spherical, homolactic, non-sporulant and facultative anaerobic bacterium, with hundreds of strains and biovariants published to date (19). L. lactis is categorized into three subspecies: i) L. lactis ssp. Lactis; ii) L. lactis ssp. Cremoris; and iii) L. lactis ssp. Hordniae (19-21). Bajaj et al (11) demonstrated that L. rhamnosus GG induced a decrease in endotoxemia and systemic inflammation in patients with cirrhosis. Similar results were observed following the administration of lactulose, rifaximin and probiotics containing Lactobacillus, which partially reversed cirrhosis-associated enteric dysbiosis, together with improving the severity of encephalopathy (18). Due to its immunomodulatory properties (22-24) and ability to transit through the gastrointestinal tract, L. lactis does not colonize the intestine in the manner of other similar organisms, such as Lactobacillus spp. (24).

The primary beneficial effect reported for wild or recombinant strains of L. lactis is its anti-inflammatory potential, indicating its potential use as a therapeutic tool for chronic intestinal diseases. Cellular in vitro models, as well as mouse models of colitis, have been used to investigate the anti-inflammatory properties of L. lactis, where an increase in anti-inflammatory cytokines and a decrease in NF-κB have been reported (25-28). In the present study, the protective effects of oral administration of L. lactis were evaluated after tetrachloromethane (CCL4)-induced fibrosis in Wistar rats.

Materials and methods

Bacterial strains and culture conditions. Pure stocks of L. lactis (10 µl) in 1 ml M17 medium (10% glucose and 30% glycerol) were donated by Dr María de Jesús Loera Arias of the Autonomous University of Nuevo León (Monterrey, Mexico). To reactivate the L. lactis strain, the cells were incubated overnight in 50 ml M17 (Difco) medium (supplemented with 10% glucose) at 30°C without shaking. Subsequently, 1 ml culture was used to inoculate 50 ml M17 medium (10% glucose). The optical density (OD) was measured at 600 nm, and the cells were incubated again until they reached an OD of 0.8. The final bacterial concentration was 1x10⁸ cells/ml.

Animals. Male Wistar rats (age, 6-8 weeks; weight, 150-250 g) were obtained from the Laboratory Animal Service of the Autonomous University of Aguascalientes (Aguascalientes, Mexico). The animals were maintained on a light/dark cycle (12:12) with ad libitum access to Purina® Rodent Chow (Cargill, Inc.) and tap water. All animal experiments were approved by the Research Ethics Committee of the Autonomous University of Aguascalientes (approval no. A1-S-21375) and were conducted in accordance with institutional guidelines for caring for experimental animals and the national regulatory norm (NOM-062-Z00-1999). To prepare the intestinal environment, all animals were previously treated with neomycin sulfate and sulfadimethylpyrimidine for 7 days. For experimentation, the rats were divided into the following 4 groups (n=5 rats/group): i) Control group not treated with L. lactis or CCl4; ii) L. lactis group administered L. lactis; iii) L. lactis-CCl4 group orally treated with L. lactis and induced with CCl4; and iv) CCl4 group intraperitoneally administered CCl4 for cirrhosis induction. All animals were sacrificed by overdose of sodium pentobarbital at 6 weeks, and different sections of the liver, the terminal region of the ileum (Peyer's Patches), the ascending portion of the large intestine and serum samples were obtained. The tissue sections were preserved in 10% formalin in PBS at room temperature and RNA later (Invitrogen; Thermo Fisher Scientific, Inc.) at -80°C for histological and molecular analysis, respectively, and the serum was used to conduct liver function tests.

Fibrotic induction. Fibrosis was induced in the L. lactis-CCL4 and CCl4 groups. Based on a pilot experiment in our laboratory, CCl4 was diluted in petrolatum and intraperitoneally administered 3 times per week for 4 weeks as follows
(CCl₄/petrolatum by volume): Weeks 1, 1:6; 2, 1:5; 3, 1:4; 4, 1:3. These proportions were prepared according to the number of experimental animals (n=5 for each group) and the number of applications per week (3 per week).

**Administration of L. lactis.** For 6 weeks, 1 ml L. lactis (1x10⁹ cells), was orally administered to the L. lactis and L. lactis-CCl₄ groups on a daily basis. In the L. lactis-CCl₄ group, the probiotic was administered two weeks prior to CCl₄, and was subsequently continued for an additional 4 weeks together with CCl₄. For the L. lactis group, the probiotic was administered alone for 6 weeks as a control (Fig. 1).

**Histological analysis.** Liver damage and Peyer's patches were evaluated histologically by light microscopy. Sirius red staining (with polarized light microscopy) was used to identify collagen fiber deposits (type I, red; type III, green). For histopathological analysis of Peyer's patches. The tissue sections were preserved in 10% formalin in PBS at room temperature for 24 h, and transverse cuts of 5-µm thickness were made in the terminal portion of the ileum to reveal clusters of lymphatic tissue (lymph nodes) that cover the lamina propria, which were then stained with hematoxylin and eosin. The histological preparations were visualized under a Axioskop 40/40 FL light polarized microscope (Carl Zeiss AG) and analyzed using Image-Pro Plus Software 4.5.1 (Media Cybernetics, Inc.). The percentage of fibrosis was determined as the ratio of the fibrotic area to the total tissue area.

**Markers of liver damage.** To determine the degree of liver damage, serum levels of glucose (cat. no. BSIS19-P), albumin (cat. no. BSIS02-E), bilirubin (cat. no. BSIS92-I) total protein (cat. no. BSIS30-E), urea (cat. no. BSIS35-I), alanine aminotransferase (ALT; cat. no. BEIS11-E) and aspartate transaminase (AST; cat. no. BEIS09-E) were quantified. Kits for all biochemical tests were obtained from Spinreact SAU. Each test was performed according to the manufacturer's instructions. The samples were read on a BTS-350 semi-automated spectrophotometric analyzer (BioSystems S.A.).

**Total RNA isolation and reverse transcription-quantitative PCR (RT-qPCR).** Total RNA was isolated from 100 mg liver tissues using the SV Total RNA Isolation System (Promega Corporation) according to the manufacturer's protocol. The RNA was quantified using NanoDrop-2000 (NanoDrop Technologies; Thermo Fisher Scientific, Inc.) and stored at -80°C until required. Reverse transcription was performed with 1 µg total RNA using the GoScript™ Reverse Transcription System (Promega Corporation) according to the manufacturer's instructions. Subsequently, qPCR was performed using the qPCR GreenMaster with UNG-clear (Jena Bioscience GmbH) using StepOne™ equipment (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the following thermocycling conditions: 50°C for 2 min and 95°C for 45 sec, followed by 40 cycles of 95°C for 45 sec and 60°C for 45 sec. The oligonucleotide primers are displayed in Table I. Relative expression levels were normalized to those of β-actin, and the differences were determined using the 2^ΔΔCq method (29).

**Statistical analysis.** GraphPad Prism 5.00 (GraphPad Software, Inc.) was used for statistical analysis and figures. Data are presented as the mean ± standard error of the mean for each group. Significant differences between mean values were assessed using one-way ANOVA followed by Tukey’s test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Macroscopic and histopathological analysis of the livers of control and treated animals.** At a macroscopic level, the livers of the control and L. lactis groups exhibited a smooth surface and the classic dark brown color of a healthy liver (Fig. 2A and D). By contrast, the liver tissues of the CCl₄ group were rough and irregular, with a lighter brown color (Fig. 2G). The livers of the rats on the L. lactis-CCl₄ presented with a similar coloration and texture to those of the control group (Fig. 2J). At the microscopic level (magnification, x10 and x40), the control and L. lactis groups displayed classic liver lobules, with hepatocytes and normal hepatic sinusoids (yellow arrows) that did not affect the liver histology (Figs. 2C and F). In the CCl₄ groups, steatosis (black arrows) and pyknotic nuclei (black asterisk) were observed in zone I of the liver acini (Fig. 2I). At the microscopic level (magnification, x10 and x40), the control and L. lactis groups displayed classic liver lobules, with hepatocytes and normal hepatic sinusoids (yellow arrows) that did not affect the liver histology (Figs. 2C and F). In the CCl₄ groups, steatosis (black arrows) and pyknotic nuclei (black asterisk) were observed in zone I of the liver acini (Fig. 2I). In the L. lactis-CCl₄ group (magnification, x10), a small number of hepatocytes in zone II presented with CCl₄-induced damage (to a lesser degree compared with that in the CCl₄ group) and a smaller area of steatosis, described as a microvesicular type (black arrowheads) compared with the CCl₄ group. Additionally, at x40 magnification, acidophilic cells were observed with a larger cytoplasm; it was therefore speculated that these cells exhibited a degree of incipient damage in the CCl₄ and L. lactis-CCl₄ groups (yellow asterisk; Fig. 2I and L).

The liver sections stained with Sirius Red and analyzed under a polarized light microscope exhibited normal...
histological architecture in the control and *L. lactis* groups, and type III collagen was observed (green arrow; Fig. 3A-a and b). In the CCl₄ group, an increase in type I collagen (red) was evident around blood vessels (red arrows; Fig. 3A-c) along with a low level of type III collagen (green arrow), indicating a fibrotic lesion. By contrast, a significant decrease in type I collagen fibers was observed in the *L. lactis*-CCl₄ group (red arrow; Fig. 3A-d). To confirm the degree of fibrosis, a morphometric analysis of the hepatic parenchyma was performed; an increase in collagen fibers was evident in the CCl₄ group compared with that in the control group (P<0.001; Fig. 3B). In addition, the percentage of total collagen was lower in the *L. lactis*-CCl₄ group compared with the CCl₄ group.

**Liver Function.** Liver function was evaluated by the quantification of albumin, glucose, bilirubin and total

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**Table I. Primers used for quantitative PCR.**

| Primer      | Sequence (5′→3′)                  |
|-------------|-----------------------------------|
| FoxP3       | F: CGGGAGAGTTTCTCAAGCAC R: CACAGGTTGGAGCTTTTGTA |
| IL-1β       | F: CTGTGACTCGTGAGGATGATG R: GGGATTTTTCTTTGCTTT |
| IL-10       | F: TGGCTCAGACTGCTATGTG R: CCTTCCTT |
| β-actin     | F: GTGTGATCGTGAAGCTGTA R: GCTGTGGTGGTGAAGCTGTA |

IL, interleukin; FoxP3, forkhead box protein P3.
proteins, and no significant differences were observed (Fig. 4). However, increased plasma ALT and AST (indicators of liver damage) levels were observed following induction with CCl₄. The L. lactis-CCl₄ group exhibited a significant decrease in ALT and AST levels compared with those in the CCl₄ group (P<0.001; Fig. 4A and B), indicating that L. lactis improved liver function. Urea is primarily formed in the liver as an end product of protein metabolism (6,11); a significant decrease in the urea level was observed in the CCl₄ group compared with that in the control group (P<0.05; Fig. 4C), whereas the L. lactis-CCl₄ group presented with similar levels to those of the control group, which suggested that the liver was functionally transforming ammonium to urea for excretion. The recovery of liver functional enzymes may be associated with the histological improvement presented in Fig. 2. No changes in hepatic function were observed in the L. lactis group compared with the control group.

**Histopathological analysis of Peyer's Patches.** The L. lactis-CCl₄ group induced ~8 well-defined nodules in a single tissue portion (black arrows; Fig. 5A); however, the control and CCl₄ groups possessed a mean of 3 nodules.
(P<0.01), which were smaller in size. Although few lymphoid nodules were observed in the *L. lactis* group, these were larger than those in the control group (Fig. 5A and B). To corroborate these size variations, a morphometric analysis was performed, and no significant differences were apparent between any of the groups. However, the *L. lactis*-CCl4 group exhibited the largest area of these nodules (Fig. 5B).

Histopathological analysis of the large intestine (cecum). Transverse cuts of the cecum were made in animals from each of the study groups (Fig. 6). Normal histology was observed in the control group; however, the colonic tissue of the CCl4 group presented with cellular infiltrate (black arrowheads) in the region of the mucosa layer. This infiltrate was diminished in the groups treated with *L. lactis*. L., *Lactococcus*; CCl4, tetrachloromethane.

Evaluation of inflammatory markers in the liver. *L. lactis* is known to have an immunomodulatory effect due to its association with IL-10, a potent anti-inflammatory cytokine that represses the expression of inflammatory cytokines such as TNF-α, IL-6 and IL-1β produced by macrophages activated during liver injury (30). To analyze the possible effect of intestinal *L. lactis* on CCl4-induced liver damage, the levels of specific cytokines were assessed in the liver tissue, such as pro-inflammatory IL-1β, the anti-inflammatory IL-10 and a T-cell regulatory transcription factor forkhead box protein P3 (FoxP3) (Fig. 7A-C). IL-10 expression was also assessed in intestinal tissues; in the *L. lactis*-CCl4 group, intestinal IL-10 expression was increased compared with the control group (P<0.001), and CCl4 groups (P<0.001), (Fig. 7D). No significant differences in IL-10 expression were observed among any of the experimental groups, although there was a non-significant tendency towards higher expression in the *L. lactis*-CCl4 group. IL-1β expression was decreased in the liver tissues (P<0.05). FoxP3 is the primary regulator of the development and function of regulatory T cells, and its expression was increased in the *L. lactis*-CCl4 group compared with that in the CCl4 group (P<0.05). Collectively, these results demonstrated the immunoregulatory effects of intestinal *L. lactis* on hepatic pathology.

Discussion

In the present study, the inhibitory effect of *L. lactis* NZ9000 on CCl4-induced hepatic fibrosis was analyzed. Oral administration of *L. lactis* induced a physiological change and modified the development of CCl4-induced fibrosis in the liver tissue in the following manners: i) Reducing structural liver damage; ii) reducing the area of fibrosis; iii) increasing the number of lymph nodes in the Peyer's patches; iv) decreasing ALT and AST expression; v) increasing the mRNA expression of IL-10 in small intestine samples; vi) increasing FoxP3 levels in liver samples; and vii) decreasing the expression of IL-1β in the liver.

The aim of the present study was to investigate a protective strategy to reverse liver damage using the physiological interconnection between the digestive tract and the liver via the hepatic portal system (31). The human microbiome is defined as the collective genome of >1,000 different types of microorganisms that exist in association with the human body, the vast majority of which reside in the distal intestine (32,33). This ecological system interacts with internal and external organs, factors that help to maintain the overall health of the
Taking advantage of this physiological association, anatomical-functional communication was investigated with the aim to induce an immunoregulatory response in the intestine, with ultimate effects on liver inflammation. In cases of colitis, the *L. reuteri* R2LC strain has been demonstrated to exert an anti-inflammatory effect in the large intestine (13,14,16,17). Different experimental animal models (predominantly of colitis) have demonstrated the benefits of probiotics in controlling intestinal inflammation. In an acetic acid-induced rat colitis model, the administration of *L. reuteri* R2LC immediately after induction prevented the establishment of colitis (16). Previous reports have demonstrated the effect of probiotics on the gut microbiota under inflammatory processes. The oral administration of *L. plantarum* attenuated inflammatory bowel disease in a mouse model; *L. plantarum* also affected the proportion of *Firmicutes* and *Bacteroides*, which may be associated with inflammation of the mouse gut (34). The intestinal microbiota has been reported to serve a fundamental role in homeostatic maintenance of the systemic immune system; for example, *L. johnsonii* of an intestinal origin did not induce the release of TNF-α or IL-1β following downregulation of the transcription factor NF-κB, whereas TGF-β expression was increased, resulting in a global anti-inflammatory profile (15). Specific recognition of commensal microorganisms occurs in the mesenteric lymph nodes. Most antigens or infectious agents pass into the venous system or tissues through mucous membranes, which includes the lining of the gastrointestinal, respiratory and genitourinary tracts. At these mucosal surfaces, the mucus represents the first barrier against the entry of microorganisms, while gut-associated lymphoid tissues (GALT), which include intestinal Peyer's patches, is critical for efficient protective immune response, making the GALT an attractive portion of the small intestine to study (35). In the present study, the probiotic *L. lactis* generated an anti-inflammatory environment in the small intestine by increasing the expression of IL-10, as well as the number of lymph nodes in the Peyer's patches; these findings suggested a stimulus that may potentially increase the number of regulatory, anti-inflammatory lymphoid cells. However, one of the limitations of the present study was not determining whether *L. lactis* may influence the proportions of various taxonomic and functional groups of the gut microbiota, which may increase our knowledge about the mechanisms of action of probiotics.

IL-10 decreases and regulates dendritic cell- and macrophage-associated inflammatory responses by activating STAT-3 (14). IL-10 also suppresses the adaptive immune response by inhibiting NF-κB secretion by CD4+ T cells and the production of IL-1 and TNF-α by macrophages (14). The results of the present study revealed a notable decrease in hepatic IL-1β expression in the *L. lactis*-CCL4 group compared with the CCL4 group; this was potentially due to an increase in intestinal IL-10 as a result of CCL4-induced damage, which was subsequently transported to the liver via the portal-hepatic.
system. Additionally, an increase in the expression of Foxp3 mRNA was observed in the liver, which supports the increase in intestinal IL-10 in the L. lactis-CCl₄ group, and may be affected by the downregulation of NF-κB (36,37). This supports the existence of an immunoregulatory process induced by L. lactis in the intestine, which has an inhibitory effect on CCl₄-associated fibrosis; thus, L. lactis may exert an anti-fibrotic effect in the early stages of inflammation, which potentially modifies the adhesion properties of epithelial cells, altering the local host immune response (1,38). For this reason, potential new therapies for liver fibrosis may target the recovery of the microbiota to reduce the possible adverse effects associated with pharmacological treatment (18). L. lactis may therefore be an optional co-treatment for decreasing inflammation in the early stages of cirrhosis.

In conclusion, L. lactis prevented liver damage in an animal model of CCl₄-induced liver fibrosis. The results of the present study suggested that oral administration of L. lactis in its native form may be a potential means to prevent and protect against CCl₄-induced liver damage.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

SLMH contributed to the analysis and interpretation of the histological data. CSDV developed the histological technique for the intestine and liver tissues. DCG performed reverse transcription-quantitative PCR analysis. RMDOL and MDJLA donated the L. lactis cultures and analyzed the quantitative PCR data. MGMM developed the liver function study. JVJ and MHMO contributed to study conception and design, and the writing and revision of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Research Ethics Committee of the Autonomous University of Aguascalientes (approval no. A1-S-21375) and were conducted in accordance with institutional guidelines for caring for experimental animals and the national regulatory norm (NOM-062-Z00-1999).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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