Cholecystectomy during ceftriaxone therapy. A translational study with a new rabbit model[1]

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Abstract

Purpose: To evaluate the actual incidence of both microlithiasis and acute cholecystitis during treatment with intravenous ceftriaxone in a new rabbit model.
Methods: New Zealand rabbits were treated with intravenous ceftriaxone or saline for 21 days. Ultrasound monitoring of the gallbladder was performed every seven days until the 21st day when histopathology, immunohistochemistry for proliferating cell nuclear antigen (PCNA), pro-caspase-3 and CD68, liver enzyme biochemistry, and chromatography analysis of the bile and sediments were also performed.
Results: All animals treated with ceftriaxone developed acute cholecystitis, confirmed by histopathology ($P<0.05$) and biliary microlithiasis, except one that exhibited sediment precipitation. In the group treated with ceftriaxone there was an increase in pro-caspase-3, gamma-glutamyl transpeptidase concentration, PCNA expression and in the number of cells positive for anti-CD68 ($P<0.05$). In the ceftriaxone group, the cholesterol and lecithin concentrations increased in the bile and a high concentration of ceftriaxone was found in the microlithiasis.
Conclusion: Ceftriaxone administered intravenously at therapeutic doses causes a high predisposition for lithogenic bile formation and the development of acute lithiasic cholecystitis.
Key words: Cholelithiasis. Cholecystitis, Acute. Ceftriaxone. Models, Animal. Rabbits.

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■ Introduction

Cholelithiasis, is one of the most frequent disorders in Western countries but is rare in children and is the main cause of acute cholecystitis. The etiopathogenesis is multifactorial and gallstone formation represents the main cause[1] Several studies have focused on biochemical factors, including the micellar solubility, saturation point and nucleation of biliary components or on genomic and functional factors, such as gallbladder hypomotility[2-4] There are also some significant risk factors for cholelithiasis, such as ethnic group, age, gender (women), geographic location, obesity, metabolic syndrome, diabetes mellitus, dyslipidemia, reduced physical activity, rapid weight loss, total parenteral nutrition, surgical procedures, cirrhosis, Crohn's disease, and the use of drugs, such as contraceptives, octreotide, thiazide diuretics, and ceftriaxone sodium[2,3]

It is important to note the role of ceftriaxone sodium, a third-generation and long-acting cephalosporin, in the development of lithogenic bile in consequence of this antibiotic precipitation. In the 1980s, Schaad *et al*.[5] reviewed the ultrasound examinations of children under treatment with ceftriaxone and described the morphological similarity between hyperechogenic biliary precipitates and gallstones which supposedly would disappear in all patients after discontinuing treatment. Since then, several cases of pseudolithiasis have been reported[5-7] This evidence has been mentioned in the drug's informational leaflet available to the consumer, also reporting improvement with no major risks upon the suspension of ceftriaxone use. Yet, this apparent benign outcome without any complications presents obvious contradictions. A prospective study demonstrated microlithiasis or gallstone resolution in less than half (43.8%) of the pediatric patients treated with cephalosporin[8] It is noteworthy that microlithiasis formation in the gallbladder resulting from the use of ceftriaxone associated with serious adverse events is generally neglected, especially in adult population. In the medical literature, there are few but worrisome reports of acute biliary pancreatitis, acute cholecystitis including necrotizing type and choledocholithiasis followed by cholangitis during long-term ceftriaxone therapy[9-13]

The authors performed a translational study in an original rabbit model, aiming to study the reliable incidence of and cholecystitis due to intravenous ceftriaxone use, seldom described in the medical literature.

■ Methods

This study was approved by the Ethics Committee for the Use of Animals of the Health Sciences Center under reference number 096/14. This scientific paper complies with the EU Directive 2010/63 for animal experiments and it is also in agreement with the National Institutes of Health guide for the care and use of Laboratory animals (revised in 1978).

Eleven New Zealand adult rabbits (*Oryctolagus cuniculus*) were used in this study, weighing 2.5 to 3 kg. The animals were kept in individual cages, under a circadian cycle and temperature control, in addition to standard feeding, at Experimental Surgery Center, Department of Surgery, School of Medicine, UFRJ. The animals were randomly distributed into two groups: The Placebo group (PG, n = 6), with the daily intravenous administration of 3 ml of 0.9% saline for 21 days; and the Ceftriaxone group (CF, n = 5), with the daily administration of 80 mg/kg ceftriaxone sodium for 21 days.

Anesthesia consisted of the intravenous administration of a solution containing 35 mg/kg ketamine hydrochloride and 10 mg/kg xylazine hydrochloride and animals were kept

with spontaneous ventilation. For this purpose, a central venous catheter (single-lumen 24 G x 13 cm) was surgically inserted into the left internal jugular vein.

Simple, panoramic chest radiographic studies were performed in all rabbits to control the positioning of the catheter tip in the superior vena cava upon its fixation (Figure 1).

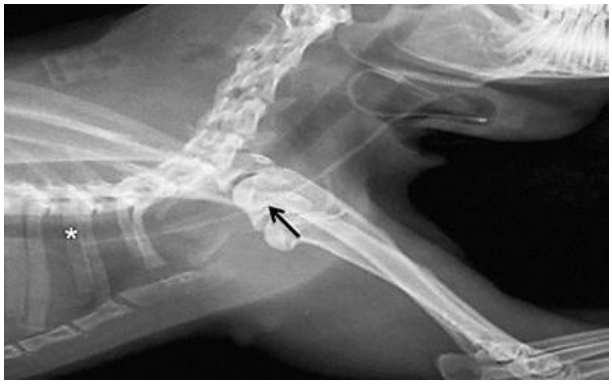

Figure 1 - Simple, panoramic control radiographic studies in rabbits. The catheter inserted (*arrow*) is observed with its distal tip located in the superior vena cava (*).

Serial ultrasound examination

Ultrasound examinations (with a 12 L-RS, 5-13 MHz linear transducer) were performed in all rabbits, while they were under sedation, at baseline and at 7, 14, and 21 days after starting treatment with ceftriaxone.

Liver enzyme biochemistry

Twenty-one days after starting the experiment, blood was collected from all animals in both the P and CF groups, and liver enzyme concentrations were evaluated using the following methods: ultraviolet (UV)-kinetic-ICC (alanine aminotransferase - ALT), modified Bowers and McComb (alkaline phosphatase - ALP), modified Szasz (gamma-glutamyl transferase - GGT), and Sims-Horn (total bilirubin- TBil); the results were expressed in the following units, respectively: UI/l, UI, U/l, and mg/dl.

Histopathology

On day 21 of the experiment, the animals were euthanized without pain with an anesthetic overdose; then, the gallbladder was extracted, and the contents were removed. Gallbladder wall samples were fixed in a 10% formaldehyde solution and stained with hematoxylin and eosin (H&E) for analysis under an optical microscope at x10 and x40 magnification.

Immunohistochemistry (proliferating cell nuclear antigen (PCNA), caspase-3 and CD68

Two preparations on StarFrost® slides were used for the P group (control) and three were used for the CF group; the preparations were deparaffinized in advance for each of the analyses performed. The primary antibodies used were a mouse monoclonal anti-PCNA antibody (F-2; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:100 and an anti-caspase-3 (anti-casp-3) antibody, a p11 mouse monoclonal antibody (Cat # SC-271759 Santa Cruz Biotechnology), and an anti-CD68 monoclonal rat antibody (Serotec, Inc., Raleigh, NC, USA) at 1:100. Staining for PCNA, caspase-3, and CD68 was detected with secondary anti-mouse IgG antibodies conjugated to Cy3 (Sigma; 1:600) or Alexa 488 (Molecular Probes, Eugene, OR, USA). The cell nuclei were stained with 4',6-diamidino-2-phenylindole, and the slides were sealed with Vectashield mounting medium (Vector Laboratories).

Bile, sediment, lecithin, cholesterol, and ceftriaxone chromatography analysis

A high-performance liquid chromatography apparatus (Shimadzu

LC-10AT®) was used for the determination of chromatographic parameters. Quantitative analyses of cholesterol, phosphatidylcholine (lecithin), and ceftriaxone in bile and microlithiasis samples from both groups were performed using thin layer chromatography (TLC silica gel 60®) and high-performance TLC (HPTLC silica gel 60®) with spectrophotometric detection.

Statistical analysis

The Mann–Whitney test and Student's t-test were used, preceded by the Shapiro–Wilk normality test. Analysis of variance was also performed. SPSS software (version 14.0 for Windows, SPSS Inc, USA) was used for statistical analysis. The results were considered significant for values of $P < 0.05$.

■ Results

Serial ultrasound

The abdominal ultrasound examination performed on all animals at baseline and 7 days after the start of ceftriaxone treatment was compatible with normality. At 14 days, all animals from the CF group (100%) showed biliary sludge/sediment characterized by low-density echoes and mobility with decubitus, but with no posterior acoustic shadowing. At 21 days, 4 animals (80%) in the CF group developed biliary microlithiasis. Another animal showed biliary sediment with low-density echoes and postural mobility (Figure 2). No animal from the saline group developed biliary sludge or microlithiasis.

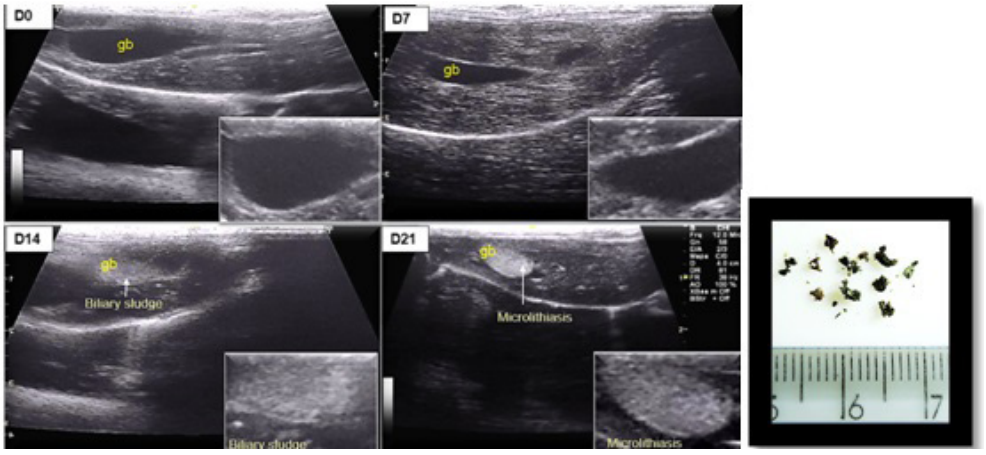

Figure 2 - Abdominal ultrasound of rabbits at 7, 14, and 21 days after the first dose of intravenous ceftriaxone. On the bottom, right of each image a more detailed incidence of the gallbladder was captured. On D0 and D7, gallbladder (gb) images with normal appearance were observed. On D14, biliary sludge with mobility on decubitus was observed, occupying part of the gallbladder lumen (arrow). On D21, four animals (80%) in the CF group developed biliary microlithiasis, which can be observed in the color image (multiple microlithiasis, some aggregated, with sizes up to 3 mm).

Histopathological examination

The five animals (100%) in the CF group showed mild-to-moderate edema in the biliary mucosa and inflammatory infiltrate composed of polymorphonuclear and mononuclear leukocytes, which was compatible with a diagnosis of acute cholecystitis (Figure 3). No animal from the saline treated group developed acute cholecystitis.

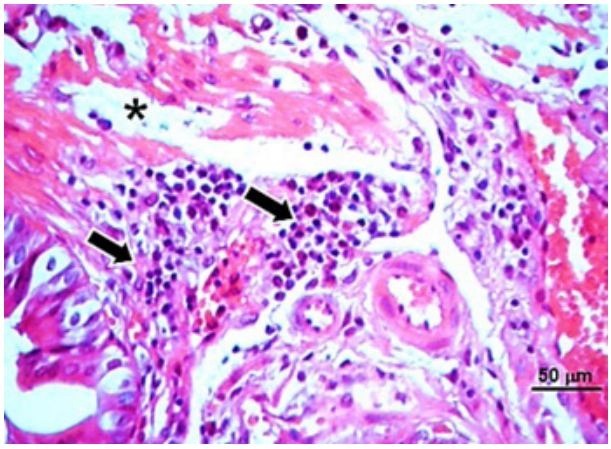

Figure 3 - Histopathology of gallbladder from the ceftriaxone group. The presence of an inflammatory leukocyte infiltrate (*arrows*) and interstitial liquid (*) was observed, compatible with a diagnosis of acute cholecystitis (H&E, x40). Scale, 50 µm.

Biochemical tests

Among the liver enzyme evaluated, only GGT had a significant difference, with serum elevation in the CF group ($P = 0.04$) (Table 1).

Table 1 - Liver enzyme tests.

Groups/ Enzymes	P Average ±SD	CF Average ±SD
ALT (UI/L)	48.07 ± 18.93	58.20 ± 15.04
AP (UI)	23.41 ± 13.17	29.72 ± 11.07
TBil (mg/dL)	0.30 ± 0.22	0.49 ± 0.4
GGT (U/L)	7.27 ± 2.11	11.54 ± 3.64*

P=Placebo group, CF=Ceftriaxone group, ALT=alanine aminotransferase, AP=alkaline phosphatase, TBil=Total bilirubin, GGT=gamma-glutamyl transferase, SD=Standard deviation, GGT: PG *vs*. CFG = P<0.05*

Immunohistochemistry

Compared to the Placebo group, there were significant increases in the CF group in the number of immunolabelled cells positive for casp-3, PCNA, and CD68 in the gallbladder mucosa ($P < 0.001$) (Figure 4).

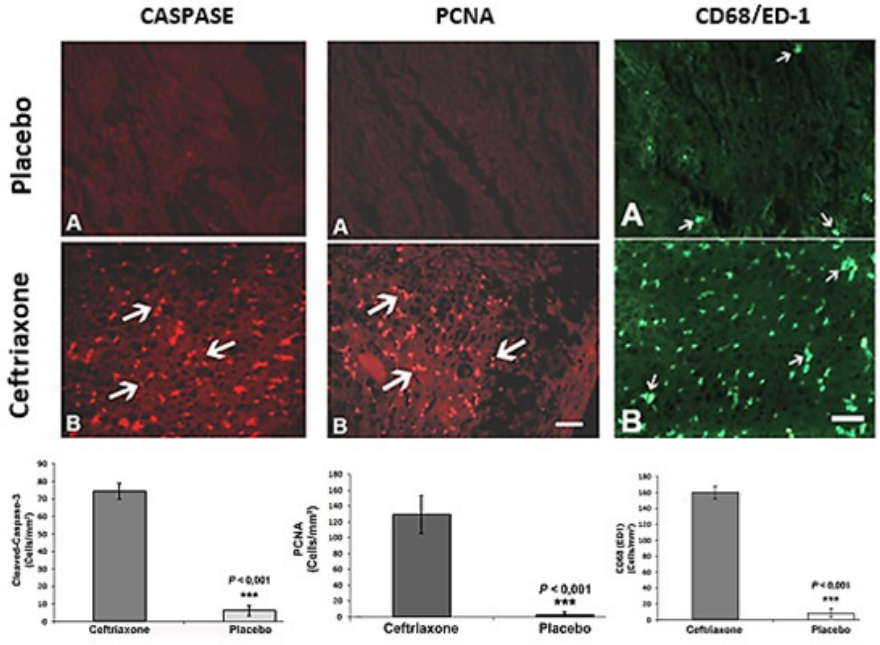

Figure 4 - Immunohistochemistry with caspase-3, PCNA, and CD68 labelling (cells/mm²) with significant increases in the numbers of positive cells for all markers were observed in the Ceftriaxone group compared with the Placebo group ($P < 0.001$).

Chromatography analysis

In the bile samples and microlithiasis composition obtained from the animals on day 21 after the beginning of the experimental study, there was a significant increase of both cholesterol (P = 0.009) and lecithin (P = 0.004) concentrations in the CF group compared to the Placebo group. In the CF group, a trend towards a higher ceftriaxone concentration in the microlithiasis in comparison to the bile was observed (P = 0.07) (Figure 5).

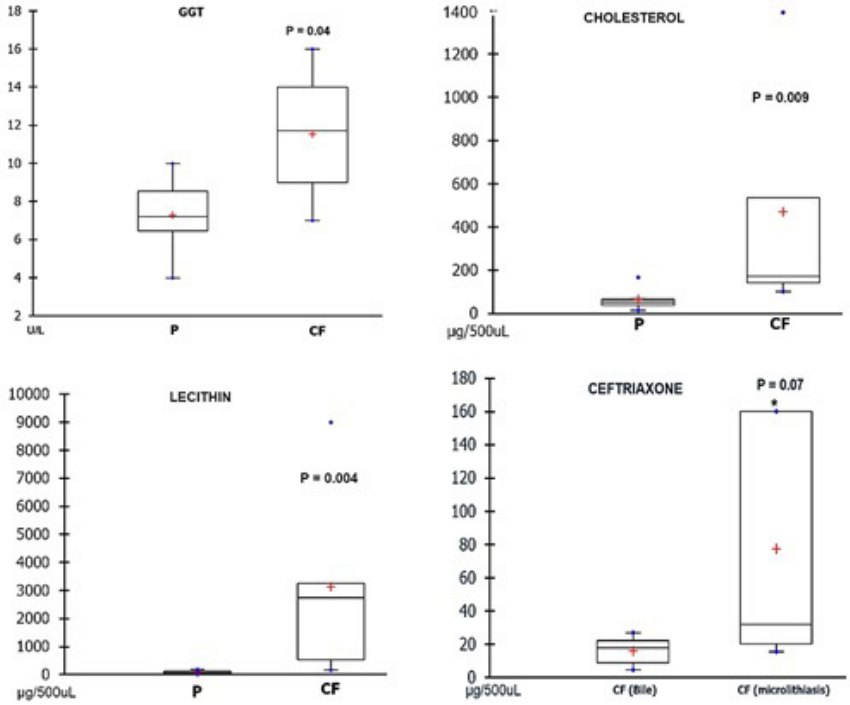

Figure 5 - GGT biochemistry and lecithin and cholesterol chromatography. Box plot with data separation, per group, into quartiles, with the top of the box representing the 75th percentile and the bottom the 25th percentile. The line inside the box represents the median. The limits of the upper and lower bars represent the maximum and minimum values. A significant increase in the GGT concentration (top left) *(P = 0.04) was observed in the Ceftriaxone (CF) group compared with the Placebo (P) group. There was a significant increase in the cholesterol concentration (top right) in the CF group relative to the P group *(P = 0.009). The blue dot represents outliers obtained in one animal. A significant increase in the chromatographic concentration of lecithin (bottom left) was also observed in the CF group *(P = 0.004) compared with the P group. In the CF group, a higher ceftriaxone concentration was found in the microlithiasis than in the bile *(P = 0.07).

■ Discussion

The incidence of cholelithiasis in the adult Western population is estimated to be 10%[14]. In approximately 60% of these cases, the carriers of biliary lithiasis are asymptomatic, but when symptoms or clinical signs are manifested, nearly 70% of the patients will undergo surgical procedures due to acute or chronic cholecystitis[15]. When biliary microlithiasis (stones up to 3 mm) is present, typically involving multiple calculi, and approximately 20% of patients develop pancreatitis, as the microlithiasis may migrate to the common bile duct and pass through the papilla causing relevant injuries associated

with an acute inflammatory process[16,17]

It must be emphasized that ceftriaxone has a broad spectrum of action with a long half-life. A long-term therapy (two or more weeks of intravenous administration) is currently indicated for septicemia, meningitis, pneumonia, osteomyelitis, severe urinary tract infection, endocarditis, typhoid fever and Lyme disease[18]. However, especially in adult patients, very few scientific studies have been associated this drug with acute cholecystitis with microlithiasis or other biliary surgical complication.

After intravenous administration, 40% to 60% of the total ceftriaxone dose is secreted in the bile and the remainder excreted in the urine, if kidney function is normal[19] The high ceftriaxone concentrations obtained in the chromatography analysis of the bile and microlithiasis confirmed the relevant role of this antibiotic in the development of biliary lithiasis in the model studied. Ceftriaxone exists in solution as a bivalent ion and may precipitate if the maximum amount dissolved in the bile exceeds the metastable zone under certain time and temperature conditions, as observed in this study with rabbits[20] This behavior differs from that of other antibiotics excreted in the bile, which rarely become insoluble[10]

The authors of this study had the opportunity to follow one case of ceftriaxone treatment for Lyme disease, in a 56-year-old male adult, previously asymptomatic and with normal routine ultrasonography. At the 21st day of antibiotic course the patient developed gallbladder microlithiasis, transient pancreatitis and acute cholecystitis requiring mandatory surgery consisting of laparoscopic cholecystectomy (unpublished data). A Medline search from the last four decades revealed only three reports of ceftriaxone-associated gallbladder lithiasis leading to acute pancreatitis in adults[11,13,21] Furthermore, only few cases of acute cholecystitis caused by ceftriaxone were reported in adults[22]

Long-term therapy with intravenous ceftriaxone in pediatric patients is usually associated with none or mild symptoms. Likewise, when complains are present the general belief, even in adult's patients, is that the diagnosis of pseudolithiasis (sometimes denominated as sediment, sludge, precipitate) outlines a transient situation which disappears when the drug is discontinued[23-26] However, a careful revision of the medical literature has showed a high incidence of symptomatology, ranging from 22% to 100% of the cases[7,10,11,12,21,22,27,28] Ettestad *et al*.[29] described 25 patients with Lyme disease diagnosis treated with ceftriaxone, of whom 14 underwent surgery with histopathological evidence of cholecystitis and 172 were diagnosed with biliary calculi. Therefore, conservative management, as a widely recommended practice, disregards the epidemiological evidence of a significant frequency of iatrogenic surgical complications in the over 18-years-age group. No well-structured prospective studies in adults were found, a situation that is likely related to underreporting owing to poor cause-effect perception or lack of knowledge. This situation overlooks the actual incidence and pathophysiological relevance of microlithiasis, acute cholecystitis or pancreatitis caused by ceftriaxone therapy.

It is also remarkable that standard information contained in the drug leaflets should be more categorical in warning of the possibility of acute abdominal complications, for instance, acute cholecystitis, pancreatitis or cholangitis, among other biliary diseases. Moreover, in such circumstances, an interventional management, including endoscopic or surgical indication may be mandatory, even if treatment is discontinued.

This scenario reinforces the impression that a conservative approach for adult patients is indicated by analogy to that adopted for pediatric patients. Hence, the authors were motivated to develop this translational experimental study in order to obtain evidences highlighting the natural history and the surgical

impact after a long-term ceftriaxone treatment.

Rabbits were chosen as an experimental model considering their size, the presence of gallbladder (non-existent in rats), the liver anatomy quite similar to that of human beings and because it is an easy specie to deal with. Moreover, the biliary clearance, approximately 8 times higher than in humans (also larger than dogs or mice), markedly increases the output of bile pigment or ceftriaxone precipitates. Thus, only in the presence of great amount of solutes, both sludge precipitation and stone formation may occur. Consequently, along with a new model for biliary lithiasis, these findings show a favorable perspective for its use as alternative in future translational studies.

The formation of microlithiasis within the observed 21-day period in the CF group corroborated the findings described by Schaad *et al*.[5] and by most reports on humans, where these findings were diagnosed in a variable period of time from 2 to 22 days. Two-dimensional ultrasound examination, for which the sensitivity (97%) and specificity (95%) parameters for human cholelithiasis are well documented, was also effective in the detection of biliary sludge/sediments and microlithiasis in all animals (100%) of the CF group, starting on the 14th day of treatment.

In the experimental study, among the liver enzymes evaluated, only GGT concentration increased. It is well known that GGT is a more specific marker than alkaline phosphatase for cholestasis, since skeletal damage is not associated with serum activity elevation[30]. It can be supposed that such cholestasis was probably due to extrahepatic biliary obstruction caused by microlithiasis.

The histological analysis of the gallbladder confirmed a diagnosis of acute cholecystitis in four animals (80%) in the CF group. In fact, the presence of an active inflammatory infiltrate in the gallbladder preparations in the CF group was confirmed by the reaction of the samples with the anti-CD68, an antibody which is associated with the phagocytic activity of locally accumulated inflammatory cells by macrophages[31]. The detection of many positive cells for this marker compared with the P group revealed that activated macrophages were persistently present at the inflammation site. This finding, together with the PCNA labeling, suggests the occurrence of chronic tissue damage apparently induced by ceftriaxone toxicity. Moreover, we can suppose that there is a close relationship between the presence of apoptotic cells (casp-3-positive) with this increased number of CD68-positive activated macrophages, which would potentially be in a state of intense phagocytosis and capable of removing the apoptotic bodies present in the tissue[31].

There are few cases in the medical literature describing in detail the physiopathology linked to physicochemical mechanism of microlithiasis caused by ceftriaxone. Shiffman *et al*.[20] observed that at total doses equal to or higher than 2 g/day, the ceftriaxone saturation index would exceed the metastability limit of this drug. In this study, substantial changes in lecithin and cholesterol concentrations, likely correlated with micelle equilibrium instability, associated with an insufficiency of solubilizing lipid aggregates, contributed to the precipitation of crystals with ceftriaxone. In addition, a large ceftriaxone concentration was found in the microlithiasis, proving a strong evidence of the antibiotic's role in gallstone formation. It seems that ceftriaxone acted as the main trigger of this metabolic derangement, being part of both bile and microlithiasis composition, causing acute cholecystitis.

The evidences collected in our translational experimental model, suggest that the adult population treated with long-term intravenous ceftriaxone, at doses greater than 1 g/day, should be closely monitored by clinical and ultrasound examinations. If the patient is asymptomatic and there is only sediment

formation, serial ultrasound examination must be considered until complete resolution of the abnormal imaging findings, even if it happens after drug suspension. In the presence of microlithiasis in asymptomatic patients, ultrasound will also be highly useful to monitor the outcome and to diagnose early complications. Signs of acute cholecystitis, choledocholithiasis, or acute pancreatitis will require an adequate decision based on common sense, professional expertise and the knowledge of the intrinsic surgical risks of complications.

In this context, the conservative treatment in adults has been usually misunderstood, based on a flawed belief that gallbladder stones disappear after ceftriaxone withdrawal. This guidance disregards the parietal inflammatory process itself when it has already associated with metabolic and systemic consequences. Nowadays, early laparoscopic cholecystectomy is the gold standard for treatment of most cases of acute cholecystitis considering its safety and efficacy, reducing the operative time, lowering of conversion rates and costs, accelerating hospital discharge, and decreasing the incidence of biliary complications[32] In addition, approximately 3% to 10% of patients with biliary lithiasis will develop acute pancreatitis, a risk that is increased up to 20% in male patients in the presence of gallbladder stones smaller than 5 mm[33] Additionally, it should be considered that the incidence of biliary microlithiasis in the adult population using ceftriaxone will more likely be higher than in the general population, which would substantially increase the incidence of acute pancreatitis. Thus, an early cholecystectomy will not only benefit most patients with gallbladder microlithiasis and cholecystitis but also prevent acute pancreatitis or recurrent biliary events[34]

There is no ideal animal model for the development of microlithiasis and acute cholecystitis which can mimic the whole spectrum of changes that occur in humans. Thus, although this new low cost experimental model presents several aspects of human being outcome, it has some limitations. Certainly, further studies of the lithiasic mechanism or signaling pathway and about the interaction of other physicochemical components of the bile may contribute to a better understanding.

In this translational study, the authors have stressed the development of biliary microlithiasis and acute cholecystitis after long-term treatment with intravenous ceftriaxone requiring surgical management. It may contribute to change routines for adult patients, enlighten surgeons to the importance of a close follow-up, targeting a safer therapy with preventive measure or early surgical indication.

■ Conclusion

Ceftriaxone sodium administered intravenously at therapeutic doses causes a high predisposition for the formation of lithogenic bile and the development of acute lithiasic cholecystitis, in grown-up animals.

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