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ABOUT COVER

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ORIGINAL ARTICLE

Basic Study

Pentadecapeptide BPC 157 resolves Pringle maneuver in rats, both ischemia and reperfusion

Marijan Kolovrat, Slaven Gojkovic, Ivan Krezic, Dominik Malekinusic, Borna Vrdoljak, Katarina Kasnik Kovac, Tamara Kralj, Domagoj Drmic, Ivan Barisic, Katarina Horvat Pavlov, Andreja Petrovic, Antonija Duzel, Mario Knezevic, Ivan Mirkovic, Antonio Kokot, Alenka Boban Blagaic, Sven Seiwerth, Predrag Sikiric

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Marijan Kolovrat, Slaven Gojkovic, Ivan Krezic, Dominik Malekinusic, Borna Vrdoljak, Katarina Kasnik Kovac, Tamara Kralj, Domagoj Drmic, Ivan Barisic, Katarina Horvat Pavlov, Andreja Petrovic, Antonija Duzel, Mario Knezevic, Ivan Mirkovic, Antonio Kokot, Alenka Boban Blagaic, Sven Seiwerth, Predrag Sikiric, Departments of Pharmacology and Pathology, School of Medicine, University of Zagreb, Zagreb 10000, Croatia

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Abstract

BACKGROUND

The Pringle maneuver [portal triad obstruction(PTO)] provides huge disturbances during ischemia and even more thereafter in reperfusion. Contrarily, a possible solution may be stable gastric pentadecapeptide BPC 157, with already documented beneficial effects in ischemia/reperfusion conditions. Recently, BPC 157, as a cytoprotective agent, successfully resolved vessel occlusions in rats (ischemic colitis; deep vein thrombosis, superior anterior pancreaticoduodenal vein; bile duct cirrhosis) through rapid collateral vessel recruitment to circumvent vessel occlusion. Thereby, medication BPC 157 regimens were administered as a single challenge before and during ischemia or, alternatively, at various time points during reperfusion.

AIM

To introduce BPC 157 therapy against pringle maneuver-damage.

METHODS

In deeply anesthetised rats, the portal triad was clamped up for 30 min. Rats then underwent reperfusion for either 15 min or 24 h. Medication [(10 μg, 10 ng/kg) regimens, administered as a single challenge] picked (a) ischemia, PTO period [at 5 min before (ip) or at 5 or 30 min of ligation time (as a bath to PTO)] or (b) reperfusion, post-PTO period [at 1 or 15 min (bath during surgery) or 24 h (ip) reperfusion-time]. We provided gross, microscopy, malondialdehyde, serum enzymes, electrocardiogram, portal, caval, and aortal pressure, thrombosis and venography assessments.

RESULTS

P wrote the paper.

Institutional animal care and use committee statement: This research was approved by local Ethic Committee (case number 380-59-10106-17-100/290) and by Directorate of Veterinary (UP/I-322-01/15-01/22).

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BPC 157 counteracts electrocardiogram disturbances (increased P wave amplitude, S1Q3T3 QRS pattern and tachycardia). Rapidly presented vascular pathway (portal vein-superior mesenteric vein-inferior mesenteric vein-rectal veins-left ileal vein-inferior caval vein) as the adequate shunting immediately affected disturbed haemodynamics. Portal hypertension and severe aortal hypotension during PTO, as well as portal and caval hypertension and mild aortal hypotension in reperfusion and refractory ascites formation were markedly attenuated (during PTO) or completely abrogated (reperfusion); thrombosis in portal vein tributaries and inferior caval vein or hepatic artery was counteracted during portal triad obstruction PTO. Also, counteraction included the whole vicious injurious circle [*i.e.*, lung pathology (severe capillary congestion), liver (dilated central veins and terminal portal venules), intestine (substantial capillary congestion, submucosal oedema, loss of villous architecture), splenomegaly, right heart (picked P wave values)] regularly perpetuated in ischemia and progressed by reperfusion in Pringle rats.

CONCLUSION
BPC 157 resolves pringle maneuver-damage in rats, both for ischemia and reperfusion.

Key words: BPC 157; Pringle maneuver; Rats; Portal hypertension; Caval hypertension; Ischemia

Core tip: Recently, cytoprotective agent BPC 157 successfully resolved vessel occlusions in rats (ischemic colitis; deep vein thrombosis, superior anterior pancreaticoduodenal vein) through rapid collateral vessel recruitment to circumvent vessel occlusion. Medication BPC 157 [(10 μg, 10 ng/kg) regimens, administered as a single challenge] picked (1) ischemia, portal triad obstruction (PTO) period [at 5 min before (ip) or at 5 or 30 min of ligation time (as a bath to PTO)] or (2) reperfusion, post-PTO period [at 1 or 15 min (bath during surgery) or 24 h (ip) reperfusion time]. Gross, microscopy, malondialdehyde, serum enzymes, electrocardiogram, portal, caval and aortal pressure, thrombosis and venography assessments demonstrated that BPC 157 successfully attenuates ischemia-reperfusion injury of the liver and other organs. In particular, BPC 157 rapidly activates portocaval shunt, normalises arterial and disturbed blood pressure (portal and caval hypertension and aortal hypotension), counteracts formation of blood clots and cardiac rhythm changes and counteracts gastrointestinal mucosal lesions, as complications that follow the Pringle maneuver.

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INTRODUCTION

We focused on the therapy of the Pringle maneuver in rats[1], so far not described severe preportal hypertension[1], the temporary portal triad obstruction (PTO), ischemia, the short and prolonged reperfusion, the lack of adequate portocaval shunting as the most detrimental feature that should be counteracted. With stable gastric pentadecapeptide BPC 157[2-6], we suggest the resolution of the damages, either those following occlusion or those following re-opening of the hepatic artery, portal vein and bile duct.

Therapy is the recovering effect it has on occluded vessels, bypassing the occlusion as the specific effect of BPC 157 in ischemia/reperfusion[7-11]. There is benefit arising from BPC 157 therapy of the deep vein thrombosis, inferior caval vein occlusion, colitis ischemia/reperfusion, duodenal venous congestion and cecum perforation[7-10].

Recently, after induction of liver cirrhosis due to both bile duct ligation and portal hypertension, prevention and reversal of the already pre-existing portal hypertension to normal values[11] have become possible.

Therefore, in the PTO-syndrome in rats, the rapidly activated way, portal vein-superior mesenteric, vein-inferior mesenteric vein-rectal vein-left iliac vein-inferior caval vein, would appear as a specific activation of the collateral circulation, as the bypassing loop that can rapidly circumvent occlusions and decompress PTO-rats upon BPC 157 administration. That solution in Pringle-rats with ischemia and reperfusion goes with the resolution of oxidative stress, hemodynamic disturbances, severe portal and caval hypertension, aortic hypotension, rapid cloth formation in the portal vein, superior mesenteric vein, lienal vein, inferior caval vein, hepatic artery, ascites, peaked P waves, tachycardia; increased serum values; gross intestine, liver, lung, spleen and heart lesions. Especially, it goes with the agent application during reperfusion.

Contrarily, in preportal hypertension studies in chronically made portal vein-stenotic rats, the high-grade portal-systemic shunting[12] is unable to decompress even the slow development of steady mild portal hypertension[13-16]. The PTO-rat studies are all without portal hypertension assessment[17-21]. They are only reperfusion-induced injury studies[16-20]. Pre-existing ischemia was not investigated[17-21]. Finally, without the agent's application during reperfusion, all require preconditioning during the ischemia (*i.e.*, purportedly attenuated ischemia to attenuate reperfusion)[17-21].

On the other hand, the resolution of all these points in Pringle rats mandates BPC 157 pleiotropic beneficial effects[2-6]. This includes those it has on the liver (including portal hypertension) and intestinal (*i.e.*, simultaneously induced lesions by NSAIDs[22-25], insulin[26], or alcohol[27]), lung[28-30], venous and arterial thrombosis[9,31] as well as heart disturbances[32-36]. BPC 157 counteracts the free radical formation and lesions in distinctive targets (*i.e.*, liver[11,37] and gastrointestinal tract[7,8,10,38], vessels[9], brain[39], sphincters[40], bladder[41]). Namely, BPC 157 is an original cytoprotective anti-ulcer peptide rapidly acting in particular to protect the endothelium, used in ulcerative colitis and now in a multiple sclerosis trial, with lethal dose (LD1) not achieved[2-6].

Ultimately, using the regimens effective in previous studies[7-11], rats before, during and after the Pringle maneuver used several distinct BPC 157 regimens to resolve ischemia (PTO-ligation-period) and reperfusion-related injury (post-PTO-period) and demonstrated a direct beneficial effect with regard to either injury.

MATERIALS AND METHODS

Animals

Study protocols were conducted in male Albino Wistar rats, body weight 200 g, 12 wk old, randomly assigned, used in all of the experiments, with six rats/group/interval, approved by the local Ethics Committee (case number 380-59-10106-17-100/290) and by the Directorate of Veterinary (UP/I-322-01/15-01/22). They were in-house bred - Pharmacology animal facility, School of Medicine, Zagreb, Croatia. The animal facility registered by the Directorate of Veterinary; Reg. No: HR-POK-007. Laboratory rats were acclimated for 5 d and randomly assigned to their respective treatment groups. They were housed in PC cages in conventional laboratory conditions at a temperature of 20 °C-24 °C, a relative humidity of 40%-70% and a noise level 60 DCB. Each cage was labelled according to study number, group, dose, number and sex of each animal. Fluorescent lighting provided illumination 12 h/d. Standard Good Laboratory Practice diet and fresh water were provided *ad libitum*. Animal care was in compliance with the SOPs of the Pharmacology Animal facility; the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123).

Ethical principles of the study ensured compliance with European Directive 010/63/E, the Law on Amendments to Animal Protection Act (Official Gazette 37/13), the Animal Protection Act (Official Gazette 135/06), Ordinance on the Protection of Animals used for Scientific Purposes (Official Gazette 55/13), FELASA recommendations and recommendations of the Ethics Committee School of Medicine, University of Zagreb. Experiments were assessed by observers unaware of the given treatment.

Drugs

As previously[15-19] medication, without carrier or peptidase inhibitor, included stable gastric pentadecapeptide BPC 157 (a partial sequence of the human gastric juice protein BPC, freely soluble in water at pH 7.0 and in saline).

It was prepared as a peptide with 99% (HPLC) purity (1-des-Gly peptide was the main impurity; manufactured by Diagen, Ljubljana, Slovenia, GEPPPGKPADDAGLV, M.W. 1419) (dose and application regimens as described previously[2-11].

Surgery

In deeply anesthetised rats [thiopental (Rotexmedica, Germany) 40 mg/kg ip, apaurin (Krka, Slovenia) 10 mg/kg ip], the portal triad was exposed *via* a midline laparotomy and then clamped up for 30 min. Rats then underwent reperfusion for either 15 min or 24 h.

Medication

To evaluate lesions and blood vessel presentation by gross, microscopic and venographic assessment, electrocardiogram (ECG), contrast ink application, thrombosis, serum enzymes level assessment and free radicals in liver tissue assessment, medication [BPC 157 (10 μg/kg, 10 ng/kg) or saline (5 mL/kg) (controls)] was applied as a bath at the clamped area immediately after portal triad clamping in rats with PTO. Likewise, the same medication [BPC 157 (10 μg/kg, 10 ng/kg) or saline (5 mL/kg) (controls)] was applied as a bath at the area that used to be clamped immediately after clamping removal and reperfusion initiation. For portal vein, caval vein and abdominal aorta pressure recording, medication [BPC 157 (10 μg/kg, 10 ng/kg) or saline (5 mL/kg) (controls)] was applied intraperitoneally in rats at 5 min before PTO, as a bath in rats with PTO at 5 min or 30 min of ligation time, as a bath in rats that had PTO, in the post-PTO-period, at 1 minute or at 24 h of reperfusion time. For portal vein, caval vein venography or yellow ink contrast application, the medication [BPC 157 (10 μg/kg, 10 ng/kg) or saline (5 mL/kg) (controls)] was immediately applied before as a bath in rats with PTO at 15 min of ligation time, as a bath in rats used that had PTO, in the post-PTO-period, at 15 min reperfusion time.

Portal vein and abdominal aorta pressure recording

In deeply anesthetised and laparatomised rats, the recording lasted 5 min, with a cannula (BD Neoflon™ Cannula) (assessed in 1-min intervals) connected to a pressure transducer (78534C MONITOR/TERMINAL Hewlett Packard), inserted into the portal vein, inferior caval vein and abdominal aorta at the level of bifurcation at 5 or 30 min of ligation time in rats with PTO or in rats that had PTO at 5 min or 24 h of reperfusion time.

Of note, normal rats exhibit a portal pressure between 3 and 5 mmHg[34] or like the pressure in the inferior caval vein (providing at least 1 mmHg higher values in the portal vein) and abdominal aorta blood pressure values between 100 and 120 mmHg at the level of bifurcation[17].

ECG recording

In deeply anesthetised rats, the ECG was recorded continuously in all three main leads by positioning stainless steel electrodes on all four limbs, using an ECG monitor *via* a 2090 Medtronic programmer (Minneapolis, MN, United States) connected to a digital oscilloscope (LeCroy waverunner LT342, Chestnut Ridge, NY, United States), which enabled precise recordings, measurements and analysis of the ECG parameters[32-36].

Vessels, intestine, liver presentation

Using a camera attached to a USB microscope (Veho discovery VMS-004 deluxe), in deeply anaesthetised rats, we directly recorded the presentation of the vessels. We assessed vessels [filled/appearance or cleared out/disappearance (hollow)] at the stomach and between the arcade vessels on the ventral and dorsal sides at a 1-cm long segment of the duodenum, jejunum, ascending colon and between 10 vessels from the proximal to the distal cecum throughout the experiment. Assessments were made at selected time points before and after therapy - with regard to the point immediately before therapy (as 100%) - in rats with PTO at 5, 15 and 30 min of ligation time and in rats that had PTO at 5, 10 and 15 min of reperfusion time.

We grossly assessed yellow or pale areas in the liver [(1): Normal liver presentation with no yellow or pale areas; (2) Only small yellow or pale areas; (3) Mild yellow or pale areas; and (4) Extensive yellow or pale areas]. We assessed hemorrhagic congestive areas in stomach, duodenum, jejunum, cecum and colon ascendens, scored upon opening 1-4, (1) normal mucosa presentation; (2) only small hemorrhagic areas; (3) advanced hemorrhagic areas; and (4) extensive and severe hemorrhagic areas. Assessments occurred before sacrifice at 30 min of ligation time in rats with PTO or at 15 min or 24 h of reperfusion time in rats that had PTO.

Using the described camera attached to a USB microscope, we monitored the application of yellow or orange ink (KOH-I-NOR HARDTMUTH, Ceske Budejovice,

Czech Republic) in rats with a PTO-ligation into the portal vein below the point of ligation or up to this point before its entry into the liver or the inferior caval vein. This was done to verify, upon application, the rapid presentation (or absence) of contrast in the liver, increased vessel-branching or tortuous veins of portosystemic shunts (of portal vein-superior, mesenteric vein-inferior, mesenteric vein-rectal, veins-left iliac, vein-inferior caval vein, azygos vein). Thereby, we used a simple scoring system (1) presentation not different from healthy; (2) presentation shows moderate increase; (3) presentation shows mild increase; and (4) presentation shows abundant increase. Assessments were performed at 15 min of ligation time in rats with PTO or at 15 min of reperfusion time in rats that had PTO. We used a total injection volume of 1 mL (0.1 mL/s) in the portal vein or of 2 mL (0.3 mL/s) in the inferior caval vein.

Venography

Venography, in a fluoroscopy unit (Shimadzu type C-VISION PLUS, Tokyo, Japan)[17], was performed (1) in rats with a PTO-ligation or (2) in rats that had PTO, during reperfusion. Warmed non-ionic contrast medium (Iohexol; OMNIPAQUE 350, GE Healthcare, Chicago, United States) was injected (A) in rats with a PTO-ligation into the (1) portal vein below obstruction [total injection volume of 1 mL (0.1 mL/s)]; (2) portal vein up to obstruction [total injection volume of 1 mL (0.1 mL/s)]; and (3) inferior volume of 2 mL (0.3 mL/s). The contrast medium was visualised under real time to assure adequate filling. The subtraction mode was used to record the images (14 frames per second). At 15 min of ligation time, or at 15 min of reperfusion-time, venograms were taken, captured and digitised onto a personal computer file, followed by analysing using the ISSA (VAMSTEC- Software Company, Zagreb, Croatia) image program. We assessed the number of rats presenting (1) full presentation of the portal vein-superior, mesenteric vein-inferior, mesenteric vein-rectal, vein-left iliac, vein-inferior caval, vein pathway (portal vein venography below obstruction); (2) complete filling of the hepatic venous vascular bed, hepatic vein, inferior caval vein and the right atrium of the heart (portal vein venography up to obstruction); and (3) blood flow through the hepatic veins into the liver, and the liver fully presented (inferior caval vein venography at the level of bifurcation) or (B) the time to liver presentation in reperfusion with inferior caval vein venography at the level of bifurcation.

Microscopy

In rats with PTO, at 30 min, and in rats after PTO, in the post-PTO period, in reperfusion at 15 min and 24 h of reperfusion time, tissue specimens from liver, spleen, stomach, duodenum, ileum, cecum, ascending colon, cecum, liver and heart were obtained. The tissue specimens were fixed in buffered formalin (pH 7.4) for 24 h, dehydrated and embedded in paraffin wax, followed by staining with hematoxylin-eosin. Tissue injury was evaluated microscopically by a blinded examiner.

Liver and spleen weight, ascites

Liver and spleen weight were expressed as percentages of the total body weight (presenting in normal rats, for liver 3.2%-4.0% and 0.20%-0.26% for spleen). Likewise, ascites (mL) were assessed.

Thrombus assessment

At death, the portal vein, the superior mesenteric vein (up to the inferior anterior pancreaticoduodenal vein), the lienal vein inferior and the caval vein, as well as a hepatic artery were removed, and the clot was weighed[17].

Bilirubin and enzyme activity

To determine the serum levels of aspartate transaminase (AST), alanine transaminase (ALT, IU/L) and total bilirubin (μmol/L), blood samples were collected immediately after euthanasia and centrifuged for 15 min at 3000 rpm. All tests were performed using an Olympus AU2700 analyser with original test reagents (Olympus Diagnostica, Lismeehan, Ireland)[28-34,42]. Since bilirubin levels were not increased, the data are not shown.

Oxidative stress in liver

At the end of the experiment and at 30 min of PTO ligation time or in the post-PTO ligation period, at 15 min and 24 h of reperfusion time, oxidative stress in the collected tissue samples was assessed by quantifying thiobarbituric acid-reactive species as malondialdehyde (MDA) equivalents, as described previously[15-19,43,44].

For this, the tissue samples were homogenised in PBS (pH 7.4) containing 0.1 mmol/L butylated hydroxytoluene (Tissue Ruptor, Qiagen, United States) and sonicated for 30 s in an ice bath (Ultrasonic bath, Branson, United States).

Trichloroacetic acid (10%) was added to the homogenate, the mixture was centrifuged at 3000 rpm for 5 min, and the supernatant was collected. Then, 1% TBA was added, and the samples were boiled (95 °C, 60 min). The tubes were then kept on ice for 10 min. Following centrifugation (14000 rpm, 10 min), the absorbance of the mixture was determined at a wavelength of 532 nm. The concentration of MDA was estimated based on a standard calibration curve plotted using 1,1,3,3'-tetraethoxypropane. The extent of lipid peroxidation was expressed as MDA, using a molar extinction coefficient of 1.56×10^5 mol/L/cm. The protein concentration was determined using a commercial kit; the results are expressed in nmol per mg of protein.

Statistical analysis

Statistical analysis was performed by parametric one-way ANOVA with post hoc Newman-Keuls test and non-parametric Kruskal-Wallis followed by the Mann-Whitney *U*-test to compare groups. Values are presented as the mean ± SD and as the minimum/median/maximum values. To compare the frequency difference between the groups, the χ^2 test or Fischer's exact test was used; $^aP < 0.05$ was considered statistically significant.

RESULTS

We focused on the stable gastric pentadecapeptide BPC 157 and the recruitment of the portal vein-superior, mesenteric vein-inferior, mesenteric vein-rectal, vein-left iliac, vein-inferior caval vein pathway to recover Pringle rats in ischemia. Likewise, the focus was on BPC 157 and the counteraction of the reperfusion-induced worsening when applied in reperfusion.

All BPC 157 administration regimens (μg- and ng-regimens) were effective in ischemia and reperfusion (Figures 1-14). The portal hypertension assay (Figure 1) and the disturbances course documented a marked attenuation when it was given before (5 min) PTO, much like in the rats with PTO and pre-existing severe portal hypertension and systemic hypotension (seen in the abdominal aorta) (at 5 or 30 min of ligation time). In reperfusion, the worsening that simultaneously appeared and persisted, the huge portal hypertension and, even more, the caval hypertension and aortic pressure not compensated (increasing to values of 80 mmHg) completely disappeared with BPC 157 medication (given at 1 min and 24 h of reperfusion time) (Figure 1). Likewise, unlike the controls with peaked P waves and tachycardia, QRS complexes such as right bundle branch block (RBBB) pattern in all rats, in ischemia and reperfusion, the peaked P waves and tachycardia either did not appear or, if pre-existing, they rapidly disappeared with all BPC 157 regimens (Figure 1). The RBBB pattern was absent (Fisher´s exact probability test $^aP < 0.05$ at least *vs* control) and sinus rhythm appeared in the normal range of heart frequency (Figure 2). As visualised grossly, in ischemia and in reperfusion, with BPC 157, increased blood vessel branching rapidly appeared in the serosa of all organs affected (Figures 3-5), while the splenic veins were particularly less congested and tortuous (Figure 2), much like the azygos vein, indicating the counteraction of the right heart malfunction (Figure 5). In a period of 30 min of PTO, progressive thrombosis occurred in controls (*i.e.* in the portal vein, the lienal vein, the superior mesenteric vein and the inferior caval vein as well as in the hepatic artery) (Figure 6). Contrarily, strong attenuation occurred in the veins and artery of the BPC 157 rats presenting only considerably smaller clots (Figure 6). Likewise, in ischemia and reperfusion, BPC 157 rats had much less ascites formation (Figure 6). Serum ALT and AST values in ischemia and in reperfusion increased in controls and lessened in rats along with BPC 157 administration in either ischemia or reperfusion (Figure 7). Administration of BPC 157 in either ischemia or reperfusion markedly declined gross lesions in the liver (yellowish areas in ischemia; grey areas in reperfusion) and in the gastrointestinal tract (hemorrhagic lesions mostly exaggerated in the stomach and the duodenum in ischemia and in reperfusion) (Figures 7-9); splenomegaly was abolished in ischemia, but presented in reperfusion (Figure 7).

The MFDA-levels in the liver may be indicative. Regularly, PTO increased MDA levels in the liver, and reperfusion additionally increased them, unless BPC 157 administration resulted in MDA levels in the liver within a normal, healthy range in both ischemia and reperfusion (Figure 6).

Further, with respect to the portal vein-superior, mesenteric vein-inferior, superior mesenteric vein-rectal, veins-left iliac, vein-inferior, caval vein shunt along with portal hypertension, persisting (controls) or quickly counteracted (BPC 157), portal vein venography (Figure 10) or orange ink contrast (Figure 10) application below ligation likely revealed the portosystemic shunt non-functioning (controls) or functioning

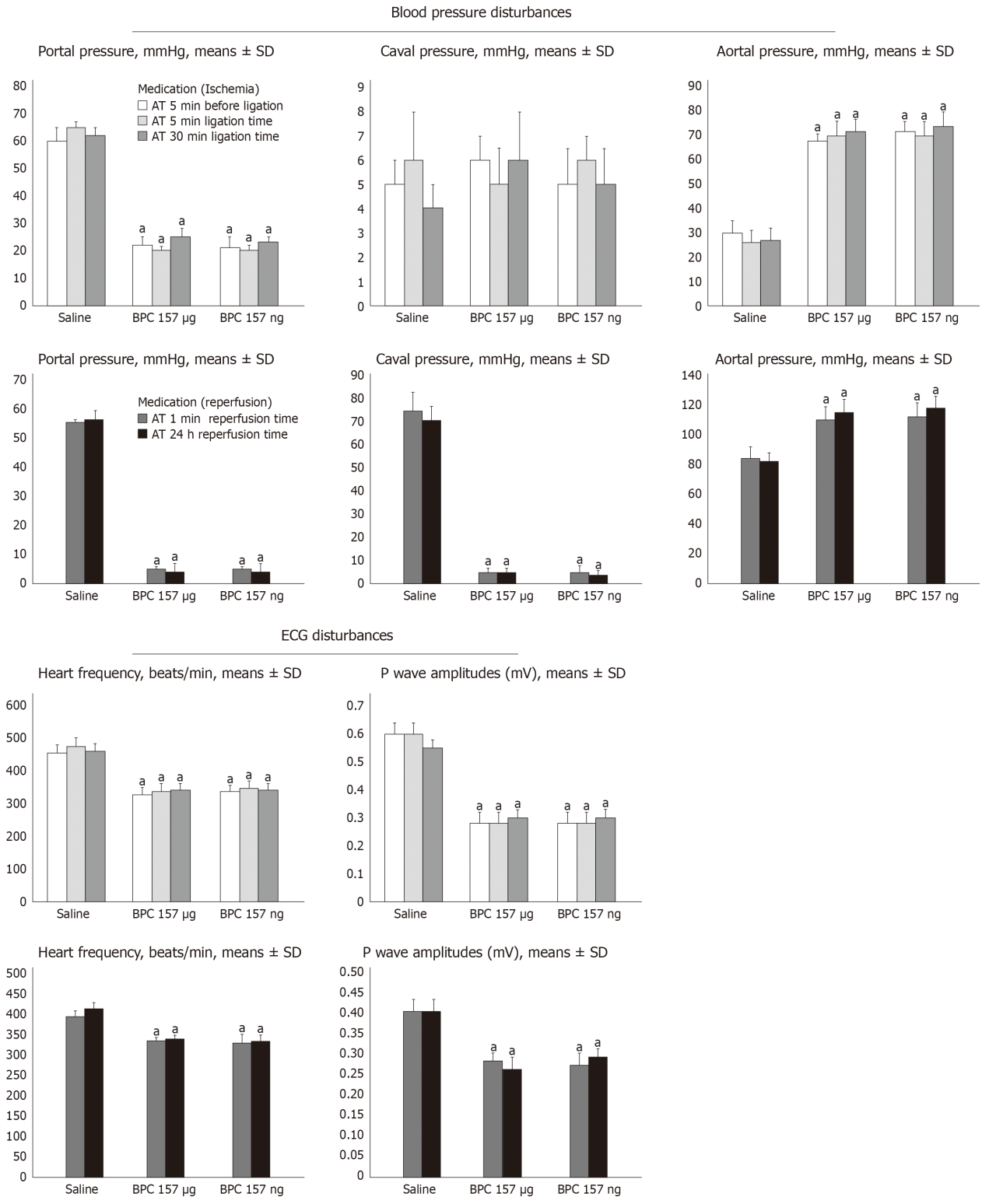

Figure 1 Counteracting effect of BPC 157 on portal, caval hypertension and aortal hypotension, sinus tachycardia, peaked P waves, in (1) ischemia and (2) reperfusion. Portal, caval and aortal pressure (mmHg); P wave amplitudes (Mv) and heart frequency (beats/min) in rats (1) with portal triad obstruction (PTO), at the end of 30 min PTO-period, or (2) after PTO, at the end of 24 h period of reperfusion, mean ± SD. Medication [BPC 157 10 µg/kg, 10 ng/kg, or saline (1 ml/rat)] was given: (1) intraperitoneally at 5 min before PTO, or as a bath administration was given at 5 min or at 30 min PTO-time; and (2) a bath administration given at 1 min or at 24 h after reperfusion initiation. The same results were obtained at 5 min reperfusion time (data not specifically shown). $^{a}P < 0.05$, at least *vs* control. ECG: Electrocardiogram.

(BPC 157) presentation. Controls presented such portosystemic shunt only weakly (Fisher´s exact probability test $^{a}P < 0.05$ at least *vs* control) (Figure 10A), inferior mesenteric vein with the tortuous presentation (Figure 10A). The BPC 157 rats fully presented portosystemic shunt as portal vein-superior, mesenteric vein-inferior, superior mesenteric vein-rectal, veins-left iliac, vein-inferior caval vein way (Figure

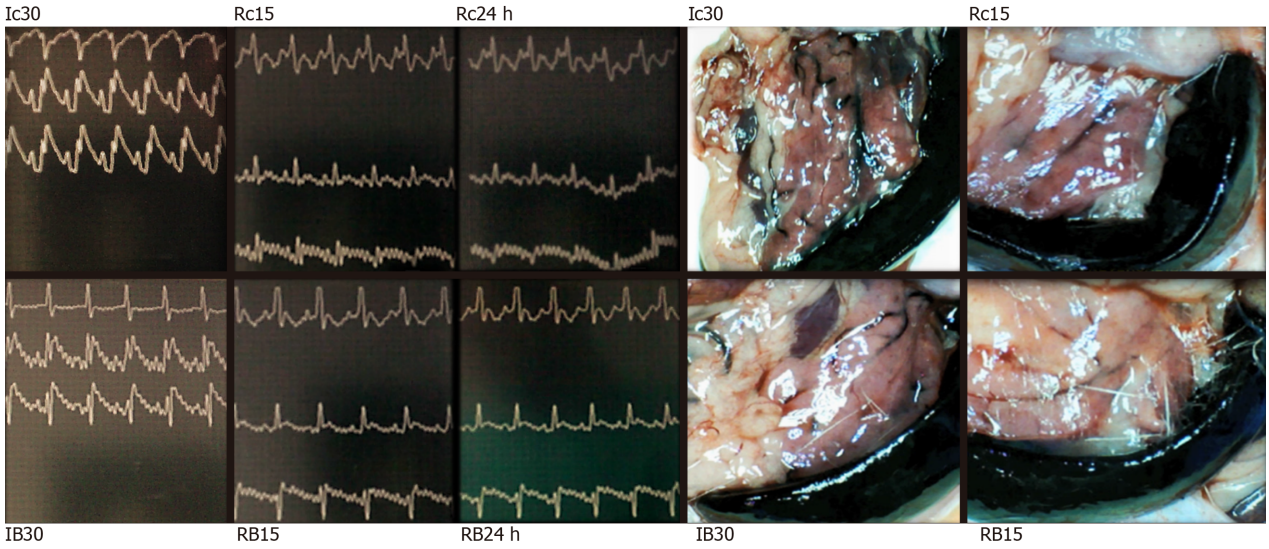

Figure 2 BPC 157 effect on electrocardiogram-disturbances (left) and lienal veins (right) presentation. Electrocardiogram presentation [lead I (upper), lead II (middle), lead III (low)] at 30 min of portal triad obstruction (PTO)-ligation (Ic30; IB30) or in reperfusion (Rc15, RB15, Rc24 h, RB24 h) at 15 min and 24 h reperfusion-time in controls (Ic30, Rc15, Rc24 h) or BPC 157 (IB30, RB15, RB24 h)-rats. Note, while PTO control rats are regularly presenting with sinus tachycardia, peaked P waves (Ic30, Rc15, Rc24 h), RBBB pattern of QRS complexes (Ic30), BPC 157 application markedly prevented or reversed all of these disturbances, having sinus rhythm in normal range of heart frequency. Presentation of lienal veins, congestion and tortuosity in control rats at 30 min PTO-ligation time (Ic30, scored 3/4/4 Min/Med/Max) and at 15 min reperfusion time (Rc15, scored 2/2/2). Marked attenuation occurs in BPC 157 rats at 30 min PTO-ligation time [IB30, scored 2/2/2 (μg), 2/2/2 (ng), $^{a}P < 0.05$, at least vs control], or in rats used to have PTO, at 15 min reperfusion time [RB15, scored 1/1/1 (μg), 1/1/1 (ng), $^{a}P < 0.05$, at least *vs* control] as well as at 24 h reperfusion time.

10B), rectal inferior mesenteric vein with increased branching (Figure 11a2). Likewise, after BPC 157 application, as a function of time, portal vein venography up to ligation (Figure 10) or yellow ink contrast (Figure 11) application, before its entry into the liver, revealed complete filling of the hepatic venous vascular bed, hepatic vein, inferior caval vein and the right atrium of the heart (Figure 10D). There was an immediate presentation of the yellow contrast in the liver (Figure 11, b2 and b4), which further progressed. Contrarily, in the controls, there was no filling of the hepatic vascular bed or any other blood vessels except for the portal vein (note, as administration of contrast continued, the portal vein ruptured) (Fisher´s exact probability test $^{a}P < 0.05$ at least *vs* control). Also, no yellow contrast appeared in the liver; as the administration of contrast continued, the portal vein ruptured (Figure 11, b1 and b3). Thus, a particular functioning of intrahepatic vasculature capacity occurs in rats that received BPC 157 therapy. Also, after BPC 157 application, inferior caval vein venography or yellow ink contrast application at the bifurcation site (Figures 7, 10E) demonstrated blood flow through the hepatic veins into the liver, and the liver fully presented. There was an immediate and highly abundant presentation of yellow contrast in the liver and in the hepatic veins. Contrarily, venography could regularly not find blood flow through the hepatic veins into the liver, and the liver was not presented in controls (Figure 7) (Fisher´s exact probability test $^{a}P < 0.05$ at least *vs* control), while the yellow contrast demonstrated an immediate, but very scant, presentation in the liver and hepatic veins, which would later disappear (Figure 10E). Consequently, it is likely that BPC 157 affects hepatic vein contribution, thereby counteracting the absence of blood flow in the liver during PTO.

In reperfusion, we assume that also intrahepatic vasculature capacity during reperfusion could be rescued. Namely, in reperfusion, portal vein venography (Figure 10) or yellow ink contrast (Figure 11) application into the inferior caval vein at the bifurcation site demonstrated a distinctive reperfusion in BPC 157 rats, which exhibited more extensive and faster reperfusion (Figures 10H and 11d2). This may be an interesting finding with respect to BPC 157 administration in reperfusion, along with the pre-existing ECG-disturbances, severe portal hypertension, and even more caval hypertension and not compensated aortal hypotension and high MDA-level liver, which were all counteracted, and organ lesions markedly attenuated. A comparative venography assessment upon contrast application showed faster liver presentation in BPC 157 rats [5.06 ± 0.1 (μg), 5.16 ± 0.2 (ng) *vs* 11.55 ± 0.1 (control) s]; $^{a}P < 0.05$, at least *vs* control. Consistently, a comparable application of yellow ink revealed that BPC 157 rats showed abundant presentation of yellow contrast in the liver before the controls.

Regularly, PTO rats, much like post-PTO rats, appeared with a considerable

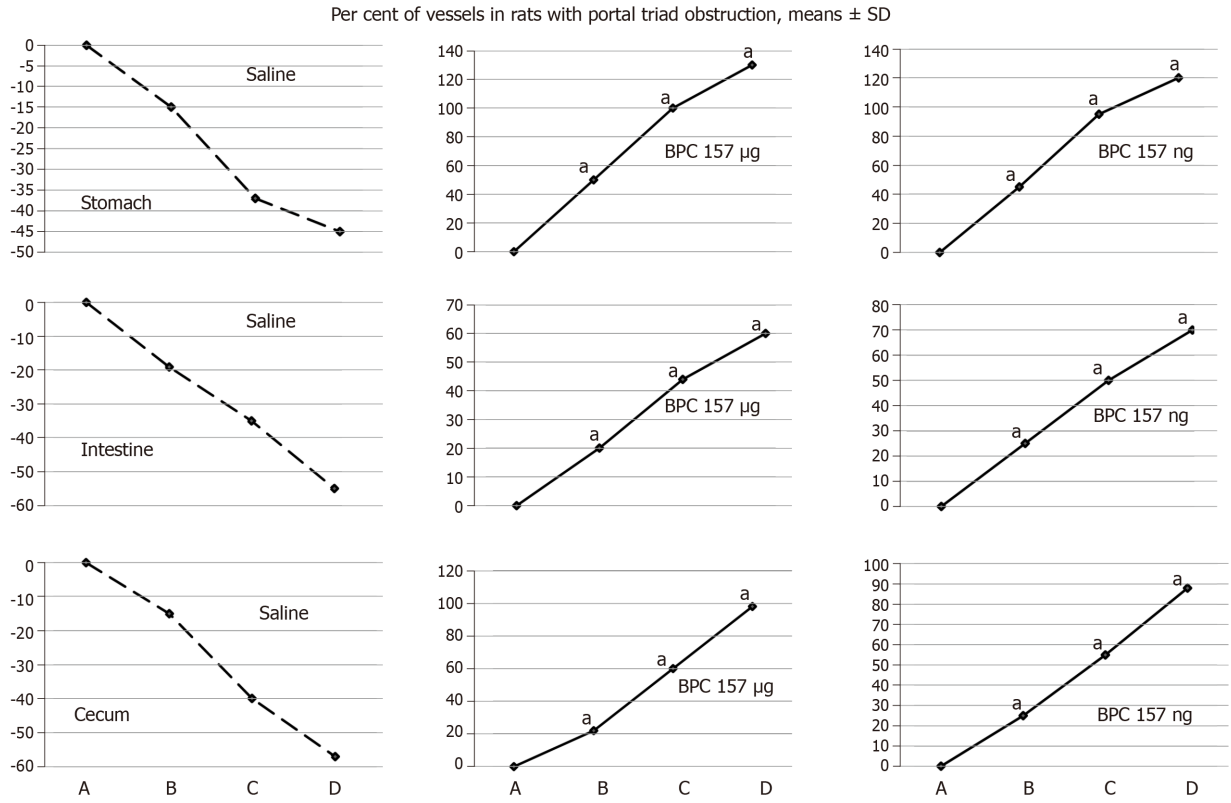

Figure 3 BPC 157 improves the vessels presentation during ischemia. Vessels presentation (filled/appearance or cleared out/disappearance) assessed at the stomach, and between the arcade vessels on the ventral side at 1 cm long segment of duodenum, jejunum, ascending colon, and between 10 vessels from proximal to distal cecum throughout the following experiment [with regard to the point immediately before therapy (as 100%) (A)] at selected time points before (A); and (i) after therapy (B, C, D), in rats with portal triad obstruction (PTO) at 5 (B), 15 (C) and 30 (D) min PTO-ligation time; mean ± SD. The gross appearance of the tissue was recorded using a USB microscope camera. At 1 min post-injury (PTO-ligation time), medication BPC 157, 10 µg/kg (BPC 157 µg), 10 ng/kg (BPC 157 ng), saline (Saline) 5 mL/kg (1 mL/rat) as a bath. For clarity, the SD is not shown on the graph; the SD was never higher than 10% of the mean. Presentation at the dorsal side corresponds with that on the ventral side. $^{a}P < 0.05$ *vs* saline.

amount of similar lesions. Illustratively, in livers, the substantial congestion of the central vein, as well as the branches of the terminal portal venules, are the most prominent findings (Figure 8), along with submucosal edema, substantial capillary congestion, extravasation of erythrocytes and erythrocytes with ischemic changes consistently present in the stomach, duodenum, jejunum, cecum and ascending colon (Figures 12 and 13). Specifically, lifting of the surface of the mucosal epithelial layer appears in the stomach, along with the loss of villous architecture, loss of surface epithelium in the duodenum, jejunum and cecum and focal loss of epithelium in the ascending colon. The BPC 157 therapy largely attenuated all of the noted changes in PTO-rats (Figures 12 and 13). In the liver, BPC 157 rats showed no congestion of the central vein or branches of the terminal portal venules (Figure 8), as well as less submucosal edema, capillary congestion and preserved mucosal architecture throughout the whole intestine (Figures 12 and 13). Lungs presented with preserved architecture, but mild to severe capillary congestion in alveolar septa, progressing during the reperfusion, particularly with a prolonged period, a course markedly counteracted in BPC 157 rats (Figure 14).

In the spleen, all rats exhibited sinusoidal congestion and enlargement of the red pulp, leading to a reduction of the white pulp at the end of the ischemia (data not shown).

Likewise, as expected, no morphological changes were found in the myocardium, mainly because changes found on an ECG were the result of acute right ventricular overload (data not shown).

Generally, these results indicate the success of BPC 157 therapy (Figures 1-14). With BPC 157 given either before or during PTO, there is the resolution of ischemia–induced disturbances. Likewise, with BPC 157 given during reperfusion, after PTO, at post-PTO time, there is a counteraction of the reperfusion-induced disturbances in rats.

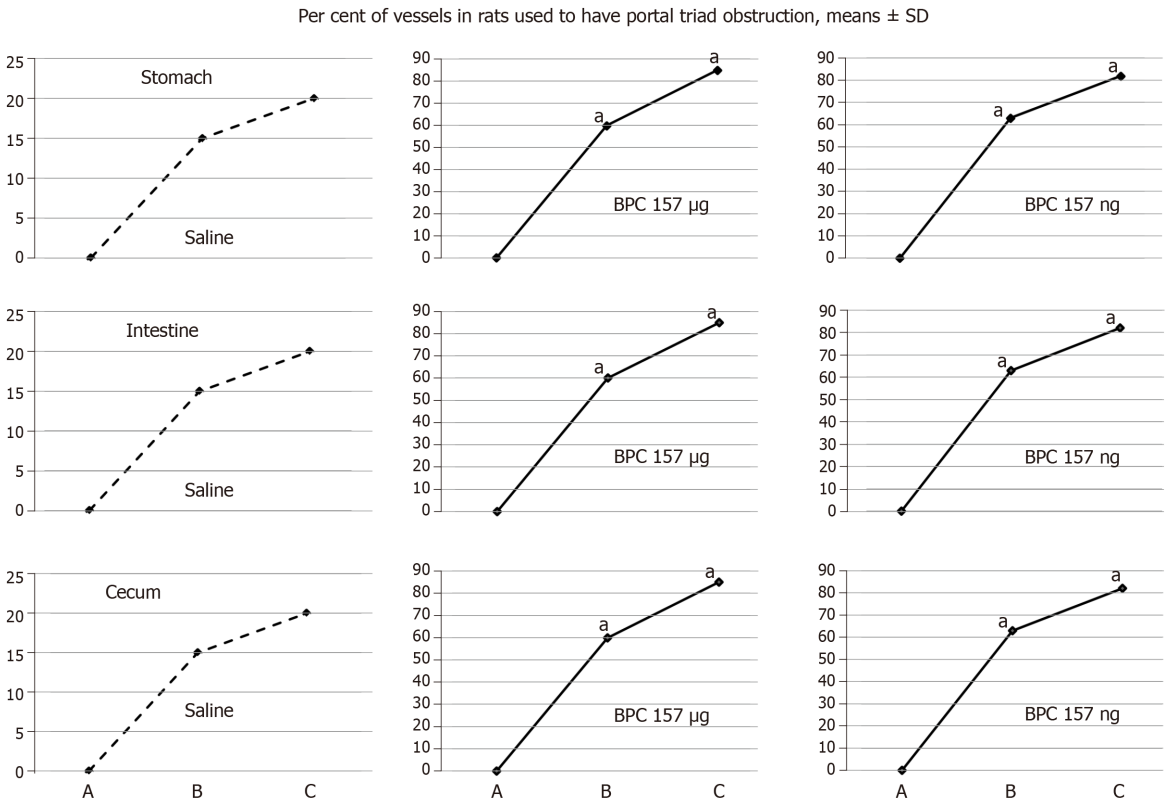

Figure 4 BPC 157 improves the vessels presentation during reperfusion. Vessels presentation (filled/appearance or cleared out/disappearance) assessed at the stomach, and between the arcade vessels on the ventral side at 1 cm long segment of duodenum, jejunum, ascending colon, and between 10 vessels from proximal to distal cecum throughout the following experiment [with regard to the point immediately before therapy (as 100%) (A)] at selected time points before (A); and after therapy (B, C), in rats used to have portal triad obstruction (PTO) at 15 min (B) and 24 h (C) post-PTO-ligation time; mean ± SD. The gross appearance of the tissue was recorded using a USB microscope camera. At at 1 min reperfusion-time (post-PTO-ligation time), medication BPC 157, 10 µg/kg (BPC 157 µg), 10 ng/kg (BPC 157 ng), saline (Saline) 5 mL/kg (1 mL/rat) as a bath. For clarity, the SD is not shown on the graph; the SD was never higher than 10% of the mean. Presentation at the dorsal side corresponds with that on the ventral side. $^{a}P < 0.05$ *vs* saline.

DISCUSSION

We emphasize the resolving of the Pringle maneuver, the stable gastric pentadecapeptide BPC 157[2-6] therapy in ischemia and in reperfusion, its portal vein-superior mesenteric vein-inferior mesenteric vein-rectal vein-left iliac vein-inferior caval vein pathway recruitment (Figures 10 and 11). This therapeutic effect rapidly decompressed portal hypertension and related disturbances in Pringle rats (Figure 1), much like its particular effect on the occluded vessels, the bypassing of the occlusion and the reestablishment of the blood flow[7-11]. Supportive effectiveness analogy goes with the successful ischemic/reperfusion therapy demonstrated in the rats with the infrarenal occlusion of the inferior caval vein, ischemic/reperfusion colitis, duodenal congestion and cecum perforation injuries[7-10], as well as bile-duct-induced liver cirrhosis with portal hypertension[11]. Here, BPC 157 administration in ischemia promptly attenuates portal hypertension (effective prophylactically (portal hypertension development prevented); a therapeutic effect against pre-existing short-lasting and long-lasting portal hypertension, both decreased instantaneously). Moreover, in the worst reperfusion condition, as therapy efficacy progressed, BPC 157 completely eliminated both portal and caval hypertension and aortal hypotension (Figure 1).

Illustratively, inferior mesenteric vein presentation is tortuous in controls, unlike in BPC 157 rats (Figure 11). In BPC 157-rats, in ischemia and in reperfusion, the end result of the oxidative stress (MDA level) in the liver is continuously within the normal values. Contrarily, in the control livers, high MDA values during PTO further progressed in reperfusion (Figure 6)[2-6]. Control rats exhibited rapid clot formation (in the portal vein, superior mesenteric vein, lienal vein, inferior caval vein, hepatic artery) (Figure 6) and ascites (consistently present in the ischemia and reperfusion period) (Figure 6). These disturbances contrast with apparently less venous and less arterial clots and less ascites in BPC 157 rats, as emphasized previously[9,11,31]. Moreover, controls presented with immediately peaked *P* values, tachycardia and an

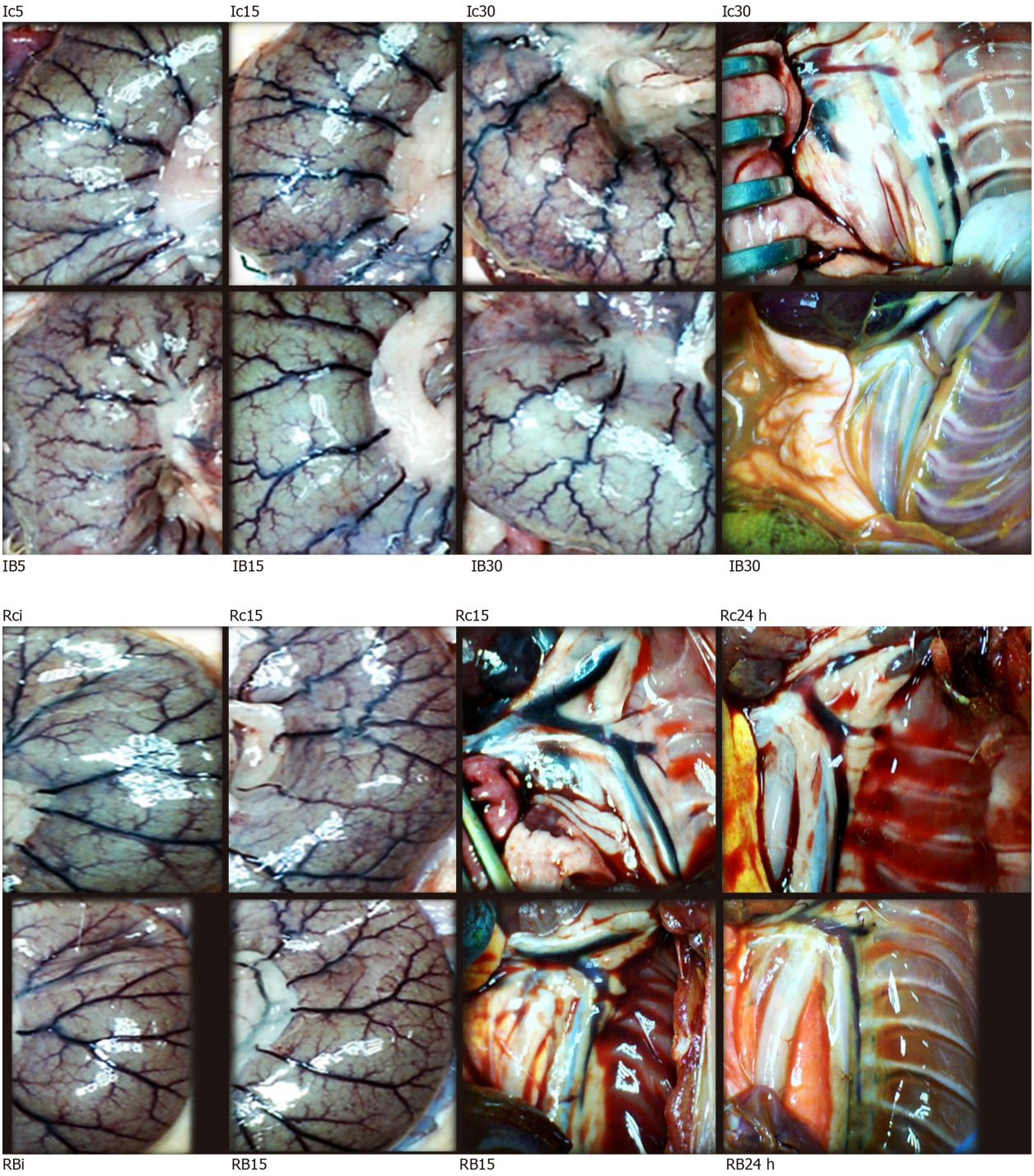

Figure 5 BPC 157 effect on cecal vessels (left, Ic5, Ic15, Ic30, Rci, Rc15, IB5, IB15, IB30, RBi, RB15) and azygos veins [right, Ic30, Rc15, Rc24 h, IB30, RB15, RB24 h (white letters)] presentation. Presentation of cecal vessels. Controls with veins congestion and tortuosity at 5 min (Ic5), 15 min (Ic15) and 30 min (Ic30) portal triad obstruction (PTO)-ligation time, and in reperfusion, immediately (Rci) and at 15 min reperfusion time (Rc15); markedly attenuated veins congestion and tortuosity, increased small vessel branching, "honeycomb" smaller vessel network, in BPC 157 rats 5 min (IB5), 15 min (IB15) and 30 min (IB30) PTO-ligation time, and in reperfusion, immediately (RBi) and at 15 min reperfusion-time (RB15) (Figures 3 and 4). Presentation of azygos veins (yellow ink contrast in inferior caval vein) controls with congestion and tortuosity at 30 min PTO-ligation time (Ic30, scored 2/2/2 Min/Med/Max likely reflecting right heart failure), at 15 min (Rc15, scored 3/3/3) and 24 h (Rc24 h, scored 3/3/3) in reperfusion time, likely reflecting persisting right heart failure. Marked attenuation occurs in BPC 157 rats at 30 min PTO-ligation time [IB30, scored 1/1/1 (μg), 1/1/1 (ng), $^{a}P < 0.05$, at least *vs* control] and in reperfusion, at 15 min (RB15, scored 1/1/1 (μg), 1/1/1 (ng), $^{a}P < 0.05$, at least *vs* control) and at 24 h [RB 24 h, scored 1/1/1 (μg), 1/1/1 (ng), $^{a}P < 0.05$, at least *vs* control] reperfusion-time.

RBBB pattern of QRS complexes as the identifiers of the right heart failure (Figures 1 and 2) [and thereby, congested azygos vein (Figure 5) and lung congestion (Figure 14)]. This failure contrasts with the ECG disturbances completely abrogated (and thereby, non-congested azygos vein) and less lung congestion in BPC 157-rats[2-6]. Therefore, the immediate presentation of adverse effects and the immediate therapeutic effect may suggest the essential immediate cause-consequence chain of

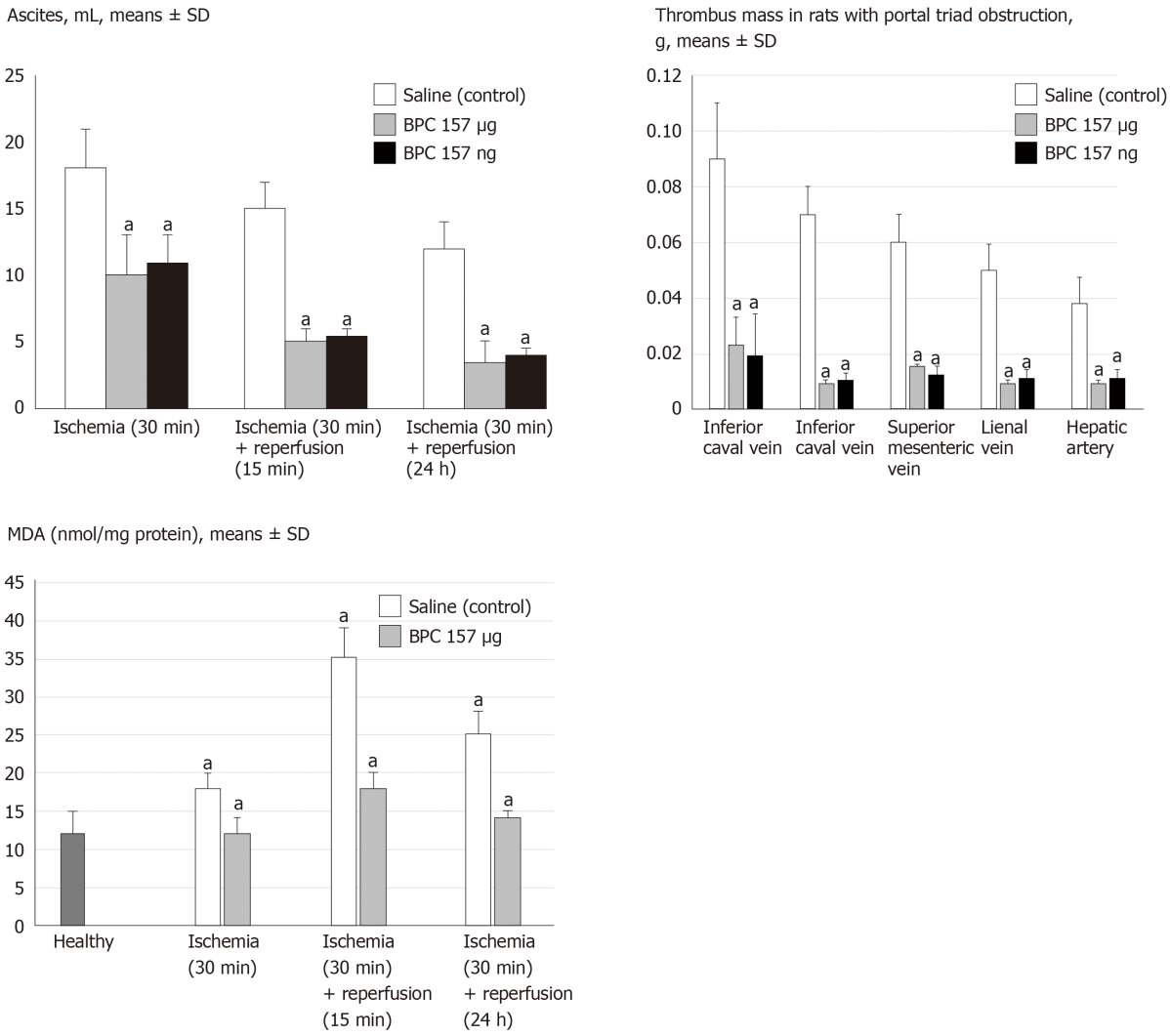

Figure 6 BPC 157 counteracts ascites (mL) (upper), increased MDA-level (nmol/mg protein) in liver (low), in ischemia and reperfusion, and thrombus presentation (thrombus mass, g) in veins [inferior caval vein, portal vein, superior mesenteric vein, lienal vein] and artery (hepatic artery) (middle) during portal triad obstruction. Assessment at 30 min portal triad obstruction (PTO)-ligation time, and in reperfusion, at 15 min and 24 h reperfusion time, mean ± SD. At 1 min post-injury (PTO-ligation time) (ischemia), or at 1 min reperfusion-time (post-PTO-ligation time) (reperfusion), medication (BPC 157, 10 µg/kg, 10 ng/kg, saline 5 mL/kg (1 mL/rat)) as a bath. $^{a}P < 0.05$ *vs* saline. $^{a}P < 0.05$, at least *vs* healthy liver.

events and, in particular, the relation to the liver as the prime organ affected[2-6], liver and liver circulation failure, presentation (controls) and counteraction (BPC 157) in Pringle rats. All controls exhibited gross lesion progression, in ischemia and in reperfusion (Figure 7), increased enzyme serum values (Figure 7) and a dilated central vein and terminal portal venules (Figure 8). This contrasts with the markedly spared gross liver presentation, less gross lesions in the ischemia and in the reperfusion, lower serum enzyme values and no congestion of the central vein or branches of the terminal portal venules in BPC 157-rats.

First, while the quick activation of the portal vein-superior mesenteric vein-inferior mesenteric vein-rectal vein-left iliac vein-inferior caval vein pathway may rapidly decompress portal hypertension, this may reflect the regular absence of blood in the sinusoid[45] and reversal with BPC 157 therapy. Unlike controls, in BPC 157 rats, there is retrograde blood entry through the hepatic veins and liver visibility or yellow contrast presentation in hepatic veins, as well as rapid abundant presentation in the liver (inferior caval vein venography or yellow contrast application in inferior caval vein, during PTO) (Figures 10 and 11). Consequently, the anoxic effect of ischemia on sinusoidal endothelial cells, Kupffer cells and hepatocytes, was accentuated (controls), or counteracted (BPC 157), and thereby, enhanced liver dysfunction was accentuated (controls) or counteracted (BPC 157), as reflected in the more (controls) or markedly less (BPC 157) increase in liver enzymes (Figure 7) (including also the consequence of reduced bile flow rate)[45].

Second, portal vein venography (or yellow contrast application) up to the ligation

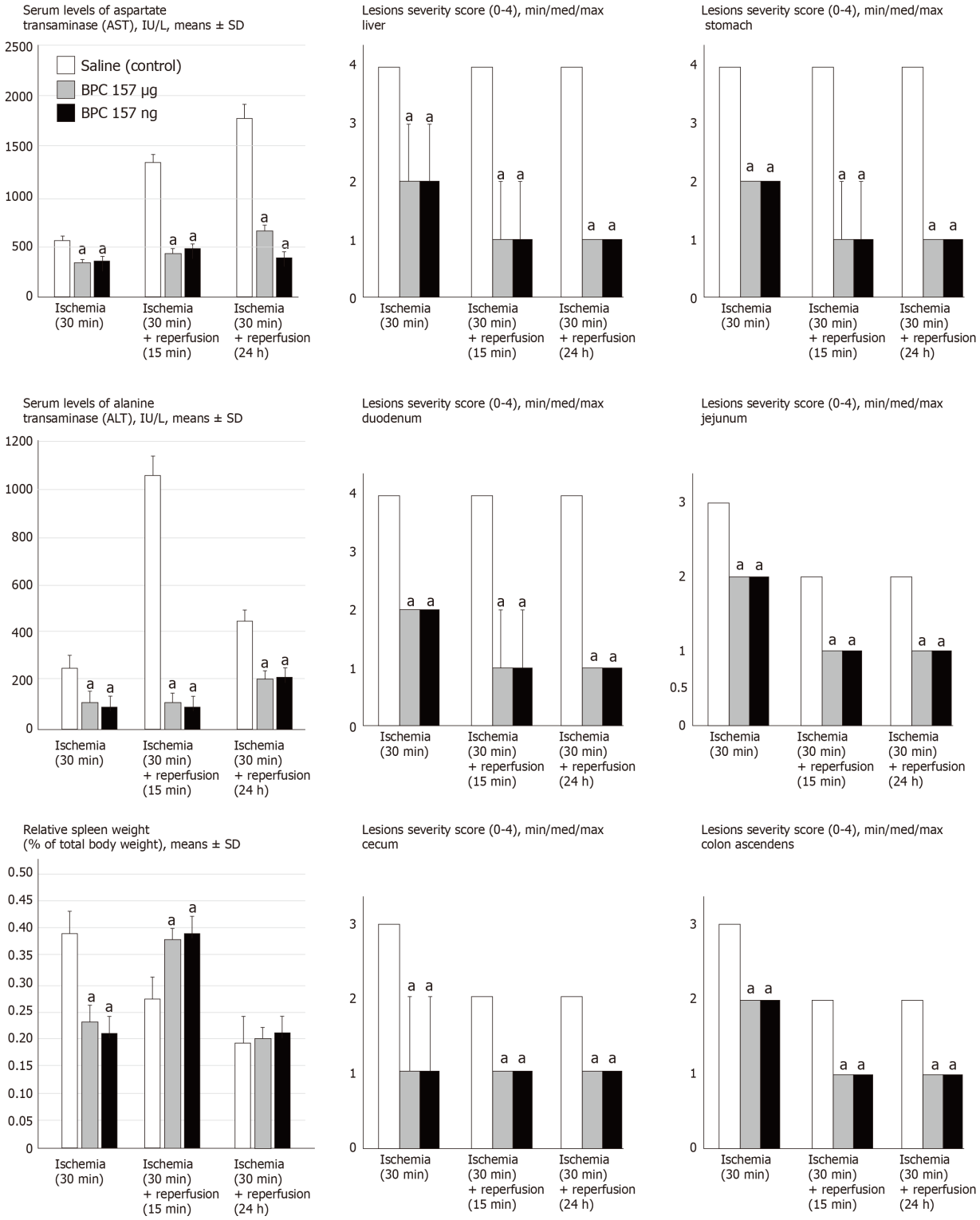

Figure 7 BPC 157 counteracts increased serum enzymes values (IU/L, mean ± SD), gross lesions severity (liver, stomach, duodenum, jejunum, cecum, ascending colon) scored 0-4, Min/Med/max, splenomegaly (relative spleen weight as % of total body weight, mean ± SD) in ischemia, (splenomegaly appears in early reperfusion). Assessment at 30 min portal triad obstruction (PTO)-ligation time, and in reperfusion, at 15 min and 24 h reperfusion time. At 1 min post-injury (PTO-ligation time) (ischemia), or at 1 min reperfusion-time (post-PTO-ligation time) (reperfusion), medication (BPC 157, 10 µg/kg, 10 ng/kg, saline 5 mL/kg (1 mL/rat)) as a bath. [a]$P < 0.05$ *vs* saline.

before its entry into the liver indicates the particular functioning (BPC 157) [or non-functioning (controls)] of intrahepatic vasculature capacity during PTO in rats. Unlike in the controls, with BPC 157 therapy, as a function of time, there was the complete filling of the hepatic venous vascular bed, the hepatic vein, the inferior caval vein and the right atrium of the heart and an immediate presentation of yellow contrast in the liver, which further progressed (Figures 10 and 11).

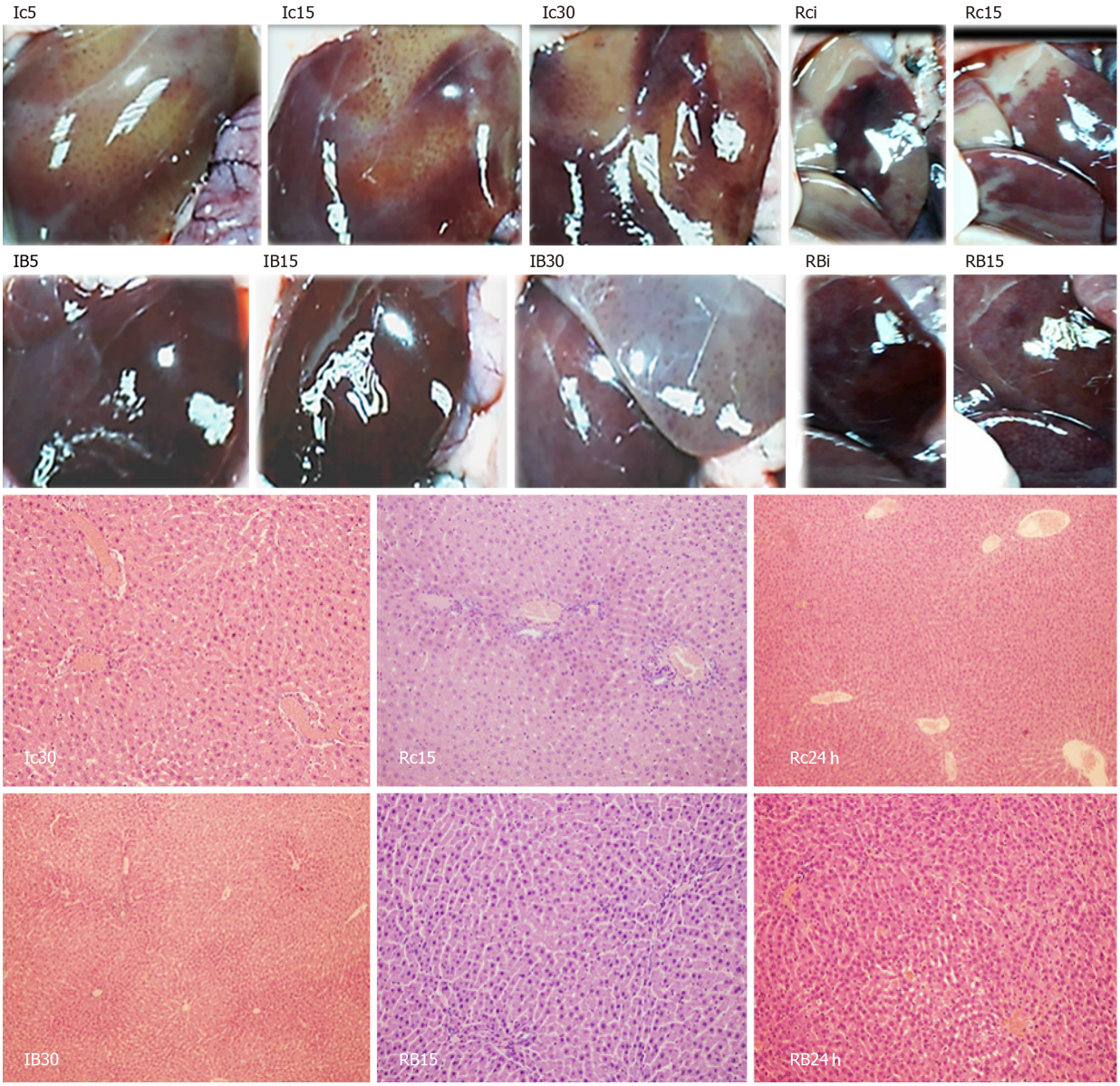

Figure 8 BPC 157 effect on liver gross presentation (left, Ic5, Ic15, Ic30, Rci, Rc15, IB5, IB15, IB30, RBi, RB15) and liver microscopy [right, Ic30, Rc15, Rc24 h, IB30, RB15, RB24 h (white letters)] presentation. Liver gross presentation. Yellowish liver presentation in control rats at 5 min (Ic5), 15 min (Ic15) and 30 min (Ic30) portal triad obstruction (PTO)-ligation time and in reperfusion with pale areas, immediately after medication (Rci) and at 15 min reperfusion time (Rc15); BPC 157 application markedly attenuated these changes, during PTO (IB5, IB15, IB30) as well as in reperfusion time (RBi, RB15) (see **Figure 7**). Liver microscopy (HE staining, × 10). Controls. Substantial congestion of central vein as well as branches of terminal portal venules present at the end of 30 min PTO ischemia period (Ic30). With reperfusion, as seen at 15 min of reperfusion (Rc15) as well as at 24 h of reperfusion, substantial congestion of central vein as well as branches of terminal portal venules remained (Rc24 h). BPC 157. Substantial congestion of central vein as well as branches of terminal portal venules counteracted at the end of 30 min PTO ischemia period (IB30). With reperfusion, as seen at 15 min of reperfusion (RB15) as well as at 24 h of reperfusion (RB24 h), the reversal of substantial congestion of central vein as well as branches of terminal portal venules.

As a final point, the acknowledged increased vascular incapacitation of the liver during reperfusion as a follow up of PTO (and thereby, persistent severe portal hypertension, complicated with even more caval hypertension), the therapy application in reperfusion could either further accentuate (saline, controls) or rapidly resolve (BPC 157) debilitated intrahepatic vascular capacity. Therefore, in BPC 157 rats, the rescue is documented with the inferior caval vein venography, much like with the yellow contrast application in the inferior caval vein during reperfusion. The BPC 157 rats exhibited more rapid and extensive full liver presentation and yellow contrast in the liver (Figures 10 and 11); they also microscopically counteracted substantial congestion of the central vein as well as branches of the terminal portal venules (Figure 5). Simultaneously, as already mentioned above, just opposite to the downhill course in controls, portal and caval hypertension and aortal hypotension disappeared (Figure 1), peaked P waves and tachycardia disappeared (Figure 1), lung congestion decreased (Figure 14) and MDA liver values became normal (Figure 6). In

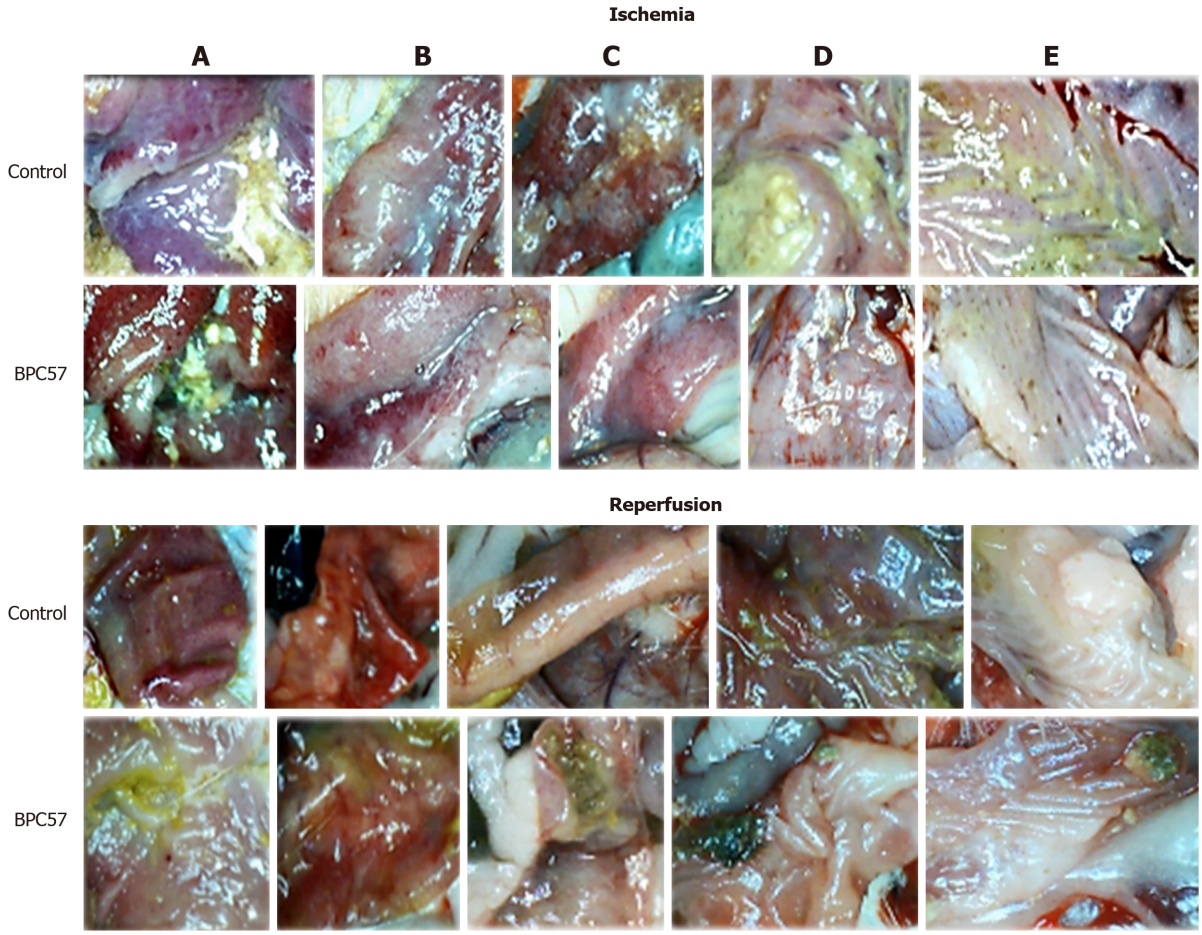

Figure 9 BPC 157 effect on gastrointestinal lesions gross presentation in ischemia (upper) and in reperfusion (lower). A: Stomach; B: Duodenum; C: Jejunum; D: Cecum; E: Ascending colon. Lesions presentation in controls, at 30 min portal triad obstruction (PTO)-ligation-time in rats with PTO, and at 24 h reperfusion-time. Lesions attenuation in BPC 157 rats, at 30 min PTO-ligation-time in rats with PTO, and at 24 h reperfusion-time (**Figure 7**).

addition, it may be the prompt antithrombotic effect of BPC 157 application[9,31,43,46] that provided the abrogated thrombosis[1] as an important factor to rescue the disturbed hepatic microcirculation and increased hepatic vascular resistance. Likely, BPC 157 acts to counteract Virchow, as it did in the rats with inferior caval vein occlusion[9]. Thus, BPC 157 may counteract the regular venous return decrease to the heart and failure of spontaneous decompression of the portal system (note, caval hypertension was consistently elevated in comparison to the portal hypertension, with a gradient of at least 10 mmHg). Such a special effect may be that BPC 157 specifically interacts with the NO-system[44], counteracts NOS-blocker L-NAME-induced hypertension as well as NOS-substrate L-arginine-induced hypotension[43], potassium over-dose and severe hyperkalemia arrhythmias and hypertension[33] or doxorubicin-induced chronic heart failure and hypotension[34].

Resolution with BPC 157 therapy abrogated all congestive hemorrhagic intestinal lesions, from the stomach to the ascending colon (Figures 7, 9, 12 and 13). Providing resolution of hemodynamic disturbances (Figure 1), in addition to the known beneficial BPC 157 effect on liver and gastrointestinal lesions[2-6], there is a supportive chain of events in the counteraction of lesions in ischemia and reperfusion. This occurs along with the rapid activation of a bypassing loop and, consequently, portal hypertension counteraction (Figure 1). Along with this is a "honeycomb" smaller vessel network, which appears at the intestinal serosa (Figures 3-5), a finding noticed in rats with ischemic/reperfusion colitis, duodenal venous congestion lesions or inferior caval vein occlusion[7-10]. Otherwise, intestinal ischemia[42] appears as the final consequence of blood pooling in the splanchnic bed, inducing portal hypertension and multivisceral edema; mesenterial venous occlusion could induce intestinal injury as early as within 5 min, both of which are inflow and outflow alterations[47].

Probably, these findings, when summarized, would need relations that are more precise. On the other hand, the recovery of the Pringle rats, in the ischemia and in the reperfusion, could explain the previously described pleiotropic effect on all of the affected systems[2-6], venous and arterial thrombosis[9,31] and free radical formation[2-6].

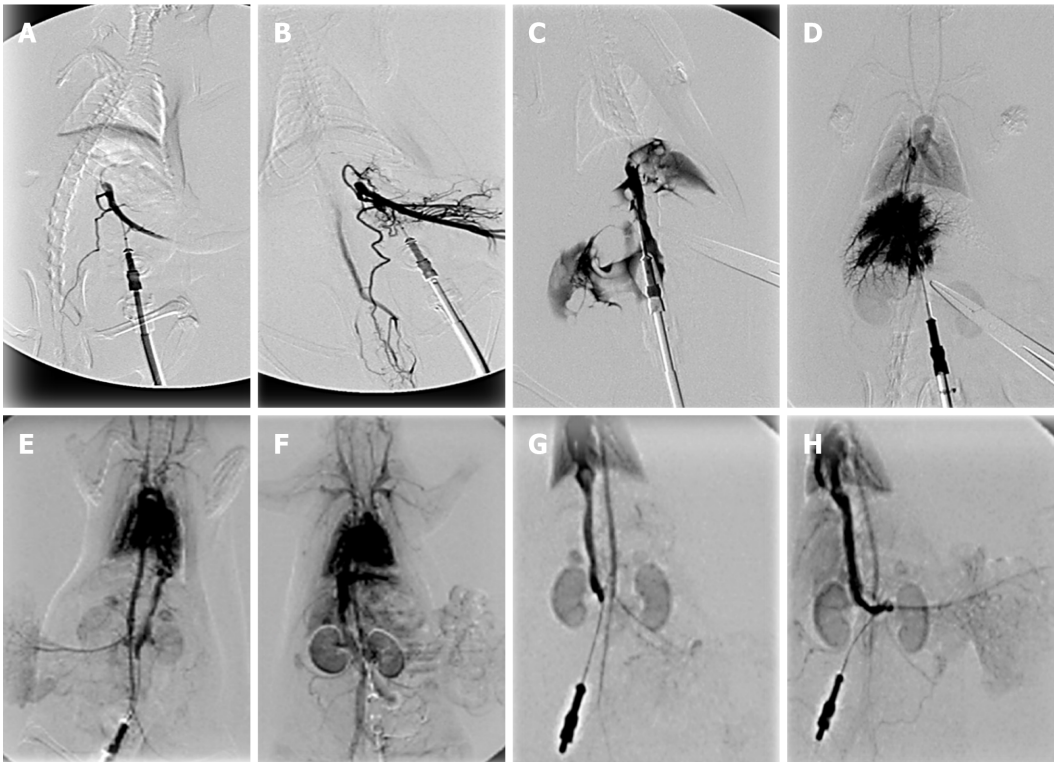

Figure 10 BPC 157 and venography assessment. Portal vein venography below obstruction. A, B: The portal vein-superior mesenteric vein-inferior superior mesenteric vein-rectal veins-left iliac vein-inferior caval vein shunt presentation in rats with a portal triad obstruction (PTO)-ligation, controls (A), BPC 157 (B); C, D: Portal vein venography up to obstruction before its entry into the liver. Presentation of filling of the hepatic venous vascular bed, hepatic vein, inferior caval vein and the right atrium of the heart in rats with a PTO-ligation, controls (C) (note, as administration of contrast continued, the portal vein ruptured), BPC 157 (D); E, F: Inferior caval vein venography at the level of bifurcation. Blood flow through the hepatic veins into the liver, and the liver presentation in rats with a PTO-ligation, controls (E), BPC 157 (F); G, H: Reperfusion with inferior caval vein venography at the level of bifurcation. Presentation at the time when liver is presented in BPC 157 rats (H), and still not presented in controls (G). Venography 15 min of ligation-time, or at 15 min of reperfusion-time a fluoroscopy unit (Shimadzu type C-Vision Plus, Tokyo, Japan), Warmed non-ionic contrast medium (Iohexol; Omnipaque 350, GE Healthcare, Chicago, United States), a total injection volume of 1 mL (0.1 mL/s) in portal vein (A-D), or 2 mL (0.3 mL/s) in inferior caval vein (E-H).

Namely, BPC 157 affects different molecular pathways noted in counteracting tumor cachexia[48], vascular occlusion[9], and various lesions (*i.e.*, skin[49,50], tendon[51,52], muscle[53]). Of note, the progressive activation of the mTOR signalling pathway is likely a pathogenic mechanism of portal hypertension, and thereby a blockade of mTOR as a possible therapy[54]. Also, a release of TNF from the liver by the injured hepatic tissue, delivered to the pulmonary circulation as the first vascular bed, could be responsible for lung lesions[55]. Therefore, it is indicative that in counteracting tumor-induced muscle cachexia, BPC 157 counteracts the expression of p-mTOR, much like it counteracts the increase of pro-inflammatory and pro-cachectic cytokines such as IL-6, TNF-α and the expression of FoxO3a, p-AKT and P-GSK-3β[48].

Thus, the administration of BPC 157 resolves the adverse effects of the Pringle maneuver. The immediate recovery of collaterals results in the bypassing of the obstruction, even in the worst conditions of PTO disturbances. Accordingly, the reversal of the signs of the right heart failure occurs. Portal hypertension markedly decreased and completely disappeared. Likely, this combined effect resolves the further circle of injuries (*i.e.*, the specific pathology in the liver, intestine, spleen, lung and heart). Specifically, the disturbed hemodynamics (*i.e.*, the severe portal hypertension and severe aortal hypotension during PTO as well as the severe portal and caval hypertension and mild aortal hypotension in reperfusion, along with refractory ascites formation), thrombosis and ischemia, perpetuated and progressed by reperfusion in the Pringle rats, would be attenuated or completely abrogated. The rapid reestablishment of blood flow in both the ischemic and reperfusion conditions accompanied the reduction of the increased MDA values in liver tissues to the normal values. Thus, these new insights into PTO and related disturbances, and into portal hypertension, suggest the effective BPC 157 use in future therapy. Otherwise, the abrupt PTO and severe portal hypertension (> 60 mmHg) [and in reperfusion, caval hypertension (> 70 mmHg)] makes spontaneous decompression of the portal system by a portocaval shunt hardly possible as well as severe aortal hypotension in the ischemia not compensated in the reperfusion. Further, these notations likely correlate

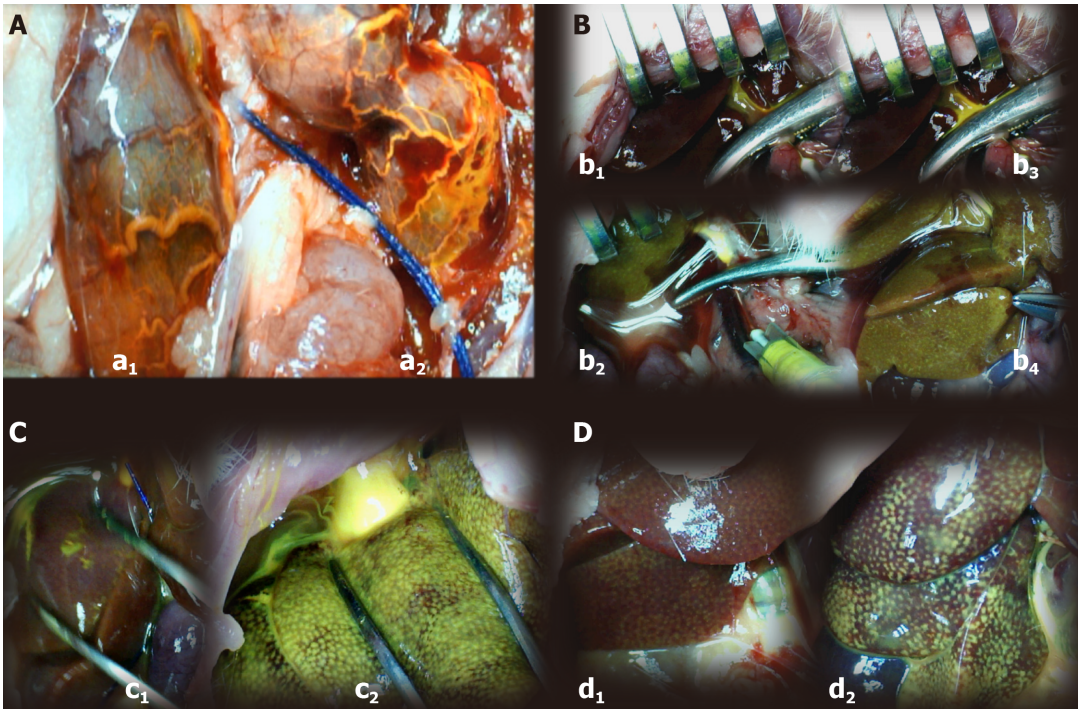

Figure 11 BPC 157 and yellow (orange) contrasts assessment. Application in portal vein below obstruction. A: The portal vein-superior mesenteric vein-inferior superior mesenteric vein-rectal veins-left iliac vein-inferior caval vein shunt presentation in rats with a portal triad obstruction (PTO)-ligation, with rectal inferior mesenteric vein with the tortuous presentation controls (a1: scored 3/4/4 Min/Med/Max)) or rectal inferior mesenteric vein with increased branching , BPC 157 [a2: scored 1/1/2 (μg), 1/1/2 (ng) Min/Med/Max, $^{a}P < 0.05$, at least *vs* control)]; B: Application in portal vein up to obstruction before its entry into the liver. Presentation of yellow contrast filling of the liver in rats with a PTO-ligation, controls [no yellow contrast in the liver (upper, b1, b3) (scored 1/1/1 Min/Med/Max)] [note, as administration of contrast continued (b3), the portal vein ruptured], BPC 157 (immediate presentation of yellow contrast (down, b2, b4) in the liver that further progressed (down, b4) scored 4/4/4 (μg), 4/4/4 (ng) Min/Med/Max, $^{a}P < 0.05$, at least *vs* control); C: Application in inferior caval vein in rats with a PTO-ligation. Presentation of the hepatic veins into the liver, and the liver presentation in rats with a PTO-ligation, controls (c1: immediate scant yellow contrast presentation in liver that would later disappear and poor presentation in hepatic veins, scored 1/1/2 Min/Med/Max)), BPC 157 (c2: immediate very abundant presentation of yellow contrast in the liver, the yellow contrast in hepatic veins abundantly seen, scored 4/4/4 (μg), 4/4/4 (ng) Min/Med/Max, $^{a}P < 0.05$, at least vs control); D: Reperfusion with yellow ink contrast application in inferior caval vein at the level of bifurcation. Presentation at the time when liver is fully presented in BPC 157 rats [d2: scored 3/4/4 (μg), 3/4/4 (ng) Min/Med/Max, $^{a}P < 0.05$, at least *vs* control)], and still not fully presented in controls (d1: scored 1/2/3 Min/Med/Max). A total injection volume of 1 mL (0.1 mL/s) in portal vein (A, B), or 2 mL (0.3 mL/s) in inferior caval vein (C, D).

with important pulmonary pathological alterations (*i.e.*, the adult respiratory distress syndrome) associated with human liver transplantation[56], portopulmonary hypertension common in cirrhosis with refractory ascites[57], as well as with a high incidence of caval hypertension in patients with portal hypertension[58].

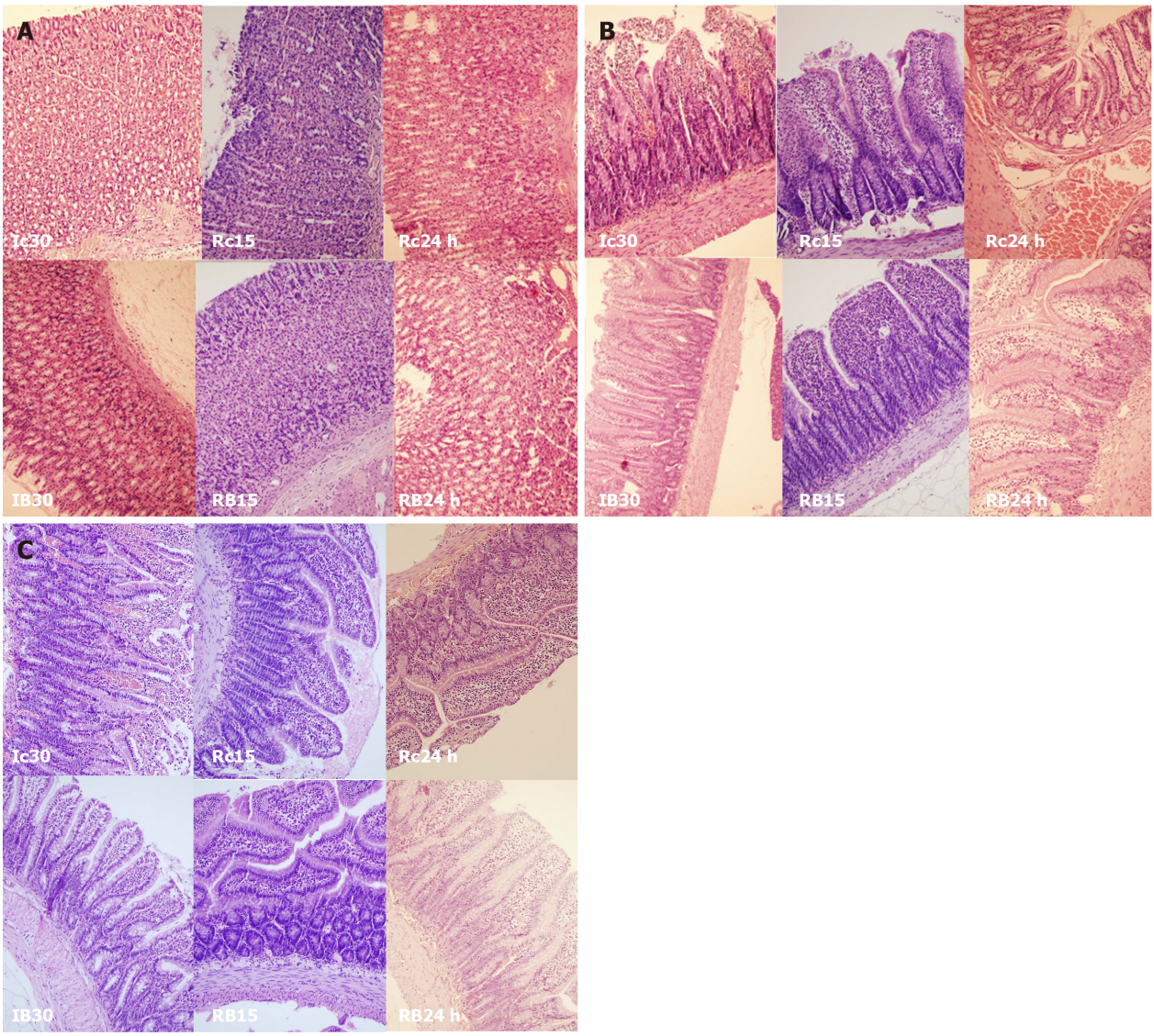

Figure 12 BPC 157 effect on gastrointestinal lesions microscopy presentation [stomach, duodenum, ileum]. A: Stomach (HE staining, × 20), Controls: Enhanced capillary congestion and erythrocytes extravasation present at the end of 30 min portal triad obstruction (PTO) ischemia period (Ic30). With reperfusion, as seen at 15 min of reperfusion the lesion progressed to enhanced capillary congestion, mild lifting of surface epithelial layer from lamina propria (Rc15). At the 24 h of reperfusion appears enhanced capillary congestion in lamina propria with erythrocytes showing ischemic changes (Rc24 h). BPC 157: Mildly villous surface, rare erythrocytes with ischemic changes present at the end of 30 min PTO ischemia period (IB30). With reperfusion, glandular architecture preserved at 15 min of reperfusion (RB15). At the 24 h of reperfusion appears only mild capillary congestion in lamina propria is seen. Architecture of glands is preserved (RB24 h). B: Duodenum (HE staining, × 20), Controls: Loss of villous architecture, loss of surface epithelium, edema of lamina propria present at the end of 30 min PTO ischemia period (Ic30). With reperfusion, the lesion progressed as seen with edema of lamina propria with extravasation of erythrocytes, some of them with ischemic changes, and focal loss of surface epithelium at 15 min of reperfusion (Rc15). At the 24 h of reperfusion appear enhanced capillary congestion, focal erythrocytes showing ischemic changes (Rc24 h). BPC 157: Mild capillary congestion in lamina propria is seen with edema of lamina propria. Extravasation of erythrocytes, elevation of surface epithelium from lamina propria at the end of 30 min PTO ischemia period (IB30). Villous architecture is preserved. Edema of lamina propria with extravasation of erythrocytes and reparatory changes of epithelium. Mild capillary congestion in lamina propria is seen at 15 min of reperfusion (RB15). At the 24 h of reperfusion villous architecture is preserved. Edema of lamina propria (RB24 h); C: Ileum (HE staining, × 20), Controls: Loss of villous architecture, enhanced elevation of surface epithelium from lamina propria with loss of surface epithelium, edema of lamina propria, marked capillary congestion present at the end of 30 min PTO ischemia period (Ic30). With reperfusion, edema of lamina propria with extravasation of erythrocytes. Some of them with ischemic changes. Mild lymphocytic infiltrate. Focal elevation of surface epithelium from lamina propria, at 15 min of reperfusion (Rc15). At the 24 h of reperfusion appear enhanced capillary congestion, focal erythrocytes showing ischemic changes (Rc24 h). BPC 157: Mild capillary congestion in lamina propria is seen with mild edema of lamina propria. Mild elevation of surface epithelium from lamina propria. Some lymphocytes infiltrating lamina propria at the end of 30 min PTO ischemia period (IB30). Villous architecture is preserved, edema of lamina propria with extravasation of erythrocytes and mild lymphocytic infiltrate is seen at 15 min of reperfusion (RB15). At the 24 h of reperfusion mild capillary congestion in lamina propria is seen, villous architecture is preserved, edema of lamina propria (RB24 h).

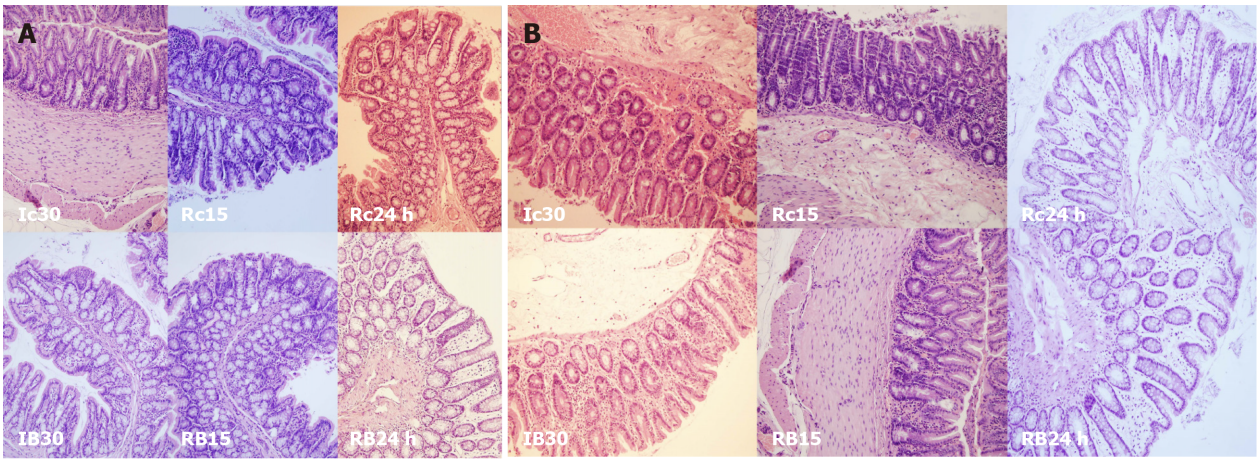

Figure 13 BPC 157 effect on gastrointestinal lesions microscopy presentation (cecum, colon). A: Cecum (HE staining, × 20), controls: Increased capillary congestion and edema of lamina propria. Extravasation of erythrocytes some of them with ischemic changes. Present at the end of 30 min portal triad obstruction (PTO) ischemia period (Ic30). With reperfusion, at 15 min of reperfusion mild capillary congestion with just focal loss of surface epithelium. Mild infiltrate of lymphocytes (Rc15). At the 24 h of reperfusion appear enhanced capillary congestion, focal erythrocytes showing ischemic changes (Rc24 h). BPC 157: Mild capillary congestion in lamina propria is seen with mild edema of lamina propria at the end of 30 min PTO ischemia period (IB30). Mild capillary congestion in lamina propria is seen at 15 min of reperfusion (RB15). At the 24 h of mild capillary congestion in lamina propria is seen. Edema of lamina propria (RB24 h); B: Colon (HE staining, × 20), controls: Increased capillary congestion and edema of lamina propria, extravasation of erythrocytes some of them with ischemic changes, focal loss of surface epithelium present at the end of 30 min PTO ischemia period (Ic30). With reperfusion, at 15 min of reperfusion mild capillary congestion with just focal loss of surface epithelium (Rc15). At the 24 h of reperfusion appear enhanced capillary congestion, focal erythrocytes showing ischemic changes (Rc24 h). BPC 157: Mild capillary congestion in lamina propria with mild edema of lamina propria at the end of 30 min PTO ischemia period (IB30). Mild capillary congestion in lamina propria is seen at 15 min of reperfusion (RB15). At the 24 h of mild capillary congestion in lamina propria is seen. Edema of lamina propria (RB24 h).

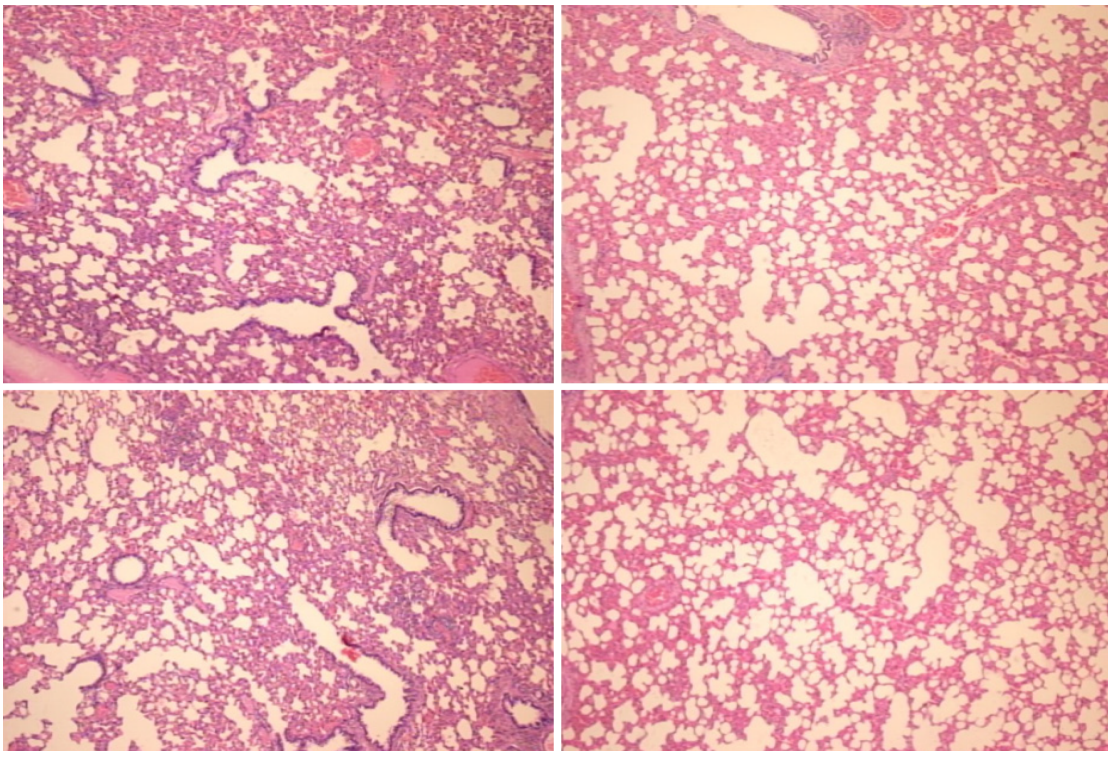

Figure 14 Lung (HE staining, × 4). Portal triad obstruction-rats, 24 h reperfusion (control, left; BPC 157 right). While architecture was preserved, there was in portal triad obstruction-rats some edematous exudate in alveoli and progressing capillary congestion, mild to severe in alveolar septa, which counteraction was clearly seen at 24 h of reperfusion.

ARTICLE HIGHLIGHTS

Research background

The Pringle maneuver (portal triad obstruction) provides huge disturbances during ischemia

and even more thereafter in reperfusion. Contrarily, a possible solution may be stable gastric pentadecapeptide BPC 157, with already documented beneficial effects in ischemia/reperfusion conditions. Recently, BPC 157, as a cytoprotective agent, successfully resolved vessel occlusions in rats (ischemic/reperfusion colitis; deep vein thrombosis, superior anterior pancreaticoduodenal vein; bile duct cirrhosis) through rapid collateral vessel recruitment to circumvent vessel occlusion. Likely, as a new effect, BPC 157 application may be useful when applied in the ischemia condition much like when given in the reperfusion condition. Thereby, medication BPC 157 regimens were administered as a single challenge before and during ischemia or, alternatively, at various time points during reperfusion.

Research motivation

We focused on the therapy of the Pringle maneuver in rats, so far not described severe preportal hypertension, the temporary portal triad obstruction (PTO), ischemia, the short and prolonged reperfusion, the lack of adequate portocaval shunting as the most detrimental feature that should be counteracted. With stable gastric pentadecapeptide BPC 157, we suggest the resolution of the damages, either those following occlusion or those following re-opening of the hepatic artery, portal vein and bile duct.

Research objectives

The first objective in the PTO-syndrome in rats is the rapidly activated way, portal vein-superior mesenteric, vein-inferior mesenteric vein-rectal vein-left iliac vein-inferior caval vein, supposed to appear as a specific activation of the collateral circulation, as the bypassing loop that can rapidly circumvent occlusions and decompress PTO-rats upon BPC 157 administration. The additional objective is verification that that solution in Pringle-rats with ischemia and reperfusion goes with resolution of the whole syndrome. In this, there are the resolution of oxidative stress, hemodynamic disturbances, severe portal and caval hypertension, aortic hypotension, rapid cloth formation in the portal vein, superior mesenteric vein, lienal vein, inferior caval vein, hepatic artery, ascites, peaked P waves, tachycardia; increased serum values; gross intestine, liver, lung, spleen and heart lesions. The final objective is demonstration that it goes also with the agent application during reperfusion.

Research methods

In the Pringle-rats with the PTO occluded or reopen, assessment includes gross (USB camera) and microscopic observations, venography, blood pressure and electrocardiogram assessment, bilirubin and enzyme activity, levels of nitric oxide, malondialdehyde in the liver. With the mentioned methods, assessed was the activated pathway, portal vein-superior mesenteric, vein-inferior mesenteric vein-rectal vein-left iliac vein-inferior caval vein. Then, we assessed the resolution of the oxidative stress, hemodynamic disturbances, severe portal and caval hypertension, aortic hypotension, rapid cloth formation in the portal vein, superior mesenteric vein, lienal vein, inferior caval vein, hepatic artery, ascites, peaked P waves, tachycardia; increased enzymes serum values; gross intestine, liver, lung, spleen and heart lesions.

Research results

BPC 157 counteracts electrocardiogram disturbances (increased P wave amplitude, S1Q3T3 QRS pattern and tachycardia). BPC 157 administration rapidly presented portal vein-superior mesenteric vein-inferior mesenteric vein-rectal veins-left ileal vein-inferior caval vein vascular pathway as the adequate shunting. As evidenced, this vascular pathway recovery means the immediately recovered disturbed hemodynamic. Portal hypertension and severe aortal hypotension during PTO, as well as portal and caval hypertension and mild aortal hypotension in reperfusion and refractory ascites formation were markedly attenuated (during PTO) or completely abrogated (reperfusion); thrombosis in portal vein tributaries and inferior caval vein or hepatic artery was counteracted during PTO. Likewise, the whole vicious injurious circle was counteracted [*i.e.*, lung pathology (severe capillary congestion), liver (dilated central veins and terminal portal venules), intestine (substantial capillary congestion, submucosal edema, loss of villous architecture), splenomegaly, right heart (picked P wave values)], otherwise regularly perpetuated in ischemia and progressed by reperfusion in Pringle rats.

Research conclusions

BPC 157 resolves Pringle maneuver in rats, both for ischemia and reperfusion.

Research perspectives

The reported evidence that the administration of BPC 157 resolves the adverse effects of the Pringle maneuver means the immediate recovery of collaterals, which results in the bypassing of the obstruction, even in the worst conditions of PTO disturbances. The key importance of this therapy effect verifies the reversal of the signs of the right heart failure much like the severe portal hypertension and severe aortal hypotension during PTO as well as the severe portal and caval hypertension and mild aortal hypotension in reperfusion, along with refractory ascites formation, thrombosis and ischemia markedly decreased and completely disappeared. The rapid reestablishment of blood flow in both the ischemic and reperfusion conditions accompanied the reduction of the increased malondialdehyde values in liver tissues to the normal values. Likely, this combined effect resolves the further circle of injuries (*i.e.*, the specific pathology in the liver, intestine, spleen, lung and heart). Thus, these new insights into Pringle maneuver and related disturbances, and into portal hypertension, suggest the effective BPC 157 use in future therapy. Otherwise, the abrupt PTO and severe portal hypertension (> 60 mmHg) [and in reperfusion, caval hypertension (> 70 mmHg)] makes spontaneous decompression of the portal system by a

portocaval shunt hardly possible as well as severe aortal hypotension in the ischemia not compensated in the reperfusion.

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ORIGINAL ARTICLE

Retrospective Study

Drug and herbal/dietary supplements-induced liver injury: A tertiary care center experience

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Author contributions: Siddique AS and Ligato S designed the study, collected and reviewed the cases and wrote the manuscript; Siddique O, Einstein M and Urtasun-Sotil E contributed to the design, analysis and final revision of the manuscript.

Institutional review board statement: This study was approved by the IRB at Hartford Hospital.

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Abstract

BACKGROUND

Drug-induced liver injury (DILI) and herbal/dietary supplements (HDS) related liver injury present unique diagnostic challenges. Collaboration between the clinician and the pathologist is required for an accurate diagnosis and management.

AIM

To report our experience on the clinical-pathological findings of hepatic injury caused by drugs/HDS.

METHODS

A retrospective review of clinically proven cases of DILI/HDS who presented to our institution from January 1, 2013 to December 31, 2017 was performed. Slides were reviewed for histopathological patterns of injury and correlated with the causative agent. Out of 600 patients presenting with unexplained rise in liver enzymes undergoing biopsy, 107 were suspected to have DILI/HDS. Of these, 53 had a directly linked exposure to drug/herbal supplements. Fifteen patients were excluded for concurrent known liver disease. Thirty-eight patients with clinically proven DILI/HDS were finally included.

RESULTS

Thirty-eight cases of DILI/HDS with a male:female of 1:1.5 and mean age of 51 ± 3 years were identified. DILI was identified in 84.2% cases while HDS injury in 15.8%. Acute hepatitis (42.1%) was the most common pattern of injury while granulomatous hepatitis (2.6%) was the least common. We found one case of acute-cholestasis due to rivaroxaban and two cases of cholestatic-hepatitis due to rizatriptan and trimethobenzamide-hydrochloride that, to the best of our knowledge, have not been previously reported. One case of steatohepatitis due to trimethoprim-sulfamethoxazole and three unusual cases of cholestatic-hepatitis

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with bile duct injury and steatosis due to dronedarone, C4-Extreme and hydroxycut, were also seen. Of our cohort, 81.6% of the patients fared well with discontinuation of drug and 18.4% underwent transplant; of which 42.9% were deceased.

CONCLUSION
We describe the clinical findings, histopathological patterns of injury and clinical outcomes caused by drugs. In particular, we report a few previously unreported/rarely observed clinical and histopathological patterns of hepatic injury.

Key words: Drug-induced liver injury; Herbals; Dietary supplements; Liver enzymes; Biopsy; Supplements

Core tip: This a retrospective study to evaluate the clinicopathological patterns of drug and herbal/dietary supplement related injury with only reports of proven cases from a large cohort of patients. We describe many unusual patterns of injury as well as newer drugs/herbals causing previously unreported patterns of injury. Drug/Herbal dietary supplements related liver injury is a well-reported topic, but it is important to report newer patterns in this changing era of medicine.

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INTRODUCTION

Drug-induced liver injury (DILI) and herbal/dietary supplements (HDS) related liver injury (DILI/HDS) represent a rare idiosyncratic reaction with potentially devastating clinical outcomes[1]. It may present unique diagnostic challenges, and require a close collaboration between the clinician and the pathologist for an accurate diagnosis and management[1,2]. The true incidence of DILI/HDS is challenging to discern due to difficulties in gathering and arbitrating cases in a quantitative way. However, Icelandic data from 2013 suggests an estimated annual incidence of 19.1 per 100000[3]. The DILI network of the United States reported 300 idiosyncratic DILI cases in 2007 of which 8% died, 2% required a transplant and 14% had continued liver test abnormalities at 6 mo[4]. Additionally idiosyncratic DILI/HDS accounts for 11% of the cases of acute liver failure in the United States[5]. The United States Food and Drug Administration have issued a black box warning and safety alerts for a number of drugs that have been implicated in serious liver injury[6]. Despite these data, new reports on DILI/HDS keep emerging contributing to the annual 23 billion dollar expenditure on hepatitis alone in the United States[7].

For the clinician, the assessment of the presence, pattern and degree of hepatic injury starts by determining the liver function tests (LFTs), and the markers of hepatocellular function (albumin, prothrombin time and bilirubin). The R ratio helps to classify the liver injury into hepatocellular, cholestatic and mixed patterns of injury[8,9]. On the other end, the pathologist by examining the liver biopsy provides information on the histopathological pattern of injury and its severity. Finally and ideally, by a multidisciplinary approach, the clinician and pathologist correlate all the clinical and pathological data, and after exclusion of other causes of liver injury (such as viral hepatitis or autoimmune diseases), the determination of the likelihood of DILI/HDS and the possible drugs or HDS responsible for the damage is made.

The purpose of this study was to report our experience on the clinical-pathological findings including patterns of hepatic injury and its severity caused by drugs and HDS identified in our tertiary care center during the last five years. Additionally, we report a few previously unreported/rarely observed cases of DILI/HDS describing their clinico-pathologic findings at presentation and clinical outcomes.

MATERIALS AND METHODS

Study material

A retrospective review was done of all patients who presented at our institution with unexplained rise in liver enzymes and subsequently underwent a biopsy from January 1, 2013 to December 31, 2017. Patients who were suspected to have DILI/HDS were included. Medical records were reviewed for a rigorous evaluation of exposure to drug/herbal supplements and only those patients who had a directly linked exposure were accepted for the study. The age, gender and liver enzyme results were obtained, and R ratio scoring was calculated on each case. Strict exclusion criteria were used after review of medical records, and patients with concurrent history of known liver disease such as viral hepatitis, autoimmune hepatitis (AIH), Wilson's disease, alcoholic liver disease, non-alcoholic fatty liver disease were excluded. Figure 1 summarizes the inclusion criteria of patients for this study.

The biochemical classification of the DILI/HDS was performed using the R ratio[8,9] defined as [Alanine aminotransferase (ALT)/upper limit of normal]/[Alkaline phosphatase (ALP)/upper limit of normal]. An R > 5.0 was considered as an expression of hepatocellular injury, R < 2.0 was consistent with cholestatic injury and R 2.0-5.0 was considered as an expression of a mixed pattern of injury.

The causal factors of the patterns of injury were subdivided into anti-infective agents, analgesics, psychotropic medications, antineoplastic, antilipidemic, immunomodulatory agents, HDS and others. The drugs and/or HDS were then analyzed according to the pattern of injury.

Histopathology

In each case, slides were independently reviewed by two pathologists (Siddique AS and Ligato S) for assessment of the histopathological pattern of injury and the results subsequently correlated with the clinical findings and the potential drug or HDS responsible for the hepatic injury. The histopathological patterns of injury were divided into necroinflammatory pattern (hepatitis), cholestatic injury pattern, combined cholestatic hepatitic pattern, steatotic injury pattern and mixed injury pattern.

Necroinflammatory pattern was subdivided into acute hepatitis (AH), acute hepatitis with extensive necrosis, AIH, chronic hepatitis (CH) and granulomatous hepatitis (GH). AH and CH patterns were similar to viral hepatitic patterns consisting of lobular-dominant inflammation in AH and portal predominant inflammation in CH cases. Acute hepatitis with extensive necrosis was characterized by confluent or coagulative necrosis of hepatocytes, with variable amounts of inflammation. These cases often revealed massive necrosis with collapse of parenchyma between the portal areas. Areas of regeneration or ongoing injury patterns were also observed in some of these cases. AIH cases displayed moderate to severe portal inflammation with interphase necroinflammatory activity with abundant plasma cells and eosinophils along with hepatocyte rosettes and emperipolesis. GH was characterized by non-necrotizing epithelioid granulomas in the parenchyma and portal areas.

Cholestatic injury pattern was subdivided into acute cholestasis (AC) and chronic cholestasis (CC). AC cases displayed canalicular or hepatocellular bile accumulation while CC cases showed cholate stasis along with copper accumulation. Duct injury was often seen in these cases with secondary biliary ductular proliferation. Mixed cholestatic hepatitis (MCH) demonstrated a combination of various patterns of hepatocellular injury, inflammation and cholestasis.

Steatotic injury pattern included small and large droplet macrovesicular steatosis with varying degrees of hepatocellular injury and inflammation. Mixed pattern of injury was a mixture of various degrees of cholestatic pattern, steatotic pattern and hepatitic patterns.

Histochemical and immunohistochemical methods

Histochemical stains included trichrome, Periodic Acid–Schiff–diastase, iron and reticulin stain. Trichrome stain helped establish fibrosis. Periodic Acid–Schiff–diastase highlighted the multiple aggregates of pigmented laden macrophages with ceroid material within the cytoplasm suggestive of ongoing necroinflammatory activity. Iron stain demonstrated accumulation of iron in the Kupffer cells and/or hepatocytes. Reticulin displayed the architecture of liver plates such as expansion in regenerative conditions, compression in nodular regenerative hyperplasia and collapse in necrosis. Immunohistochemical stain for cytokeratin 7 helped highlight bile duct loss/damage and secondary biliary ductular proliferation.

Statistical analysis

The mean, median, ranges, percentages of the age, gender, liver function enzymes,

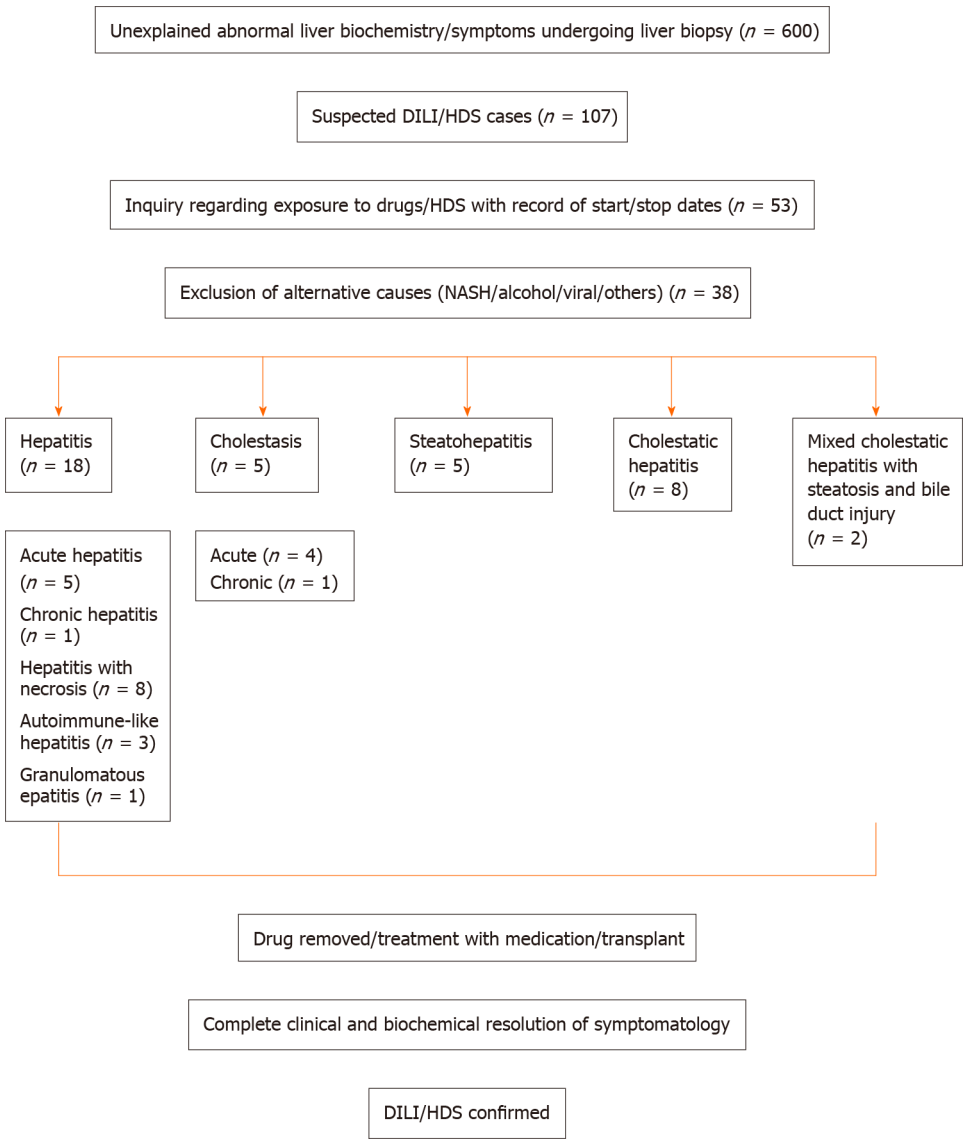

Figure 1 Inclusion criteria for our study. HDS: Herbal dietary supplements; NASH: Non-alcoholic steatohepatitis; DILI/HDS: Drug/herbal dietary supplement induced liver injury.

patterns of injury, causative agents were done on Microsoft Excel 2010 (Seattle, WA, United States) and so was the R ratio.

The statistical methods were reviewed by the Biostatistics Department at Hartford Hospital.

This study was performed under a protocol approved by the Institutional Review Board of Hartford Hospital.

RESULTS

Clinical features

Out of 600 patients presenting with unexplained rise in liver enzymes from January 1, 2013 to December 31, 2017, 107 patients were suspected to have DILI/HDS due to polypharmacy. Of these, 53 patients had a recent change in use of drug/herbal supplement explaining a directly linked exposure. Fifteen patients were excluded since they had a history of known liver disease alongside the possible concurrent drug injury. Thirty-eight cases of DILI/HDS were identified with the help of a liver biopsy with a male:female ratio of 1:1.5; and mean age of 51 ± 3 years. The initial ALT at the time of presentation ranged from 12 to 3555 (median 432) U/L. The initial ALP ranged from 66 to 802 (median 178) U/L. The initial aspartate aminotransferase levels ranged from 27 to 1925 (median 310) U/L. The total bilirubin at presentation ranged from 0.4 to 36.7 (median 5.7) mg/dL. The mean platelet count at presentation was 230000 ±

170000. The clinical features of the group subdivided by the histopathological pattern of injury are summarized in Table 1. As displayed in Table 2, a variety of drug categories were seen as causative agents of each pattern of injury including anti-infective agents, analgesics, psychotropic agents, antineoplastic, antilipidemic, immunomodulatory agents, HDS and others. Analgesics were found to be the most common (38.9%) causative agents of hepatocellular injury while anti-infective agents (26.7%) were the most common causal factors of mixed pattern of injury.

Biochemical and histopathological correlation was done for all the patients and an R ratio > 5 was identified in cases with a hepatitic histopathological pattern of injury, an R ratio < 2 corresponded to a cholestatic histopathological pattern and an R between 0.4 and 12.7 was observed in cases with mixed histopathological patterns listed in Table 3.

In our cohort, 32 (84.2%) patients had DILI and 6 (15.8%) had HDS. The unique cases in our study included one patient with rivaroxaban related injury who presented with an ALT of 76 U/L, ALP of 214 U/L, R 0.9, total bilirubin of 6.8 mg/dL and a platelet count of 123000/μL. Another patient with rizatriptan related injury presented with an ALT of 147 U/L, ALP of 135 U/L, R 2.7, total bilirubin of 1.2 mg/dL and a platelet count of 132000/μL. Trimethobenzamide hydrochloride related DILI in our patient presented with an ALT of 630 U/L, ALP of 135 U/L, R 11.4, total bilirubin of 1.5 mg/dL and a platelet count of 350000/μL. One patient who used trimethoprim-sulfamethoxazole (TMP-SMX) presented with an ALT of 152 U/L, ALP of 198 U/L, R 1.9, total bilirubin of 10.9 mg/dL and a platelet count of 116000/μL. DILI caused by dronedarone in our patient presented with an ALT of 93 U/L, ALP of 477 U/L, R 0.5, total bilirubin of 3.3 mg/dL and a platelet count of 114000/μL. C4 extreme related injury presented with ALT of 3384 U/L, ALP of 175 U/L, R 49.5, total bilirubin of 22.5 mg/dL and a platelet count of 144000/μL. Cholestatic hepatitis caused by hydroxycut presented with ALT of 344 U/L, ALP of 500 U/L, R 1.7, total bilirubin of 0.7 mg/dL and a platelet count of 350000/μL. The features are listed in Table 4.

The HDS agents identified in our study were: Black cohosh (2 cases), hydroxycut (2 cases), green tea extract (1 case) and C4 extreme (1 case). Black cohosh, green tea extract and C4 extreme displayed an R ratio of greater than 5 while hydroxycut displayed an R ratio of less than 2.

Histopathology

Histopathological patterns of injury and corresponding causative drugs/HDS are summarized in the Table 3. Hepatitic pattern was observed in 18 (47.4%). AH was the most common histopathological pattern of injury caused by nitrofurantoin, sertraline, lamotrigine, diclofenac and acetaminophen. AH with autoimmune-like features was caused by infliximab and isoniazid. CH was caused by nitrofurantoin. GH was the least common pattern of injury caused by Bacilli Calmette Guerin (BCG).

Cholestatic pattern of injury was found in 5 (13.2%) of the cases. AC was caused by ceftriaxone, methimazole, total parenteral nutrition, and rivaroxaban. CC was caused by triazolam and statins.

Mixed patterns 15 (39.5%) included macrovesicular steatohepatitis 5 (13.2%), cholestatic hepatitis 8 (21.1%) and MCH with steatosis and bile duct injury 2 (5.3%). The causal factors for steatohepatitis were TMP-SMX (Figure 2), L-asparaginase, valproic-acid, highly-active-antiretroviral-therapy and amiodarone. Drugs causing cholestatic hepatitis included ezetimibe, cefazolin, rizatriptan, methyldrostanolone, trimethobenzamide hydrochloride (Figure 3), C4 extreme (Figure 4) and mesalamine. MCH with steatosis and bile duct injury was caused by dronedarone.

In our cohort amongst HDS, black cohosh and green-tea extract caused AH while two cases of hydroxycut (Figure 5) displayed cholestatic hepatitis and an MCH with steatosis and bile duct injury (Table 4) respectively.

Follow-up

The clinical course and follow-up of all the patients with confirmed DILI/HDS was available. Thirty-one (81.5%) patients fared well with discontinuation of drug and/or treatment with N-acetyl cysteine/prednisone, while 7 (18.4%) underwent transplant due to severe acute liver injury. Patients with this AH with extensive necrosis were found to be mainly secondary to acetaminophen toxicity and less commonly black cohosh use; they had the worst outcome requiring transplant; of which 3 (42.9%) were deceased.

DISCUSSION

In this retrospective study we investigated the clinical and histopathological patterns

Table 1 Clinical features of patients with DILI/HDS according to the histopathological pattern of injury

	Hepatocellular	Cholestatic	Mixed
n (%)	18 (49)	5 (14)	15 (39)
Age (mean ± SD)	50 ± 8	65 ± 5	49 ± 18
Gender (M:F)	5:13	3:2	7:8
Initial ALT (mean ± SD)	870 ± 280	37 ± 32	470 ± 190
Initial AST (mean ± SD)	780 ± 110	44 ± 54	360 ± 600
Initial ALP (mean ± SD)	190 ± 93	300 ± 25	220 ± 91
R ratio (mean ± SD)	12 ± 0.4	0.5 ± 0.1	6.4 ± 0.9
Total bilirubin (mean ± SD)	5.5 ± 0.4	4.3 ± 4.5	3.7 ± 1.5
Platelet count/μL (mean ± SD)	230000 ± 92000	230000 ± 130000	220000 ± 31000

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase.

of DILI/HDS at a single institution over a period of five years. The majority of the histopathological patterns of injury identified in our cohort were consistent with those previously reported in the literature[10] with 32 (84.2%) cases demonstrating DILI and 6 (15.8%) HDS. The histopathological spectrum of hepatic injury identified in our cohort was similar to those identified in other studies on DILI[10] and included: AH, CH, AC, CC and cholestatic hepatitis. Kleiner *et al*[10] reported that 83% of their DILI cases could be classified into one of the above patterns. In our analysis, AH (*n* = 16; 45.7%) was the most common histopathological pattern of injury while GH (*n* = 1; 2.6%) was the least common.

Analgesics were found to be the most common (*n* = 7; 38.9%) causative agents of hepatocellular injury while antimicrobial agents (*n* = 4; 26.7%) were the most common causal factors of mixed pattern of injury. Antimicrobials are the most frequent etiologic agents of DILI in the United States accounting for 45% of the cases[11] and analgesic induced DILI is a well-studied topic specifically those caused by acetaminophen[12]. Acetaminophen amongst analgesics is the most common single cause of acute liver failure in western nations with severe cases requiring liver transplantation[13]. In our study 15.8% of all DILI was caused by acetaminophen alone of which 5 (83.3%) patients underwent transplant. Additionally, acetaminophen-opiate or opioid combinations are declining in popularity but were responsible for at least 50% of serious acetaminophen related liver injury cases in the United States[13] compared to only 6 (15.8%) patients with acetaminophen related injury in our study. While most DILI cases are idiosyncratic, acetaminophen toxicity is dose related. In subtoxic cases, the toxic metabolite of acetaminophen (N-acetyl-p-benzoquinone imine) is rendered harmless by conjugation with glutathione, abundantly available within the hepatocytes. However, once the glutathione stores are depleted during higher levels of acetaminophen intake, fasting, malnutrition or chronic alcohol ingestion, N-acetyl-p-benzoquinone imine binds to cysteine residues in cellular proteins and causes cell necrosis, subsequently elevating aspartate aminotransferase and ALT levels in the blood[14]. The cell necrosis also releases acetaminophen adducts that can be measured by liquid chromatography[15] and recent immunoassays appear to be promising in detecting these adduct in acute liver failure[16,17].

Another interesting finding was GH secondary to BCG treatment for urothelial carcinoma. Disseminated BCG disease is a rare but life-threatening complication of BCG administration with symptoms similar to that of tuberculosis infection, including persistent fever, night sweats and weight loss[18]. The pathogenesis is thought to involve a combination of mycobacteraemia and local inflammatory hypersensitivity at various sites including the liver[18]. Our case of non-necrotizing GH secondary to BCG along with previously reported cases[18] emphasizes the significance of a complete history and clinicopathologic correlation of diagnosing DILI.

In addition, we identified some unusual patterns of hepatic injury including one case of cholestatic hepatitis with bile duct injury and steatosis due to dronedarone, and one case of steatohepatitis due to TMP-SMX. Dronedarone is a well-known antiarrhythmic agent and DILI related to it has been rarely reported. Most cases however, describe a hepatocellular pattern of injury similar to amiodarone unlike our case[19,20]. TMP-SMX is a fixed antibiotic combination widely used for mild to moderate bacterial infections and has been reported for cholestatic or mixed patterns of injury[21,22]. Our case was unique in that we identified steatohepatitis (Figure 2) similar to type-2 non-alcoholic steatohepatitis often identified in children. Since alcoholic

Table 2 Causative agents summarized according to the histopathological pattern of injury

Drugs	Hepatocellular (*n* = 18)	Cholestatic (*n* = 5)	Mixed (*n* = 14)
Anti-infective agents	3	1	4
Analgesics	7	0	0
Psychotropic agents	2	0	0
Antineoplastic agents	1	0	1
Antilipedemic agents	0	1	1
Immunomodulatory agents	2	0	0
Others	0	Antithyroid: 1; Thrombolytic: 1; Total parenteral nutrition: 1	Triptan: 1; Antiemetic: 1; Methyldrostanolone: 1; Anti-inflammatory: 1; Antiarrythmic: 2
Herbal/Dietary supplements	3	0	3

liver disease and Non-alcoholic steatohepatitis were excluded in all our cases and the injury improved with removal of drug and symptomatic treatment, the diagnosis of DILI was confirmed.

Furthermore, we report two cases of cholestatic hepatitis, and one of AC that, to the best of our knowledge, have not been previously reported in the literature. The two cases of cholestatic hepatitis were secondary to the use of trimethobenzamide hydrochloride (Tigan) and rizatriptan (Maxalt). The patient with trimethobenzamide hydrochloride related DILI presented with altered liver function and a histopathological pattern displayed in Figure 3. The LFTs downtrended after 60 d of removal of drug. Trimethobenzamide hydrochloride was introduced in 1959 as an antinauseant-antiemetic. Chemically unrelated to the phenothiazines it is structurally closer to the dimethylaminoethoxy antihistamines. It was widely used prophylactically and therapeutically in vomiting induced by drugs, radiation therapy, infection, and motion sickness[23]. One case of hepatitis secondary to trimethobenzamide hydrochloride was seen in 1967[24]. However, no recent data with detailed histopathological pattern of injury are available; in this report we provide detailed histopathological findings caused by this drug. Rizatriptan belongs to a group of serotonin receptor agonists that are useful in the therapy of vascular headaches and migraine. Cholestatic hepatitis secondary to triptans specifically rizatriptan has been unproven in the literature[25,26]. Our case presented with elevated liver enzymes and moderate to severe AH associated with cholestasis and bile duct damage on histology. LFTs downtrended after 45 days of drug removal. Lastly, the case of AC was caused by rivaroxaban (Xarelto) and displayed a histopathological pattern characterized by acute hepatocellular and canalicular cholestasis associated with mild steatohepatitis. LFTs downtrended after 15 d of drug removal. Rivaroxaban is a recently introduced oral anticoagulant and direct Factor Xa inhibitor. DILI secondary to rivaroxaban has been described as early as the preclinical licensure trials, but the details of patterns of injury and severity of disease are still lacking[27,28]. Hence it is important to report histopathology and outcome in such cases in order to become familiar with the newer patterns of injury.

One interesting case of C4 extreme presented with cholestatic hepatitis with sub massive necrosis. C4 extreme is an HDS and has the synephrine-containing *Citrus aurantium* extract. Synephrine is an analog of ephedra, which has previously been associated with liver toxicity[29]. However, DILI from C4 extreme in particular has just been reported in one series to date[30]. Our case resulted in a 24-year-old patient undergoing transplant (Table 4) displaying the significance of reporting such cases.

The diagnosis of DILI/HDS presents a daunting task for the clinician and pathologist alike. However, the collaboration of the clinician and pathologist in obtaining a complete medical and drug/alternative medication history along with the assessment of the histopathological findings and the review of previously reported cases in the literature (with the help of online resources such as "Livertox") can help guide the way to a prompt and successful management of a DILI/HDS affected patient.

In conclusion, we present a five-year experience on the diagnosis of DILI/HDS in our institution. Despite the final inclusion being limited to 38 proven cases of DILI/HDS, we reported previously unreported, or rarely observed, drug-induced histopathological patterns of hepatic injury, reaffirming the pivotal role of the clinician and pathologist, in the diagnosis and care of patients with DILI/HDS.

Table 3 Histopathological patterns, R ratios and Drugs/HDS in our study

Histopathological pattern	R ratio	Total, *n* = 38 (%)	Drugs/HDS (*n*)
Hepatitis	12 ± 0.4	18 (47.4)	
Acute	16 ± 0.1	5 (13.2)	Nitrofurantoin (1)
			Black cohosh[1] (1)
			Sertraline (1)
			Lamotrigine (1)
			Green tea extract (1)
Acute with extensive necrosis	11 ± 3.3	8 (21.1)	Diclofenac (1)
			Acetaminophen (6)
			Black cohosh[1] (1)
Autoimmune-like	6.2 ± 0.1	3 (7.9)	Infliximab (2)
			Isoniazid (1)
Chronic	8.8	1 (2.6)	Nitrofurantoin (1)
Non-necrotizing granulomatous hepatitis	14	1 (2.6)	BCG (1)
Cholestasis	0.5 ± 0.1	5 (13.2)	
Acute	0.4 ± 0.5	4 (10.5)	Ceftriaxone (1)
			Methimazole (1)
			Total parenteral nutrition (1)
			Rivaroxaban (1)
Chronic	0.8	1 (2.6)	Triazolam and statin (1)
Cholestatic hepatitis	9.0 ± 5.6	8 (21.1)	Ezetimibe (1)
			Cefazolin (1)
			Hydroxycut[2] (1)
			Rizatriptan[5] (1)
			Methyldrostanolone (1)
			Trimethobenzamide Hydrochloride[5] (1)
			Mesalamine (1)
			C4 extreme[3] (1)
Macrovesicular steatohepatitis	1.4 ± 1.2	5 (13.2)	Trimethoprim-sulfamethoxazole[6] (1)
			L-asparaginase (1)
			Valproic acid (1)
			Haart[4] (1)
			Amiodarone (1)
Mixed cholestatic hepatitis with steatosis and bile duct injury	1.1 ± 0.8	2 (5.3)	Dronedarone[6] (1)
			Hydroxycut[26] (1)

[1]Actaea racemosa or cimicifuga racemose.
[2]Contains caffeine, lady's mantle extract (Alchemilla vulgaris), wild olive extract (Olea europaea), cumin extract (Cuminum cyminum), wild mint extract (Mentha longifolia), and, in some products, green coffee bean extract (Coffea canephora).
[3]CarnoSyn Beta Alanine to support muscular endurance, TeaCrine to prolong tolerance buildup, Rauwolfia, and Toothed Clubmoss.
[4]Highly active antiretroviral therapy.
[5]Not previously reported.
[6]Unusual patterns for this drug/HDS. HDS: Herbal/Dietary supplements; BCG: Bacillus Calmette Guerin.

Table 4 Clinical features, histopathological patterns and outcomes of patients presenting with previously unreported drugs/HDS or with unusual pattern of injury

Cases	ALT (U/L)	ALP (U/L)	R ratio	Total Bilirubin (mg/dL)	Platelet count (/μL)	Histopathological pattern	Outcome
Rivaroxaban	76	214	0.9	6.8	123000	Acute hepatocellular and canalicular cholestasis associated with bile duct damage, mild steatohepatitis with portal, periportal and pericentral fibrosis (stage 2 of 4), and nodular regenerative hyperplasia	LFTs downtrended after 15 d of drug removal
Rizatriptan	147	135	2.7	1.2	132000	Moderate to severe acute hepatitis with associated cholestasis and bile duct damage	LFTs downtrended after 45 d of drug removal
Trimethobenzamide hydrochloride	630	135	11.4	1.5	350000	Cholestatic hepatitis with portal and lobular mononuclear inflammatory infiltrate, bile duct damage and bile ductular proliferation	LFTs downtrended after 60 d of drug removal
Trimethoprim-sulfamethoxazole	152	198	1.9	10.9	116000	Panacinar macrovesicular steatosis/mild steatohepatitis, cholestasis and mild portal tract fibrous expansion. (steatohepatitis necroinflammatory grade I; fibrosis stage 0-1)	LFTs downtrended after 90 d of drug removal
Dronedarone	93	477	0.5	3.3	114000	Pericentral hepatocyte and canalicular cholestasis, bile duct injury with bile ductular proliferation, mixed portal inflammatory infiltrates, mild interface hepatitis, lobular necroinflammatory activity with ballooning degeneration; mild macro and microvesicular steatosis	LFTs downtrended after 85 d of drug removal

C4 Extreme	3384	175	49.5	22.5	144000	Severe cholestatic hepatitis with sub massive hepatic necrosis involving approximately 70% of the liver parenchyma accompanied by severe centrilobular congestion, necrosis and extravasation of red blood cells	Underwent transplant; Alive and doing well
Hydroxycut	344	500	1.7	0.7	350000	Cholestatic hepatitis with bile duct injury and steatosis	LFTs downtrended after 45 d of drug removal

LFTs: Liver function tests; HDS: Herbal dietary supplements; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase.

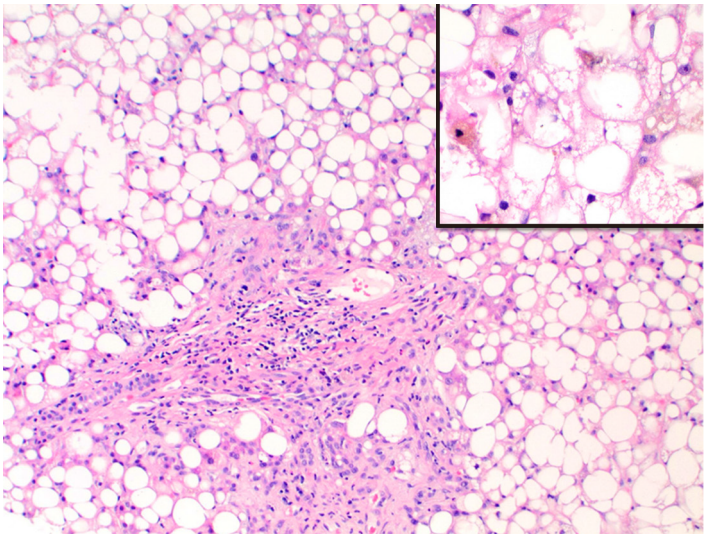

Figure 2 Liver injury secondary to Trimethoprim sulfamethoxazole. Liver core biopsy with marked pan-acinar macrovesicular steatosis and periportal fibrosis with mild portal chronic inflammation (20 ×). Additionally, hepatocellular cholestasis is observed in the insert (40 ×).

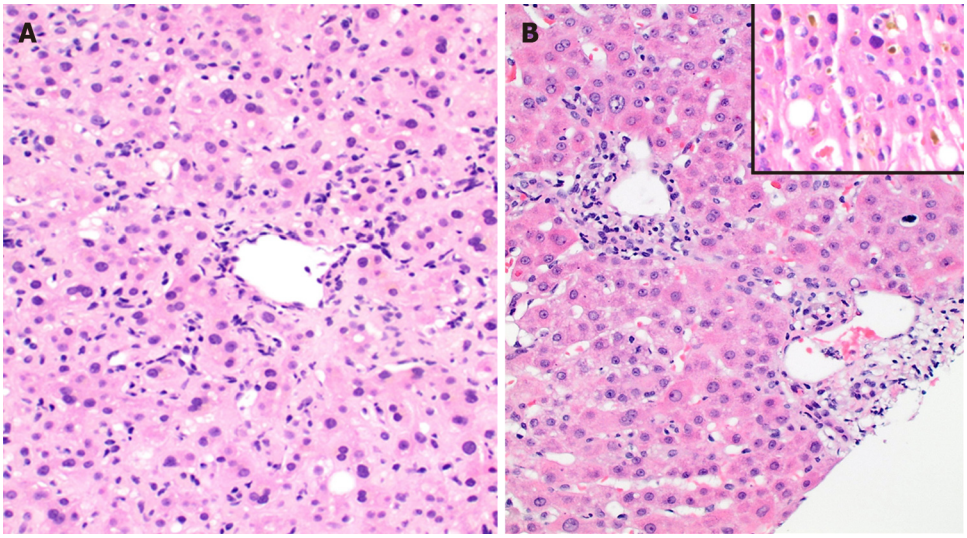

Figure 3 Liver injury secondary to trimethobenzamide hydrochloride. A: Acute cholestatic hepatitis characterized by pericentral and lobular necrotic inflammation (40 ×); B: Bile duct damage and canalicular cholestasis (insert) (40 ×).

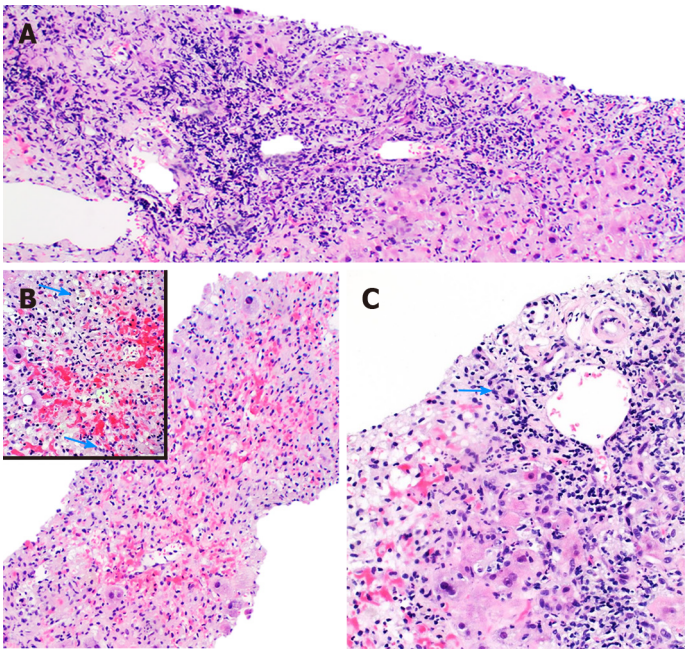

Figure 4 Liver injury secondary to C4 extreme. A: The liver parenchyma demonstrates a dense mononuclear inflammatory infiltrate involving the portal tracts with severe interface necroinflammatory activity (10 ×); B: In the pericentral and midzonal area of the hepatic lobules (zone 2 and 3), there is extensive hemorrhagic necrosis with extravasation of red blood cells from the central vein into the sinusoidal spaces (10 ×). Hepatocellular and canalicular cholestasis is seen (arrow) (insert 40 ×); C: The portal areas display extensive bile duct damage (arrow) (20 ×).

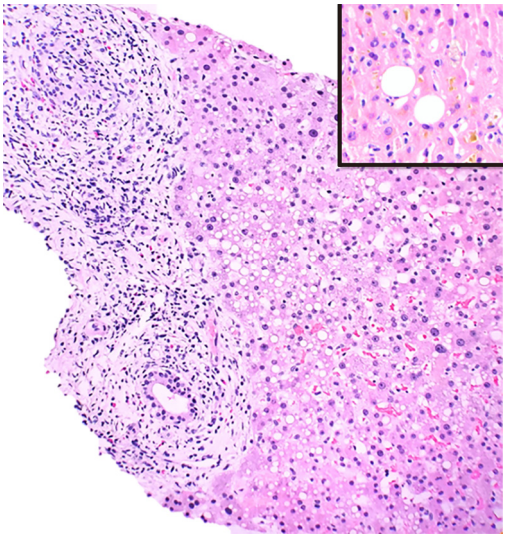

Figure 5 Liver injury secondary to hydroxycut. Liver parenchyma with expansion of the portal areas by a mixed inflammatory infiltrate comprising lymphocytes, eosinophils, rare plasma cells and scattered neutrophils. There is evidence of bile duct injury with infiltration of the bile duct epithelium by inflammatory cells (20 ×). The lobules show macrovesicular steatosis, feathery degeneration of hepatocytes, minimal lobulitis, and focal cholestasis (insert 40 ×).

ARTICLE HIGHLIGHTS

Research background

Drug-induced liver injury (DILI) is a continuously evolving theme especially with the availability of over the counter medications and herbal/dietary supplements (HDS). The annual expenditure in the United States of hepatitis alone is 23 billion dollars and 11% of all cases of acute liver failure are related to DILI. These statistics alone highlight the importance of reporting the course of DILI cases and new confirmed cases with complete clinicopathological correlation. We believe these findings will be of interest to the readers of your journal.

Research motivation

The main idea behind this study is to continuously report previously unreported but proven cases of DILI/HDS and their patterns of injury for hepatologists and pathologists to consider

while dealing with such cases.

Research objectives
The purpose of this study was to report our experience on the clinical-pathological findings including patterns of hepatic injury and its severity caused by drugs and HDS identified in our tertiary care center during the last five years.

Research methods
A retrospective review of clinically proven cases of DILI/HDS who presented to our institution from January 1, 2013 to December 31, 2017 was performed. Slides were reviewed for histopathological patterns of injury and correlated with the causative agent. Out of 600 patients presenting with unexplained rise in liver enzymes undergoing biopsy, 107 were suspected to have DILI/HDS. Of these, 53 had a directly linked exposure to drug/herbal supplements. Fifteen patients were excluded for concurrent known liver disease.

Research results
Thirty-eight cases of DILI/HDS with a male:female of 1:1.5 and mean age of 51 ± 3 years were identified. DILI was identified in 84.2% cases while HDS injury in 15.8%. Acute hepatitis (42.1%) was the most common pattern of injury while granulomatous hepatitis (2.6%) was the least common. We found one case of acute-cholestasis due to rivaroxaban and two cases of cholestatic-hepatitis due to rizatriptan and trimethobenzamide-hydrochloride that, to the best of our knowledge, have not been previously reported. One case of steatohepatitis due to trimethoprim-sulfamethoxazole and three unusual cases of cholestatic-hepatitis with bile duct injury and steatosis due to dronedarone, C4-extreme and hydroxycut, were also seen. Of our cohort, 81.6% of the patients fared well with discontinuation of drug and 18.4% underwent transplant; of which 42.9% were deceased.

Research conclusions
We report our experience on the hepatic injury caused by drugs and herbals at a tertiary care center including clinical findings, histopathological patterns of injury and clinical outcomes caused by these agents. In particular, we report on a few previously unreported/rarely observed clinical findings and histopathological patterns of hepatic injury caused by specific drugs and herbals. This implies that reporting of newer drugs/HDS and their related injury is important in this constantly changing era of medicine. Despite using a large cohort, we report only the proven cases of DILI/HDS increasing the impact of this study on clinical practice.

Research perspectives
This study highlights a topic that has been addressed several times in the past but with the upcoming drugs/herbals it is important to continually work as a team of clinician and pathologist and report newer cases. Future prospective studies might reassure our findings further since these haven't been reviewed in the past 10 years.

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ORIGINAL ARTICLE

Retrospective Study

Usefulness of Mac-2 binding protein glycosylation isomer in non-invasive probing liver disease in the Vietnamese population

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Author contributions: Pham TTT and Ho DT conceived the investigative study and recruited patients; Nguyen T performed all experimental runs; Pham TTT, Ho DT and Nguyen T analyzed the data and prepared the manuscript.

Institutional review board statement: The experimental procedure strictly follows the ethical codes of conduct as laid out by the Declaration of Helsinki. Institutional review board approval was waived as residual blood was used.

Informed consent statement: Our research follows strictly the Declaration of Helsinki for ethical compliance. Residual blood samples were used for biomarker evaluation and informed consent was waived. No patient identifiers were used and standard of care accorded to patients was not affected by the results of this study.

Conflict-of-interest statement: All authors declare no conflicts of interest.

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Abstract

BACKGROUND

Early diagnosis is critical for successful intervention before liver disease progresses to cirrhosis and hepatocellular carcinoma.

AIM

To examine a novel biomarker for probing early liver disease quickly using an automated immunology system.

METHODS

This was a cross-sectional study. 140 patients at various stages of liver disease were randomly selected. The cohort consisted of patients who were treatment naïve and currently undergoing therapy. We included patients with diverse liver disease etiologies. Mac-2 binding protein glycosylation isomer (M2BPGi) levels in addition to different clinical parameters, co-morbidities and transient elastography results were collected and compared.

RESULTS

M2BPGi levels were significantly correlated with transient elastography for liver fibrosis staging across all disease etiologies. Statistically significant differences were observed in patients with F0-1; F2 and > F3 liver fibrosis. Further examination showed that M2BPGi levels were two-fold higher in F4 than F3 hepatitis C (HCV) patients. M2BPGi was observed to be etiology-specific and HCV patients had higher mean M2BPGi levels. We also observed significant correlations with aspartate aminotransferase to platelet ratio index and fibrosis-4 index as well as HBV DNA levels. Mean M2BPGi levels for HBV patients with a viral load lower than 2000 IU/mL was 1.75-fold lower than those with a viral load greater than 2000 IU/mL.

CONCLUSION

M2BPGi was observed to be a good indicator of early liver disease in patients with different etiologies. Our results provide reference cut-offs for different causes of liver disease and demonstrated the utility of this marker for early

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disease monitoring. This is useful for remote regions in developing countries.

Key words: Hepatitis B; Hepatitis C; Noninvasive fibrosis markers; Mac-2 binding protein glycosylation isomer; Liver disease

Core tip: Mac-2 binding protein glycosylation isomer levels can be used for non-invasive liver fibrosis staging in the Vietnamese population with mixed etiologies. In early evaluations, significantly higher levels of this marker were observed in cirrhotic patients and showed good correlations with viral load testing in hepatitis B. This marker is convenient and useful, especially in resource limited countries for fast turnaround to assess liver disease.

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INTRODUCTION

Chronic liver disease resulted in a high global burden of 1.5 billion people in 2017, which was mostly due to non-alcoholic fatty liver disease (NAFLD, 60%), hepatitis B (HBV, 29%), hepatitis C (HCV, 9%), and alcoholic liver disease (ALD, 2%)[1]. Chronic liver disease progressively leads to liver fibrosis, cirrhosis, and finally hepatocellular carcinoma (HCC). Globally, liver disease accounts for 2 million deaths per year, of which 1 million are due to cirrhosis and 1 million are due to hepatocellular carcinoma[2]. Vietnam has a high prevalence of hepatitis B and C, with a high HBV surface antigen (HBsAg) rate of 8.8%-19.0% and a high anti-HCV rate of 1.0%-3.3%[3]. It is estimated that HBV-related liver cancer and HCC will increase to 9400 and 25000, respectively, and HBV-related mortality will increase to 40000 in 2025 in Vietnam[4]. Given the disease impact in Vietnam, efforts to provide early detection of liver disease are critical.

Early diagnosis and treatment are key to improve disease outcomes as it can halt further liver disease progression and save lives. At present, pan-genotypic drug combinations of direct acting antivirals such as sofosbuvir/velpatasvir and glecaprevir/pibrentasvir have demonstrated high efficacy against different HCV genotypes[5]. Long-term HBV antivirals have been effective in managing the disease[6]. Nonetheless, most patients are in a chronic state and different degrees of liver fibrosis have manifested. Management of these patients requires clear understanding of their liver fibrosis staging[7,8]. At present, liver biopsy is considered the gold standard for liver fibrosis staging. However, liver biopsy has its limitations such as sampling bias and inter-observer variations[9]. In addition, liver biopsy is invasive and it is not feasible to carry out repeated liver biopsies for regular monitoring. In advanced stage liver disease patients (> F3), a 3-6 monthly evaluation is required. In response to these limitations, non-invasive tests using imaging methods such as transient elastography (TE)[10], magnetic resonance elastography[11], ultrasound-based elastography[12], and acoustic radiation force impulse[13] have emerged. Serum biomarkers have also been explored. These included hyaluronic acid, type IV collagen, type III procollagen-N-peptide[14], soluble Axl[15], and lincRNA-p21[16] among others. Surrogate markers (aspartate aminotransferase to platelet ratio index (APRI)[17], and Fibrosis-4 index (FIB-4)[18] are also some of the commonly used tests to assess liver disease. A number of these tests require costly equipment, and patient results turnaround and waiting times may limit their usefulness. On the other hand, other tests lack sufficient clinical sensitivity and specificity.

In recent years, Mac-2 binding protein glycosylation isomer (M2BPGi) was identified in patients with liver fibrosis[19]. Serum M2BPGi levels were found to correlate with liver fibrosis stage[20], reflect impaired liver function or cirrhosis and hepatectomy-related complications after surgery[21], correlate with liver stiffness[22], and was negatively correlated with sustained virological response (SVR)[23]. In

addition to liver fibrosis in HCV and HBV, M2BPGi can be used to assess liver fibrosis in primary biliary cirrhosis[24], biliary atresia[25], primary sclerosing cholangitis[26], and NAFLD[27]. In this study, we examined M2BPGi as a biomarker for early monitoring of liver disease in a Vietnamese population. We compared M2BPGi levels with different clinical parameters, co-morbidities and TE results. M2BPGi was measured from blood samples using a sandwich immunoassay. The aim of the current study was to examine this novel diagnostic biomarker for probing early liver disease quickly and easily using an automated immunology system.

MATERIALS AND METHODS

Study design and patient demographics

A cross-sectional study design was employed in this study. Institutional review board approval was waived as residual blood samples were used in the evaluation. The results of this study did not alter the course of treatment in these patients. Other than patient history and current laboratory results, no other patient identifiers were used. We randomly selected patients with diverse liver disease etiologies who were treated in MEDIC Medical Center, Ho Chi Minh City, Vietnam from January to April 2019. Patients with confirmed liver cancer were excluded. Routine clinical investigations as part of standard of care included liver function tests, TE and other associated fibrosis measurements. A total of 140 patient samples were analyzed.

Liver disease probing and measurements

As part of standard of care, TE (Fibroscan, Echosens, France) was performed on all patients. Staging of liver fibrosis in patients was based on the METAVIR scoring system (F0 to F4, where F0: No fibrosis and F4: Cirrhosis) and cut-offs adopted from manufacturer's recommendations. Plasma and serum blood samples were taken from each patient for complete blood count and clinical biochemistry investigations. Liver function tests including alanine aminotransferase (ALT), aspartate aminotransferase (AST) alkaline phosphatase (ALP), albumin and bilirubin were measured using a routine laboratory chemistry analyzer (Roche, Switzerland). APRI was calculated using the following equation: AST (/upper limit of normal) × 100/platelet count (PLT) (× 10^9/L)[17]. FIB-4 was calculated using the following equation: FIB-4 = age (years) × AST (U/L)/[PLT (10^9/L) × ALT (U/L)$^{1/2}$][18]. PLT was obtained using a hematology analyzer among other blood parameters (Sysmex, Japan). Serum M2BPGi levels were measured on the HISCL 5000 automated immunoassay analyzer (Sysmex, Japan). 10 μL of sample were used and M2BPGi levels were measured by a sandwich immunoassay. Each reaction took 17 min and was performed automatically alongside HBsAg and anti-HCV assays. M2BPGi was expressed as the cut-off index (COI) and calibrated using the manufacturer's calibrators.

Statistical analysis

All categorical variables were expressed as mean ± SD. Student *t* tests were used to compare two categorical variables and one-way analysis of variance (ANOVA) was used to compare multiple groups of liver fibrosis stages. A difference of $P < 0.05$ was considered statistically significant. Analysis was computed using Prism version 7.0 (Graphpad Inc., United States).

RESULTS

Patient characteristics and experimental design

Patient demographics are shown in Table 1. A total of 140 patients were enrolled. This was a cross-sectional study involving randomly selected patients with diverse liver disease etiologies to assess the versatility of the marker M2BPGi. All patients were treated in the MEDIC Medical Center and assessed by their attending physicians for liver disease stage. The median age of the study cohort was 52 years and all suffered from chronic hepatitis. Among the patient group, the number of male and female patients was almost equal (52.9% *vs* 47.1%, respectively). Most of the selected patients were HBV or HCV patients, of which around 60% were HBV patients, and 2 were patients co-infected with HBV and HCV. The HBV and HCV cohorts, 35% and 13%, respectively, had immediate family members with the same condition or had progressed to HCC. In Vietnam, HBV is the predominant cause of HCC and was critical in this study. The range of TE, APRI, FIB-4 and M2BPGi measurements were 3–46.4 kPa, 0.07–7.02, 0.23–9.97 and 0.07- > 20 COI, respectively. Among the 140 patients, 35% of patients had higher than normal AST, ALT or Gamma glutamyl

transferase. Alpha fetoprotein (AFP) measurements were obtained in these patients as part of an early indication of HCC. Only 1 patient (112 ng/mL) in this cohort had higher than normal AFP level.

M2BPGi levels were correlated with other fibrosis markers in HCV patients

As shown in Figure 1A, M2BPGi levels increased with increasing fibrosis stages (as measured by TE) in HCV patients. The results were statistically significant when comparing the F2 cohort with F0-1 subjects. Patients with significant fibrosis require regular monitoring and this provides a quick method to stratify patient groups for clinicians. We also observed significantly more patients with severe fibrosis or cirrhosis than patients with normal liver in HCV positive individuals. In an attempt to further evaluate the discriminatory role of M2BPGi, we divided patients with > F3 fibrosis into the F3 and F4 groups (Figure 1B). The results showed a statistically significant difference in M2BPGi levels between F3 and F4 patients. The cirrhotic group (F4) had two-fold higher M2BPGi levels. These data supported the accurate staging of liver fibrosis using M2BPGi levels. More data are required to ensure statistical power for medical decisions; however, preliminary data demonstrated strong evidence of a clear discriminatory role. Within this cirrhotic patient group, some patients were currently undergoing treatment and some were treatment naïve. M2BPGi levels were observed to be insignificant between these two groups (Figure 1C). Previous reports have shown that M2BPGi may be affected by the treatment for viral hepatitis. In a comparison of fibrosis staging methods, we selected only treatment-naïve patients (Figure 2) to ensure an unbiased correlation. As shown in Figure 2, we found that M2BPGi levels showed good correlation with TE (A), FIB-4 (B), and APRI (C) in untreated patients ($P < 0.001$).

M2BPGi levels were correlated with other fibrosis markers in HBV patients

Similar to the results in the HCV group, we observed significant correlations in HBV patients. The limitation of the current study was that the distribution among this cohort leaned towards the early liver disease group with fewer severe fibrosis and cirrhosis patients. Nonetheless, from Figure 3A, it can be seen that M2BPGi levels increased with increasing fibrosis stage. The maximum detected M2BPGi level in patients with > F3 fibrosis staging was 4.0 COI with a mean value of 2.0 COI. This was more than 2-fold higher than early stage patients (F0-1) with a mean M2BPGi level of -0.8 COI. Compared with a similar early disease group in HCV patients, the mean levels were significantly lower in the HBV cohort indicating the need for etiology-specific cut-offs using M2BPGi. Further division of > F3 patients into F3 and F4 groups showed the same trend as previously seen in the HCV cohort (Figure 3B). More datapoints are required for better statistical comparison in advanced stage patients. In the same cohort, we attempted to understand if M2BPGi correlated with HBV DNA levels. HBV viral load is a required parameter during treatment monitoring especially in addressing treatment discontinuation and efficacy. From Figure 4, it can be seen that the mean M2BPGi level for hepatitis B patients with a viral load of < 2000 IU/mL was 1.75-fold lower than the group with a viral load greater or equal to 2000 IU/mL.

M2BPGi levels are etiology-specific for early liver disease

During early analysis, it was observed that M2BPGi levels were significantly different among the HBV and HCV cohorts within the same fibrosis stages. To address these differences further, the patient cohort was divided into HCV, HCV with NAFLD, HBV, HBV with NAFLD, NAFLD, and ALD as shown in Figure 5 for early disease patients (F0-1). ANOVA showed significant differences among these major groups. Viral hepatitis related liver disease alone resulted in higher M2BPGi levels compared with alcoholic liver disease ($P < 0.01$) and NAFLD ($P < 0.01$). Interestingly we did not observe any statistical significance in viral hepatitis patients with NAFLD comorbidity (HCV *vs* HCV and NAFLD; HBV *vs* HBV and NAFLD). These findings show that M2BPGi levels may be dominated by the presence of viral hepatitis in patients. Overall, we found that in early disease patients, M2BPGi levels were highest in HCV groups, followed by HBV and subsequently alcoholic liver disease and finally NAFLD cases.

DISCUSSION

Non-invasive tests in probing liver disease are gaining attention as liver biopsy is insufficient in addressing critical disease management roles. These methods are also more patient friendly and allow repeated sampling. However, equipment investment is not easy for medical facilities in remote regions of Vietnam in relation to advanced

Table 1 Evaluated subjects' demographics and corresponding liver fibrosis marker results

Parameters		Parameter value
Sample size (*n*)		140
Age (yr)	Range (mean ± SD)	26-81 (52 ± 16)
Gender, *n* (%)	Male	74 (52.9%)
	Female	66 (47.1%)
Etiology, *n* (%)	HBV	54
	HBV + NAFLD	31
	HCV	37
	HCV + NAFLD	13
	HCV + HBV	2
	NAFLD	1
	Alcoholic	2
M2BPGi (COI)	Minimum	0.065
	Median	0.863
	Maximum	> 20
TE (kPa)	Minimum	3
	Median	6.4
	Maximum	46.4
APRI	Minimum	0.0737
	Median	0.2997
	Maximum	7.0192
FIB-4	Minimum	0.2328
	Median	1.119
	Maximum	9.9664

HBV: Hepatitis B virus; HCV: Hepatitis C virus; NAFLD: Non-alcoholic fatty liver disease; M2BPGi: Mac-2 binding protein glycosylation isomer; TE: Transient elastography; COI: Cut-off index; APRI: Aspartate aminotransferase to platelet ratio index; FIB-4: Fibrosis-4 index.

elastography methods. Hence, it is crucial to have a quick and straightforward method to detect and stratify liver disease. Therefore, serum biomarkers derived from automated analyzers which are easy to interpret without skilled operators are desirable.

The results of the present study showed that M2BPGi levels were correlated with fibrosis stage derived from TE measurements and positively correlated with other surrogate markers (FIB-4 and APRI). This concurs with other studies[28,29]. However, no correlation was found between M2BPGi levels in treatment naïve and treated cirrhotic HCV patients. Treatment failure is typically associated with cirrhotic cases and the current results may be representative of this phenomenon. Ura *et al*[30] found that SVR is negatively correlated with M2BPGi levels, highlighting that treatment response may be reflected in serial M2BPGi measurements. Cirrhotic patients remain a key target group for treatment and surveillance, of which M2BPGi may be useful. One limitation of our study is the small sample size of cirrhotic patients (n = 16), which may be insufficient to demonstrate the effects of anti-viral treatment on M2BPGi levels in cirrhotic patients. Future studies will examine the effects of cirrhosis regression on M2BPGi levels in hepatitis patients, and patients can be further stratified based on time of onset of cirrhosis[31], which affects regression.

In this study other than METAVIR-based staging, we found that HBV viral load was correlated with M2BPGi levels, showing that M2BPGi may be used as a surrogate marker for HBV viral load and disease severity. In fact, some studies have shown that M2BPGi level is a predictor of HCC and death[32], HBV-related HCC recurrence[33], and liver function and prognosis[34]. Hence, M2BPGi may complement HBV viral load testing to better understand disease severity and prediction of disease outcomes. Several studies have shown that other than liver fibrosis staging, M2BPGi level is a predictor of HCC after SVR[35], HCC survival[36], HCC recurrence[37], and cirrhosis survival[38].

M2BPGi is etiology-specific and our results showed that M2BPGi levels are higher in HCV than HBV patients, which is consistent with Nishikawa *et al*[39] and patients with viral hepatitis have higher M2BPGi levels than those with NAFLD and ALD.

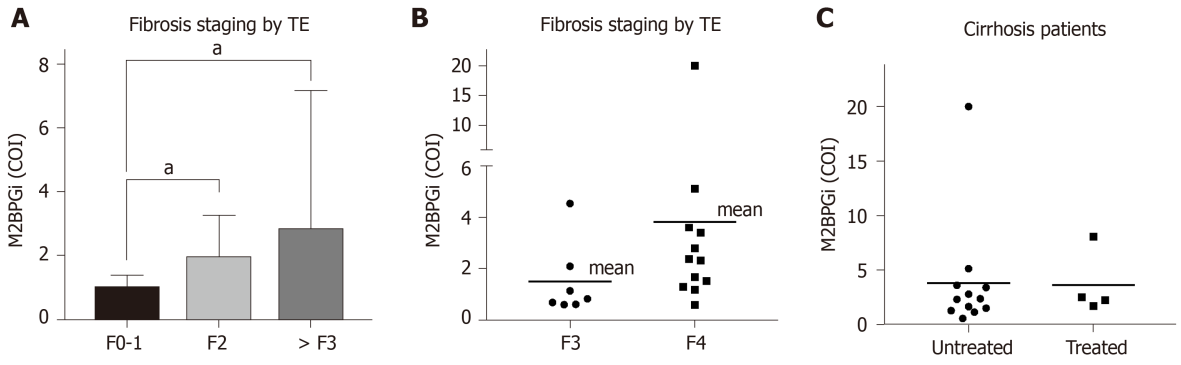

Figure 1 Comparison of Mac-2 binding protein glycosylation isomer levels against fibrosis staging by transient elastography in hepatitis C patients. M2BPGi levels were plotted against fibrosis stages based on transient elastography. A: Mac-2 binding protein glycosylation isomer (M2BPGi) levels against fibrosis stages (F0-1, F2, and >F3); B: M2BPGi levels against fibrosis stages (F3 and F4); C: M2BPGi levels in treated and untreated cirrhosis patients. [a]$P < 0.05$. M2BPGi: Mac-2 binding protein glycosylation isomer; COI: Cut-off index; TE: Transient elastography.

Therefore, different cut-off values should be assigned to different types of patients. The mechanism of this difference in M2BPGi level between HBV and HCV patients remains unclear, but our observational study showed that M2BPGi is dominated by the presence of viral hepatitis. With larger sample sizes in future investigations, we will be able to establish a clear disease-based cut-off to implement M2BPGi in routine clinical use.

The present study demonstrated the feasibility of using M2BPGi level as a surrogate marker for liver fibrosis in a Vietnamese population. This method is convenient, easy to use, and can guide prevention and treatment efforts for viral hepatitis in remote regions. At present, there is no national program against HBV and HCV in Vietnam[3] and mortality due to liver cancer is high in Vietnam[40]. In contrast, M2BPGi tests are reimbursable in South Korea and Japan. In future, national programs for treating HCV and HBV could be rolled out along with such tests to reduce the prevalence of liver fibrosis, cirrhosis, and HCC in Vietnam and thereby reduce mortality.

In conclusion, this study is the first to investigate mixed etiology testing of this marker and demonstrated a significant correlation with existing routine assays and treatment monitoring. Our results provide reference cut-offs for different causes of liver disease and demonstrated the utility of this marker for early disease monitoring. This is useful for remote regions in developing countries.

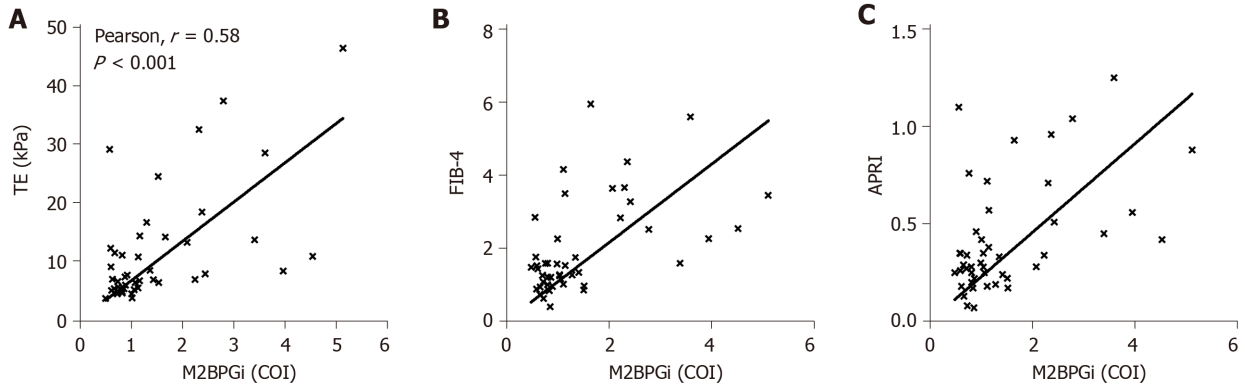

Figure 2 Comparison of Mac-2 binding protein glycosylation isomer levels against other fibrosis markers in treatment naïve patients. A: Mac-2 binding protein glycosylation isomer (M2BPGi) levels against transient elastography; B: M2BPGi levels against FIB-4; C: M2BPGi levels against aminotransferase to platelet ratio index. M2BPGi: Mac-2 binding protein glycosylation isomer; COI: Cut-off index; TE: Transient elastography; FIB-4: Fibrosis-4 index; APRI: Aspartate aminotransferase to platelet ratio index.

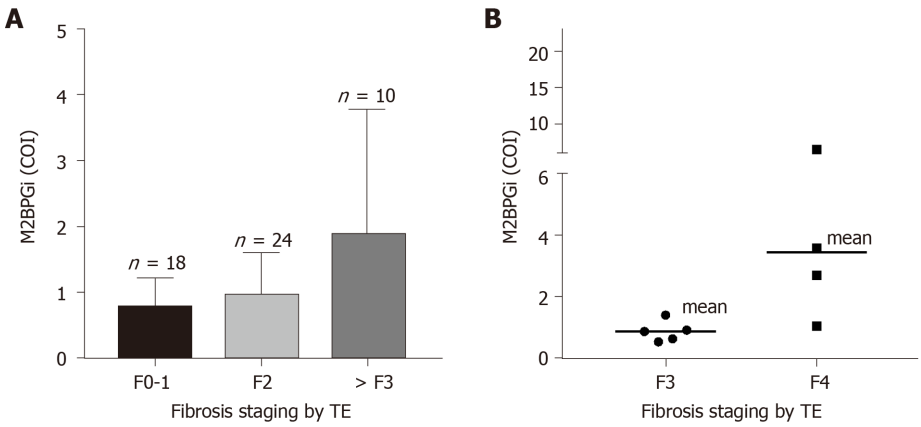

Figure 3 Comparison of Mac-2 binding protein glycosylation isomer levels against fibrosis staging by transient elastography in hepatitis B patients. A: Mac-2 binding protein glycosylation isomer (M2BPGi) levels against fibrosis stages (F0-1, F2, and > F3); B: M2BPGi levels against fibrosis stages (F3 and F4). M2BPGi: Mac-2 binding protein glycosylation isomer; COI: Cut-off index; TE: Transient elastography.

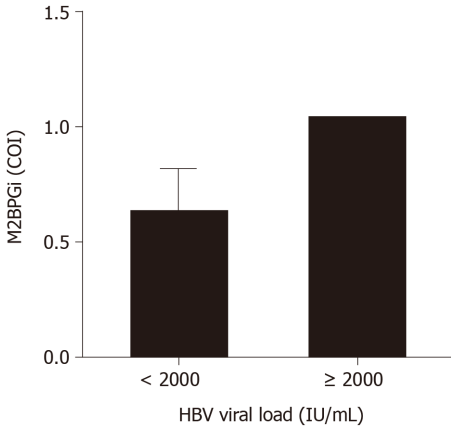

Figure 4 Comparison of Mac-2 binding protein glycosylation isomer levels against hepatitis B viral load. M2BPGi: Mac-2 binding protein glycosylation isomer; COI: Cut-off index; HBV: Hepatitis B virus.

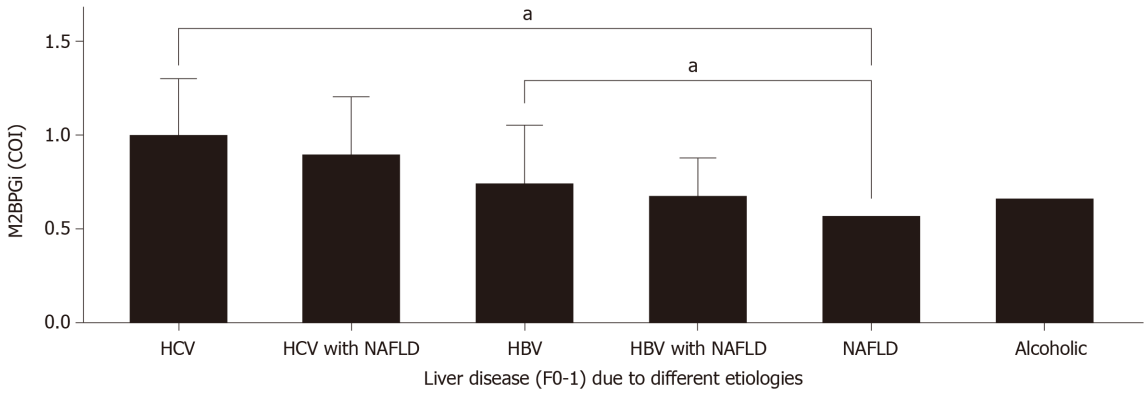

Figure 5 Mac-2 binding protein glycosylation isomer levels in early liver disease (F0-1) due to different etiologies. [a]*P* < 0.01. M2BPGi: Mac-2 binding protein glycosylation isomer; COI: Cut-off index; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NAFLD: Non-alcoholic fatty liver disease.

ARTICLE HIGHLIGHTS

Research background

Non-invasive and rapid testing of liver disease for chronic hepatitis patients is crucial given its high prevalence in Vietnam.

Research motivation

Liver disease can be managed properly with timely treatment and control. Mac-2 binding protein glycosylation isomer (M2BPGi) offers the capability to stage fibrosis severity quickly and assess treatment response with longitudinal measurements.

Research objectives

This study aims to compare M2BPGi, a blood-based biomarker with existing methods of non-invasive testing and elastography in chronic hepatitis. In a preliminary assessment of treatment response, hepatitis B DNA concentrations were correlated with M2BPGi levels in respective patients.

Research methods

In patients with liver disease of different etiologies, M2BPGi levels in residual blood samples were measured. Comparisons with transient elastography (TE) were made to establish preliminary clinical cut-offs. Pearson correlations were tested using different liver disease markers to establish any significant trends. M2BPGi levels in early disease patients were compared to show etiology specificity.

Research results

We established clear correlations between M2BPGi with TE and other non-invasive biomarkers. For fibrosis staging of both hepatitis B and C patients, we observed statistically significant correlations with M2BPGi. M2BPGi levels in early disease were higher in viral hepatitis patients indicating the need to establish different cut-offs. The results were also significantly correlated with hepatitis B viral load, which established the possibility of treatment assessment.

Research conclusions

M2BPGi level is a useful addition to the current routine assessment of chronic hepatitis patients. This is performed by a routine immunoassay that enables fast turnaround time and quicker reporting for patients.

Research perspectives

We envision this research to have clinical potential to improve treatment monitoring procedures and rapid assessment of liver disease. In a resource limited setting, this marker presents useful results to ensure patients are linked to care promptly.

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ORIGINAL ARTICLE

Observational Study

Epidemiological profile of alcoholic liver disease hospital admissions in a Latin American country over a 10-year period

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Author contributions: Lyra AC developed the idea, mentored and reviewed the study; de Almeida LMC and Mise YF were responsible for collecting, analyzing the data and writing the manuscript; Cavalcante LN analyzed the data and reviewed the study.

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Conflict-of-interest statement:

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Abstract

BACKGROUND

Alcoholic liver disease (ALD) is a major cause of chronic liver disease worldwide.

AIM

To describe the epidemiological profile and mortality rates of patients with ALD admitted to public hospitals in different regions of Brazil from 2006 to 2015.

METHODS

This is a descriptive study that evaluated aggregate data from the five Brazilian geographic regions.

RESULTS

A total of 160093 public hospitalizations for ALD were registered. There was a 34.07% increase in the total number of admissions over 10 years, from 12879 in 2006 to 17267 in 2015. The region with the highest proportion (49.01%) of ALD hospitalizations was Southeast (n = 78463). The North region had the lowest absolute number of patients throughout the study period, corresponding to 3.9% of the total (n = 6242). There was a 24.72% increase in the total number of ALD deaths between 2006 and 2015. We found that the age group between 50 and 59 years had the highest proportion of both hospitalizations and deaths: 28.94% (n = 46329) of total hospital admissions and 29.43% (n = 28864) of all deaths. Men were more frequently hospitalized than women and had the highest proportions of deaths in all regions. Mortality coefficient rates increased over the years, and

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simple linear regression analysis indicated a statistically significant upward trend in this mortality (R^2 = 0.744).

CONCLUSION
Our study provides a landscape of the epidemiological profile of public hospital admissions due to ALD in Brazil. We detected an increase in the total number of admissions and deaths due to ALD over 10 years.

Key words: Alcoholic liver disease; Epidemiology; Mortality; Liver; Cirrhosis; Hospital admissions

Core tip: Alcoholic liver disease (ALD) is one of the main causes of chronic liver disease worldwide. Many patients with ALD present for medical care after they have developed advanced liver disease and its complications. It is important to know the epidemiology of ALD within a specific region/country to better understand which resources might be necessary to improve management. This study provides a landscape of the epidemiological profile of hospital admissions due to ALD in different regions of Brazil from 2006 to 2015, including the mortality rates and admissions according to age range. We detected a 34.07% increase in the total number of hospital admissions for ALD and a 24.72% increase in the total number of ALD deaths over these 10 years. Therefore, this study signals the need to be alert to this liver illness and to possibly revisit policies related to alcohol marketing, sales, and consumption.

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INTRODUCTION

Alcoholic liver disease (ALD) is one of the main causes of chronic liver disease worldwide. Alcohol is also a frequent co-factor in patients with other types of liver diseases, including hepatitis C virus infection among others. Alcohol-related morbidity and mortality have wide geographical variation. Within each country, there is an excellent correlation between the level of alcohol consumption and the prevalence of alcohol-related injury. Individuals with long-term significant alcohol consumption remain at risk for liver disease that may range from alcoholic steatohepatitis to cirrhosis and hepatocellular carcinoma[1-4].

Many patients with ALD present for medical care after they have developed advanced liver disease and its complications[1-4]. It is important to know the epidemiology of ALD within a specific region/country to better understand which resources might be necessary to improve management[5,6]. ALD might have been overlooked in recent years due to recent therapy advances in the viral hepatitis field. Therefore, few pharmacological developments have been made in the management of patients with this illness. Furthermore, ALD needs more than just a pharmacological intervention to be cured. It is also important to educate the population about the potential harm of alcohol usage. Given its high prevalence, economic burden, and clinical repercussions, ALD should receive significant attention from health authorities, research funding organizations, the population, and academic liver associations[1-3,7,8].

This study describes the epidemiological profile of hospital admissions due to ALD in different regions of Brazil from 2006 to 2015, including the mortality rates and admissions according to age range.

MATERIALS AND METHODS

This is a descriptive study that evaluated aggregate data. Data from the five Brazilian

geographic regions were used for the study (Figure 1).

The public health care information system (SUS hospital information system [SIH/SUS]) was used as the data source regarding admissions to public hospitals as well as death rates. It is available on the Ministry of Health online platform: www.datasus.gov.br[9]. Demographic data for the coefficient calculations were collected in an electronic database maintained by the Brazilian Institute of Geography and Statistics (http://www.ibge.gov.br)[10]. Data from the period spanning 2006 to 2015 were analyzed.

Study variables

For this study, all reported cases of ALD (International Statistical Classification of Diseases and Related Health Problems 10th Revision code K70) were evaluated. The analyzed variables were the number of hospitalizations (hospital admissions) and deaths due to ALD according to sex (male and female), age range (< 29, 30 to 39, 40 to 49, 50-59, 60-69, 70-79, 80 years-old or more) and year (2006 to 2015). Data are presented as their absolute and relative values. The proportional distribution and mortality coefficient were used as indicators. Subsequently, the data were organized into spreadsheets and presented in tables and graphs using Microsoft Office Excel 2016. The ALD mortality coefficient per year and per region was calculated as follows: mortality coefficient = number of deaths/population × 100000. The analysis of temporal evolution of mortality from ALD in Brazil (2006 to 2015) was performed by describing the magnitude and fluctuations of this indicator during this period. Thereafter, the temporal trend in ALD mortality was evaluated using simple linear regression, with mortality from ALD as the dependent variable (Y) and the calendar year as the independent variable (X). The values of β, R^2, and P value were evaluated with SPSS version 21.0. $P < 0.05$ was considered statistically significant.

Ethical issues

This study was performed according to Resolution 466/2012 of the National Health Council. Since this study was conducted using a secondary database in the public domain, which is available on the internet, it was not necessary to obtain written consent or approval from the Ethics and Research Committee. None of the authors have conflicts of interest.

RESULTS

Hospitalizations for ALD

During the period between 2006 and 2015, 160093 hospitalizations for ALD were registered. There was a 34.07% increase in the total number of hospital admissions for ALD in the Brazilian regions over these 10 years, from 12879 in 2006 to 17267 in 2015. The region with the highest proportion of hospitalizations during the study period was the Southeast, with 49.01% (n = 78463). Nevertheless, compared to the other regions, there was a decrease in this proportion from 2012 to 2015. The North region had the lowest absolute number of patients throughout the study period, corresponding to 3.9% of the total (n = 6242). The Midwest region experienced the greatest proportional increase over the years; this region accounted for 5.30% (n = 683) of hospitalizations in 2006 and increased to 8.70% (n = 1502) in 2015. The northeastern region had an increased number of hospitalizations from 2508 (19.47%) hospital admissions in 2006 to 3936 (22.79%) hospital admissions in 2015. The North region had a mild proportional increase from 3.14% (n = 404) in 2006 to 4.53% (n = 782) in 2015. On the other hand, although the South and Southeast regions also showed an increase in the total number of patients admitted to SUS hospitals over the analyzed period, there was a proportional reduction compared to the other regions. The Southeast region had the greatest reduction, from 52.69% (n = 6786) in 2006 to 45.34% (n = 7828) in 2015. In the South region, the rate of ALD admissions decreased from 19.4% (n = 2498) in 2006 to 18.64% (n = 3219) in 2015 (Table 1).

ALD deaths

When the total number of ALD deaths between 2006 and 2015 was evaluated, there was an increase of 24.72% over the years, with 8429 deaths in 2006 and 10513 in 2015. While all regions had an increase of deaths, the Southeast and Northeast regions had the highest mortality in the country in 2015 with 39.47% (n = 4150) and 31.82% (n = 3345), respectively. The North region had the lowest proportion of deaths, corresponding to 4.25% (n = 447) of the total number in 2015 (Table 2).

When the number of hospitalizations and deaths due to ALD was analyzed according to age group, we found that the age group between 50 and 59 years had the highest proportion of both hospitalizations and deaths [28.94% (n = 46329) of the total

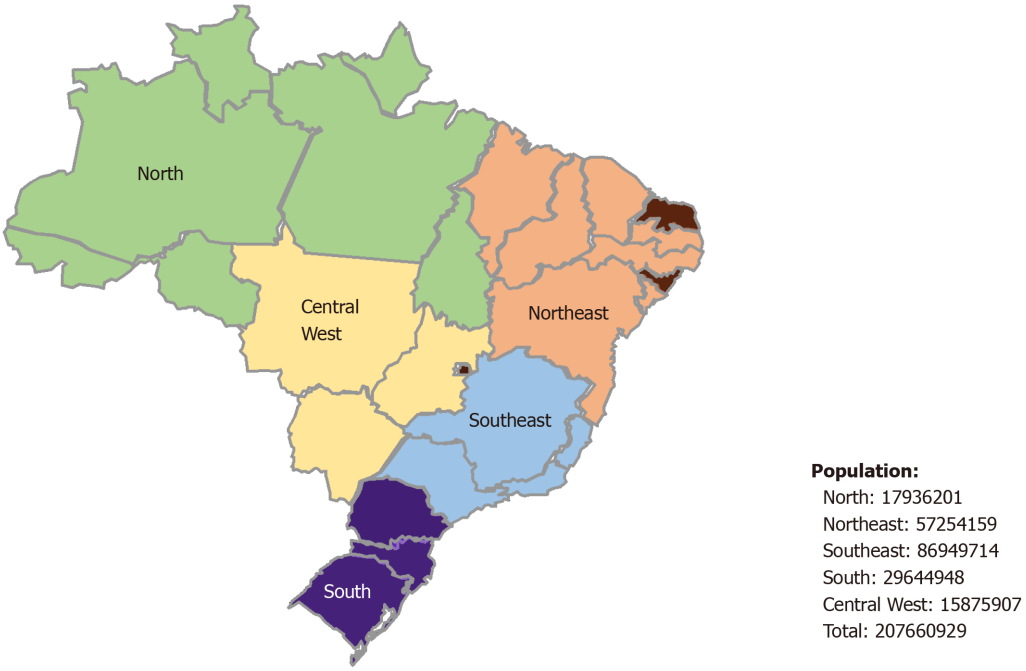

Figure 1 Brazilian map with the five geographic regions and their population (https://ibge.gov.br/).

hospital admissions (data not shown) and 29.43% (n = 28864) of all deaths (Table 3)], followed by the age range of 40 to 49 years [27.33% (n = 43751) of the total hospital admissions] (data not shown) and 28.05% (n = 27509) of all deaths (Table 3)]. On the other hand, the subgroup over 80 years presented with the lowest proportion of hospitalizations and deaths, corresponding to 1.99% (n = 3184) (data not shown) of admissions and 2.18% of deaths (n = 2135) (Table 3). Men were more frequently hospitalized than women in all Brazilian regions (81.68% *vs* 18.32%) (Table 4). Males also presented with the highest proportions of deaths in all regions, ranging from 86.22% (n = 6293) in the Midwest to 89.70% (n = 15282) in the South region (Table 5).

Mortality rate coefficients

The mortality rate coefficients increased over the years in the North, Northeast, South and Midwest regions, while a lower coefficient was detected in the Southeast region. The highest mortality coefficients were observed in the Midwest (5.99 in 2015) and South (6.49 in 2011). The lowest coefficient in 2015 was found in the North region (2.56). Simple linear regression analysis indicated that the upward trend of this mortality was statistically significant (y = 0.072 x– 140.62). The coefficient of determination was R^2 = 0.744 (Figure 2, Table 6). The mortality coefficient was highest in the age groups of 50 to 59 years, 60 to 69 years, and 70 to 79 years. The mortality coefficient for the ≤ 29 years old and ≥ 80 years or older age groups remained stable. When analyzing the mortality coefficients according to sex, men had the highest values throughout the study period, ranging from 7.99 to 9.26; women had the lowest values, ranging from 1.07 to 1.30 (data not shown).

DISCUSSION

Our study showed a burden of more than 150000 registered hospitalizations for ALD over 10 years. We also detected an increase in the total number of hospital admissions for ALD during the study period. We are unsure if this increase reflects an actual augmentation of the disease's burden or an increase in the number of admitted patients. If it reflects the first situation, it would be interesting to speculate that alcohol consumption could be growing in Brazil and public health authorities should be alerted to consider undertaking measures to mitigate this problem and its consequences.

Notably, as expected, our study detected a sharp increase in the admission rate for ALD in the population over 30 years old, reaching its peak in the 40- and 59-year-old age group, the most afflicted age range. On the other hand, the elderly population appears to be less affected, probably because most patients with ALD will die or be

Table 1 Number and proportion of hospitalizations due to alcoholic liver disease in the Brazilian regions between 2006 and 2015

Region		North	Northeast	Southeast	South	Midwest	Total
2006	*n*	404	2508	6786	2498	683	12879
	%	3.14	19.47	52.69	19.40	5.30	100
2007	*n*	420	2995	7008	2725	647	13795
	%	3.04	21.71	50.8	19.75	4.70	100
2008	*n*	389	2613	7085	3169	926	14182
	%	2.74	18.43	49.96	22.35	6.52	100
2009	*n*	503	2900	8284	3322	980	15989
	%	3.14	18.14	51.81	20.78	6.13	100
2010	*n*	618	3211	8952	3471	1142	17394
	%	3.55	18.46	51.47	19.96	6.56	100
2011	*n*	757	3245	8737	3131	1181	17051
	%	4.44	19.03	51.24	18.36	6.93	100
2012	*n*	774	3771	8181	3224	1088	17038
	%	4.54	22.13	48.02	18.92	6.39	100
2013	*n*	724	4223	7958	3279	1277	17461
	%	4.15	24.18	45.58	18.78	7.31	100
2014	*n*	871	3983	7644	3156	1383	17037
	%	5.11	23.38	44.87	18.52	8.12	100
2015	*n*	782	3936	7828	3219	1502	17267
	%	4.53	22.79	45.34	18.64	8.70	100
Total	*n*	6242	33385	78463	31194	10809	160093
	%	3.90	20.85	49.01	19.49	6.75	100

Data source: Ministry of Health–SUS hospital information system.

transplanted earlier in the course of the illness or will stop drinking and improve.

As expected, the region with the highest proportion of hospitalizations during the study period was the Southeast because it concentrates the greatest population of the country. Nevertheless, a decrease in this proportion from 2012 to 2015 was observed. In other words, while there was an overall 34.07% increase in the total number of admissions over these 10 years, this rise was approximately 15% in the Southeast region. This might have occurred for one or more of the following reasons: An increase in the number of disease notifications of other regions, an improvement of the regional health care system of the Southeast region, or a decrease in the disease incidence in that particular area.

It is interesting to note that the North and Midwest regions have similar population sizes. However, the North region had the lowest absolute number of patients throughout the study period, corresponding to 3.9% of the total (n = 6242) compared to 6.75% (n = 10809) in the Midwest region. We are unsure if this difference is due to better disease reporting or a finer regional health care system in the Midwest or a lower disease incidence in the North.

The mortality rate was higher in the 40- and 59-year-old age groups as well. Therefore, it is interesting to speculate that excessive beverage ingestion starts during young adulthood, and this population should be educated regarding the hazards of the disease[5,11-13]. It is also interesting to mention that men accounted for the majority of the affected patients across all regions. Mortality coefficient analysis showed an increase of the death rates in the North, Northeast, South and Midwest regions, while there was a decrease in the Southeast region. The highest mortality coefficients were observed in the Midwest and Northeast. The mortality rate among males increased while it remained stable among females.

It is expected that men's drinking habits are greater than women's because culturally, in Brazil and possibly worldwide, women are not supposed to socially drink more than men. Nevertheless, our study still detected ALD in a reasonable number of females, and this issue should be addressed by public authorities.

Some countries have banned household alcohol production, increased taxation on factory-produced alcohol, specified the legal age of consumption of alcohol at 21 years of age, and identified and monitored one dry day a week on weekdays[14,15]. Meta-

Table 2 Number and proportion of deaths due to liver disease in the Brazilian regions between 2006 and 2015

Region		North	Northeast	Southeast	South	Midwest	Total
2006	*n*	204	2260	3953	1494	518	8429
	%	2.42	26.81	46.90	17.72	6.15	100
2007	*n*	244	2487	3926	1686	542	8885
	%	2.75	27.99	44.19	18.98	6.10	100
2008	*n*	286	2627	4097	1745	639	9394
	%	3.04	27.96	43.61	18.58	6.80	100
2009	*n*	305	2676	4131	1596	610	9318
	%	3.27	28.72	44.33	17.13	6.55	100
2010	*n*	350	2829	4310	1769	660	9918
	%	3.53	28.52	43.46	17.84	6.65	100
2011	*n*	317	2991	4424	1838	741	10311
	%	3.07	29.01	42.91	17.83	7.19	100
2012	*n*	391	3133	4303	1716	834	10377
	%	3.77	30.19	41.47	16.54	8.04	100
2013	*n*	411	3169	4142	1800	950	10472
	%	3.92	30.26	39.55	17.19	9.07	100
2014	*n*	459	3215	4153	1746	880	10453
	%	4.39	30.76	39.73	16.70	8.42	100
2015	*n*	447	3345	4150	1646	925	10513
	%	4.25	31.82	39.47	15.66	8.80	100
Total	*n*	3414	28732	41589	17036	7299	98070
	%	3.48	29.30	42.41	17.37	7.44	100

Data source: Ministry of Health–SUS hospital information system. SIM: Mortality Information System.

analyses and reviews have detected that a price increase for alcohol beverages was associated with a decrease in its consumption. It is estimated that a 10% price increase might be associated with a 5% reduction in consumption, on average[5,8,11,14-16].

Another possible action could be focused on marketing interventions and regulations. It has been reported that for each 10% increase in advertising expenditure, there is a 0.3% increase in adult alcohol consumption[7,11,17,18]. Therefore, the implementation of policies reinforcing explanations of the ill effects of alcohol the same way it is performed for smoking could be effective. The Brazilian government has conducted massive television broadcasts to clarify the potential consequences of smoking, including lung cancer and other types of malignant neoplasia. Additionally, the manufacturers are obliged to expose the outcomes of smoking as well during cigarette advertisements. Therefore, a similar procedure could be performed to educate the population about the potential hazards of excessive alcohol ingestion, including the development of ALD, alcoholic hepatitis, hepatic cirrhosis, and liver cancer.

It is important to be aware that this study was conducted using a government secondary database in the public domain which is fed by numerous health professionals in each region. Therefore, the accuracy of the provided information cannot be evaluated. Also, the database does not provide further details regarding the studied population. Therefore, we were unable to look in-depth if the reason for hospital admission was only due to ALD or other associated conditions might have influenced. Nevertheless, it is interesting to note that usually the main reason for the hospital admission is inserted in the database.

In summary, our study has provided a landscape of the epidemiological profile of hospital admissions due to ALD in different regions of Brazil from 2006 to 2015, including the mortality rates and admissions according to age range. We detected a 34.07% increase in the total number of hospital admissions for ALD and a 24.72% increase in the total number of ALD deaths over these 10 years. Notably, the Brazilian population increased by only 10% during the same period. Therefore, this study signals the need to be alert to this liver illness and to possibly revisit policies related to alcohol marketing, sales, and consumption.

Table 3 Number and proportion of deaths due to liver disease according to age range

Region	Age group, in years																	
	< 29		30-39		40-49		50-59		60-69		70-79		≥ 80		Missing data		Total	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
North	109	3.19	467	13.68	865	25.34	876	25.66	604	17.69	326	9.55	145	4.25	22	0.64	3414	100
Northeast	997	3.47	4502	15.67	8119	28.26	7335	25.53	4604	16.02	2273	7.91	835	2.91	67	0.23	28732	100
Southeast	704	1.69	4725	11.36	11594	27.88	13273	31.91	7553	18.16	2897	6.97	729	1.75	114	0.27	41589	100
South	234	1.37	1624	9.53	4706	27.62	5345	31.37	3389	19.89	1412	8.29	307	1.80	19	0.11	17036	100
Midwest	186	2.55	1083	14.84	2225	30.48	2035	27.88	1156	15.84	460	6.30	119	1.63	35	0.48	7299	100
Total	2230	2.27	12401	12.65	27509	28.05	28864	29.43	17306	17.65	7368	7.51	2135	2.18	257	0.26	98070	100

Table 4 Number and proportion of hospitalizations due to alcoholic liver disease, by sex, in the Brazilian regions between 2006 and 2015

Region	Male		Female		Total	
	n	%	*n*	%	*n*	%
North	4846	77.64	1396	22.36	6242	100
Northeast	26694	79.96	6691	20.04	33385	100
Southeast	64329	81.99	14134	18	78463	100
South	26308	84.34	4886	15.66	31194	100
Midwest	8590	79.47	2219	20.53	10809	100
Total	130767	81.68	29326	18.32	160093	100

Data source: Ministry of Health–SUS hospital information system.

Table 5 Number and proportion of deaths, by alcoholic liver disease by sex, in the Brazilian regions between 2006 and 2015

Region	Male		Female		Total	
	n	%	*n*	%	*n*	%
North	3020	88.48	393	11.51	3413	100
Northeast	25260	87.92	3463	12.05	28723	100
Southeast	36273	87.22	5311	12.77	41584	100
South	15282	89.70	1753	10.29	17035	100
Midwest	6293	86.22	1005	13.77	7298	100
Total	86128	87.82	11925	12.16	98053	100

Data source: Ministry of Health–SUS hospital information system. SIM: Mortality Information System. Missing data: $n = 17$.

Table 6 Mortality rate (per 1000) of alcoholic liver disease in Brazil in the Brazilian regions between 2006 and 2015

Region	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015
North	1.35	1.59	1.83	1.91	2.16	1.92	2.34	2.42	2.66	2.56
Northeast	4.30	4.69	4.91	4.95	5.19	5.44	5.66	5.68	5.72	5.91
Southeast	4.98	4.90	5.06	5.06	5.23	5.32	5.14	4.90	4.88	4.84
South	5.51	6.16	6.32	5.73	6.30	6.49	6.01	6.25	6.02	5.63
Midwest	3.88	3.99	4.63	4.34	4.62	5.10	5.65	6.34	5.78	5.99
Total	4.50	4.69	4.90	4.81	5.07	5.22	5.21	5.21	5.16	5.14

Data source: Ministry of Health–SUS hospital information system (SIH/SUS). SIM: Mortality Information System; IBGE: Brazilian Institute of Geography and Statistics.

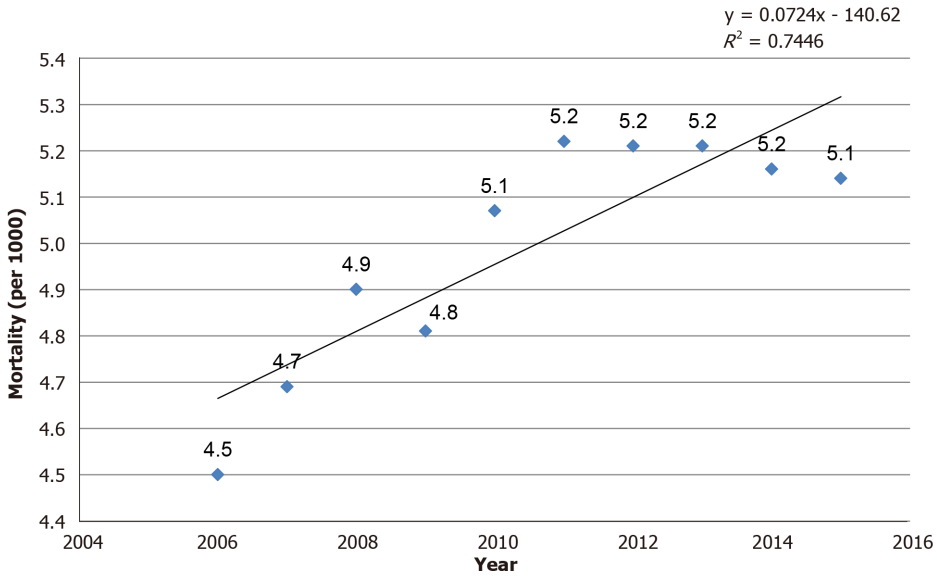

Figure 2 Mortality rate coefficients. Simple linear regression analysis (y = 0.072x – 140.62). The coefficient of determination was R^2 = 0.744.

ARTICLE HIGHLIGHTS

Research background

Alcoholic liver disease (ALD) is one of the main causes of chronic liver disease worldwide. Individuals with long-term significant alcohol consumption remain at risk for liver disease that may range from alcoholic steatohepatitis to cirrhosis and hepatocellular carcinoma.

Research motivation

It is important to know the epidemiology of ALD within a specific region/country to better understand which resources might be necessary to improve management. ALD might have been overlooked in recent years due to recent therapy advances in other Hepatology fields.

Research objectives

To describe the epidemiological profile of hospital admissions due to ALD in different regions of Brazil from 2006 to 2015, including the mortality rates and admissions according to age range.

Research methods

This is a descriptive study that has evaluated aggregate data. Data from the five Brazilian geographic regions were used for the study.

Research results

There was a 34.07% increase in the total number of admissions over these 10 years, from 12879 in 2006 to 17267 in 2015 as well as a 24.72% increase in the total number of ALD deaths between 2006 and 2015. We found that the age group between 50 and 59 years had the highest proportion of both hospitalizations and deaths: 28.94% (n = 46329) of total hospital admissions and 29.43% (n = 28864) of all deaths. Men were more frequently hospitalized than women and had the highest proportions of deaths in all regions. Mortality coefficient rates increased over the years, and simple linear regression analysis indicated a statistically significant upward trend in this mortality (R^2 = 0.744).

Research conclusions

Our study has provided a landscape of the epidemiological profile of public hospital admissions due to ALD in Brazil. We detected an increase in the total number of admissions and deaths due to ALD over 10 years.

Research perspectives

This study signals the need to be alert to this liver illness and to possibly revisit policies related to alcohol marketing, sales, and consumption.

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META-ANALYSIS

Systemic review and network meta-analysis: Prophylactic antibiotic therapy for spontaneous bacterial peritonitis

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Abstract

BACKGROUND

Spontaneous bacterial peritonitis (SBP) is an important prognostic factor for outcomes in patients with cirrhosis. Antibiotic prophylaxis is recommended in patients at high risk for developing SBP, but the choice of antibiotics remains unclear.

AIM

To evaluate the efficacy of various antibiotics for prophylaxis of SBP based on randomized control trials (RCTs).

METHODS

Electronic databases were searched through November 2018 for RCTs evaluating the efficacy of therapies for primary or secondary prophylaxis of SBP. The primary outcome was the development of SBP. Sensitivity analyses limited to studies of primary or secondary prophylaxis and studies reported after 2010 were performed. The secondary outcome was the risk of all-cause mortality or transplant. The outcomes were assessed by rank of therapies based on network meta-analyses. Individual meta-analyses were also performed.

RESULTS

Thirteen RCTs (1742 patients) including norfloxacin, ciprofloxacin, rifaximin, trimethoprim-sulfamethoxazole (TMP-SMX), or placebo/no comparator were

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identified. Individual meta-analyses showed superiority of rifaximin over norfloxacin as well as norfloxacin and TMP-SMX over placebo. Network meta-analysis demonstrated the rank of efficacy in reducing the risk of SBP as: Rifaximin, ciprofloxacin, TMP-SMX, norfloxacin, and placebo/no comparator. Rifaximin ranked highest in sensitivity analyses limited to studies of primary or secondary prophylaxis and studies reported after 2010. Similarly, rifaximin ranked highest in reducing the risk of death/transplant.

CONCLUSION
The present comprehensive network meta-analysis provides RCT based evidence for superior efficacy of rifaximin compared to other antibiotics for the prophylaxis of SBP and reducing risk of death/transplant. Further RCTs are warranted to confirm our findings.

Key words: Spontaneous bacterial peritonitis; Prophylaxis; Antibiotics; Network meta-analysis; Systemic review; Cirrhosis

Core tip: Spontaneous bacterial peritonitis (SBP) is an important prognostic factor for outcomes in patients with cirrhosis. We performed a systematic review and network meta-analysis of randomized control trials evaluating the efficacy of antibiotics for primary or secondary prophylaxis of SBP. Rifaximin ranked highest in reducing the risk of SBP as well as the risk of death/transplant. Our comprehensive network meta-analysis provides randomized control trials-based evidence for superior efficacy of rifaximin compared to other antibiotics for the prophylaxis of SBP and reducing the risk of death/transplant.

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INTRODUCTION

Spontaneous bacterial peritonitis (SBP) is the most common infection seen in patients with advanced liver cirrhosis and ascites[1,2]. Development of SBP can lead to renal dysfunction, hepatic encephalopathy, and deterioration of hepatic function, which adversely affect survival. Despite advances in treatment, in-hospital mortality of patients with SBP remains as high as 25%-30%[3]. Risk factors for the development of SBP include ascites protein levels < 1 g/dL, high serum bilirubin, prior episodes of SBP, and advanced liver disease[4,5]. Recurrences are also common following a single episode of SBP and are seen in up to 69% of infected patients within one year[6]. Thus, the first onset of SBP is an important prognosticator for health outcomes in patients with advanced liver disease. The use of antibiotics in patients with variceal bleeding and as secondary prophylaxis of SBP is recommended by the American Association for Study of Liver Diseases[7] and European Association for the Study of the Liver[8] guidelines[7,9,10]. However, evidence for the role and choice of antibiotics in both primary and secondary prophylaxis in the absence of gastrointestinal (GI) bleeding remains unclear.

Antibiotic prophylaxis has been shown to reduce the incidence of SBP in patients who are at high risk[11,12]. Overgrowth, translocation, and dissemination of intestinal bacteria are early steps in the pathogenesis of SBP and are more prevalent in cirrhotic patients compared to non-cirrhotic controls[13,14]. The majority of SBP are caused by *Escherichia coli* or other gram-negative bacteria, though gram-positive bacteria have been increasingly seen in the setting of antibiotic resistance[15,16]. Antibiotic prophylaxis primarily works via decontamination of the gut, thus lowering the bacterial reserves available for translocation. Guidelines recommend ceftriaxone for patients with advanced cirrhosis and GI bleeding or norfloxacin twice daily for seven days with severe liver disease as these patients are at high-risk for developing SBP.

Trimethoprim/sulfamethoxazole (TMP-SMX) and ciprofloxacin are also listed as effective alternatives[7,10]. Additionally, two recent meta-analysis by Goel *et al*[17] and Sidhu *et al*[18] suggested a benefit for primary or secondary SBP prophylaxis in using rifaximin, a gut-selective antibiotic, compared to norfloxacin.

Several randomized control trials (RCTs) and cohort studies have demonstrated efficacy of various antibiotics, either in comparison to placebo or other antibiotics for prophylaxis of SBP[19]. Yet the number of trials remains small, and comparisons between antibiotics remains sparse, thus limiting our ability to compare treatments which have been studied separately. A network meta-analysis can be used to study outcomes of multiple interventions within the same disease process[20,21]. This study uses a network meta-analysis method to rank and provide a comprehensive evaluation of recommended options for primary and secondary antibiotic prophylaxis of SBP based on RCTs.

MATERIALS AND METHODS

Search strategy and study selection

We performed this study according to a previously defined protocol and in accordance with the PRISMA for Network Meta-Analyses (PRISMA-NMA) guidelines[22]. The protocol of this meta-analysis has been registered to the International prospective register of systematic reviews (PROSPERO)[23]. We conducted a systemic literature search of PubMed/MEDLINE, Google scholar, Scopus, EMBASE and Cochrane Central Register of Controlled Trials (inception to November 1, 2018) for studies assessing the efficacy of antibiotic prophylaxis for SBP. For Google scholar, only the first 1000 articles were reviewed at each search as results are not provided past this number. We also searched abstracts from medical conferences (Digestive Disease Week, American College of Gastroenterology, United European Gastroenterology Week, and AASLD) and bibliographies of identified articles for additional references.

Only RCTs evaluating the efficacy of one or more antibiotic interventions for prophylaxis (primary or secondary) of SBP or reported it as an outcome were eligible for inclusion. Studies of SBP prophylaxis in the setting of GI bleeding were excluded. Control arms were placebo, no treatment, or alternative treatments. For the purpose of this study, placebo and no treatment arms were combined and are aggregately referred to as placebo from this point forward. Inclusion was not restricted based on age, sex, or duration of study. No geographic restrictions were placed on eligible articles and articles in languages other than English were translated if necessary. Studies were searched with a combination of terms including "spontaneous bacterial peritonitis", "prophylaxis", "antibiotics" and "randomized". Terms were searched as both medical subject headings and free text and were combined using the set operators. Two authors (Faust N and Yamada A) independently screened potential titles and abstracts in the primary search in order to identify articles addressing the question of interest. The full text of selected articles was then evaluated for eligibility and content areas of disagreement or uncertainty were resolved based on discussion and consensus between the two authors and principal investigator.

Data extraction and quality assessment

Data was abstracted using a standardized data abstraction form. Study characteristics including the authors, location, year of study, study period, sample size, mean age of patients, sex of patients, inclusion and exclusion criteria, antibiotics used, and endpoints were collected. Outcomes and adverse events were extracted for each study when reported. The Jadad scale, a validated method for assessing the methodological quality of a clinical trial, was used to assess the quality of each included study[24]. Cochrane scores were also used as a qualitative measure for bias[25].

Outcome assessment

The primary outcome for this study was the proportion of patients who developed SBP in each intervention arm. Incidence of SBP was determined in each study by a combination of clinical characteristics (fever, abdominal pain), cytologic criteria, and ascitic fluid cultures. The secondary outcome was the risk of death/transplant as assessed by the proportion of patients who died or were transplanted in each intervention arm due to any cause. Data was extracted as intention-to-treat whenever allowed by individual RCT reporting. Outcomes were assessed by risk difference between the two treatment arms.

We performed the following subgroup analyses: (1) Excluding studies with low quality as assessed with the Jadad scale ≤ 2; (2) Analysis of primary prophylaxis,

including only patients without a history of SBP; (3) Analysis of secondary prophylaxis including only patients with a history of SBP; and (4) Analysis of studies performed after 2010 (after rifaximin was approved by United States Food and Drug Administration to reduce the risk of hepatic encephalopathy).

Statistical analysis

The network meta-analysis is a technical method which allows readers to visualize and interpret data for the relative merits of multiple interventions in a given condition. This synthesis of data allows preservation of the randomization within each trial[26]. Two assumptions necessary for the validity of the network meta-analysis' mixed comparisons are that the data across sets is transitively related and consistent[27].

In the framework of this study, transitivity is a measure of methodological homogeneity and can be assumed when the data sets for two direct comparison studies are similar in their distributions. Such is the case when subject demographics for the included studies are similar in distribution, and subjects for any given study eligible for any of the interventions based on eligibility and exclusion criteria across all studies. Still, some clinical and methodological heterogeneity is expected across studies. The Bayesian Markov chain Monte Carlo method was used account for this[28]. Our model contained parameters describing the relative treatment effect of each treatment compared with each other and a common comparator (placebo). Other treatment comparisons were derived by analyzing differences between model parameters.

Consistency refers to statistical heterogeneity, or the degree to which disagreements in study specific treatment effects exist beyond what can be explained by chance[29]. RCT consistency in this study was measured using the node-splitting method. The results were presented as median effect sizes along with 95% confidence intervals (CIs). No significant inconsistency was present when 95%CIs of inconsistency factors included zero or when a large P value (> 0.05) for the comparison between direct and indirect effects in the node splitting analysis was found.

Each Bayesian Markov chain Monte Carlo cycle provided a ranking of the treatments according to the estimated effect size and the full set of simulations. We calculated the surface under the cumulative ranking (SUCRA) probabilities[20]. SUCRAs expressed as percentages compare each intervention to an imaginary intervention that is always the best without uncertainty. The ranking probability for each drug, *i.e.*, the most efficacious, the second-best, the third-best, and so on, was calculated and the overall ranks were interpreted by SUCRA technique. The larger SUCRAs denote more effective interventions.

For direct meta-analysis, we evaluated the presence of heterogeneity across trials of each therapy by using the I^2 statistic. An I^2 value of < 25% indicates low heterogeneity, 25%-75% moderate heterogeneity, and > 75% high heterogeneity, respectively[30]. We also evaluated the presence of heterogeneity across trials of each therapy by using the statistic Q and used a P value of < 0.10 as evidence of statistically significant heterogeneity[31]. All analysis was performed with ADDIS 1.× (drugis.org)[32]. We followed the Cochrane Handbook for Systematic Reviews of Interventions in the report of this meta-analysis[25].

RESULTS

Study characteristics

Literature review identified 171 citations through the initial search. We excluded 154 titles and abstracts after initial screening and assessed 18 articles for eligibility (Figure 1). Ultimately, 13 RCTs, including a total of 1757 patients, were included in the evaluation of 5 interventions for SBP prophylaxis: Norfloxacin, ciprofloxacin, rifaximin, trimethoprim-sulfamethoxazole (TMP-SMX), and placebo. All studies were parallel studies and 5 were placebo-controlled trials. Inclusion criteria for participants among each study included diagnosis of cirrhosis by clinical diagnosis, imaging, liver biopsy, laboratory values and/or presence of ascites. Exclusion criteria included documented anaphylaxis to one of the study interventions, hepatocellular carcinoma or other neoplasias that could shorten life expectancy, bacterial infection at admission, HIV infection or hepatic encephalopathy, and pregnant and lactating women. All trials included ascitic fluid PMN count in the diagnosis of SBP. The majority of trials diagnosed SBP with PMN ≥ 250, with one study using diagnostic criteria of polymorphonuclear cells ≥ 350. The majority of the studies included advanced cirrhotic patients (Child-Pugh class B or C) with alcoholic or viral hepatitis as its cause. The study by Assem *et al*[33] included a treatment group that alternated

norfloxacin and rifaximin, but it was excluded from our analysis. Five studies used antibiotics for primary prophylaxis (excluded patients with a history SBP and the remainder contained a mixed cohort of patients with or without a history of SBP. Seven and 6 studies were published before and after 2010, respectively. All 3 studies that included rifaximin were published after 2015 and compared its efficacy to norfloxacin in a non-double-blinded manner[33-35]. A summary of individual study characteristics and outcome data for the included studies are summarized in Table 1. The median JADAD for all included studies was 3, with individual scores for each study ranging from 1 to 4. JADAD scores and Cochrane meta-analysis bias scores are shown in Table 2.

Individual meta-analyses of SBP risk

Individual meta-analyses were performed to compare the efficacy between each antibiotic. It should be noted that the number of studies in each meta-analysis was small ranging from 1-5. Superiority of norfloxacin and TMP-SMX over placebo were demonstrated in meta-analyses including 5 and 1 study, respectively (Supplementary Figure 1B and C). One study comparing ciprofloxacin to placebo demonstrated a non-significant superiority of ciprofloxacin over placebo (Supplementary Figure 1A). Three studies compared rifaximin to norfloxacin, and the meta-analysis showed superiority of rifaximin over norfloxacin with no heterogeneity (Supplementary Figure 1D). Two studies and one study compared TMP-SMX to norfloxacin and ciprofloxacin to norfloxacin, respectively, and the meta-analyses showed no difference between the two agents (Supplementary Figure 1E and F).

Network meta-analysis of SBP risk

There were 5 studies comparing norfloxacin to placebo, 3 studies comparing norfloxacin to rifaximin, and two studies comparing norfloxacin to TMP-SMX. The remainder of comparisons (ciprofloxacin *vs* placebo, norfloxacin *vs* ciprofloxacin, TMP-SMX *vs* no treatment) included only one study each. The network of all intervention comparisons analyzed for efficacy of SBP prophylaxis is shown in Figure 2A. The network meta-analysis for the relative effects of each treatment for SBP prophylaxis is shown in Figure 2B. SUCRA interpretations of the rank probability for efficacy is shown in Figure 2C, with larger SUCRA scores indicating higher efficacy. In ascending order, the treatments ranked as (1) rifaximin; (2) ciprofloxacin; (3) TMP-SMX; (4) norfloxacin; and (5) placebo. Most of the 95%CIs of SUCRA for active treatments overlapped with each other, but none of those overlapped with the one of placebo. Similar results were found when we excluded studies with low quality (Jadad scale ≤ 2) (Supplementary Figure 2).

The results were shown to meet criteria for consistency based on the inconsistency model analyses and node-splitting analyses. The median inconsistency factors for norfloxacin/placebo/TMP-SMX and ciprofloxacin/norfloxacin/placebo were -0.26 [95%CI: (-2.85, 1.36)] and 0.06 [95%CI: -1.92, 2.41]. Comparison data from the node split model did not show significant differences between the direct and indirect effects (ciprofloxacin *vs* norfloxacin, $P = 0.72$; ciprofloxacin *vs* placebo, $P = 0.91$; norfloxacin *vs* placebo, $P = 0.64$; norfloxacin *vs* TMP-SMX, $P = 0.35$; placebo *vs* TMP-SMX, $P = 0.35$) supporting the consistency of the network meta-analysis.

As part of the subgroup analysis, we performed a network meta-analysis among the 5 studies that used antibiotics for primary prophylaxis of SBP (Supplementary Figure 3). In ascending order, the treatments ranked as (1) rifaximin; (2) norfloxacin; (3) ciprofloxacin; and (4) placebo. There was no study that used TMP-SMX for primary prophylaxis, thus, it was not included in this particular network meta-analysis. Network meta-analysis undertaken among the 8 studies that included patients who used antibiotics for secondary prophylaxis of SBP (Supplementary Figure 4), demonstrated that the treatments ranked as (1) rifaximin; (2) ciprofloxacin; (3) TMP-SMX; (4) norfloxacin; and (5) placebo. When network meta-analysis was performed among the 6 studies that were published after 2010 (Supplementary Figure 5), the treatments ranked in ascending order as (1) rifaximin; (2) TMP-SMX; (3) ciprofloxacin; (4) norfloxacin; and (5) placebo.

Individual and network meta-analyses of the risk of death/transplant

Individual meta-analyses assessing the risk of death/transplant are shown in Supplementary Figure 6. One study compared ciprofloxacin to placebo and 3 studies compared rifaximin to norfloxacin, and the meta-analyses showed superiority of ciprofloxacin and rifaximin over their comparators in reducing the risk of death, respectively (Supplementary Figure 6A and D). The remainder of the individual meta-analyses demonstrated no significant superiority between each treatment arm. It should be noted that the number of studies in each meta-analysis was small ranging from 1-5.

Table 1 Characteristics of the studies included in the network meta-analysis

Therapy/control	Ref.	Yr	Dosage of therapy	Control	Mean follow-up	History of SBP/%	ITT/PP	SBP diagnosis criteria	*n* (total)	Therapy			Control		
										n	Developed SBP	Deaths/ transplant	*n*	Developed SBP	Death/ transplant
Ciprofloxacin *vs* placebo	Terg *et al*[45]	2008	Ciprofloxacin 500 mg daily	Placebo	7.6-7.8 mo	Excluded	ITT	Ascites PMN > 250/mm^3	100	50	2/50	6/50	50	7/50	15/50
Norfloxacin *vs* placebo	Ginés *et al*[46]	1990	Norfloxacin 400 mg daily	Placebo	6.4 mo	Included/20.0%	ITT	Abdominal pain, fever, ascites PMN > 350/mm^3	80	40	5/40	7/40	40	14/40	11/40
Norfloxacin *vs* no treatment	Novella *et al*[47]	1997	Norfloxacin 400 mg daily	No treatment	43 wk	Excluded	ITT	Ascites PMN > 250/mm^3, positive ascitic fluid culture	109	56	1/56	13/56	53	9/53	16/53
Norfloxacin *vs* placebo	Grangé *et al*[48]	1998	Norfloxacin 400 mg daily	Placebo	6 mo	Excluded	ITT	Ascites PMN > 250/mm^3, positive ascitic fluid culture	107	53	0/53	8/53	54	5/54	11/54
Norfloxacin *vs* placebo	Fernández *et al*[49]	2007	Norfloxacin 400 mg daily	Placebo	12 mo	Excluded	ITT	Ascites PMN > 250/mm^3	68	35	2/35	16/35	33	10/33	19/33
Norfloxacin *vs* placebo	Moreau *et al*[50]	2018	Norfloxacin 400 mg daily	Placebo	6 mo	Included/88.3%	ITT	Ascites PMN > 250/mm^3	291	144	10/144	36/144	147	17/147	42/147
Norfloxacin *vs* ciprofloxacin	Yim *et al*[51]	2018	Norfloxacin 400 mg daily	Ciprofloxacin 750 mg weekly	12 mo	Included/88.7%	ITT/PP	Ascites PMN > 250/mm^3	124	62	4/55	15/62	62	3/57	16/62
Rifaximin *vs* norfloxacin	Mostafa *et al*[34]	2015	Rifaximin 800 mg daily	Norfloxacin 400 mg daily	6 mo	Included/100%	ITT	Ascites PMN > 250/mm^3	70	40	0/40	0/40	30	5/30	0/30
Rifaximin *vs* norfloxacin	Elfert *et al*[35]	2016	Rifaximin 1200 mg daily	Norfloxacin 400 mg daily	6 mo	Included/ 100%	ITT/PP	Ascites PMN > 250/mm^3	262	131	4/103	18/131	131	13/92	32/131
Rifaximin *vs* norfloxacin	Assem *et al*[33]	2016	Rifaximin 1100 mg daily	Norfloxacin 400 mg daily	6 mo	Excluded	ITT/PP	Ascites PMN > 250/mm^3	334	82	8/64	8/82	78	13/57	11/78
Trimethoprim-sulfamethoxazole *vs* no treatment	Singh *et al*[52]	1995	TMP-SMX 160/800 mg 5 d per week	No treatment	90 d	Included/73.3%	ITT	Ascites PMN > 250/mm^3	60	30	1/30	2/30	30	8/30	6/30
Trimethoprim-sulfamethoxazole *vs* norfloxacin	Alvarez *et al*[53]	2005	TMP-SMX 160/800 mg 5 d per week	Norfloxacin 400 mg daily	163-182 d	Included/38.6%	NA	Ascites PMN > 250/mm^3	57	25	4/25	5/25	32	3/32	7/32
Trimethoprim-sulfamethoxazole *vs* norfloxacin	Lontos *et al*[54]	2014	TMP-SMX 160/800 mg daily	Norfloxacin 400 mg daily	208-251 d	Included/26.2%	ITT	Ascites PMN > 250/mm^3	80	40	2/40	22/40	40	2/40	18/40

ITT: Intention to treat; NA: Not available; PMN: Polymorphonuclear cells; PP: Per protocol; SBP: Spontaneous bacterial peritonitis.

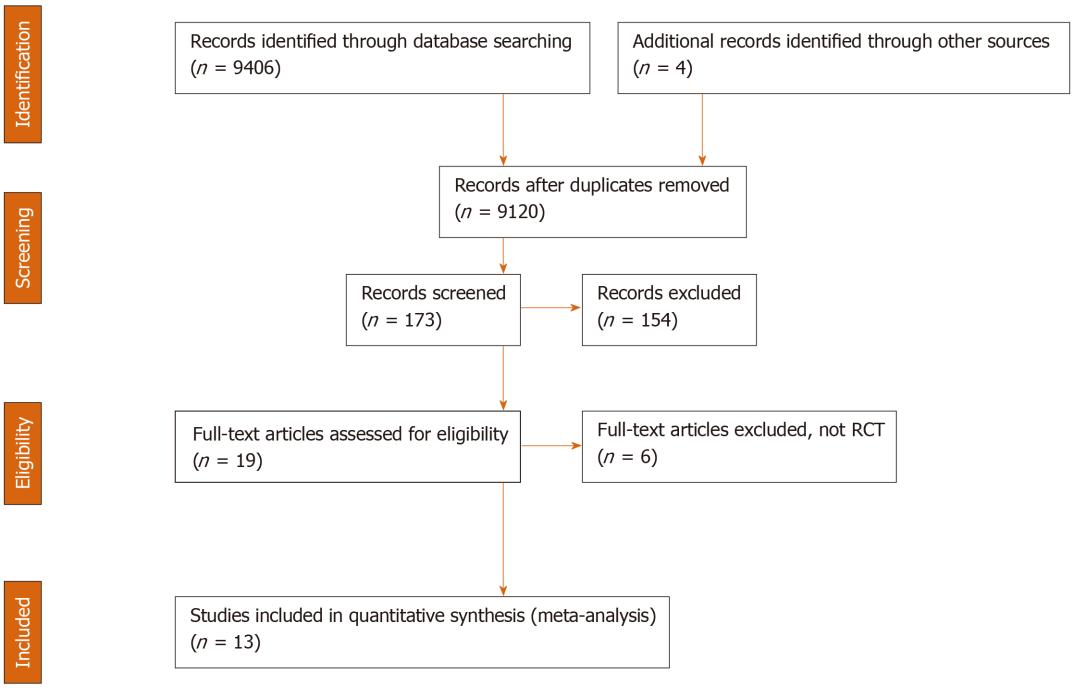


Figure 1 Flow chart of assessment of studies identified in the network meta-analysis.

The network of all intervention comparisons analyzed for efficacy of risk reduction of death is shown in Figure 3A. The network meta-analysis for the relative effects of each treatment is shown in Figure 3B. SUCRA interpretations of the rank probability for efficacy is shown in Figure 3C and, in ascending order, the treatments ranked as (1) rifaximin; (2) ciprofloxacin; (3) norfloxacin; (4) TMP-SMX; and (5) placebo. The median inconsistency factors for norfloxacin/placebo/TMP-SMX and ciprofloxacin/norfloxacin/placebo were -0.22 [95%CI: (-1.64, 0.56)] and -0.20 [95%CI: (-1.39, 0.50)], which met the criteria for consistency. Comparison data from the node split model did not show significant differences between the direct and indirect effects (ciprofloxacin *vs* norfloxacin, $P = 0.25$; ciprofloxacin *vs* placebo, $P = 0.21$; norfloxacin *vs* placebo, $P = 0.09$; norfloxacin *vs* TMP-SMX, $P = 0.35$; placebo *vs* TMP-SMX, $P = 0.20$) supporting the consistency of the network meta-analysis.

DISCUSSION

In this systematic review and meta-analysis, we compared and assessed the efficacy of different antibiotic treatments for SBP prophylaxis in individuals with advanced cirrhosis. This was done in order to validate current treatment recommendations and to perform indirect comparisons of active treatments where no or few direct randomized comparison trials existed. Among the four antibiotics and placebo included in the meta-analysis, rifaximin was the most effective in preventing SBP, followed by ciprofloxacin, TMP-SMX, norfloxacin, and placebo. Similarly, rifaximin ranked highest in reducing the risk of death.

Current guidelines from the AASLD and EASL recommend prophylactic treatment with intravenous ceftriaxone or oral norfloxacin for the prevention of SBP in the setting of GI bleeding and severe liver disease[10]. Norfloxacin is recommended for primary prophylaxis in cirrhotic patients with low ascitic fluid protein concentration and/or high serum bilirubin levels as they are at high risk of developing a first episode of SBP. Furthermore, norfloxacin is also recommended for secondary prophylaxis because recurrent SBP is common[7,10]. Our study validates results from two meta-analyses by Goel *et al*[17] and Sidhu *et al*[18], which found a reduction in the development of SBP with the use of rifaximin compared to the recommended norfloxacin regimens. A recent network meta-analysis by Facciorusso *et al*[2] reported moderate evidence for norfloxacin and ciprofloxacin in primary prophylaxis of SBP, and low quality evidence for the use of rifaximin. This difference may be accounted for by the inclusion of studies that included both patients with primary prophylaxis and with a history of SBP in our study. Such studies were included in our primary outcome of combined primary and secondary prevention, but not in our subgroup

Table 2 JADAD and Cochrane meta-analysis bias scores

Study	1 Was the study described as randomized?	2 Was the randomization scheme described and appropriate?	3 Was the study described as double-blind?	4 Was the method of double blinding appropriate?	5 Was there a description of dropouts and withdrawals?	Total JADAD Score	Sequence generation (for arm randomization)	Allocation concealed	Blinding of outcomes	Incomplete outcome data addressed	ITT	Sample size calculation
Ginés *et al*[46]	O	NA	O	O	O	4	X	X	O	O	O	O
Singh *et al*[52]	O	NA	X	NA	X	1	?	?	X	?	O	X
Novella *et al*[47]	O	NA	X	NA	O	2	?	?	X	?	O	X
Grangé *et al*[48]	O	NA	O	NA	O	3	?	?	?	O	O	O
Alvarez *et al*[53]	O	NA	X	NA	X	1	O	O	X	?	?	X
Fernández *et al*[49]	O	O	O	NA	O	4	O	O	O	O	O	O
Terg *et al*[45]	O	O	O	NA	O	4	O	O	O	O	O	O
Lontos *et al*[54]	O	O	X	NA	O	3	O	X	X	O	O	O
Mostafa *et al*[34]	O	NA	X	NA	X	1	O	X	X	?	O	O
Elfert *et al*[35]	O	O	X	NA	O	3	O	X	X	?	O	O
Assem *et al*[33]	O	O	X	NA	O	3	O	X	X	?	O	O
Yim *et al*[51]	O	O	X	NA	O	3	O	X	X	?	O	O
Moreau *et al*[50]	O	O	O	O	O	5	O	O	O	O	O	O

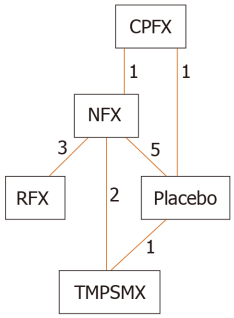
ITT: Intention to treat; NA: Not applicable.

analyses due to lack of subgroup randomization and incomplete information. Analyses of treatment effects in these subgroups are therefore subject to additional biases when compared to complete cohorts[36]. Our network meta-analysis provides evidence for superiority of rifaximin over the other studied antibiotics, which could otherwise not be compared by direct meta-analysis. Furthermore, ciprofloxacin and TMP-SMX ranked higher than norfloxacin in reducing the risk of SBP. Ciprofloxacin also ranked higher than norfloxacin in reducing the risk of death. Selective decontamination likely reduces the incidence of bacterial translocation of causative microflora through the gut. Evidence suggests that this effect may be compounded by or contributed to decreased expression of bacterial virulence factors and adhesion molecules[37,38]. Increased antibiotic efficacy with rifaximin has been seen in other GI diseases such as small intestinal bacterial overgrowth and traveler's diarrhea, which may also be working preferably in the setting of SBP prophylaxis[39,40].

Rifaximin also has a favorable side effect profile compared to other antibiotics, particularly with respect to the development of antibiotic resistant flora. The use of fluoroquinolones such as norfloxacin, which have traditionally been used for SBP prophylaxis, is associated with the development of resistant bacterial strains. Concurrently, there has been a recent shift in cases of documented SBP from being caused by gram-negative organisms to being caused by gram-positive organisms[41,42]. This is particularly seen in cases of SBP in patients on norfloxacin prophylaxis and may contribute to the increased efficacy of rifaximin seen in trials *vs* norfloxacin, as the infective organisms are more likely to be may be gram-positive that fall under the spectrum covered by rifaximin.

A major limitation of our study is the rather sparse geometry of the network due to

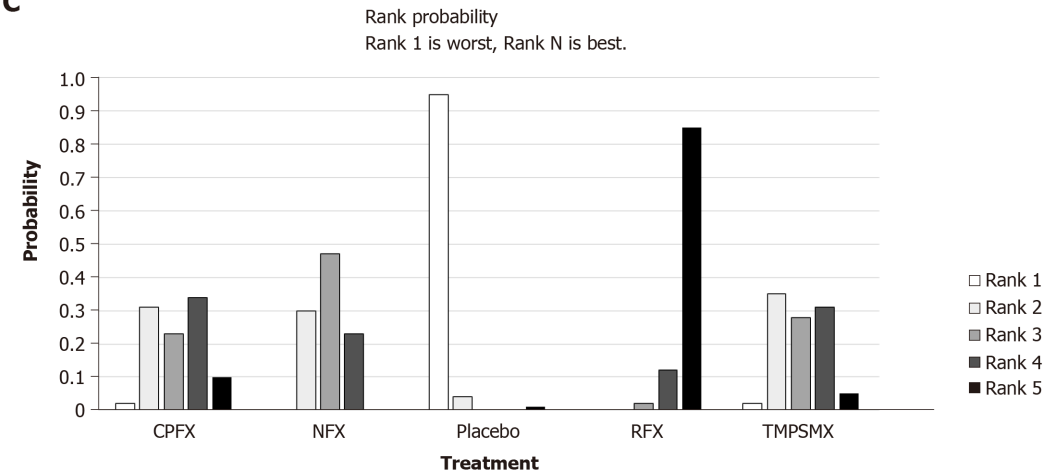
A



B

CPFX	1.14 (0.17, 7.37)	6.09 (0.99, 45.36)	0.25 (0.02, 2.32)	1.12 (0.09, 11.93)
0.88 (0.14, 6.03)	NFX	5.18 (2.24, 18.58)	0.23 (0.04, 0.78)	0.97 (0.20, 5.15)
0.16 (0.02, 1.01)	0.19 (0.05, 0.45)	Placebo	0.04 (0.00, 0.18)	0.19 (0.03, 0.96)
3.96 (0.43, 55.25)	4.43 (1.29, 23.21)	22.52 (5.41, 225.70)	RFX	4.25 (0.61, 45.72)
0.90 (0.08, 11.67)	1.03 (0.19, 5.02)	5.33 (1.04, 37.17)	0.24 (0.02, 1.63)	TMPSMX

C

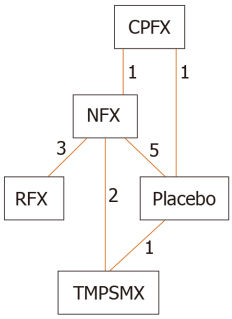


Drug	Rank 1	Rank 2	Rank 3	Rank 4	Rank 5	SUCRA	95%CI		SUCRA rank
CPFX	0.02	0.31	0.23	0.34	0.1	0.55	0.184	0.91	2
NFX	0	0.30	0.47	0.23	0	0.48	0.04	0.93	4
Placebo	0.95	0.04	0	0	0	0.010	-0.010	0.030	5
RFX	0	0	0.02	0.12	0.85	0.95	0.88	1.02	1
TMPSMX	0.02	0.35	0.28	0.31	0.05	0.51	0.12	0.90	3

Figure 2 Network meta-analysis of studies assessing the risk of spontaneous bacterial peritonitis. A: Network of treatment comparisons between antibiotics. Numbers indicate the number of studies comparing the two connected treatment arms; B: Relative effects of between each treatment. The numbers in the cell represent the odds ratio (95%CI) of the column defining modality relative to the row defining treatment; C: Rank probability (consistency model) for each antibiotic. Indicated is the possibility of each rank (No. 5 is the best). The overall rank interpreted by surface under the cumulative ranking technique is shown in the table. surface under the cumulative rankings in the table are reordered in the conventional ascending sequence. CPFX: Ciprofloxacin; NFX: Norfloxacin; RFX: Rifaximin; TMPSMX: Trimethoprim-sulfamethoxazole; SUCRA: Surface under the cumulative ranking.

the small number of RCTs. This effect is compounded by the range in quality of the studies used, with a JADAD score of less than three in 4 of the 11 studies used. Scores were most often lowered by the fact that practitioners and participants were not blinded to treatments and outcomes in some of the studies. However, we confirmed that a similar result was found when excluding studies with low quality scores. Several studies also contained elements indicative of bias and heterogeneity as determined by Cochrane meta-analysis criteria, and many of the studies analyzed were relatively smaller in size. The studies that included rifaximin all compared its efficacy to norfloxacin and there were no studies comparing rifaximin to placebo or other antibiotics, therefore limiting direct comparison with other agents. This further affirms the need for network meta-analyses in order to simultaneously compare the efficacy of multiple agents. Disagreement between direct and indirect comparisons may raise concerns for the validity of a network meta-analysis, however, the

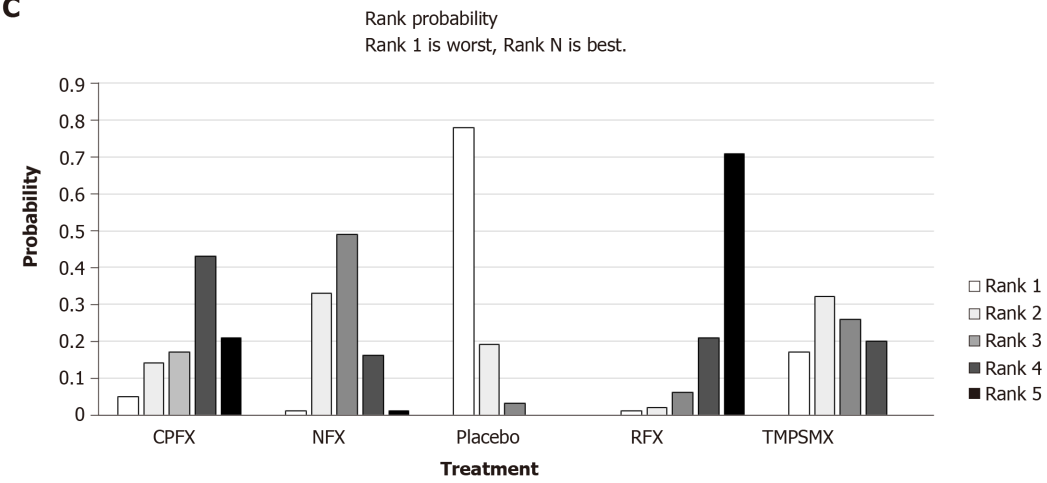
A



B

CPFX	1.27 (0.58, 2.66)	1.95 (0.88, 4.51)	0.68 (0.24, 1.98)	1.30 (0.44, 3.72)
0.79 (0.38, 1.74)	NFX	1.52 (1.02, 2.46)	0.55 (0.26, 1.12)	1.04 (0.48, 2.12)
0.51 (0.22, 1.14)	0.66 (0.41, 0.98)	Placebo	0.35 (0.14, 0.81)	0.68 (0.28, 1.60)
1.46 (0.51, 4.25)	1.82 (0.90, 3.85)	2.83 (1.24, 6.94)	RFX	1.89 (0.65, 5.31)
0.77 (0.27, 2.30)	0.96 (0.47, 2.08)	1.48 (0.62, 3.53)	0.53 (0.19, 1.53)	TMPSMX

C



Drug	Rank 1	Rank 2	Rank 3	Rank 4	Rank 5	SUCRA	95%CI		SUCRA rank
CPFX	0.05	0.14	0.17	0.43	0.21	0.65	0.34	0.97	2
NFX	0.01	0.33	0.49	0.16	0.01	0.46	0.017	0.90	3
Placebo	0.78	0.19	0.03	0	0	0.063	-0.041	0.17	5
RFX	0.01	0.02	0.06	0.21	0.71	0.90	0.77	1.03	1
TMPSMX	0.17	0.32	0.26	0.20	0.06	0.42	0.09	0.75	4

Figure 3 Network meta-analysis of studies assessing the risk of death/transplant. A: Network of treatment comparisons between antibiotics. Numbers indicate the number of studies comparing the two connected treatment arms; B: Relative effects of between each treatment. The numbers in the cell represent the odds ratio (95%CI) of the column defining modality relative to the row defining treatment; C: Rank probability (consistency model) for each antibiotic. Indicated is the possibility of each rank (No. 5 is the best). The overall rank interpreted by surface under the cumulative ranking technique is shown in the table. surface under the cumulative rankings in the table are reordered in the conventional ascending sequence. CPFX: Ciprofloxacin; NFX: Norfloxacin; RFX: Rifaximin; TMPSMX: Trimethoprim-sulfamethoxazole; SUCRA: Surface under the cumulative ranking.

robustness of our network meta-analysis was supported by the inconsistency model that demonstrated no such inconsistency. Rank probabilities identified in this network meta-analysis can be plotted against the possible ranks for all competing treatments[43,44]. We used SUCRA as a numerical summary to supplement the cumulative ranking[44], however, the results should be interpreted with caution as there is no means to statistically assess the difference of the SUCRA values[44]. Most studies did not differentiate between primary and secondary prophylaxis, but we found similar results when network meta-analysis was limited to studies using antibiotics for either primary or secondary prophylaxis. The time span of included studies ranged from the 1990s to 2018 which may have seen a change in bacteriology of organisms causing SBP, however subgroup analysis including studies that were reported after 2010 demonstrated similar outcome. The results of the secondary

outcome in our network meta-analysis, the reduction in the risk of death/transplant, needs to be approached with caution as it was not a primary outcome in any of the included studies. The included studies did not take other decision points into account, such as cost or quality of life. Furthermore, other factors such as demographics, concomitant proton inhibitor use, or past antibiotic use, which could confound outcomes, could not be assessed in the present study.

In conclusion, this systematic review and network meta-analysis of RCTs comparing multiple antibiotics for prophylaxis of SBP suggests that rifaximin is the most effective for the outcomes of preventing SBP and reducing all-cause mortality in high risk cirrhotic patients. Further comparative studies, particularly with appropriate randomization and larger power, are warranted to confirm these findings.

ARTICLE HIGHLIGHTS

Research background

Spontaneous bacterial peritonitis (SBP) confers significant mortality with high rates of recurrence. Prevention is therefore indicated and of great importance in cirrhotic individuals with ascites and either significant hepatic disease, gastrointestinal (GI) bleeding, or history of SBP.

Research motivation

Yet data is sparse regarding the choice of antibiotic when comparing the previous gold standard, norfloxacin, to other agents including ciprofloxacin, trimethoprim-sulfamethoxazole (TMP-SMX), and the GI selective agent rifaximin. The network meta-analysis technique allows us to make indirect comparisons across studies using common comparators.

Research objectives

Our present study uses this technique to rank and evaluate recommended therapies for primary and secondary prophylaxis of SBP.

Research methods

Thirteen randomized control trials including a total of 1757 patient were analyzed. Individual meta-analyses showed superiority of rifaximin over norfloxacin as well as norfloxacin and TMP-SMX over placebo. Network meta-analysis demonstrated the rank of efficacy in reducing the combined primary and secondary risk of SBP as: Rifaximin, ciprofloxacin, TMP-SMX, norfloxacin, and placebo/no comparator. Rifaximin ranked highest in sensitivity analyses limited to studies of either primary or secondary prophylaxis alone, and in studies reported after 2010. Similarly, rifaximin ranked highest in reducing the risk of death/transplant.

Research results

This study provides new evidence for superiority of rifaximin compared to norfloxacin in both primary and secondary SBP prophylaxis. In summary, this conclusion is supported by decreased mortality when rifaximin is used for primary or secondary prophylaxis compared to norfloxacin, ciprofloxacin, and TMP-SMX as shown in individual and network meta-analyses. Other new insights from this study were that rifaximin still performed best in a subgroup analysis of studies done after the year 2010, after the recommendation was made for rifaximin use in hepatic encephalopathy.

Research conclusions

Therefore, this study proposes the new hypothesis that the common use of rifaximin for hepatic encephalopathy in decompensated cirrhosis does not decrease its effectiveness in SBP prophylaxis. Additional molecular and biochemical data is needed to explain the beneficial effect of rifaximin. However, our data supports the hypothesis that rifaximin's selective decontamination of the GI tract, favorable resistance profile, and ability to decrease bacterial translocation across the gut may all contribute to its superiority for prophylaxis. Implications of these results for clinical practice include reconsideration of current AASLD guidelines to recommend rifaximin over norfloxacin as the first line agent for SBP prophylaxis.

Research perspectives

The next steps in this area of study should include additional data from large studies with direct comparisons between each antibiotic. Randomized control trial methods should be used in future research studies in order to confirm our meta-analysis findings.

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CASE REPORT

Transmission of cryptococcosis by liver transplantation: A case report and review of literature

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Abstract

BACKGROUND

Cryptococcosis is a fungal infection caused by the yeast-like encapsulated basidiomycetous fungus of the *Cryptococcus neoformans* (*C. neoformans*) species complex. These fungi are ubiquitous in soil and bird droppings, and infection by them is an important global health concern, particularly in immunosuppressed patients, such as organ transplant recipients and those infected by the human immunodeficiency virus. The fungus usually enters the body through the respiratory tract, but extremely rare cases of infection acquired by transplantation of solid organs have been reported.

CASE SUMMARY

We report a case of disseminated cryptococcosis in a liver transplant recipient, diagnosed 2 wk after the procedure. The patient initially presented with fever, hyponatremia and elevated transaminase levels, manifesting intense headache after a few days. Blood cultures were positive for *C. neoformans*. Liver biopsy showed numerous fungal elements surrounded by gelatinous matrix and sparse granulomatous formations. Magnetic resonance imaging of the brain showed multiple small lesions with low signal in T2, peripheric enhancement and edematous halo, diffuse through the parenchyma but more concentrated in the subcortical regions. Treatment with amphotericin B for 3 wk, followed by maintenance therapy with fluconazole, led to complete resolution of the symptoms. The recipients of both kidneys from the same donor also developed disseminated cryptococcosis, confirming the transplant as the source of infection. The organ donor lived in a rural area, surrounded by tropical rainforest, and had negative blood cultures prior to organ procurement.

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CONCLUSION
This case highlights the risk of transmission of fungal diseases, specifically of *C. neoformans*, through liver graft during liver transplantation.

Key words: Cryptococcosis; Liver transplantation; *Cryptococcus neoformans*; Case report; Immunosuppression; Fungal infection

Core tip: Transmission of cryptococcosis through a liver graft during transplantation is an exceedingly rare occurrence, with less than 10 cases reported in the literature. Many of these patients either died or were followed for only a short period of time prior to the report, so there is little information about long term follow-up of patients with this condition. We report the case of a patient who acquired disseminated cryptococcosis from a liver graft during transplantation and was successfully treated, along with the results of follow-up biopsies and imaging exams up to 3.5 years after the transplant.

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INTRODUCTION

Cryptococcosis is a fungal infection caused by the yeast-like encapsulated basidiomycetous fungus of the *Cryptococcus neoformans* (*C. neoformans*) species complex. This fungus is ubiquitous in soil and tree bark and is found in particularly great concentrations in the nitrogen-rich environment that is present in the droppings of birds, bats and other vertebrates. It can also be found in up to 50% of domestic dust samples[1]. There are two pathogenic species: *C. neoformans* and *Cryptococcus gattii* (*C. gattii*). Approximately 95% of reported cryptococcal infections are caused by *C. neoformans* serotype A, with the remaining 5% caused by other serotypes or by *C. gattii*[2].

The fungus presents as an oval or globular yeast at microscopy, with a diameter of 3 mm to 8 mm, and is characteristically surrounded by a mucopolysaccharidal capsule[1]. The capsule has a high content of melanin, produced as a result of the action of the phenoloxidase enzyme. Brain tissue is rich in substrates for phenoloxidase action, which may at least in part explain the tropism of Cryptococcus for the central nervous system (CNS)[1]. Cryptococcus has several characteristics that underlie increased virulence, including thermotolerance and variations in composition of its cell wall and capsule. There are reports of infection by Cryptococcus in dogs, cats, horses, sheep, snakes, and porpoises. Birds appear to be relatively resistant to this infection, possibly due to their high body temperature[3].

C. neoformans was first described in 1894, isolated from fermenting peach juice[4,5]. That same year, it was first described as a human pathogen, having infected a young woman with osteomyelitis[5]. Cryptococcosis was first reported in Brazil in 1941, in a patient with pulmonary disease[6]. Infection usually occurs by inhalation of basidiospores and dry yeasts but it can also occur through the gastrointestinal tract[2,7]. In the pulmonary alveoli, the fungus comes in contact with alveolar macrophages, which play a central role in the initial immune response. The macrophages internalize the fungal structures through phagocytosis, and Cryptococcus is able to survive and replicate inside the vacuole despite acidification. After replication, it can exit the host cell by a lytic process that destroys the host cell, or by a non-lytic process that leaves both cells intact[5].

The most common site of symptomatic cryptococcosis is the CNS, which is affected in about 80% of patients with the disease, and usually presents as a subacute or chronic meningitis with or without hydrocephalus, or less commonly as cerebral cryptococcomas, which may be confused for brain neoplasms in imaging evaluation[1]. The most common symptoms are headache, fever and confusion, but ataxia, amaurosis and cranial nerve palsies may also occur[8,9]. Signs of meningeal irritation

are present in about 50% of patients[10]. The syndrome of inappropriate antidiuretic hormone secretion may occur as a complication of cryptococcal meningitis and can cause severe hyponatremia[11]. There are reports from endemic areas in Brazil where *C. gattii* is the main causative agent of meningeal cryptococcosis, but *C. neoformans* remains the most common agent throughout the world[12].

Pulmonary disease is the second most common manifestation of cryptococcosis, presenting as pulmonary consolidations, nodular or cavitary infiltrates, miliary pattern, or rarely as pleural effusion, and may be unilateral or bilateral[1]. Symptoms of pulmonary disease include coughing, fever, pleuritic chest pain, and hemoptysis, but it can be asymptomatic in up to 30% of patients[1,13].

Cutaneous cryptococcosis can present as nodular lesions with a central umbilication, mimicking molluscum contagiosum, or as areas of swelling and erythema, similar in aspect to bacterial cellulitis. The most common sites for cryptococcal skin lesions are the lower extremities (65% of cases) and the trunk (26%)[14]. Subcutaneous abscesses are a rare manifestation of cryptococcosis, described mainly in solid organ transplant recipients[15].

Cryptococcal peritonitis is clinically similar to spontaneous bacterial peritonitis, and usually occurs in cirrhotic patients[16]. Hepatic cryptococcal infection is rare but may occur in disseminated disease, usually manifesting as cholestatic jaundice that may rapidly progress to liver failure and death[17]. Disease affecting more than one organ is considered as disseminated cryptococcosis. Vertical transmission of cryptococcosis during pregnancy is extremely rare[1].

The diagnosis of cryptococcosis can be made by direct microscopy of sputum, bronchoalveolar lavage, cerebrospinal fluid (CSF), blood, urine, or organ biopsy. The use of India ink is helpful in the identification of fungal elements, having a 50%-80% sensitivity. Fungal culture can also be obtained from the same kind of samples, and colony growth is usually observed in 48 h to 72 h using mediums such as Sabouraud agar or blood agar, and having a sensitivity of 70%-90%. A latex agglutination test can be used to detect Cryptococcus antigens in serum and CSF, having a sensitivity of 95% and a specificity of 98%. Titers above 1:8 strongly suggest active cryptococcosis, and titration can be used as a parameter to assess the response to antifungal treatment. Serum antigen does not cross the blood-brain barrier, and therefore does not interfere with titers detected in the CSF. Enzyme-linked immunosorbent assay can also be used for the detection of both antigens and antibodies to Cryptococcus, with even greater sensitivity. Analysis of the CSF usually shows pleocytosis with lymphocytosis, an increase in protein content, and a decrease in glucose[1,2]. Imaging of the brain in patients with cryptococcosis affecting the CNS can show leptomeningeal enhancement, encephalomalacia, infarcts, cerebellitis, hydrocephalus, transverse myelitis, or the presence of cryptococcomas. In these patients, magnetic resonance imaging (MRI) of the brain is more sensitive than computerized tomography (CT), which can be normal in 50% of patients[10,18,19]. Analysis of the ascitic fluid in patients with cryptococcal peritonitis is highly variable but seldom shows significant pleocytosis and usually shows an increase in lymphocytes[16].

Recommendations for the treatment of cryptococcosis vary according to the site of infection and the immunological status of the host. Amphotericin B (0.7 mg/kg daily) in combination with flucytosine or fluconazole for 2 wk to 10 wk is the recommended induction treatment for cryptococcal meningitis, followed by a maintenance treatment of 12-24 mo with daily fluconazole or itraconazole. Refractory cases should be treated with higher doses (3-6 mg/kg daily) of amphotericin B for another 6-10 wk. High doses of fluconazole (800-2000 mg daily) can be used if amphotericin B is not tolerated or unavailable. Intrathecal infusion of amphotericin B has multiple side effects and should only be used in refractory cases[20]. Voriconazole and posaconazole can also be treatment alternatives for these patients. Echinocandins appear to have no efficacy against Cryptococcus. Intermittent lumbar punctures can be of great importance in the first weeks of the treatment to alleviate intracranial hypertension, which is a significant source of mortality in cryptococcal meningitis.

There is no consensus in the current literature regarding persistent inflammatory lesions in the brain during treatment. The complete regression of these lesions can take a long time, and analysis of the CSF is of limited value to assess the presence of Cryptococcus in the brain parenchyma. If the lesions persist after the full treatment course has been completed, and the patient remains asymptomatic and with negative cultures, the suspension of the antifungal drugs appears to be safe, as long as adequate clinical and imaging follow-up can be obtained[21].

Treatment for pulmonary cryptococcosis consists of fluconazole (200 mg to 400 mg daily) for 6-12 mo.

In solid organ transplant recipients afflicted with cryptococcosis, an important adjuvant for antifungal treatment will involve reduction of the immunosuppressive drug regimen to the lowest possible levels, in order to increase host cellular immunity

against the fungus[3].

Incidence of cryptococcosis in solid organ recipients has been estimated at 0.3% up to 5%, being the third most commonly occurring invasive fungal infection in this population[8,22]. Mortality ranges from 15%-20% but may be as high as 40% when infection of the CNS is present[23,24]. Among solid organ transplant recipients, lung and liver recipients appear to the be the most vulnerable to cryptococcosis, but mortality is higher in heart transplant patients[25,26]. The onset of the disease after the transplant usually ranges from 8-21 mo but may occur many years after the procedure[6,8,27,28]. Patients who develop cryptococcosis more than 24 mo after transplantation are more likely to have CNS disease than those with early-onset disease[29].

Since many antifungals, such as fluconazole, are inhibitors of the cytochrome P450 enzymes, a reduction of about 40%-50% in the dose of tacrolimus may be warranted in order to maintain therapeutic drug levels[4].

Cirrhosis is an independent risk factor for the development of cryptococcosis, particularly for cryptococcal peritonitis, and the possibility that early-onset disease after the transplant actually represents an increase in the symptoms of preexisting disease must be considered[30-32]. If adequately treated before the transplant, cryptococcosis is not a contraindication for liver transplantation[33]. There are some reports on the transmission of Cryptococcus by organ transplantation, and early (less than 4 wk after the procedure) post-transplant cryptococcosis warrants consideration of donor transmission[6,34,35]. There are well recorded cases of transmission by corneal[36,37], lung[38], kidney[39-41] and liver transplants[42-44].

CASE PRESENTATION

Chief complaints

At 14 d after the surgery, a 57-year-old male returned to the emergency room complaining of fever and malaise.

History of present illness

The patient reported fever (39 °C), chills and loss of appetite that had started 5 d after hospital discharge.

History of past illness

A 57-year-old male diagnosed with cryptogenic cirrhosis – with hepatic encephalopathy being the main manifestation of the disease – underwent liver transplant in a Brazilian tertiary care hospital. At the time of transplantation, the patient's model for end-stage liver disease score was 14 and Child-Pugh classification was class B (8 points). Prior to the procedure, the patient had been admitted to the hospital several times for hepatic encephalopathy and suffered two major episodes of upper gastrointestinal bleeding. Investigation for viral hepatitis and autoimmune disease had negative results, and there was no previous history of significant alcohol consumption. The patient worked as a farmer in a rural area located in the North of Brazil, had no comorbidities, and suffered from frequent and severe symptoms of hepatic encephalopathy in spite of optimal clinical treatment. He had a previous CT scan of the brain, with no abnormal findings, and both an ultrasound and a CT scan of the abdomen, which were unremarkable except for portal hypertension and liver cirrhosis.

The patient was submitted to orthotopic liver transplantation with a graft obtained from a cadaveric donor (53-year-old male), in whom brain death had been caused by a hemorrhagic stroke that occurred 3 d before organ procurement. The donor worked as a lumberjack in the rural area of the North region of Brazil and was reported as being previously healthy, having normal biochemical tests, and no evidence of ongoing infection at the moment of organ procurement. He had positive results for serological tests for anti-HBs and anti-HBc but negative result for the serological test for HBsAg; these findings suggested a resolved hepatitis B infection. The transplantation procedure was uneventful, with a total ischemia time of 9 h and 9 min, no intraoperative complications, and no need for blood product transfusions. The patient was discharged from the intensive care unit 3 d after the procedure and from the hospital 8 d after the transplant.

The patient had no significant comorbidities, other than the recent transplant. He had no pulmonary, neurological or genitourinary symptoms. His immunosuppressive drug regimen consisted of tacrolimus, mycophenolate and prednisone, with a blood level of tacrolimus of 11.4 ng/mL.

Physical examination

Physical examination was unremarkable, with absence of skin lesions, vital signs

within the normal range of values, and no signs of infection in the surgical wound. Neurological examination was completely normal. The patient was admitted to the hospital for investigation. As he remained hemodynamically stable, admission to the intensive care unit was not deemed to be necessary.

Laboratory testing

Blood and urine cultures were obtained, producing growth of *C. neoformans* after 48 h in two separate blood cultures. Biochemical tests showed normal leukocyte and platelet counts, and levels of blood urea nitrogen and creatinine within the normal range. Remarkable findings were anemia (hemoglobin concentration of 8.1 g/dL; normal range: 13.5-17.5 g/dL), hyperbilirubinemia (total bilirubin of 5.3 mg/dL; normal range: 0.1-1.2 mg/dL), hyponatremia (sodium concentration of 119 mmol/L; normal range: 136-145 mmol/L), increased gamma-glutamyl transferase (referred to as GGT) (1470 UI/L; normal range: 9-48 UI/L), alanine aminotransferase (referred to as ALT) (45 UI/L; normal range: 7-56 UI/L) and aspartate aminotransferase (referred to as AST) (132 UI/L; normal range: 10-40 UI/L). A lumbar puncture was obtained, with the analysis of CSF showing values of glucose, protein and cytology all within normal range. CSF culture was negative for fungal growth.

Imaging examination

An ultrasound of the liver with doppler evaluation of the hepatic vessels was performed and gave normal results. An X-ray of the chest was also normal. An MRI of the brain was obtained, showing multiple small lesions with low signal in T2, peripheric enhancement and edematous halo, diffuse through the parenchyma but more concentrated in the subcortical regions (Figure 1).

MULTIDISCIPLINARY EXPERT CONSULTATION

A liver biopsy was obtained and investigated by histological analysis carried out by an expert in liver pathologies (Dr. ESM).

FINAL DIAGNOSIS

Histology of the liver biopsy showed a large number of fungal elements immersed in gelatinous matrix, in both liver parenchyma and portal spaces, with some of them surrounded by a loose histiocytic response (Figure 2). The patient was diagnosed with disseminated cryptococcosis, affecting both the CNS and the liver graft.

TREATMENT

Treatment was initiated with liposomal amphotericin B (3 mg/kg) in combination with fluconazole for 3 wk, followed by maintenance treatment with oral fluconazole (450 mg daily). Mycophenolate was discontinued and tacrolimus dosage was adjusted according to serum levels, which eventually stabilized around 8 ng/mL, with the patient taking 1 mg of tacrolimus on alternate days. The patient remained afebrile and asymptomatic after the amphotericin B treatment, showing marked decrease in total bilirubin levels (1 mg/dL) and progressive decrease in GGT levels (1162 UI/L).

OUTCOME AND FOLLOW-UP

Early disseminated cryptococcosis was also reported in the recipients of both kidneys obtained from the same donor of our liver transplant recipient; those transplants had occurred in hospitals located in different parts of the country. We concluded that the donor, while reportedly asymptomatic, had disseminated cryptococcosis that was transmitted by the grafts to the recipients. Our patient remained asymptomatic and with laboratory tests showing normal range of values for the 3.5 years of outpatient follow-up. The fluconazole dosage was progressively reduced to the current dosage of 150 mg daily. Control MRIs of the brain were obtained at 6, 12, 18 and 36 mo, all showing persistence of the brain lesions but with gradual reduction of the contrast enhancing halo around them. Liver biopsies were obtained at 4 and 16 mo, initially showing numerous granulomas containing oval fungal structures (Figure 3) and finally the absence of fungal structures, respectively.

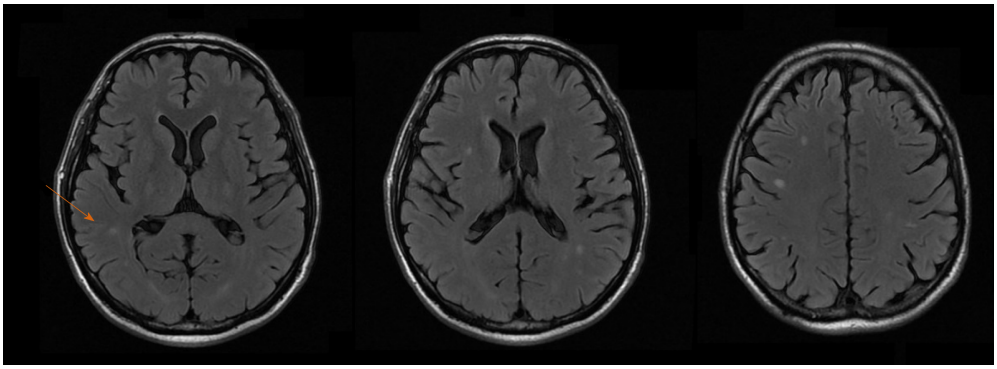

Figure 1 Magnetic resonance imaging of the brain. Multiple diffuse small lesions (arrow) in brain cortex and subcortical regions, with edematous halo and peripheral enhancement, suggestive of fungal infection (cryptococcomas).

DISCUSSION

Diagnosis and treatment of disseminated cryptococcosis in recipients of solid organ transplantation remains a clinical challenge, given the unspecific nature of the initial symptoms, the need for reduction in immunosuppression – which may cause rejection, and the adverse reactions associated with antifungal treatment. In the case we report, diagnosis of disseminated disease was obtained by growth of Cryptococcus in blood cultures. The CSF cultures and direct microbiology tests were negative despite the presence of multiple brain lesions, highlighting the poor correlation between the presence of the fungus in the CSF and in the brain parenchyma. Serological tests were unavailable at our institution at that time. The patient also presented with severe hyponatremia, which may have been associated with CNS cryptococcosis infection, and increases the morbidity of the disease. The patient also had a marked increase in bilirubin and liver enzymes caused by fungal infiltration of the liver, which was diagnosed by liver biopsy and quickly improved after antifungal treatment was initiated. The fact that all recipients (both kidneys and liver) who received organs from the same donor reported here developed early disseminated cryptococcosis, makes transmission by the transplant the most likely means of contagion in this case. This impression is reinforced by the presence of Cryptococcus in the biopsies of all three transplanted organs. In the cases of suspected or confirmed transmission of cryptococcosis through a liver transplant previously reported in the literature, the most common outcome was the death of the organ recipient[17,31,44]. Chang *et al*[42] reported on a patient who developed hepatic and pulmonary cryptococcosis one week after a liver transplant and was successfully treated with amphotericin B. We have found no previous reports in the literature of long-term follow-up of a liver transplant recipient with disseminated cryptococcosis, which allowed us to document the complete clearance of fungal structures in the liver 16 mo after treatment was started, and the long-term persistence of brain lesions in a asymptomatic patient with no other signs of infection.

CONCLUSION

Cryptococcosis is a relatively common fungal infection in patients that undergo liver transplantation, causing significant morbidity and mortality in this population. The most severe form of disease is disseminated cryptococcosis, with infection reaching multiple organs through the bloodstream. Treatment involves both a reduction of the immunosuppressive drug regimen and prolonged antifungal treatment. While most cases of cryptococcosis in solid organ recipients is caused by reactivation of their latent disease due to immunosuppression, in rare cases, the infection may be acquired through the liver graft, which may be suspected in cases of early infection after the transplant. Maintenance of prolonged antifungal treatment, even in asymptomatic patients, is important, since complete elimination of viable fungal elements in liver tissue may take more than a year of treatment to be achieved.

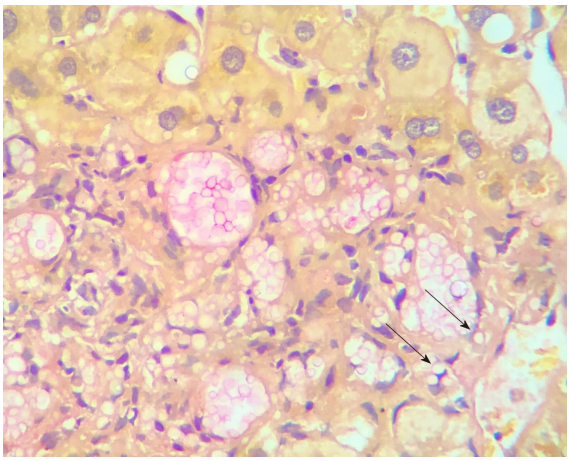

Figure 2 Liver biopsy at presentation. A large number of fungal elements (arrows) are immersed in gelatinous matrix, in both liver parenchyma and portal spaces, with some of them surrounded by a loose histiocytic response (400 ×).

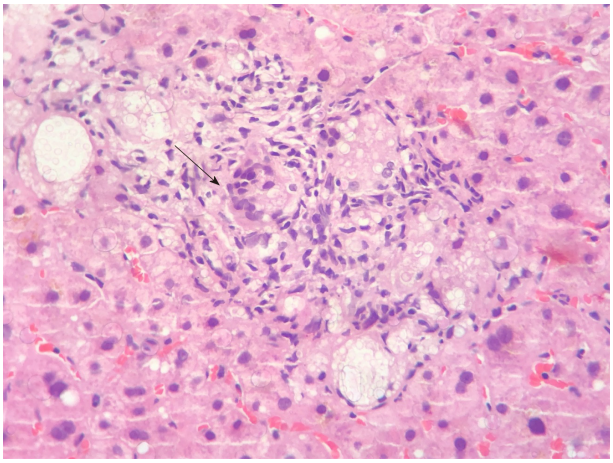

Figure 3 Follow-up liver biopsy at 4 mo. Numerous granulomas (arrow) containing oval fungal structures (400 ×).

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