The Impact of Spironolactone on the Severity of Portal-Systemic Collaterals and Hepatic Encephalopathy in Cirrhotic Rats

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ABSTRACT

Liver cirrhosis and portal hypertension are accompanied by portal-systemic collaterals formation and lethal complications. Angiogenesis participates in the development of collaterals. Spironolactone is an aldosterone receptor antagonist used to control fluid overload in cirrhotic patients although recent studies suggest that it also inhibits angiogenesis. This study investigated the effect of spironolactone on abnormal angiogenesis and portal-systemic collaterals in cirrhosis. Liver cirrhosis was induced in Sprague-Dawley rats by common bile duct ligation (BDL), and sham-operated rats were the controls. The BDL and sham rats received spironolactone (20 mg/kg/d, oral gavage) or vehicle from day 15 to 28 after the operations. Spironolactone did not influence the portal and systemic hemodynamic, and the renal and hepatic biochemistry data, but it significantly ameliorated hepatic fibrosis, portal-systemic shunting, and mesenteric angiogenesis. Plasma vascular endothelial growth factor (VEGF) levels and the mesenteric protein expression of VEGF and phosphor-vascular endothelial growth factor receptor 2 (VEGFR-2) decreased in the spironolactone group. Spironolactone did not affect motor activity or plasma ammonia levels. The down-regulation of VEGF pathway participates, albeit partly, in the antiangiogenic effect of spironolactone. Thus, spironolactone treatment in patients with liver cirrhosis may provide additional benefits aside from ascites control.

Introduction

During the progression of liver cirrhosis, portal hypertension develops due to increased splanchnic inflow and hepatic resistance. The formation of portal-systemic collaterals may divert the stagnant blood flow in the portal system. However, the shunting itself has detrimental complications like esophageal variceal bleeding and hepatic encephalopathy. Accumulating evidence demonstrates that angiogenesis, the formation of new vessels from a pre-existing vasculature, plays a pivotal role in the development and maintenance of portal hypertension (Fernandez et al., 2004). In addition, amelioration of splanchnic angiogenesis effectively ameliorates portal-systemic shunting (Huang et al., 2012; Hsu et al., 2015).

The renin-angiotensin-aldosterone system activation is a compensatory response to arterial vasodilation and “inadequate” intravascular volume associated with cirrhosis. Aldosterone is a mineralocorticoid that mediates salt and water reabsorption in the distal tubules of the kidney. Thus, spironolactone, a potent aldosterone blocker, is frequently prescribed for cirrhotic patients to control fluid overload and ascites. A previous study indicated that spironolactone significantly alleviates sodium retention when renal failure is not yet present (Wilkinson et al., 1979). But beyond the sodium and fluid status homeostasis, aldosterone may also participate in angiogenesis. Another study indicated that aldosterone mediates the actions of mineralocorticoid receptors in the pathologic angiogenesis of the retina and this is attenuated by spironolactone (Wilkinson-Berka et al., 2009). Spironolactone induced a dose-dependent reduction of capillary tube formation. The fibrin gel chamber study performed in rats also demonstrated that spironolactone significantly reduces the numbers of both peripheral and central neovessels (Miternique-Grosse et al., 2006). Spironolactone reduces neo-vascularization and varicocele-induced angiogenesis in rats (Köse et al., 2012).

Because the renin-angiotensin-aldosterone system is activated in liver cirrhosis with portal hypertension, the potential of aldosterone receptor antagonist to alleviate angiogenesis has been noted. However, its relevant influence on portal-systemic collaterals in cirrhosis has not been studied, so our study elucidates the aforementioned aspects in rats with bile duct ligation (BDL)-induced liver cirrhosis.

ABBREVIATIONS: BDL, bile duct ligation; COX, cyclooxygenases; DW, distilled water; eNOS, endothelial NO synthases; HR, heart rate; iNOS, inducible nitric oxide synthases; NO, nitric oxide; PP, portal pressure; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.
Material and Methods

Animal Model. Male Sprague-Dawley rats weighing 240–270 g at the time of surgery were used for experiments. Secondary biliary cirrhosis was induced by common BDL as previously described elsewhere (Huang et al., 2012; Hsu et al., 2015). Under ketamine anesthesia (100 mg/kg, intramuscularly), the common bile duct was exposed through a midline abdominal incision, catheterized by a PE-10 catheter, and doubly ligated with 3-0 silk. The first ligature was made below the junction of the hepatic ducts and the second ligature above the entrance of the pancreatic duct followed by section of the common bile duct between the ligatures. The rats were allowed to recover. A high yield of secondary biliary cirrhosis was noted 4 weeks after the ligation. To avoid the coagulation defects, BDL rats received daily oral gavage of 50 mg/kg intramuscularly) for 4 weeks after the operation. The subjects received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985).

Therapeutic Effects of Spironolactone on BDL Rats. The BDL and sham treatment rats received spironolactone (20 mg/kg/d, oralavage) or vehicle from days 15 to 28 after surgery.

Measurement of Systemic and Portal Hemodynamics. The right femoral artery was cannulated with a PE-50 catheter connected to a Spectramed DTX transducer (Spectramed, Oxnard, CA). Continuous recordings of mean arterial pressure, heart rate, and portal pressure (PP) were performed on a multichannel recorder (model RS 3400; Gould, Cupertino, CA). The external zero reference was placed at the level of the midportion of the rat. The abdomen was then opened with a midline incision, and a mesenteric vein was cannulated with a PE-50 catheter connected to a Spectramed DTX transducer. The abdominal cavity was closed, and the portal pressure was recorded.

Hepatic Fibrosis Determination with Sirius Red Staining. A liver paraffin section was stained with a Sirius red staining kit (Polysciences, Warrington, PA). ImageJ (http://image.nih.gov/ij/) was used to measure the percentage of Sirius red-stained area. Briefly, grayscale images were used, then the red-stained collagen was isolated using thresholding function. After that, the thresholded area was measured and shown as the percentage of threshold area per image.

Immunofluorescent Study for Liver Macrophage. Liver macrophage and Kupffer cells were evaluated by CD68 staining. In brief, the liver paraffin sections were dewaxed and retrieved with heat citrate buffer. The sections were then incubated with monoclonal anti-CD68 (1:100; AbD Serotec, Oxford, United Kingdom) for 1 hour, followed by fluorescent conjugated second antibody. The macrophage and Kupffer cell amounts were determined by thresholding the ×200 images using ImageJ software. Four areas from each sample were quantified.

H&E Staining. Tissue was fixed in 10% formalin, embedded in paraffin, sliced in 5-μm sections, and stained with H&E.

Color Microsphere Method for Portal-Systemic Shunting Degree Analysis. Portal-systemic shunting degree was determined using the technique described by Chojkier and Groszmann (1981) with substituted color for radioactive microspheres: 30,000 of 15-μm yellow microspheres (Dye Track; Triton Technology, San Diego, CA). Portal-systemic shunting was calculated as Lung Microspheres/Liver Microspheres + Lung Microspheres. Assuming a worst-case scenario in which two-thirds of the microspheres remained trapped in the spleen, this technique detected a minimum shunt of 3.5%. Studies using color microspheres have shown to provide results similar to those using radioactive microspheres (Hodeige et al., 1999).

Immunofluorescent Study for Mesenteric Vascular Density. Splanchnic angiogenesis was evaluated by assessing the CD31-labeled microvascular networks in rat mesenteric connective tissue windows, which was performed as previously described elsewhere (Huang et al., 2012; Hsu et al., 2015). Immunofluorescent images at 100× magnification were assessed using an upright fluorescent microscope (AX80; Olympus, Tokyo, Japan) and thresholded by ImageJ software. The vascular length was manually measured with the pencil tool, and the vascular area was automatically measured with histogram function. The unit of vascular length per unit area of mesenteric window would be μm/μm², and the vascular area per unit area of mesenteric window could be recorded as pixel/pixel without being converted to μm²/μm².

Plasma Vascular Endothelial Growth Factor Determination. Plasma vascular endothelial growth factor (VEGF) levels were measured using an enzyme-linked immunosorbent assay kit (R&D Systems, MN) according to the manufacturer’s instructions. The intensity of the color was measured at the absorbance of 450–600 nm with a Bio-kinetics Reader (BioTek Instruments, Winooski, VT). The intra-assay and interassay variations of these assays were less than 10%.

Western Blot Analysis. Protein expression was analyzed by Western blotting as previously described elsewhere (Huang et al., 2012; Hsu et al., 2015). Blots were incubated with the primary antibody: inducible nitric oxide synthases (iNOS), endothelial nitric oxide synthases (eNOS), cyclo-oxygenase-1 (COX-1), and cyclo-oxygenase-2 (COX-2) (all Cell Signaling Technology, Beverly, MA), and VEGF and phospho-vascular endothelial growth factor receptor 2 (VEGFR-2) (Santa Cruz Biotechnology, Santa Cruz, CA).

Motor Activities. Motor activities in an open field were determined by an opto-Variox activity meter (Columbus Instruments, Columbus, OH) as previously stated elsewhere (Hsu et al., 2012). The Opto-Variox activity sensors used high-intensity, modulated infrared light beams to detect animal motion. Animals were housed in transparent cages (17 × 17 × 8 inches; 43 × 43 × 20 cm) through which 30 infrared beams passed in the horizontal plane, 15 on each axis. This device differentiated nonambulatory movements (scratching, gnawing) from ambulation on the basis of consecutive interruption of the infrared monitoring beams. An additional row of infrared beams in the horizontal plane (15 on each axis) about 10 cm above the floor was used to count the vertical movements. During the activity measurements, animals had no access to food or chow. All studies were performed under strictly standardized conditions in the dark room for 30 minutes. The total, ambulatory, and vertical movements were recorded to reflect the motor activities of the rats.

Drugs. Spironolactone was purchased from Sigma-Aldrich (St. Louis, MO). The sources of the other drugs were specified in previous studies.

### TABLE 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham-D (n = 7)</th>
<th>Sham-S (n = 5)</th>
<th>BDL-D (n = 7)</th>
<th>BDL-S (n = 7)</th>
</tr>
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<tbody>
<tr>
<td>BW (g)</td>
<td>481 ± 14</td>
<td>473 ± 13</td>
<td>421 ± 7</td>
<td>403 ± 11</td>
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<tr>
<td>MAP (mm Hg)</td>
<td>113 ± 4</td>
<td>104 ± 9</td>
<td>90 ± 6</td>
<td>90 ± 3</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>337 ± 31</td>
<td>317 ± 22</td>
<td>318 ± 17</td>
<td>284 ± 17</td>
</tr>
<tr>
<td>PP (mm Hg)</td>
<td>7.93 ± 0.25</td>
<td>6.00 ± 0.78</td>
<td>15.52 ± 1.12</td>
<td>15.53 ± 1.15</td>
</tr>
</tbody>
</table>

BDL, bile duct ligation; BW, body weight; D, distilled water (control); HR, heart rate; MAP, mean arterial pressure; PP, portal pressure; S, spironolactone.

*P* < 0.05, BDL-D group versus Sham-D group.
Data Analysis. All results are expressed as mean ± S.E.M. Statistical analyses were performed using an independent Student’s t test or analysis of variance (ANOVA) with Tukey’s test as appropriate. Two-tailed $P < 0.05$ was considered statistically significant.

Results

Body Weight and Hemodynamics. The hemodynamic results of the sham or BDL-operated rats treated with either distilled water (DW, control) or spironolactone are shown in Table 1. Compared with the sham-treatment rats, the BDL rats had statistically significantly lower body weight, lower mean arterial pressure, and higher PP, which was not significantly modified by spironolactone.

Plasma Liver and Kidney Biochemistry Parameters.

Table 2 depicts the liver and kidney biochemistry parameters of the experimental groups. There was no statistically significant difference between the spironolactone and the parallel DW treatment groups.

Liver Fibrosis. Figure 1A reveals the Sirius red–stained fibrotic area ratio in the liver. Spironolactone statistically significantly attenuated liver fibrosis in cirrhotic rats: BDL-DW versus BDL-spironolactone (%): 16.1 ± 0.5 versus 12.2 ± 0.5 ($P < 0.001$).

![Fig. 1. Liver histology. (A) Spironolactone significantly reduced the ratio of liver fibrosis in cirrhotic rats. (B) Spironolactone significantly decreased the amount of liver macrophages over the periportal area in cirrhotic rats. (C) Top and middle: Representative Sirius red staining and H&E staining, respectively. Bottom: Representative CD68 staining. D, distilled water; S, spironolactone. *$P < 0.05$.](image-url)
Liver Macrophage. Figure 1B shows the CD68 staining area ratio in the periportal area of the liver. Compared with the DW-treated control group, spironolactone statistically significantly decreased the macrophage content in the periportal area of cirrhotic livers: BDL-DW versus BDL-spironolactone (%): 1.04 ± 0.10 versus 0.51 ± 0.05 (P < 0.036).

Portal-Systemic Shunting. Figure 2 depicts the severity of portal-systemic shunting in all groups. Compared with vehicle, cirrhotic (BDL) rats treated with spironolactone had statistically significantly less shunting: BDL-DW versus BDL-spironolactone shunting degree (%): 77.5 ± 2.6 versus 33.4 ± 6.6 (P < 0.001).

Mesenteric Vascular Density. The parameters of mesenteric vascular density are shown in Fig. 3A. In the BDL groups, spironolactone statistically significantly decreased the vascular length and vascular area: BDL-DW versus BDL-spironolactone vascular length per unit window area (μm⁻¹): 0.0182 ± 0.0009 versus 0.0111 ± 0.0007 (P < 0.001), and vascular area per unit window area (%): 12.91 ± 0.77 versus 7.79 ± 0.58 (P < 0.001).

Plasma VEGF Determination. Figure 4A depicts the levels of plasma VEGF. Spironolactone statistically significantly reduced the plasma VEGF level in BDL rats: BDL-DW versus BDL-spironolactone: 25.8 ± 1.1 versus 19.7 ± 1.8 μg/ml (P = 0.007).

Mesenteric Angiogenic Protein Expression. The expression of mesenteric angiogenic proteins is shown in Fig. 4B. The protein main angiogenic factor VEGF and the activation of its target receptor phospho-VEGFR-2 were down-regulated significantly in spironolactone-treated BDL rats compared with DW-treated BDL rats: BDL-DW versus BDL-spironolactone VEGF: 0.92 ± 0.69 versus 0.42 ± 0.03 (P = 0.014); and phospho-VEGFR-2: 1.75 ± 0.15 versus 1.11 ± 0.15 (P = 0.029). There was no statistically significant difference in iNOS, eNOS, COX-1, or COX-2 protein expression between the two groups.

Motor Activity and Plasma Ammonia Concentration. Figure 5A shows the motor activity of all groups. Compared with the sham-operated group, the BDL rats had lower motor activities. Consistently, the plasma ammonia concentration increased in the BDL rats (Fig. 5B). On the other hand, spironolactone treatment in BDL rats had no statistically significant effect on motor activities or plasma ammonia concentration.

Discussion

Common BDL is a well-established animal model widely applied to evaluate the pharmacologic aspects of chronic liver injury. It also reflects the pathophysiologic changes of the renin-angiotensin-aldosterone axis during the progression of liver cirrhosis (Jonassen et al., 1998; Kim et al., 2006). In terms of pharmacologic studies, prophylactic and therapeutic strategies can be designed with this model, depending on the timing of treatments (Hsu et al., 2015). Because liver fibrosis usually develops on day 14 and liver cirrhosis on day 28 after BDL (Kountouras et al., 1984), therapeutic agents administered...
immediately after BDL are usually regarded as a prophylactic strategy. On the other hand, agents provided after fibrosis formation also may serve as a therapeutic strategy, one more relevant to clinical conditions as patients with chronic liver disease–related symptoms and complications first seek help then receive treatment.

Fig. 4. Angiogenic factors expression. (A) Spironolactone significantly reduced plasma VEGF concentration in BDL rats. (B) In the BDL rats with spironolactone treatment, the mesenteric VEGF and phospho-VEGFR-2 protein expression statistically significantly decreased compared with distilled water-treated BDL rats. *P < 0.05.
In our study, spironolactone treatment as the therapeutic strategy in cirrhotic rats significantly ameliorated hepatic fibrosis, portal-systemic collateral shunting, and mesenteric angiogenesis, but did not influence PP. A recent survey indicated that the spironolactone prophylactic strategy alleviated liver fibrosis and reduced PP (Luo et al., 2012). Another study showed that prophylactic spironolactone treatment significantly decreased PP and portal-systemic shunts (Oberti et al., 1997). These different PP results with spironolactone suggest that a portal hypotensive effect can be exerted by early spironolactone treatment at the start of liver injury. However, the use of spironolactone at the very beginning of hepatic damage is not prevalent in the clinical setting. In our study, even though spironolactone treatment begun after the development of liver fibrosis failed to modify PP, it still statistically significantly attenuated liver fibrosis and portal-systemic collaterals in BDL rats. These results taken together suggest that spironolactone treatment as a therapeutic rather than a prophylactic strategy may be beneficial and clinically relevant, but the appropriate regimen for patients requires further investigation.

Angiogenesis plays a pivotal role in the formation of portal-systemic collaterals in portal hypertension (Fernandez et al., 2004). Regarding the potential influence of aldosterone on angiogenesis, a human study found that tumor vascularization was positively associated with aldosterone (Bernini et al., 2002). Aldosterone also enhanced ischemia-induced neovascularization (Michel et al., 2004). Consistently, spironolactone treatment in our study ameliorated abnormal splanchnic angiogenesis in cirrhosis.

Spironolactone is also a testosterone antagonist, and a study by Klauber et al. (1996) found that spironolactone attenuated neovascularization through inhibition of the VEGF pathway. However, cyproterone, another testosterone antagonist, was not effective in antiangiogenesis. Furthermore, although spironolactone had antimineralocorticoid effects, it suppressed neovascularization in an aldosterone-free culture medium. The antiangiogenesis activity of spironolactone could be unrelated to its antiandrogenic and antimineralocorticoid effects (Klauber et al., 1996; Gökhan-Köse et al., 2014).

Among the possibly implicated angiogenic factors, VEGF is a potent mitogen that stimulates endothelial proliferation and differentiation, acting mainly via its binding to VEGFR-2 (Terman et al., 1992). Inhibition of the VEGF signaling pathway has been proved to reverse portal hypertension-related pathologic angiogenesis (Fernandez et al., 2005). Aldosterone-producing adrenal adenomas expressed higher levels of VEGF (Bernini et al., 2002). Aldosterone also increased VEGF protein expression in a mouse model with ischemia (Michel et al., 2004). Moreover, spironolactone inhibited both basic fibroblast growth factor- and VEGF-stimulated endothelial cell proliferation and capillary endothelial cell chemotaxis in vitro (Klauber et al., 1996). In this study, spironolactone significantly decreased both plasma VEGF levels and mesenteric VEGF protein expression. Furthermore, the phosphorylation of its target receptor, VEGFR-2, diminished at the same time. This finding suggests that spironolactone attenuated splanchnic and collateral angiogenesis by down-regulating the VEGF pathway.

The potential detrimental effects of dampening the VEGF pathway cannot be ignored, but it may be clinically advantageous for the following reasons. First, sorafenib, a multikinase inhibitor that inhibits angiogenesis via VEGF pathway blockade, is presently a recommended target therapy for hepatocellular carcinoma, which is usually developed in cirrhotic patients. Until now, no remarkable adverse effects have been reported related to antiangiogenesis. Second, clinical experience with spironolactone for patients with cirrhosis or heart failure has indicated that the major side effects of spironolactone treatment are hyperkalemia, gynecomastia, and prerenal azotemia, none of which has been attributed to

Fig. 5. Motor activity and plasma ammonia concentration. (A) BDL rats had lower levels of motor activity compared with sham rats. Spironolactone did not influence motor activity in BDL-cirrhotic rats. (B) Cirrhotic rats had a higher plasma ammonia concentration. Spironolactone did not affect the plasma ammonia concentration. *P < 0.05.
antiangiogenesis. Third, in the current study, the mesenteric vascular density of the spironolactone-treated sham rats was not significantly different from that of the vehicle-treated sham rats, suggesting that spironolactone did not affect relatively normal vasculature. As a result, we think aldosterone application in cirrhosis can be considered safe in terms of its antiangiogenesis effect.

Prostacyclin and nitric oxide (NO) are synthesized by cyclooxygenases (COX-1 and COX-2) and NO synthases (eNOS and iNOS), respectively, and participate in the hyperdynamic circulation and vascular derangement in portal hypertension. They are, in fact, also angiogenic factors (Sumanovski et al., 1999; Gupta et al., 2003; Klenke et al., 2006). Regarding the interrelationship between mineralocorticoid and NO or prostaglandins, Schäfer et al. (2003) demonstrated that down-regulated expression of eNOS protein in the aorta of failing rats with myocardial ischemia could be restored with eplerenone administration. Aldosterone also has been found to increase COX-2 expression in bovine retinal endothelial cells and retinal pericytes (Wilkinson-Berka et al., 2009). Nevertheless, in our study, spironolactone did not affect the mesenteric protein expression of COX-1, COX-2, iNOS, or eNOS. The varying study results may be related to different animal models and experimental settings.

Antifibrotic and the antiangiogenic effects may be mediated by different mechanisms. In the heart, several studies have suggested that mineralocorticoid antagonists prevent cardiac fibrosis by inhibiting inflammatory cells infiltration. In cirrhosis, spironolactone ameliorates hepatic fibrosis by inhibiting the activation of hepatic stellate cells through mineralocorticoid–transforming growth factor-β pathway (Luo et al., 2012; Wang et al., 2014). It is interesting that inflammatory cytokines decreased at the same time (Luo et al., 2012). Furthermore, eplerenone, a selective mineralocorticoid antagonist, attenuates inflammatory cell infiltration in mice with steatohepatitis (Wada et al., 2013). In our study, spironolactone significantly attenuated hepatic fibrosis and the amount of macrophages. This is consistent with the previous studies, suggesting that spironolactone ameliorated hepatic fibrosis at least partly through suppressing macrophage infiltration.

The finding that PP is not affected by spironolactone also reflects the complicated interplay of hemodynamic factors in liver cirrhosis. Portal hypertension is determined by three major factors: augmented hepatic resistance, increased splanchic inflow, and portal-systemic collaterals (Hsu and Huang, 2013). Among them, the collateral vascular bed is a double-edged sword. The shunting vessels release stagnant blood in portal system. However, they are responsible for lethal complications such as esophageal variceal bleeding and hepatic encephalopathy. In this study, spironolactone decreased hepatic fibrosis and mesenteric angiogenesis, which reduced the hepatic resistance and portal inflow and potentially reduced PP. However, this might have been offset by the decreased portal-systemic collaterals found in this study, which reduced the diversion of portal blood flow.

Although spironolactone ameliorated portal-systemic collaterals in our present study, the ammonia level and motor activities were not influenced, suggesting that the severity of hepatic encephalopathy was not alleviated along with the diminished collaterals. In addition, the liver biochemistry data were not modified by spironolactone. Hepatic encephalopathy is driven by two major factors: the escape of ammonia and noxious substances from collaterals to central venous system, and the failure of compromised hepatocytes to manage ammonia via the urea cycle. In line with this finding, we identified previously that in BDL rats the serum total bilirubin levels, but not the severity of collaterals, was significantly and negatively correlated with the motor activity count. That is, the portal-systemic shunting plays a more minor role than liver function in the development of hepatic encephalopathy (Hsin et al., 2012).

In conclusion, spironolactone treatment initiated at the onset of hepatic fibrosis can significantly attenuate fibrosis progression, portal-systemic collateral shunting, and mesenteric angiogenesis. The antiangiogenic effect of spironolactone is accomplished by VEGF pathway down-regulation. Spironolactone does not influence liver biochemistry or hepatic encephalopathy. Spironolactone treatment in cirrhotic patients may exert additional benefits other than ascites control.

Authorship Contributions

Participated in research design: Wang, Huo, Chang, Hsin, Ho, Lin, S. Lee.

Performed data analysis: Hsu, Huang.

Wrote or contributed to the writing of the manuscript: Hsu, F. Lee, Huang.

References


The selective Cox-2 inhibitor celecoxib suppresses angiogenesis and growth of secondary bone tumors: an intravital microscopy study in mice. 


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