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Microcirculatory Stasis Precedes Tissue Necrosis in Ethanol-Induced Gastric Mucosal Injury in the Rat

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The relation of blood flow stasis to the development of unequivocal histologic necrosis (loss of parietal cells from the column of contiguous cells) in ethanol-induced gastric mucosal injury was studied in anesthetized rats. The most rapid vascular change that occurred when the gastric mucosa was exposed to 100% ethanol was a severe segmental constriction of the large submucosal venules. At 22 sec, the average venular diameter was 52.2 ± 6.0% of the original one. This was followed by complete superficial mucosal blood flow stasis at 49 ± 4 sec and appearance of histologic evidence of necrosis in one of seven rats at 2.5 min, four of six rats at 10 min, and seven of seven rats at 60 min. We conclude that in ethanol-induced gastric mucosal injury, submucosal venular constriction occurs first, followed by cessation of mucosal blood flow to be followed later on with histologic evidence of necrosis.

KEY WORDS: gastric microcirculation; ethanol injury; gastric mucosal injury; *in vivo* microscopy; stomach blood flow.

Ethanol-induced gastric mucosal injury is a widely used model to study the pathophysiological mechanisms underlying the formation of mucosal lesions. Gastric mucosal necrosis is the most severe aspect of ethanol injury (1–4). Lacy and Ito (1) have demonstrated that the necrotic lesions were accompanied by mucosal hyperemia. Guth et al (2) have shown that the hyperemia was due to stasis of blood flow with microvascular engorgement. Other studies done in our laboratory have revealed that the latter phenomenon is very rapid and occurs within minutes after the mucosa is exposed to absolute ethanol (5). In still another study, Szabo et al (3) have suggested that vascular injury, as evidenced by increased vascular permeability and endothelial cell damage, is an early pathogenetic factor in the development of ethanol-induced gastric lesions. Ito and Lacy (4) demonstrated that a stomach with no blood flow developed extensive mucosal damage within a very short period of time of contact (3 min) with absolute ethanol, whereas an intact blood supply would afford a significant degree of protection.

In this present investigation, the time sequence of blood flow stasis in relation to ethanol-induced gastric mucosal necrosis in the rat was studied. Utilizing *in vivo* microscopy for the direct visualization of gastric microcirculation, red blood cell

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velocity measurement, submucosal vessel diameter measurement, and histology, studies were designed to test the hypothesis that cessation of blood flow in the mucosa is an early, essential step in the pathogenesis of ethanol-induced gastric mucosal injury in the anesthetized rat.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (230–280 g body weight) (Simonsen Lab. Inc., Gilroy, California) were fasted overnight before the experiments but allowed free access to water. Each control and experimental group consisted of six to seven rats unless otherwise stated. The rats were anesthetized with sodium pentobarbital (Abbott Lab., North Chicago, Illinois) 50 mg/kg intraperitoneally. Rectal temperature was maintained at 37–38°C by a heating pad. The abdomen was opened via a midline incision and the stomach exposed.

In Vivo Microscopy Preparations. An *in vivo* microscopy technique was used to study the mucosal microvasculature (6). A long incision was made in the anterior wall of the forestomach just proximal and parallel to the "limiting ridge" (which separates the squamous cell-lined forestomach from the more distal glandular stomach). A portion of the posterior wall of the corpus was then everted through the incision, thus exposing the mucosal surface to direct visualization. A fiberoptic light carrier rod 5 mm in diameter was placed beneath the serosa of the everted portion of the corpus. The exposed area was transilluminated by passing a cool light from a 150-W tungsten halogen projector lamp (GTE Products Corp., Winchester, Kentucky) through a green filter (Kodak Wratten gelatin filter No. 61) to this rod. A lightweight concave brass disk (2.5 cm in diameter and 5 mm deep) with a 5-mm-diameter hole in the center was fixed, water tight, with a silicone plastic adherent (Silly Putty, Binney & Smith Inc., Easton, Pennsylvania) to the area under study for applications of different solutions. All solutions were maintained at 38°C prior to application. Krebs solution (pH 7.4) was continuously superfused to maintain temperature and moisture of the exposed surface. The preparation was allowed to stabilize for 10–15 min before starting the experiment. The flow of red blood cells through the capillaries surrounding the glands and into collecting venules could be observed readily. Just before the experiment, the superfusion was stopped, and the Krebs solution remaining in the disk was gently aspirated.

To study the submucosal vasculature during the intragastric instillation of noxious agents, a modification of the *in vivo* microscopy technique developed by Hakkinen and Oates (7) was used. A tracheostomy was performed and the trachea intubated with a PE 250 tubing to ensure a patent airway. The esophagus was ligated at the same level as the tracheostomy to avoid including nerves and blood vessels. The duodenum was ligated just distal to the pylorus. A small incision was made in the wall of the forestomach, and the fiberoptic rod was inserted along with a PE 90 tubing for instillation and removal of solutions. The stomach was then gently distended with 3 ml of warm Krebs solution. An incision was made through the serosal and muscle layers in order to expose the submucosal vascular plexi to direct microscopic viewing (6). A continuous drip of Krebs solution at 38°C was applied to the exposed submucosal surface for the duration of the study. The preparation was allowed to stabilize for about 10 min before the start of the experiments.

Optical and Television Systems. An American Optical (A/O) Microstar microscope with long working distance objectives—Leitz UM 32×/0.4 (mucosal studies) and A/O 4 ×/0.12 (submucosal studies)—were used for transmitted light *in vivo* microscopy. The microscope was connected to a closed circuit television system which consisted of a CCD (charge coupled device) video camera (TM-34K; Pulnix America, Inc., Sunnyvale, California), a Hitachi monitor (Hitachi Denshi Ltd.) and a videotape recorder (JVC-HR-7100U). All *in vivo* microscopy studies were videotaped for later analysis. The final magnification on the TV screen was 750× (mucosal studies) and 120× (sub-mucosal studies).

Red Blood Cell (RBC) Velocity Measurement. The technique described by Holm-Rutili and Obrink (8) was used to measure red blood cell velocity in the mucosal capillary vessels. Briefly, on playback of the videotape, two videophotometric windows (upstream and downstream) were positioned along the monitored vessel lumen. The voltage output would change as red blood cells passed each window; the resulting signals were fed into a red blood cell velocity tracking correlator (model 102B, Instrumentation for Physiology and Medicine, San Diego, California), which would determine the time lag for similar voltage patterns between the upstream and downstream signals. By continuously dividing this time lag into the preset distance between the two windows, the correlator continuously outputs the red blood cell velocity in millimeters per second.

Submucosal Vessel Diameter Measurement. On playback of the videotape, the diameter of the submucosal vessels was measured using an image-splitting technique (9). Since the width of the column of red blood cells was measured, the measurement reflects the internal diameter of the vessel. Turning a knob on the image-splitter (Instrumentation for Physiology and Medicine, model 907) shears the image horizontally. When the vessel is split exactly one diameter, the change in the meter readout on the image-splitter (which reflects the amount of turn of the knob) is proportional to the vessel diameter.

Histologic Studies. The same mucosal preparation employed for *in vivo* microscopy was used for all histologic studies. At various times after the placement of experimental solutions in the disk, the area under study (5 mm in diameter) was marked by two points using a microsurgical cautery (Vari-i-Stat, Concept Inc., Clearwater, Florida). The marked piece of tissue (the 5-mm circle from the center of the disk) was immediately removed, placed in 10% buffered formalin, and later embedded in paraffin, sectioned (6 μm thick), and stained with hematoxylin and eosin. Slides were evaluated microscopically in a blinded fashion by coding, so that the examiner (G.P.) was unaware of the treatment group from which each slide came. Only after evaluation of all slides was complete were they decoded. Using a graded micrometer eyepiece, the overall length of each tissue section was measured,

Fig 1. Photomicrograph of stained (H&E) section of corpus mucosa demonstrating type III damage with necrosis. In addition to surface and mucous neck cell damage, the injury involves parietal and even chief cells with marked glandular disruption (necrosis) with spaces of varying length separating the remaining damaged parietal cells (black arrow). The bar in the lower right-hand corner of the figure represents 200 μm.

and the percent length of mucosal surface with each type of injury was determined.

The types of mucosal injury were assessed using the criteria of injury established previously by Lacy and Ito (1), damage to the gastric mucosa being defined as follows: O = all gastric mucosal cells appeared intact and had normal shape, location, appearance, and density; I = surface mucous cell damage—these cells were vacuolated, had pyknotic nuclei, and brightly stained or lysed cytoplasm; II = extensive surface cell damage plus disruption and exfoliation of cells lining the gastric pits; III = surface and pit cell damage plus gastric gland damage—the parietal cells had pyknotic nuclei and lucent cytoplasm; necrosis = a variant type III damage with severe damage extending through the middle third (parietal cell area) and occasionally the inner third (chief cell area) of the mucosa. In addition to the greater depth of mucosal injury, in necrosis there was marked glandular disruption with the damaged parietal cells no longer forming a continuous column of contiguous cells, but with empty spaces of varying length separating isolated cells (Figure 1). Lacy and Ito (1) found that hemorrhage and/or hyperemia accompanied the necrotic injury. Hemorrhage and/or hyperemia could not be used as criteria for necrosis in the present study because the temporal relationship between stasis and necrosis was being studied. Stasis occurs early in ethanol injury and could produce hyperemia and possibly hemorrhage whether or not deep necrosis was present. Hence the criteria for necrosis of type III damage with marked glandular disruption as described above was used.

Study I: Effect of 100% Ethanol on Superficial Gastric Mucosal Blood Flow. One hundred percent EtOH was placed in the disk and flow of blood was observed in the mucosal microvessels. Times for slowing and stasis were determined using the time display on the screen of the monitor. Ethanol was left in place, and observation was continued for at least 5 min after blood flow stasis. Application of Krebs solution for 1 hr was used as control.

One hundred percent EtOH was placed in the disk for 1 or 10 min and then gently aspirated and replaced by Krebs solution for 59 or 50 min, respectively, and the changes in blood flow were observed.

Study II: Effect of Intraluminal 100% Ethanol on Submucosal Microvasculature. After stabilization of the preparation, 2 ml of Krebs were aspirated from the stomach and 2 ml of either 100% EtOH or Krebs solution were instilled. The large vessels of the submucosa (average diameter 100 μm) were observed.

Study III: Time Course of Development of Histologic Necrotic Lesions. Absolute ethanol was placed in the disk overlying the exposed surface of the gastric mucosa for different periods of time: 1, 2.5, 5, 7.5, 10, 30, and 60 min, and then the tissue was marked, removed, and processed for histology. Another group was treated similarly for 1 or 10 min and then EtOH was gently aspirated and replaced by Krebs solution for 59 or 50 min, respectively. In still another group, 50 or 75% EtOH was applied for 10 min. Application of Krebs solution for 10 or 60 min was used as control.

RESULTS

Study I: Effect of 100% Ethanol on Superficial Gastric Mucosal Blood Flow. The application of Krebs solution into the disk for 60 min resulted in no significant changes in blood flow throughout the whole hour as measured by the velocity tracking correlator (initial velocity was 0.59 ± 0.02 mm/sec and velocity in the last 10 min was 0.57 ± 0.03).

In contrast, the topical application of 100% EtOH to the exposed gastric mucosal surface resulted in prompt cessation of flow. The superficial mucosal microvessels remained filled with red blood cells, but they were no longer flowing (Figure 2). Red blood cell flow gradually slowed and reached complete stasis in all vessels in less than 1 min. Slowing was defined as the time when 50% of the vessels in the field showed gross flow slowing, and this occurred in 22 ± 2 sec.

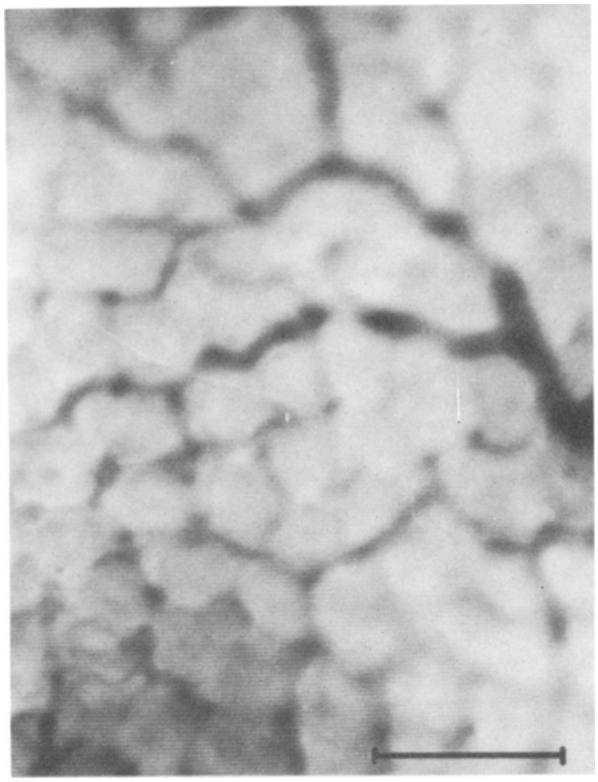

Fig 2. Photograph of the superficial mucosa taken from the face of the videomonitor. Blood flow has ceased following the topical application of 100% ethanol. The mucosal capillaries surrounding the glands and the postcapillary venules and collecting venule into which they drain are filled with blood. The bar in the lower right-hand corner of the figure represents 100 μm.

Stasis was defined as the time when flow completely stopped in all the vessels in the field, and this occurred in 49 ± 4 sec (range = 32–60 sec).

In the second part of this study, the mucosa was exposed to 100% EtOH for either 1 or 10 min. In both groups the flow reached complete stasis in less than 1 min and when the solution was changed to Krebs for 59 or 50 min respectively, the flow did not resume again.

Study II: Effect of Intraluminal 100% Ethanol on Submucosal Microvasculature. The instillation of 2 ml of Krebs solution resulted in no change of diameter in any of the submucosal vessels. The instillation of 2 ml of absolute ethanol into the stomach resulted in a very rapid constriction of the large submucosal venules (average diameter = 108.2 ± 6.8 μm) (Figure 3). The constriction was usually observed to be segmental and very severe (Figure 3B). At 10 sec, the average diameter was 74.8 ± 6.8 μm or 69.1 ± 6.3% of the initial value. At 22 sec, the average diameter was 56.5 ± 6.5 μm or 52.2 ± 6.0% of the initial value (Figure 4). Observations could not be extended beyond 90 sec in most of the rats because the field progressively became more obscure from the increasing redness of the overlying mucosa. Typically at 150 sec, the entire stomach became grossly red because of severe hyperemia. Because of the low magnification and the increased respiratory motion, flow of blood could not be observed in these experiments. Smaller venules were not observed in the fields studied. The diameter of the larger arterioles did not change from their baseline values after the experimental procedure (baseline diameter 97.83 ± 7.46 μm and 99.89 ± 8.21 μm when venular constriction was maximal).

Study III: Time Course of Development of Histologic Necrotic Lesions. Since this investigation was concerned with the temporal relationship between cessation of blood flow and the development of necrosis, only necrosis (the most severe form of type III) will be reported. Because of the small piece of tissue we were studying, we noted that whenever a rat mucosa was involved with necrotic lesions it would include the whole length of the exposed mucosa. Therefore the percent length of mucosa involved with necrosis will not be specified.

Results are presented in Figure 5. In the group treated with 100% EtOH, there was no evidence of necrosis in any of the rats at 1 min. The first evidence of necrosis appeared at 2.5 min, with necrosis in one rat out of seven. The number of rats with necrosis increased with increasing duration of exposure to ethanol. At 5 min, two of six rats; at 10 min, four of six rats; and by 60 min, all of the seven rats, had necrotic lesions. The resultant curve of time versus percent of rats whose mucosa was involved with necrotic lesions consisted of two parts: a rapidly rising component from 2.5 to 10 min and a slowly rising component from 10 to 60 min. In the group treated with 100% EtOH for 1 min and then Krebs solution for the rest of the hour, no rats developed necrotic lesions. In the group treated with 100% EtOH for 10 min and then Krebs solution for the rest of the hour, four of eight rats developed necrotic lesions. In the control group, application of Krebs solution for 60 min resulted in no rats with necrotic lesions.

DISCUSSION

In this investigation, we examined the time relationship between blood flow stasis and the development of necrotic lesions in the ethanol-induced

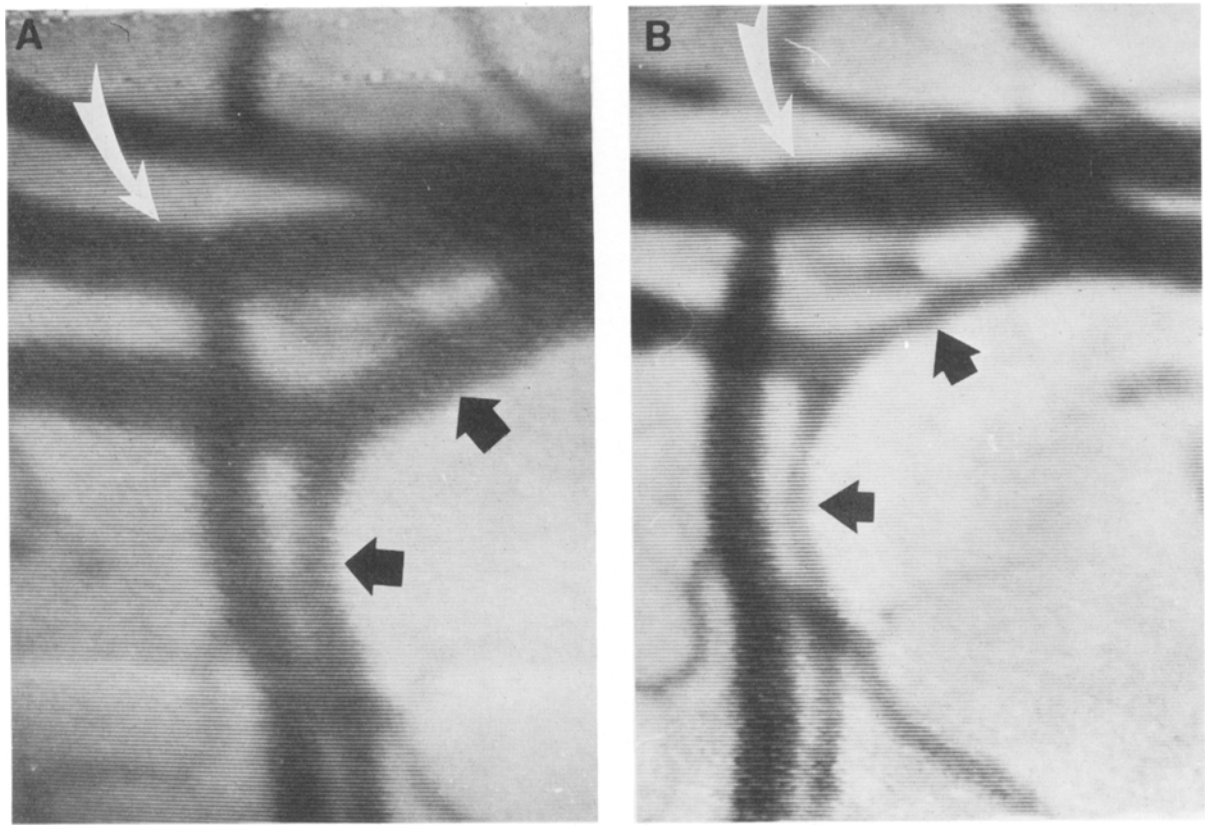

Fig 3. Photographs of the submucosa taken from the face of the videomonitor before (A) and after (B) the intragastric instillation of 2 ml of 100% ethanol. The black arrows point to a submucosal venule and the white arrow points to a submucosal arteriole. Figure 2B, taken 30 sec after ethanol instillation, reveals severe constriction of the venule, but no change in arteriolar diameter. The bar represents 200 μm.

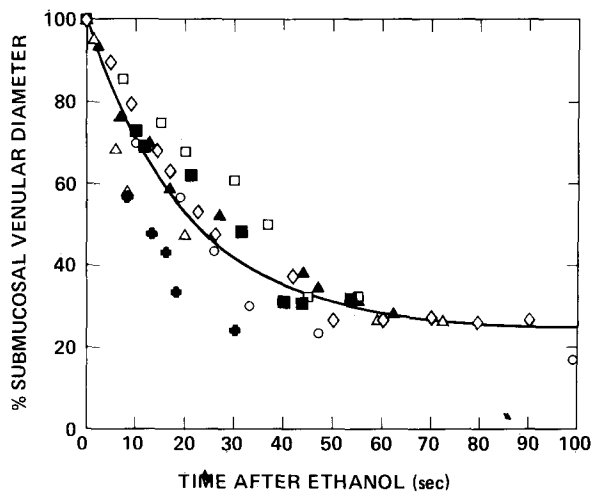

Fig 4. Time course of change in submucosal venular diameter (as percent of control) after the intragastric instillation of 100% ethanol. Each symbol refers to a different rat (total: seven rats). A nonlinear least-square regression analysis revealed an exponential time course with an initial, very rapid, and steep decrease in diameter which levels off at approximately 25% of the initial diameter at about 55 sec.

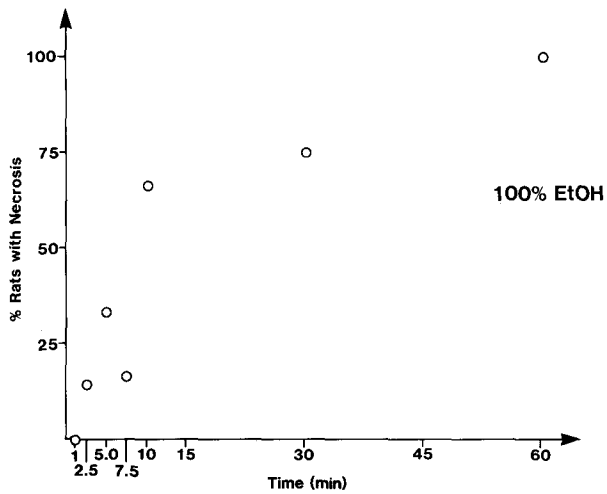

Fig 5. Time course of development of gastric mucosal necrosis after exposure to 100% ethanol. The percent of rats with necrotic lesions is presented on the ordinate. Six or more rats were studied at each time period. At 2.5 min, the first necrotic lesion appeared; at 10 min, four of six rats, and at 60 min, all the rats had necrosis. The resultant curve consists of two parts: a steep rise from 2.5 to 10 min and a more gradual one from 10 to 60 min.

gastric mucosal injury model. The most rapid vascular change that occurred when the gastric mucosa was exposed to 100% ethanol was a severe segmental constriction of the large submucosal venules accompanied by a constant arteriolar diameter. These findings are in accord with those of Hakkinen and Oates (7), except that we did not observe any dilatation of the arterioles. The reason might be that only large arterioles could be observed in our preparation, whereas their observations did include much smaller ones. In a preliminary report from our laboratory, Ohya and Guth (5), using a different experimental technique to study the submucosal vessels, did not observe the same phenomenon. They perfused a small area of the mucosa rather than filling the stomach with ethanol, and only smaller venules were observed. In the present study, at 22 sec, the submucosal venular diameter decreased to approximately 50% of baseline, which could account for the slowing of mucosal blood flow we observed at that time. The second chronological event in ethanol-induced gastric mucosal injury is stasis of superficial mucosal blood flow that occurred at 49 sec. It seems likely that the rapid superficial mucosal blood flow stasis was the result of a combination of slowed flow secondary to submucosal venular constriction plus superficial mucosal microvascular factors induced by ethanol.

When mucosal flow ceased, necrosis did not develop immediately. No necrotic lesions were present at 1 min after ethanol application, and it was not until 2.5 min that the first necrotic lesion appeared in one rat out of six. The number of rats with necrotic gastric mucosa increased with time to involve 100% of the rats by 1 hr. One explanation of this time course could be that histological evidence of necrosis needs time to develop and the increase with time is merely an effect of time on an injury already present in the early stages. For that purpose, two groups of rats were treated for 1 or 10 min with 100% ethanol and then Krebs solution was applied for the rest of the hour. The results were identical with that of 1 or 10 min of 100% ethanol alone. This was not due to any further protection by resumption of blood flow because the stasis was not reversible within 1 hr (study I). Therefore both stasis and the presence of ethanol for 10 min were needed for the development of necrotic lesions in half or more of the rats.

These findings are consistent with, but do not prove, the hypothesis that cessation of blood flow is an early, essential step in the pathogenesis of ethanol-induced gastric mucosal injury. Further studies are warranted to determine more precisely the role of microcirculatory stasis in ethanol injury—is it failure to deliver needed oxygen and nutrients, failure to dilute and carry away back-diffusing alcohol, or is it release of a mediator such as leukotriene C_4 (10) by the action of ethanol on blood elements?

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