Microcirculation in Intestinal Villi
A Comparison between Hemorrhagic and Endotoxin Shock

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Using intravital microscopy, we studied the effects of hemorrhagic and endotoxin (ETX) shock on the velocity of erythrocytes (red blood cells [RBC]) and the density of perfused villi in mouse small intestine. The mice were divided into four groups: control, normotensive sepsis (a low ETX group, 1.5 mg/kg intravenously), hypotensive sepsis (a high ETX group, 10 mg/kg intravenously), and a hemorrhagic group. One hour after endotoxemia or hemorrhage was induced, mean arterial pressure significantly decreased in the high ETX and hemorrhagic groups (72.5 ± 1.0 mm Hg in the control group, 71.0 ± 2.4 in the low ETX group, 42.7 ± 1.8 in the high ETX group, 43.0 ± 1.4 in the hemorrhagic group, respectively). We found significant decreases in RBC velocities in the villous tip and capillaries in both ETX groups but not in the hemorrhagic group (in villus tip arteriole, 1.25 ± 0.02 mm/s, 0.80 ± 0.02, 0.15 ± 0.01, 1.20 ± 0.08; in villus capillaries, 0.55 ± 0.02, 0.38 ± 0.02, 0.10 ± 0.01, 0.61 ± 0.02, for control, low ETX, high ETX, and hemorrhagic groups, respectively). We also found a significant decrease in the density of RBC-perfused villi in the high ETX and hemorrhagic groups but not in the low ETX or control groups. However, the change in the hemorrhagic group was much less than in the high ETX group (100.0 %, 95.2 ± 1.6, 32.5 ± 1.9, 87.3 ± 3.4, for control, low ETX, high ETX, and hemorrhagic groups, respectively). We concluded that ETX induces a significant decrease in mucosal perfusion characterized by a diminution in RBC velocity and flux in villi found even in a normotensive situation. At a high dose of ETX, these changes are associated with a significant decrease in the number of perfused villi. In addition, we found that at the same level of hypotension, hemodynamics and mucosal perfusion disorders are considerably larger in ETX-induced hypotension than in hemorrhagic hypotension.

Keywords: shock, septic; shock, hemorrhagic; microscopy; fluorescence; mucosa, intestinal; erythrocytes

Through the recent decade, the knowledge regarding all types of shock has increased explosively. In the critical period such as trauma or postoperative stage, the perfusion of the gut mucosa is important to prevent the translocation of intraluminal bacteria and toxins. As noted by different investigators (1, 2), microcirculation disturbances play a crucial role during endotoxemia. It is particularly important to understand how the intestinal microcirculation is affected during the first stages of endotoxemia as hyperperfusion might induce disturbance of the mucosal barrier and result in facilitating the entrance of endotoxin (ETX) into systemic circulation. In this regard, several investigators have reported the changes of the blood flow in the intestinal tissue under septic conditions. Schmidt and coworkers (3) reported that the blood flow and the diameters of arterioles were significantly decreased in the rat intestinal villi after receiving as much as 15 mg/kg/h of lipopolysaccharide (LPS). Lam and coworkers (4) observed a decrease in intestinal villous blood flow during endotoxemia even if systemic blood pressure was maintained within normal range.

With regard to hemorrhagic shock, some investigators reported that villous blood flow remained unchanged or only moderately changed in the small intestine when perfusion pressures were reduced (5–8). Indeed, it is known that many compensatory responses, involving activated sympathetic stimuli, prostaglandins, and neural responses, appeared when systemic pressure was decreased in a hemorrhagic shock model. These mechanisms are markedly altered in ETX shock, thus probably inducing differences in intestinal perfusion even for the same level of blood pressure decrease.

Blood flow measurements by global methods do not always reflect the effective oxygen transport by red blood cells (RBC) in mucosa (9). In contrast, by using intravital microscopy RBC velocity and flux can be measured simultaneously in individual villi. However, these measurements in individual villi could also be insufficient to estimate the quality of the perfusion for the mucosa if they are taken alone as the shock-induced changes are generally heterogeneous. For this reason, we developed a model for the intravital study of the intestinal mucosal microcirculation in mice. It allows one not only to measure hemodynamic properties in the individual villus but also to evaluate the density of the perfused villi, a more recently proposed parameter that reflects the proportion of the mucosa that can be hypoxic in a given situation.

Using this model, the aims of our study were to evaluate the changes in mucosal perfusion in intestinal villi during normotensive and hypotensive endotoxemia, and to compare the effect of the same level of decrease in blood pressure associated with either ETX or hemorrhagic shock.

METHODS

Fluorescent Labeling of Erythrocytes
We used the modified procedure described by Butcher and Weissmann (10) for labeling erythrocytes with fluorescein isothiocyanate (FITC). Erythrocytes were obtained from separate donor mice by heart puncture after administration of pentobarbital. They were labeled with FITC (No. 18H5075; Sigma Diagnostics, St. Louis, MO). Erythrocytes were separated by centrifugation (∗× 1,000, 5 min) and washed four times in a phosphate-buffered saline adjusted to pH 7.4 (PBS, No. 1000-3; Sigma Diagnostics) containing 100 mg/L ethylenediaminetetraacetic acid (EDTA), resuspended in PBS (pH 8.0) containing FITC and incubated at 25°C for 2 h. The labeled erythrocytes were again flushed with the PBS and centrifuged several times to free the supernatant from fluorescent dye. The labeled cells were stored in darkness in a refrigerator at 5°C. Time of storage for labeled RBC was less than 48 h.

Animal Preparation and Surgical Procedure
All the mice used were female (Balb/c) weighing between 18 and 21 g. The animals were fed standard mouse diet and had free access to water. All procedures were approved by the Institutional Animal Committee. Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (90 mg/kg body weight; Sanofi Santé Nutrition
An unrestrained blood supply to the bowel segment under investigation was verified by the pulsation of the ileal artery and its color. The bowel segment was then grafted on a specially designed pedestal to facilitate observation of the villi with transfusiluminating and epifluorescent microscopy. The bowel segment was gently fixed with two small pins on the outer fringes of each site to a frame with the mucosa on the upper side.

The ileum was placed in a supine position on a specially designed Plexiglas microscope stage equipped with a glass slide to allow for transfusiluminating microscopy. An abdominal midline incision was done and a segment of 2 to 3 cm of the ileum was exteriorized (7, 11). The ileum was opened along the antimesenteric border over a distance of 1 cm and placed on a specially designed pedestal to facilitate observation of the villi with transfusiluminating and epifluorescent microscopy. The prepared bowel segment and mesentery were superimposed with a polyethylene catheter (PE10, interior diameter [i.d.] 0.28 mm) for the injection of labeled erythrocytes and ETX or saline.

Animale, La Ballastière, France). A tracheostomy was performed to facilitate spontaneous breathing, and the right jugular vein was cannulated with a polyethylene catheter (PE10, interior diameter [i.d.] 0.28 mm) for the injection of labeled erythrocytes and ETX or saline. Another catheter was inserted in the right carotid artery. To monitor mean systemic arterial pressure, the catheter was connected to a transducer (Uniflow; Baxter, Kent, UK) to derive the pressure signal which was stored in the computer using a data acquisition system (MP-30; Biopack Systems Inc, Santa Barbara, CA). Then the animal was placed in a supine position on a specially designed Plexiglas microscope stage equipped with a glass slide to allow for transfusiluminating microscopy. An abdominal midline incision was done and a segment of 2 to 3 cm of the ileum was exteriorized (7, 11). The ileum was opened along the antimesenteric border over a distance of 1 cm and placed on a specially designed pedestal to facilitate observation of the villi with transfusiluminating and epifluorescent microscopy. The temperature of the solution was maintained at 37 ± 0.5°C with a feedback-controlled heating device (Polystat; Böblott Scientific, Illkirch, France). After completing the preparation, FITC-labeled erythrocytes (15 μl) were administered intravenously and the exposed intestine was gently covered by Saran-Wrap and allowed to stabilize for at least 30 min. All animals received 0.9% saline at 0.3 ml/h to compensate for fluid loss (Syringe Pump 33; Harvard Apparatus Ltd., Kent, UK). The animals were breathing ambient air. Mean arterial blood pressure (P a) was monitored and recorded continuously. The esophageal temperature and the mesenteric area temperature were also monitored and held constant at 37°C with a servo-controlled heating mat (Homeothermic Blanket Control Unit 50-7061; Harvard Apparatus Ltd.).

**Experimental Protocol**

After surgical procedure, the mice were randomly divided into four groups: one group (control, n = 6) was injected with saline; the second group (low ETX group, n = 6) was injected with 1.5 mg/kg of ETX (LPS 0127:B8, Sigma, St. Louis, MO) over 5 min; the third group (high ETX group, n = 6) received 10 mg/kg of ETX over 5 min; and the fourth group (hemorrhagic group, n = 6) underwent induced hemorrhagic shock. In the hemorrhagic group, the systemic blood pressure was reduced by drawing blood from the carotid artery to obtain P a at approximately 40 mm Hg for 1 h. The dose of ETX in the second group was determined by a preliminary study so as not to alter the systemic arterial pressure after ETX injection. In a separate set of experiments, we checked the values of P a, heart rate (HR), respiratory rate (RR), and arterial blood gases after 60 min under the four types of experimental conditions with the same surgical procedures (n = 3 in each group).

**Intravital Microscopy**

After preparation of the intestine was completed, the stage was placed under a fluorescence microscope (Leitz; Wetzlar, Germany) to allow for transfusiluminating and epifluorescence microscopy. The microscope was equipped with ×10 and ×25 water immersion lenses. Epifluorescence was performed with an epifluorescence illuminator equipped with a xenon lamp, a heat protecting filter, and an excitation filter. Transillumination was achieved using a cold light fountain. The images were captured using a charge-coupled device (CCD) camera (DXC-101P; Sony, Tokyo, Japan) and recorded on video recorder (S-VHS; HR-S8000MS, JVC, Tokyo, Japan). The signal of a time generator (VTG-33; For-A Company, Tokyo, Japan) was superimposed to facilitate evaluation of video images.

In each condition, we recorded three sequences of video images of three different parts of the mucosa using the ×10 lens for evaluation of villous density and recorded three sequences of images in each villus using the ×25 lens to measure the velocity and flux of RBC in villus tip arteriole and villus capillaries. For off-line analysis, the videotape was replayed at a conventional rate of 30 frames/s and frame-to-frame analysis was performed with the help of a personal computer. Magnification was confirmed with a reference micrometer slide. We showed representative images from the videotape in Figure 1.

**Quantification of Microcirculatory Parameters**

We used the methods described by Sarelius and Duling (12). The velocity and the flux were observed at the villous tip arteriole and three capillaries in each villus. For calculations, we observed three randomly selected villi in each animal. Velocity was calculated from the time required for an FITC-labeled RBC to traverse a measured length of vessel during videotape playback. To estimate the flux, the number of labeled RBC in a portion of capillary per unit time was measured. Flux was estimated by dividing this number by the labeled cell fraction. This fraction was measured in blood drawn by postocular sinus puncture on completion of the microcirculatory observations. A drop of diluted blood with saline was placed on a covered microscope slide, and sites for cell counting were selected randomly using transillumination. Additionally, intraluminal tip arterial diameters were retrospectively obtained simultaneously from video images.

We also evaluated the villus density, expressed by the fraction of perfused villi versus total number of villi during each observation. We

![Figure 1](image_url)

*Figure 1.* Photomicrographs taken from the video monitor illustrating the characteristic appearance of villi with labeled erythrocytes during in vivo microscopy. **Left panel:** intestinal villi traced with low magnification for evaluating villous density. **Right panel:** intestinal villus with high magnification for measuring velocity and flux. 1: FITC-labeled erythrocyte flowing in tip arteriole, 2: erythrocyte in villous capillary.
TABLE 1. PHYSIOLOGIC VALUES DURING THE EXPERIMENT*

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Low ETX</th>
<th>High ETX</th>
<th>Hemorrhage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight, g</td>
<td>19.0 ± 0.4</td>
<td>18.8 ± 0.4</td>
<td>18.8 ± 0.3</td>
<td>19.8 ± 0.6</td>
</tr>
<tr>
<td>Body Temperature, °C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before</td>
<td>37.0 ± 0.04</td>
<td>37.0 ± 0.04</td>
<td>37.1 ± 0.04</td>
<td>37.0 ± 0.04</td>
</tr>
<tr>
<td>1 h</td>
<td>37.0 ± 0.04</td>
<td>36.9 ± 0.04</td>
<td>37.1 ± 0.08</td>
<td>37.1 ± 0.04</td>
</tr>
<tr>
<td>Pao2, mm Hg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before</td>
<td>73.2 ± 2.0</td>
<td>74.7 ± 1.7</td>
<td>75.3 ± 0.9</td>
<td>75.3 ± 1.2</td>
</tr>
<tr>
<td>1 h</td>
<td>72.5 ± 0.9</td>
<td>71.0 ± 2.4</td>
<td>42.7 ± 1.8*</td>
<td>43.0 ± 1.4*</td>
</tr>
<tr>
<td>Arteriolar diameter, μm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before</td>
<td>5.5 ± 0.1</td>
<td>5.3 ± 0.1</td>
<td>5.5 ± 0.1</td>
<td>5.3 ± 0.1</td>
</tr>
<tr>
<td>1 h</td>
<td>5.4 ± 0.1</td>
<td>5.3 ± 0.1</td>
<td>5.4 ± 0.1</td>
<td>5.3 ± 0.1</td>
</tr>
</tbody>
</table>

* Values are expressed as mean ± SEM (n = 6).
† Arteriolar diameter was measured at intestinal villous tip.
‡ p < 0.05 versus the control and the low ETX groups.

counted the number of villi directly at low magnification in three sites randomly selected at each time of the study.

Statistical Analysis
All the physiologic values were expressed as mean ± SEM. These data were compared by analysis of variance (ANOVA) using Bonferroni/Dunn test for post hoc analysis (Statview 5.0 for Macintosh). Values of p < 0.05 were considered significant.

RESULTS
Systemic Parameters
Baseline values for the systemic parameters of the four groups were not significantly different (Table 1). After ETX administration, Pao2 remained constant in the low ETX group, but decreased significantly in the high ETX group. In addition, there were no significant differences in Pao2 between the high ETX and the hemorrhagic groups. HR and RR increased in the hemorrhagic and in both ETX-treated groups (HR: 374.2 ± 10.4, 446.4 ± 7.0, 474.8 ± 11.7, 490.2 ± 11.4; RR [n/min]: 134.8 ± 0.9, 150.9 ± 2.3, 150.7 ± 3.3, 155.0 ± 1.3 for the control, low ETX, high ETX, and hemorrhagic groups, respectively) and we found that the value of Pao2 was slightly lower in both ETX-treated groups (Pao2: 99.5 ± 8.1, 87.5 ± 9.3, 81.6 ± 11.4, 101.0 ± 6.9 for the control, low ETX, high ETX, and hemorrhagic groups, respectively). Esophageal and mesenteric area temperatures remained constant between 36.8 and 37.3°C in all the groups throughout the study.

RBC Velocity, RBC Flux, and Vessel Diameter in Villus Microcirculation
At baseline, no differences among the groups in RBC velocity, RBC flux, or vessel diameter were found. As shown in Figures 2A and 2B, after ETX administration, the velocities in both ETX groups decreased significantly within 60 min RBC velocities in villus tip arteriole and villus capillaries in a dose-dependent manner. We observed a similar effect of ETX for the flux (Figures 2C and 2D). It decreased to 30% or 50% of the control value (depending on the site) in the low ETX group and 15% (in both sites) in the high ETX group. In contrast, after hemorrhage no change was observed in velocity or flux in both sites studied. Statistical distributions of each RBC parameter are shown in Figure 3. After ETX 1.5 mg/kg injection, histograms showed slight shifts to the left, which reflect the decrease in mean velocity or flux. With 10 mg/kg of ETX, the peaks of distribution shifted toward zero and the relative spatial heterogeneity of the velocities and fluxes expressed by the coefficient of variation (standard deviation/mean value) largely increased. Indeed, the coefficients of variation increased by only 10% in the hemorrhagic and 1.5 mg/kg ETX groups but increased by more than 40% in the 10 mg/kg ETX group.

With regard to tip arteriolar diameter, we did not find any significant difference among the four groups (Table 1).

Villus Density
We did not observe any differences in the RBC-perfused villus density among the four groups at baseline values (Table 2). After 1 h, in the low ETX group, we did not find any significant difference with the control group. Indeed, we observed only very few villi without perfusion. In contrast, in the high ETX group, the number of RBC-perfused villi dramatically decreased in comparison with the other groups. It is noteworthy that after 1 h of ETX administration, we also observed a swelling and a slight decrease of transparency in each villus in this group. We also observed a significant decrease of perfused villi in the hemorrhagic group in comparison with the control value but the change was much more limited than that found in the high ETX group.

DISCUSSION
The present study showed that blood hemodynamics in intestinal villi is affected even in nonseptic sepsis. In addition, we found that the density of perfused villi, RBC velocity, and flux were dramatically decreased in hypotensive sepsis whereas for

![Figure 2. Changes in RBC velocity (A, B) and flux (C, D) in villous tip and capillaries after 1 h of administration of ETX or hemorrhage. All values are expressed as mean ± SEM. *The high ETX versus the low ETX group, p < 0.05; †versus the hemorrhagic group; ‡versus the control group, p < 0.01.](image-url)
the same level of hypotension only moderate changes were observed in hemorrhagic shock.

Our results showing that villus RBC velocity and flux decrease even in normotensive sepsis are in accordance with those of Whitworth and coworkers (13) who reported hypoperfusion in intestinal microcirculation without decreased cardiac output during endotoxemia. Theuer and coworkers (14) also reported vasoconstriction in the larger arterioles and severe decreases of microvascular blood flow during endotoxemia without changes of systemic blood pressure and superior mesenteric artery flow. We found a dose-dependent decrease in RBC velocity and flux in the ETX groups. This is in line with the findings by Schmidt and coworkers (3) who used intravital microscopy to measure blood flow in villus central arteriole for 2 h after ETX administration. They reported faster and larger decreases of blood flow in rats receiving 15 mg/kg/h of ETX than in the group receiving 1.5 mg/kg/h of ETX. They also observed the decrease of villus central arteriolar diameter, but in contrast, we did not find any changes in tip arteriolar diameter. Moreover, it should be noted that the high-dose ETX group not only reduced the average values of velocity or flux but also changed the statistical distribution of these parameters. Indeed in the present study, the absolute heterogeneity expressed by the range between extreme values is reduced owing to a compression of the statistical distribution toward zero, but the relative spatial heterogeneity of these parameters expressed by the coefficient of variation increased (15).

With regard to density of perfused vessels, Drazenovic and coworkers (16) using a carbon-colloid technique reported that

| TABLE 2. VILLOUS PERFUSED DENSITY IN INTESTINAL MUCOSA* |
|----------------|----------------|----------------|----------------|----------------|
|                | Control        | Low ETX        | High ETX       | Hemorrhage     |
|                | Before 1 hr    | Before 1 hr    | Before 1 hr    | Before 1 hr    |
| N, mm²         | 86.8 ± 1.9     | 85.8 ± 2.4     | 87.6 ± 1.0     | 81.7 ± 2.1     | 89.8 ± 2.4     | 85.6 ± 1.9     |
| n, mm²         | 86.8 ± 1.9     | 85.8 ± 2.4     | 87.6 ± 1.0     | 81.4 ± 2.1     | 87.3 ± 1.5     | 26.4 ± 1.2     | 89.8 ± 2.4     | 74.6 ± 2.9     |
| Ratio, † %     | 100            | 100            | 100            | 95.2 ± 1.6     | 100            | 32.5 ± 1.9†§   | 100            | 87.3 ± 3.4†§   |

Definition of abbreviations: N = number of villi/mm²; n = number of RBC-perfused villi/mm².
* Values are expressed as mean ± SEM.
† Ratio was calculated as n/N for proportion of perfused villi.
§ p < 0.01, before versus 1 hr.
† p < 0.01, versus the control group.
†§ p < 0.01, versus the hemorrhagic group.
perfused capillary density was reduced after 5 mg/kg of ETX administration in dogs and hypothesized that failure of capillary recruitment could be induced by ETX. On the other hand, using intravital microscopy, Farquhar and coworkers (17) described the method for measuring the “intercapillary area” expressed as the areas surrounded by perfused capillaries in a single villus. They found that these values were increased in normotensive sepsis induced by cecal ligation and perforation, and that heterogeneity in mucosal perfusion calculated by the coefficient of variation of this parameter was also increased in this condition. However, it is important to note that the studies mentioned previously reported the effect of ETX on the density of capillaries perfused within each villus. In the present study we used a more restrictive parameter, because we counted the number of villi without RBC perfusion, thus certainly in hypoxic conditions. Using this parameter, it appears that there is a clear difference between normotensive and hypertensive sepsis. In the former case, the hemodynamics within each individual villus were altered but almost all villi remained perfused.

In contrast, in the latter case we also found a dramatic decrease in the density of perfused villi because only 32% of the villi remained perfused. This was not the effect of hypotension per se because at the same level of hypotension, the intestinal villus microcirculation was considerably more affected in endotoxic than in hemorrhagic shock. Indeed, we did not observe any significant decreases of RBC velocity or flux in the hemorrhagic group compared with the control group, and the statistical distribution of RBC velocity and flux in the hemorrhagic group was also very close to that in the control group. Even if significant, the decrease in density of perfused villi was much smaller than that observed in the high ETX group for the same decrease of blood pressure. Our observations in hemorrhagic shock are generally in line with those by Lundgren and Svanvik (5) who reported that villous plasma flow did not change in the small intestine of the cat when perfusion pressure decreased to 30 mm Hg. Gosche and Garrison (6) observed vasoconstriction of arterioles in the small intestine, but stressed that autoregulation and redistribution in favor of the mucosa occurred in the moderate hemorrhage model.

We found a lower value of arterial PO2 in both ETX groups than those of the control and hemorrhagic groups. However, it is unlikely that this difference can explain the present results because values in both ETX groups were similar but changes in microcirculatory parameters were largely different, and because the lowest value of PO2 measured was 69.4 mm Hg and Sababi and Holm (7) reported that a much lower PO2 (approximately 45 mm Hg) did not affect RBC velocity in villus microcirculation. To elucidate the mechanisms involved in the differences between the effects of the two types of shock in intestinal microcirculation is beyond the scope of the present study. Factors that can possibly affect the microcirculatory response to shock or the redistribution of blood flow toward the mucosa have been identified by others. The involvement of inflammatory mechanisms mediated by leukocyte activation (18) and cytokine release is possibly more important in sepsis than in hemorrhagic shock (even if also present in this latter). For instance, Barroso-Aranda and coworkers (19) reported plasma tumor necrosis factor (TNF) activity in LPS-treated rats but not in hemorrhagic shock. In addition, immediately after ETX is administered, perivascular mast cells begin degranulation and release many substances as kinins (2) which can affect local blood flow regulation and be responsible for changes in permeability, which indirectly affects villi perfusion. Moreover, hemorrheological factors can also contribute to deleterious changes in villi perfusion during sepsis. Indeed, Sugiyama and coworkers (20) observed the loss of deformability of RBC after ETX injection even when systemic blood pressure was maintained. In contrast, they found that deformability was not impaired during hypotension (approximately 40 mm Hg).

In summary, we observed significant decreases in RBC velocity and flux in the small intestine of mice during normotensive or hypertensive sepsis. The density of RBC-perfused villi was maintained during normotensive sepsis but in hypertensive sepsis a dramatic decrease in this parameter was found. In contrast, when the same level of hypotension was achieved by hemorrhage, no changes in the hemodynamics of villus blood flow were found and changes in the density of perfused villi were very limited.

References