Activation and Decreased Deformability of Neutrophils After Intermittent Claudication

Franz-Josef Neumann, MD, Wolfgang Waas, MD, Curt Diehm, MD, Thomas Weiss, MD, Heide-Marlen Haupt, Rainer Zimmermann, MD, Harald Tillmanns, MD, and Wolfgang Kübler, MD

This study investigated local alterations in neutrophil activation and deformability after intermittent claudication. In 17 patients with one-sided peripheral arterial occlusive disease, neutrophil count, proportion of activated neutrophils (by nitro blue tetrazolium test), and neutrophil filterability as a measure of passive deformability were assessed in the femoral arterial and venous blood of the diseased leg and in the femoral venous blood of the healthy leg (n=10). The values were obtained at rest, immediately after claudication, and 10 minutes after claudication induced by repetitive toe stands. Immediately after exercise, the arterial and venous blood differences in the diseased leg were 1) neutrophil count, 9% (95% confidence interval [CI], 5–14%; relative increase in the venous blood compared with arterial blood); 2) the proportion of activated neutrophils, 26% (CI, 10–42%); and 3) the neutrophil filterability, −10% (CI, −4% to −15%). At rest and 10 minutes after exercise, neutrophil parameters did not differ significantly between the femoral arterial and venous blood. Furthermore, no arterial and venous blood differences in the neutrophil parameters were found in the healthy leg. In addition to local changes, systemic changes occurred immediately after exercise. In the femoral arterial blood, the total neutrophil count had risen by 13% (CI, 8–18%), the proportion of activated neutrophils had risen by 41% (CI, 25–58%), and average neutrophil rigidity had risen 17% (CI, 11–22%) compared with the values obtained before exercise. At 10 minutes after exercise, all neutrophil parameters were still elevated. We conclude that even short periods of ischemia, as in intermittent claudication, cause local alterations in neutrophil function and distribution. (Circulation 1990;82:922–929)

Recent experimental results suggest that neutrophils may contribute to the pathogenesis of ischemic tissue damage.1–3 The principal mechanisms are an increase in microvascular resistance by entrapment of neutrophils4–7 and by secretion of arachidonic acid metabolites,8–10 microvascular damage by oxygen-derived free radicals,11–14 and activation of platelets.13,15 Activation of neutrophils during ischemia and reperfusion is an essential prerequisite for their deleterious effects. Activation not only triggers the various release reactions,10 but also causes stiffening of the cells16–18 and promotes neutrophil adhesion.19

The effect of ischemia on local neutrophil function has been investigated only to a limited extent.20 It is still unknown whether short-term ischemia can cause a local modification of neutrophil function in the clinical setting. The purpose of the present study was to determine whether local alterations in neutrophil activation and deformability in peripheral arterial occlusive disease can be detected after intermittent claudication.

Methods

Patients

The study includes 17 male nondiabetic patients with predominantly one-sided peripheral arterial occlusive disease and who could walk up to 100 m without experiencing pain. All had angiographically proven occlusions of the superficial femoral artery in one leg. Patients were assumed to have predominantly one-sided disease if the contralateral control leg met each of the following criteria21: 1) no reduction in arterial diameter of more than 25% on arteriography as judged visually by two experienced
radiologists unaware of one another’s evaluations; 2) a Doppler systolic ankle to arm pressure ratio above 1 at rest; and 3) a Doppler systolic ankle to arm pressure ratio above 0.8 after exercise that caused prohibitive claudication pain in the diseased leg. Results of the Doppler examination at the time of entry into the study are summarized in Table 1.

The patients were primarily investigated to evaluate the individual effectiveness of an intra-arterial treatment with prostaglandin E_2. The studies in neutrophil alterations were performed as part of the baseline examination before administration of prostaglandin E_1. The study was approved by the institutional ethics committee for human subjects. Written, informed consent was obtained from all patients.

Study Protocol

The femoral artery and vein of the affected leg were retrogradely cannulated with an 18G flexible catheter (outer diameter, 1.2 mm; length, 10 cm). In 10 patients, the femoral vein of the healthy leg was also cannulated. After 20 minutes of rest, blood samples were drawn from the femoral artery and vein(s). The patient then performed repetitive toe stands until prohibitive claudication pain occurred. Immediately after stopping the exercise, a second set of blood samples was taken from the femoral artery and vein(s). Ten minutes after exercise, a third set of blood samples was obtained. All blood samples were obtained from the patient in a supine position.

Neutrophil Tests

The nitro blue tetrazolium (NBT) test was performed immediately on fresh blood with a modification of the method of Park et al. In brief, 0.2 ml heparinized blood (20 units heparin per ml) were incubated at 37°C for 10 minutes. Then, 0.2 ml of a 0.1% solution of NBT (Sigma, Deisenhofen, F.R.G.) in phosphate-buffered saline (pH 7.2) was added. The mixture was incubated at 37°C for 15 minutes and then kept at room temperature (21–25°C) for another 15 minutes. Smears were prepared by allowing a drop of the NBT and blood mixture to rapidly run down a precleaned slide. After air drying, the smears were stained by Wright’s stain (Sigma). At least three smears of each sample were evaluated without knowledge of their origin. On each slide, 100 neutrophils were counted. The percentage of neutrophils containing formazan deposits of at least the size of a lobe of the nucleus was designated as positive NBT score.

Neutrophil preparation and filtration was performed according to Nash et al. For separation of neutrophils, EDTA anti-coagulated blood was layered on a two-step density gradient of Histopaque 1077 (Sigma) layered on top of Histopaque 1119 (Sigma) and centrifuged for 10 minutes at 400g. Then, the platelet-rich top layer was discarded, and after further centrifugation at 800g for 10 minutes, the lower white cell band was harvested. This layer contained mainly neutrophils (on average 97%) with more than 95% viable cells (trypan blue exclusion). The neutrophils were washed twice and finally resuspended in phosphate-buffered saline containing 1 g/l EDTA and 5% autologous plasma. The cell concentration was adjusted to 5 × 10^6 neutrophils/ml. Neutrophil filtration was performed in the St. George’s filtermometer (Carri-Med Ltd., Dorking, U.K.) with a 1-ml sample reservoir. Polycarbonate membrane filters (Nuclepore GmbH, Tübingen, F.R.G.) with a nominal pore diameter of 8 μm (actual mean pore diameter, 7.2 μm) were used. The neutrophil suspension was filtered under constant pressure of 294 Pa (=3 cm H_2O). With a microcomputer linked to the filtermometer (software by Carri-Med Ltd.), the initial relative flow rate (with respect to buffer alone), the initial rate of decrease in flow, and the late relative flow rate, which is the relative flow rate after filtration of 1 ml of neutrophil suspension, were determined as parameters of neutrophil filtration.

Other Methods

The cell counts were performed with a Coulter Counter (Model ZF, Coulter Electronics Ltd., Herts, U.K.). Blood smears were examined by an experienced technician. Total neutrophil count was obtained by multiplying the white cell count by the differential neutrophil count, and the count of NBT positive neutrophils was calculated by multiplying total neutrophil count by the NBT percentage. Lactate levels were measured enzymatically with a standard kit (Boehringer, Mannheim, F.R.G.).

Statistical Analysis

Results are expressed as mean±SEM unless otherwise indicated. Differences between matched sam-

<p>| Patient/ | Pressure ratios at rest | Pressure ratios after exercise |</p>
<table>
<thead>
<tr>
<th>age (yr)</th>
<th>Affected leg</th>
<th>Control leg</th>
<th>Affected leg</th>
<th>Control leg</th>
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<tr>
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<td>TR 48</td>
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<tr>
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<td>0.23</td>
<td>1.10</td>
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</tr>
<tr>
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<td>0.38</td>
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<tr>
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<td>1.04</td>
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<td>0.28</td>
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<td>1.07</td>
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</tr>
<tr>
<td>PD 54</td>
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<td>1.04</td>
<td>0.22</td>
<td>0.93</td>
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<tr>
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<td>1.10</td>
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<td>1.03</td>
</tr>
<tr>
<td>MI 68</td>
<td>0.31</td>
<td>1.09</td>
<td>0.03</td>
<td>1.09</td>
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</table>
samples were tested by Wilcoxon’s matched-pairs signed-ranks test, and the 95% confidence intervals (CI) for the mean difference were calculated assuming a normal distribution. A $p$ value less than 0.05 in the two-tailed test was regarded as significant.

**Results**

**Lactate Concentrations**

During repetitive toe stands, all patients experienced progressive claudication pain only in the diseased leg, which forced them eventually to stop exercising. Before exercise, lactate levels were within normal ranges in all blood samples, and there was no arterial and venous blood difference in lactate concentration in either leg. Immediately after exercise, all patients showed a lactate release in the diseased leg, with an average arterial and venous blood difference in lactate concentration of 1.77 mmol/l (95% CI, 1.10–2.43 mmol/l; mean relative difference 86.8%; two-tailed $p=0.001$) (Figure 1). In the contralateral control leg, however, lactate concentrations in the femoral venous blood did not differ significantly from those in the femoral arterial blood (95% CI, –0.05–0.52 mmol/l; two-tailed $p=0.09$). Ten minutes after exercise, lactate concentrations in the femoral blood had not completely returned to control levels, but there were no significant arterial and venous blood differences in lactate concentrations in either leg.

**Changes in Neutrophil Parameters**

Compared with the values before exercise, total neutrophil count (Figure 2), count of activated neutrophils (Table 2), and NBT score had increased (Figure 3), and the late relative flow rate (Figure 4) had decreased in all samples immediately after exercise. The alterations in neutrophil parameters in the femoral venous blood of the diseased leg significantly

**Figure 1.** Plot of arterial and venous blood differences in lactate concentration immediately after claudication. $p$ value by two-tailed test. V. fem, femoral vein; A. fem, femoral artery.

**Figure 2.** Plot of changes in total neutrophil count in the femoral venous (solid line) and arterial blood (broken line) before ($t_0$), immediately after ($t_1$), and 10 minutes after ($t_2$) claudication. **Two-tailed $p<0.001$, for the difference to total neutrophil count in the respective vessel before exercise.

**Figure 3.** Plot of changes in nitro blue tetrazolium (NBT) score in the femoral venous (solid line) and arterial blood (broken line) before ($t_0$), immediately after ($t_1$), and 10 minutes after ($t_2$) claudication. *Two-tailed $p=0.018$, **two-tailed $p<0.001$, for the difference to NBT score in the respective vessel before exercise.
TABLE 2. Changes in Neutrophil Parameters Immediately After Intermittent Claudication

<table>
<thead>
<tr>
<th></th>
<th>D</th>
<th>95% CI</th>
<th>Drel (%)</th>
<th>95% CI</th>
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<td>a₁-a₀</td>
<td>tPMC(1/nl)</td>
<td>0.66</td>
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<td>13.1</td>
<td>8.2–17.9</td>
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<td>aPMC(1/nl)</td>
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<td>0.13–0.30</td>
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<tr>
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<td>NBT (%)</td>
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<td>1.77–4.11</td>
<td>41.3</td>
<td>24.9–57.7</td>
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<td>−0.08–0.16</td>
<td>−16.7</td>
<td>−11.4–22.1</td>
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<tr>
<td>v₁−v₀</td>
<td>tPMC(1/nl)</td>
<td>1.29</td>
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<td>25.9</td>
<td>18.8–33.0</td>
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<tr>
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<td>aPMC(1/nl)</td>
<td>0.42</td>
<td>0.33–0.52</td>
<td>115.5</td>
<td>89.1–141.9</td>
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<tr>
<td></td>
<td>NBT (%)</td>
<td>5.47</td>
<td>3.61–7.33</td>
<td>75.6</td>
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<tr>
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<td>IFR</td>
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<td>−23.3</td>
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<td>c₁−c₀</td>
<td>tPMC(1/nl)</td>
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<td>aPMC(1/nl)</td>
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<td>0.05–0.25</td>
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<td>13.8–64.7</td>
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<td>NBT (%)</td>
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<td>21.6</td>
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<tr>
<td></td>
<td>IFR</td>
<td>−0.08</td>
<td>−0.04–0.12</td>
<td>−11.0</td>
<td>6.1–16.0</td>
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</tbody>
</table>

**tPMC, total neutrophil count; aPMC, count of activated neutrophils; NBT, NBT score; IFR, late relative flow rate; D, mean absolute difference; Drel, mean relative difference; 95% CI, 95% confidence interval; p, two-tailed level of significance; a₁−a₀, changes in the femoral arterial blood compared with control before exercise; v₁−v₀, changes in the femoral venous blood of the diseased leg compared with control before exercise; c₁−c₀, changes in the femoral venous blood of the control leg compared with control before exercise.**

Ten minutes after exercise, neutrophil counts and NBT scores in the femoral arterial and venous blood of both legs were still elevated, and late relative flow rates of neutrophils were still diminished. All these parameters showed a trend toward normalization.

Exceeded those in the femoral arterial and venous blood of the contralateral control leg (Table 2).

Initial relative filtration flow rates of neutrophils, and initial rates of decrease in flow rates remained unaffected by intermittent claudication (not shown).

**Arterial and Venous Blood Differences in Neutrophil Parameters**

Before exercise and 10 minutes after exercise, blood samples from the femoral arterial and venous blood of the diseased leg and from the femoral venous blood of the contralateral control leg did not differ significantly with respect to any of the neutrophil parameters examined.

Immediately after exercise, significant arterial and venous blood differences in the neutrophil parameters were found in the diseased leg but not in the contralateral control leg (Table 3). In the femoral venous blood of the diseased leg, total neutrophil counts (Figure 5), activated neutrophil counts (Table 3), and NBT scores (Figure 6) were significantly higher than those in femoral arterial blood, and late relative flow rates (Figure 7) were significantly lower than those in the femoral arterial blood. In contrast, in the contralateral control leg, neutrophil parameters in the femoral venous blood did not differ significantly from those in femoral arterial blood (Table 3). Compared with the femoral venous blood of the diseased leg, however, total neutrophil counts, activated neutrophil counts, and NBT scores in the femoral venous blood of the contralateral control leg were significantly lower, and late relative flow rates were significantly higher (Table 3). Thus, immediately after exercise, the arterial and venous blood differences in neutrophil parameters differed significantly between the two legs.

In the diseased leg, significant correlations between the arterial and venous blood differences in neutrophil parameters and Doppler systolic ankle to arm pressure...
ratios did not occur after exercise, even though the arterial and venous differences tended to increase when Doppler ankle pressures dropped markedly.

Discussion
The present study reveals for the first time that alterations occur in neutrophil activation and deformability in peripheral arterial occlusive disease after intermittent claudication. Immediately after claudication, the venous blood of the ischemic leg contains more activated neutrophils with reduced deformability than does the arterial blood. At the same time, the neutrophil count in the venous blood exceeds that in the arterial blood, indicating a release of neutrophils from the local intravascular pool. This pool is the most likely source of the activated, rigid neutrophils that are detected in the venous blood after ischemia of the diseased leg.

Neutrophil parameters in the contralateral control leg show consistent changes in the same direction as in the ischemic leg when values before exercise are compared with those after exercise (Table 2). Because of recirculation, however, the changes in the femoral venous blood of the contralateral control leg will be the same as the changes in the arterial blood, even if there are no local changes. Analysis of the

![Figure 5](image)

**Figure 5.** Plot of arterial and venous blood differences in total neutrophil count immediately after claudication. p value by two-tailed test. V. fem, femoral vein; A. fem, femoral artery.

![Figure 6](image)

**Figure 6.** Plot of arterial and venous differences in nitro blue tetrazolium (NBT) score immediately after claudication. p value by two-tailed test. V. fem, femoral vein; A. fem, femoral artery.
arterial and venous and of the ischemic and nonischemic venous differences (Table 3) reveals that in the control leg neutrophil parameters in the femoral venous blood do not differ significantly from those in the femoral arterial blood. On the other hand, there is a consistent difference between neutrophil parameters in the femoral venous blood of the ischemic and control legs that is of the same order of magnitude as the difference between neutrophil parameters in the arterial and venous blood of the ischemic leg. We therefore conclude that a detectable local modification of neutrophil function only occurs in the ischemic leg. The alterations in neutrophil parameters in the femoral venous of the control leg and in the arterial blood, however, reflect systemic changes in neutrophil parameters. They may be attributed to recirculation of neutrophils from the ischemic leg and to the systemic effects of exercise on neutrophil kinetics.27-29

In previous studies, local and global alterations in leukocyte deformability have been described in patients with peripheral arterial occlusive disease and chronic ischemia of the leg.20 The present study shows that local changes in neutrophil function and deformability immediately after claudication are more pronounced than global changes. Although the circulating pool of neutrophils only contains about 10% of the activated neutrophils, the arterial and venous blood difference in activated neutrophils immediately after claudication amounts to almost 50% of the total arterial and venous blood difference in neutrophils. This surplus of activated neutrophils causes sizable curtailment of the overall neutrophil deformability in the femoral venous blood, although it only accounts for about 3% of total neutrophils.

**Methodological Considerations**

In the NBT test, neutrophils that reduce phagocytosed NBT to formazan are stained. This intracellular reduction of NBT has been attributed to the action of superoxide anions, which are formed by nicotinamide adenine dinucleotide phosphate oxidase catalysis of oxygen.13,23,30 Superoxide anion production is the pivotal phenomenon of the “respiratory burst” after neutrophil activation.10,31 The NBT test, therefore, assesses the proportion of circulating neutrophils having undergone “respiratory burst.”

The filtration measurements had to be performed on separated neutrophils. The possibility has to be considered that handling of the cells before filtration might have altered their mechanical properties. It has been shown, however, that separated and recombined white cells basically show the same filterability as unfractionated white cells.16 To analyze only the passive mechanical properties of the neutrophils, EDTA was used as an anticoagulant.32-34 The use of EDTA in neutrophil filtration studies also minimizes artifacts due to spontaneous activation and neutrophil aggregation.16 The mechanical responses of neutrophils to experimental stimulation, however, are preserved by EDTA.16,17 Nevertheless, the use of EDTA may result in some underestimation of the native neutrophil filterability because the cells may become partially deactivated by the chelation of calcium ions. To ensure that any artifacts due to the preparative procedures would not interfere with the principal results of the study, care was taken that handling of the cells was identical in all samples.

The filtration measurements in the present study can be interpreted with a model of three different cell populations: a large majority (≥90% in normal samples) of rapidly flowing neutrophils (pore passage time, 0.02 seconds), a smaller number of slowly flowing neutrophils (pore passage time, 1 second), and a small population of pore-blocking cells.25 The initial relative flow rate is determined by neutrophils with a quick transit, whereas the initial rate of decrease in flow and, in particular, the late relative flow rates reflect the rheological behavior of the more rigid neutrophils.25 The alterations in neutrophil filterability after intermittent claudication observed in this study, therefore, can be attributed to alterations in a small population of highly rigid neutrophils. The alterations in neutrophil deformability correspond to changes in the number of activated neutrophils that are known to be more rigid than resting neutrophils.16-18

Blood samples could not be obtained from a vein exclusively draining ischemic tissue. The femoral vein receives an unknown amount of blood from nonischemic tissue. Lactate production found in the femoral venous blood of the ischemic leg after exercise suggests drainage of larger ischemic areas. Nevertheless, even a minor admixture of blood from nonischemic tissue will cause underestimation of the local alterations in neutrophil function and deformability.
The neutrophils that are washed out from the ischemic leg after intermittent claudication may represent only a part of the local intravascular pool. The most rigid and adhesive neutrophils may remain trapped and, therefore, are not available for the analysis applied in this study. Hence, the changes in neutrophil function and deformability in the ischemic muscle can be assumed to be even more pronounced than those observed in the femoral venous blood.

Pathophysiological Implications

Equilibrium considerations suggest that the neutrophils that are washed out in the ischemic leg after intermittent claudication must have accumulated there at some previous stage. Because patients with peripheral arterial disease of stage II (according to Fontaine’s classification) have a normal resting circulation in the diseased leg, it is conceivable that neutrophils accumulated in the ischemic leg during exercise. This interpretation is supported by recent evidence from animal experiments. In the normal microcirculation of the skeletal muscle, most leukocytes pass the network through flow-dependent preferential channels at the downstream end of the arterioles and through the transverse arteriole serving the fascia. Lowering of the arterial pressure causes redistribution of the leukocytes within the network toward the more proximal nutritive nonpreferential branches. Furthermore, mean capillary passage time of leukocytes increases with decreasing arterial pressure. Both effects result in an increase in leukocyte concentration within the microcirculatory network of the muscle and in a prolonged stay of leukocytes within the network.

By the same mechanisms, during intermittent claudication, the drop of poststenotic arterial pressure in the ischemic leg may lead to an increase in the intravascular pool of neutrophils in the ischemic leg during intermittent claudication. Furthermore, because of slower passage through the microvascular network, the neutrophils may be exposed to various ischemia-related chemotraectants for a sufficient period of time to become activated. At the instant after intermittent claudication, however, part of the increased intravascular pool of neutrophils may be washed out, as the perfusion tends to normalize again. This may explain the arterial and venous blood differences in neutrophil counts early after intermittent claudication. The surplus of activated, stiffened neutrophils in femoral venous blood of the ischemic leg may then reflect local neutrophil activation, a predominant intravascular accumulation of previously activated neutrophils during ischemia, or both.

In conclusion, the present study shows that even short periods of ischemia, as in intermittent claudication, cause local alterations of function and microvascular distribution of neutrophils. These alterations may contribute considerably to the pathogenesis of ischemic tissue damage.

Acknowledgment

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References


KEY WORDS • neutrophils • nitro blue tetrazolium • ischemia • peripheral arterial occlusive disease
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