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Microvascular *in-vivo* Analysis of Retrograde Venous Arterialization of Ischemic Skeletal Muscle

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Objective: Nearly 20% of patients with critical limb ischemia will not be suitable for arterial bypass due to distal small vessel occlusion, and venous arterialization of the distal venous bed might be a valuable surgical option. This study demonstrates the in-vivo microcirculatory effects of this type of intervention.

Methods: Using intravital video microscopy, the authors studied the distal skeletal microcirculatory characteristics following venous arterialization of critical hindlimb ischemia in the rat. 25 Wistar rats underwent proximal ligation of the femoral arteries followed by venous arterialization carried out by anastomosing the saphenous vein to the femoral artery using microsurgery techniques. Microcirculatory hemodynamic conditions of the soleus muscle were observed under normal, ischemic, and arterialized conditions. Fluorescein-labeled red cells were used to measure red cell velocities (Vrbc) at the capillaries, and acridine orange injections used to stain endothelial cell nuclei to measure microcirculatory diameters, and leukocyte nuclei to measure leukocyte adhesion. Laser Doppler Perfusion (LDP) units at the distal limb were measured continuously throughout the procedure.

Results: Proximal femoral arterial ligation resulted in drastic reductions in LDP and Vrbc. Following distal venous arterialization, LDP returned to an average of 41% of baseline. Vrbc returned to near baseline values in 70% of the capillaries. Flow at the capillary and venular system showed frequent reversals and great variations in velocities. Venules and venu-venular anastomoses diameters increased by 50%. There was immediate macromolecular tracer leakage and leukocyte activation was significantly increased in both ischemic and arterialized groups (15 cells vs 156 and 178 cells respectively).

Conclusion: Venous arterialization may provide an improvement in microcirculatory velocities but is accompanied by microcirculatory injury and dysfunction in the acute phase. These results suggest that mechanisms besides microcirculatory hemodynamics play a role in the overall picture of clinical effectivity of the procedure.

Key words: Retrograde venous arterialization, ischemic skeletal muscle

The mainstay of treatment for peripheral arterial occlusive disease is the restoration or improvement of distal perfusion. This may be accomplished either through surgical means with bypass of obstructing lesions, or by pharmacological strategies which improve hemorrheological conditions or promote revascularization by angiogenesis into ischemic tissues. In many cases, however, distal occlusive lesions preclude effective surgical bypass because of the absence of normal vessel segments for bypass anastomosis. In addition, the disease at presentation usually is at a state that mandates immediate intervention that cannot wait for revascularization by angiogenesis or is at best only marginally improved by hemorrheological manipulation.

Arterialization of the distal veins through the creation of an arteriovenous shunt (venous arterialization) may conceivably provide microcapillary perfusion through retrograde venular flow that can effectively bypass distal microangiopathic lesions. Although the principle of retrograde perfusion has been recognized nearly a century ago and presently routinely applied in cardiac surgery[1], the strategy has not gained wide support and has only sporadically been reported for the reperfusion of the ischemic peripheral limb. The current role of the surgically created arteriovenous fistula in peripheral vascular reperfusion is mainly as an adjunctive procedure in peripheral bypass procedures to increase the blood flow velocity through the graft above the thrombotic threshold level.[2] Although the reports of improved foot salvage rates were implicitly associated with the

increased patency rates of the grafts with adjuvant arteriovenous fistulas, there is reason to believe that the arteriovenous shunt itself contributes to the improvement in distal perfusion through retrograde venular flow.[3]

Arterialization and retrograde flow strategies have been shown to be effective in a number of clinical and experimental situations. Myocardial protection during cardioplegia routinely utilizes retrograde perfusion via the coronary sinus that effectively supplies nutrients to left ventricle and apex.[1,4] Retrograde cerebral perfusion has been demonstrated to occur in experimental conditions. The retrograde venous perfusion route delivery of therapeutic agents to the microangiopathic limb has been reported to shorten duration of conservative therapy.[5] At least one center has reported clinical improvement of foot lesions with the performance of a surgically created arteriovenous fistula as a last-ditch means for foot salvage.[6] Although there is much experimental evidence of retrograde flow through the microcirculation, there is none in the literature that demonstrates the microcirculatory flow characteristics that occur when retrograde flow is induced beyond physiological conditions. This study demonstrates the in-vivo microcirculatory conditions that follow induction of arterial retrograde flow in the distal venous microcirculatory bed.

Methods

The animals used in this study were cared for in accordance with the Guidelines for Animal Care and Use (NIH publication 1995). Twenty-five male Wistar rats 350-400 grams weight underwent unilateral femoral artery ligation followed by femoral artery to saphenous vein anastomosis. The animals were anesthetized with an intraperitoneal injection of pentobarbital (5mg/100g body weight). Once withdrawal to pain reflexes were abolished, cannulation of the trachea for airway control, the jugular vein for tracer infusion, and the carotid artery for continuous arterial pressure recording were carried out. A laser Doppler probe and flowmeter (Advance ALF21, USA) was applied at the distal foot pad for continuous measurement of laser doppler flux.

The groin incision was then performed and extended distally to expose the infrageniculate muscles of the leg. The soleus muscle was identified and separated, and the superficial microcirculation exposed as previously described.[7] Throughout the procedure and during observation, the surface of the exposed tissue was continuously irrigated with warmed (34ºC-35ºC) phosphate buffered solution. The animal was then allowed to equilibrate for two hours prior to observation for control values.

After two hours of equilibration the prepared soleus muscle was placed under an intravital microscope (Nikon, Tokyo) equipped and modified for incident light and fluorescence microscopy and continuously irrigated with superfusion. All observations were carried out with an intravital microscope (Nikon Eclipse, Tokyo) equipped with a silicon-intensified target (SIT) tube camera (Hamamatsu Photonics, Hamamatsu) and a 100-W HBO mercury vapor lamp epifluorescence illuminator (Nikon, Tokyo). The image was displayed on a TV monitor (Hamamatsu Photonics, Hamamatsu). Recordings were documented on videotape with a videorecorder (AG-7300, Panasonic, Osaka). The measurements were then done off-line during video playback and capture after computer digitization (Adobe Premiere, Adobe, CA).

The tracers were injected, and the resultant image observed and recorded. Video recordings running at 30 frames per second over a period of 10 minutes were made of four contiguous examination fields per animal containing capillaries from two to three most superficial layers of muscle fibers.

Subsequently, the femoral artery was dissected out and isolated and all branches from the inguinal ligament to the trifurcation were ligated. After applying a vascular clip proximally at the level of the inguinal ligament, the femoral artery was then ligated and cut just before to the trifurcation, thus resulting in interruption of femoral inflow to the hindlimb from the level of the inguinal ligament. This period of hindlimb ischemia (HI) was maintained for two hours, after which intravital observations and measurements were carried out as in the control situation.

During this period while the proximal clip is applied, the microsurgical arteriovenous anastomosis was performed. The saphenous vein was isolated and divided,

the cut end brought around and anastomosed end-to-end to the femoral artery. While the clip is in place, intravital observations and measurements during ischemia were taken, after which the clip was removed and reperfusion through arterialization was allowed. After two hours of arterialization reperfusion (AR), the intravital observations and recording were once more carried out.

In eight animals, capillary red cell velocities (Vrbc) were measured. Fluorescein-labeled red cells were prepared as follows: 1ml of blood was drawn from each animal after vessel cannulation and washed four times in pH 7.4 phosphate buffered solution (PBS). The red cells were then incubated in 1:1 with fluorescein isothiocyanate (FITC-Sigma, MO) 1 mg/ml of pH 7.8 PBS at room temperature for one hour. After incubation, the labeled cells are washed successively four times and then used no later than 6 hours after incubation. 0.3ml of the labeled cells are infused into the animal and intravital microscopy performed. V_{rbc} was measured by sequential single-frame analysis of the recorded data showing the FITC-labeled cells, after calibration of the video monitor with a graticule slide recorded through the system. Observations were recorded at the three time periods separated two hours apart: prior to intervention, with the femoral artery ligated, and with the arteriovenous shunt in place. Flow directions and changes were also noted. Values were noted in absolute numbers irrespective of flow direction.

The microcirculation network and permeability data were collected from another eight animals. After vessel cannulation, FITC-dextran 150 (M.W. 150,000, 1g/kgBW in 0.3ml PBS, Sigma, MO) was infused. Recordings were made at the three time periods as previously described.

Vessel diameters and leukocyte adhesion measurements were taken from fluorescent images of acridine orange stained cells. Since sequential interventions are not possible using this tracer, the data were obtained from three separate groups. The first group served as the control. The second underwent ligation of the femoral artery and its branches and served as the ischemic group. Arteriovenous anastomosis was performed in the third group that served as the arterialized group. (Figure 1) Two hours following intervention, intravital microscopy was performed and suitable network fields identified. Each animal then received an infusion of acridine orange (0.01mg/kg, Sigma, MO), and the resultant stained endothelial cells and leukocytes were visualized and recorded. Third order venules (3V) were identified and their internuclei luminal diameters measured.

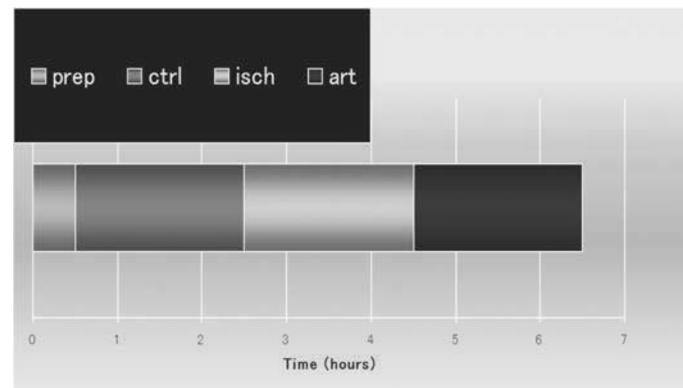


Figure 1. Time line of events carried out in the course of each experiment. Observations and recordings were performed for ten minutes at the end of each time period. (Prep, preparation; ctrl, control; isch, ischemia; art, arterialization.)

Results

The data from 25 experiments were included in the study. Animals that exhibited significant variations in heart rate or mean arterial pressures during the two-hour control observation period were excluded. Eight animals per fluorescent tracer used were studied, except for the group that received acridine orange infusions, which consisted of nine animals. Four fields were studied in each animal; each field contained three to six capillaries for a total of 128 capillaries.

The percent change in laser doppler perfusion (LDP) is shown in Figure 2. Femoral artery ligation resulted in hind limb ischemia (HI) demonstrated by an immediate drop in LDP units that slowly rose to a plateau level that was still significantly below baseline. Arterialization reperfusion (AR) resulted in an increase in LDP that was still significantly below baseline levels.

The mean values for Vrbc are given in Figure 3. Baseline V_{rbc} averaged 0.467 ± 0.092 mm/sec, while V_{rbc}

during ischemia and arterialization averaged 0.133 ± 0.013 mm/sec and 0.367 ± 0.107 mm/sec respectively ($p<0.05$). V_{rbc} after AR did not differ significantly from baseline. Capillary perfusion density rose from 34% during HI to 83% after AR. However, it was observed that 17% of capillaries that did not exhibit flow during HI remained unperfused after AR (Figure 4). The proportion of capillaries having slow flow (<0.3mm/sec) also increased from 4% at baseline to 32% during ischemia and 13% after arterialization.

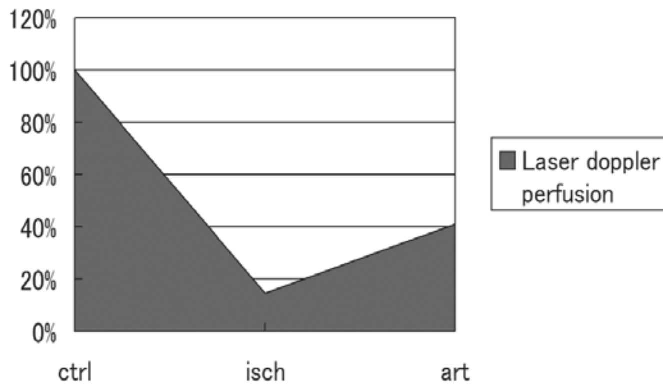


Figure 2. Percent of laser Doppler perfusion units at the distal limb after ischemia and arterialization. Ctrl, baseline/control = 100%; isch, ischemia = 13%; art, arterialization = 41%.

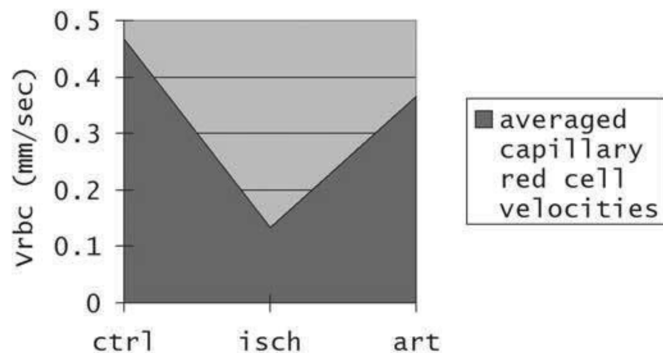


Figure 3. Averaged capillary red cell velocities (V_{rbc}) at baseline (ctrl), after ischemia (isch), and arterialization (art). V_{rbc} during hindlimb ischemia was significantly less than baseline and after arterialization (0.133 ± 0.013 mm/sec vs 0.467 ± 0.692 mm/sec and 0.367 ± 0.107 mm/sec respectively, $p<0.05$) while Vrbc after arterialization resulted in velocities comparable to baseline.

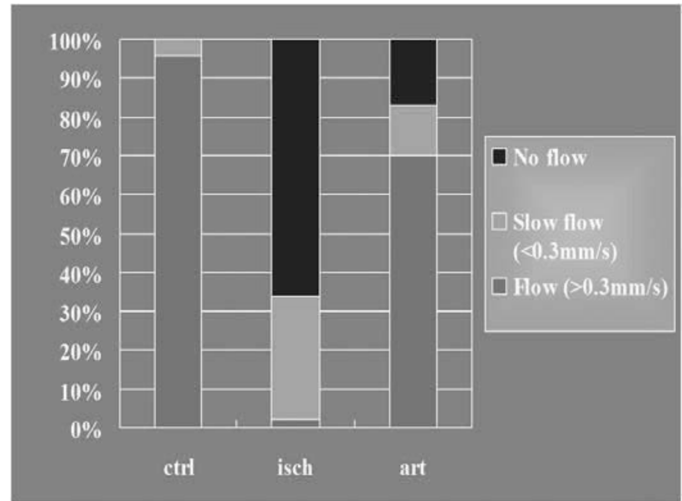


Figure 4. Percent capillary perfusion. Arterialization resulted in reperfusion of 83% of observed capillaries, however, slow perfusion (capillaries with Vrbc < 0.3mm/sec) was observed in a greater proportion of capillaries after arterialization as compared with baseline (4% and 13% respectively).

Flow directions were maintained during HI (Figure 5), but after AR, bidirectional flow reversals and distinct pulsatile retrograde flows in a number of venules and capillaries. Frequent flow loops along venu-venular anastomoses were also evident (Figure 6).

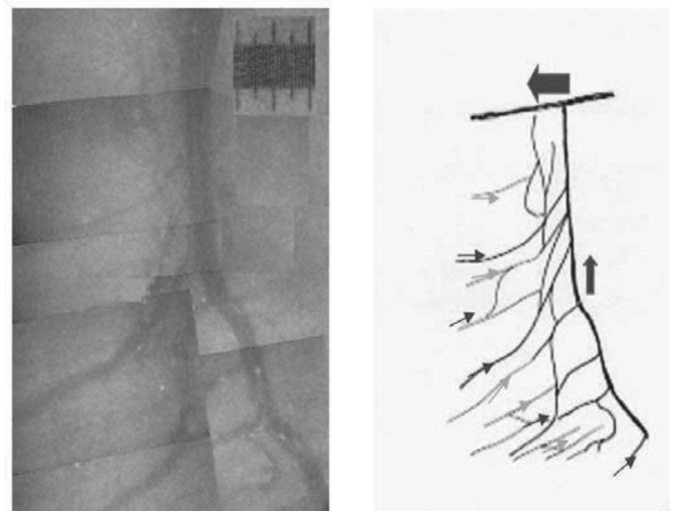


Figure 5. Composite and diagrammatic reconstruction of a sector of muscle microcirculation. Arrows denote direction of flow. light arrows, capillary; dark arrows, venule.

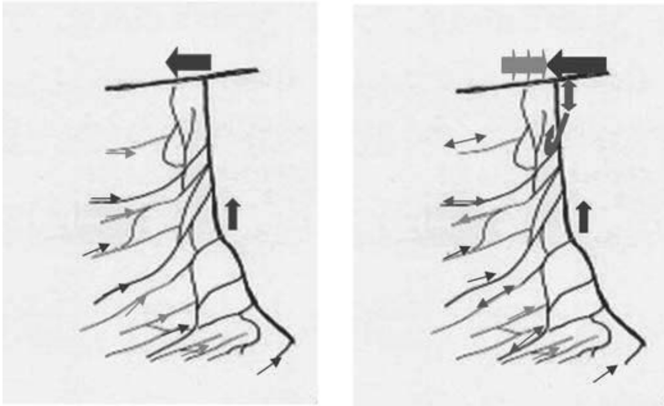


Figure 6. Diagrammatic representation of flow along a sector of muscle microcirculation. A. Flow directions in control and ischemic groups. B. Flow directions after arterialization. Numerous flow reversals and flow loops can be observed.

A total of 32 third order venules were identified and measured. At baseline, averaged venular diameters was 8.00 ± 0.04 microns. After HI there was an increase in the average venule diameter (10 ± 0.02 microns), which increased further following AR (12 ± 0.04 microns, $p<0.05$) (Figure 7).

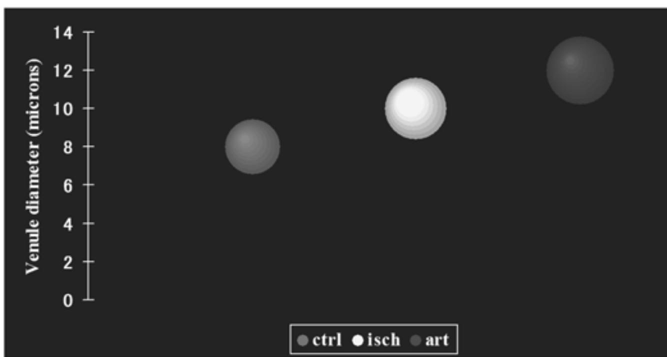
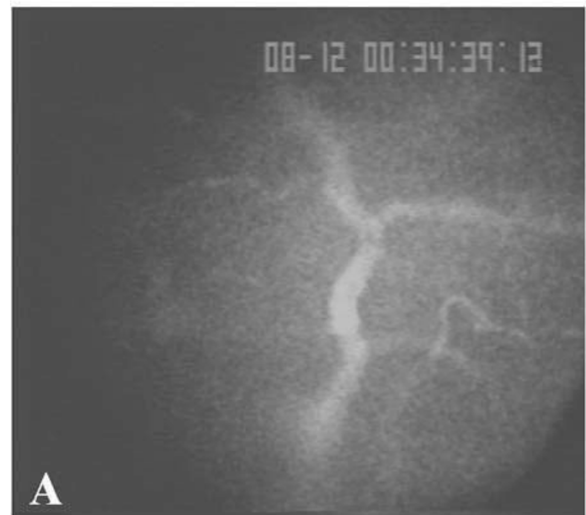


Figure 7. Averaged diameters of third order (3V) post capillary venules. Diameters increased in both ischemic and arterialized venules over baseline (10.00 ± 0.02 and 12.00 ± 0.04 microns respectively vs 8.00 ± 0.04 microns).

The total values for leukocyte adhesion are given in Figure 9. HI and AR resulted in significantly increased leukocyte-endothelial interaction compared with control (156 cells and 178 cells, respectively, vs. 15 cells).

FITC-dextran 150 remained intraluminally all throughout control conditions. Macromolecular leakage can be observed after HI; however, rapid extravasation of the fluorescent macromolecule into the interstitium occurred almost immediately after AR (Figure 8) that resulted in interstitial light intensities that exceeded the camera capabilities and prevented quantification.



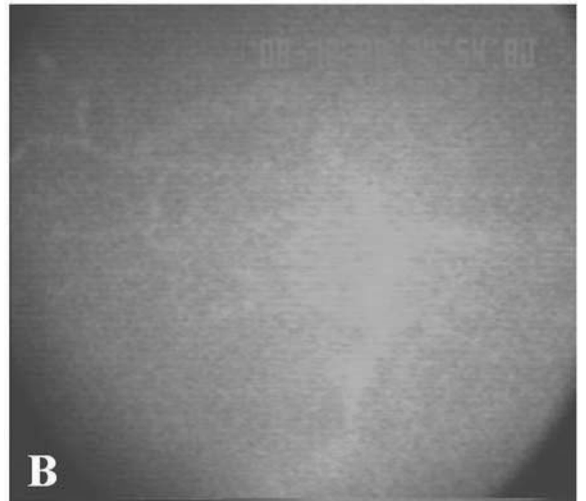


Figure 8. Macromolecular (FITC-dextran m.w. 150,000) leakage from post capillary venules. A. After 2 hours of ischemia. Fluorescent macromolecules are still intraluminal. B. After 2 minutes of arterialization reperfusion. Extravascular leakage of fluorescent macromolecules was immediately apparent.

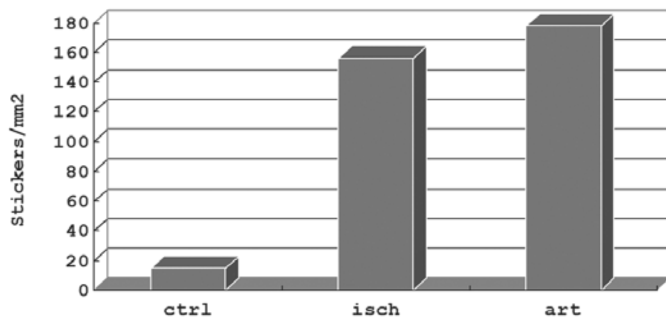


Figure 9. Total number of adherent leukocytes of observed fields. Both ischemic and arterialized groups led to leukocyte activation and therefore an increase in number of adherent cells. Sticker, adherent leukocyte.

Discussion

Distal peripheral arterial occlusive disease not amenable to conventional surgical bypass is responsible for nearly 80% of limb amputations. Limb amputations result in significant disability both physical and economic to the patient and society, and revascularization has been shown to have better long-term results than amputation.[8] Distal venous arterialization has been proposed as an option for limb salvage in some non-bypassable limbs. Limb salvage rates of 80% at 2 to 22 months have been reported[9], as well as improved wound healing rates[6,10], and abolition of pain.[11] This in-vivo study demonstrates the acute microcirculatory events that occurred following arterialization of an ischemic limb.

Capillary red cell velocities were significantly increased by arterialization of the ischemic limb and were not significantly different from those in normal limbs. However, the functional capillary density, or the number of perfused capillaries, significantly less than normal in the ischemic group, remained significantly less than normal even after arterialization. Therefore, even while there were normal velocities in the perfused capillaries, the number of perfused capillaries was significantly lower than normal. This may be attributable to the phenomenon of "no-reflow" occurring in ischemia-reperfusion.

No-reflow is thought to occur as one major consequence of reperfusion injury where postischemic microvasculature and muscle tissue are believed to be progressively damaged throughout reperfusion.[12] Primary no-reflow occurs when there is absence of capillary perfusion at the start of reperfusion. Secondary capillary perfusion failure occurs when there is progressive cessation of flow in previously functional capillaries at later stages of reperfusion. Reperfusion and no-reflow at the early phases have been shown to be reversible to a small degree, in that 10 minutes after reperfusion, some blood flow in previously unperfused areas is restored later in reperfusion; conversely, some areas exhibiting flow at the beginning of reperfusion develop no-reflow 10 minutes later. It is not known when these temporal variabilities, which are believed to be progressive, stabilize or reach a plateau. The two hours of reperfusion in this study is thought to be sufficient to allow partial stabilization, and that the results seen after this period of reperfusion are indicative of a general state of increased red cell velocity but decreased capillary functional density occurring in the microcirculation during these interventions.

Ischemia time of two hours has been demonstrated previously to not result in a significant amount of reperfusion injury[13,14], and that reperfusion results in no significant increase in damage to the microcirculation. In these previous studies, reperfusion was induced in a prograde manner and in physiologic settings. This study differs in that reperfusion was induced through the venular system under nonphysiologic conditions of retrograde flow and arterial pressures. Venous arterialization resulted in an almost instantaneous extravasation of macromolecules into the interstitium and is accompanied by a significant increase in leukocyte adhesion. This is indicative of a significant degree of endothelial disruption and damage possibly initiated by ischemia, potentiated by the arterial pressures in the venules, and perpetuated by activated leukocytes. This degree of damage to the microcirculation may lead to edema formation and loss of perfusion and exchange in the remaining capillaries that further contributes to the no-reflow phenomenon.

These evidences of microcirculatory injury occurring acutely in experimental venous arterialization reperfusion seem not in agreement with the promising results of improved wound healing and limb salvage rates obtained in the clinical setting. However, it is not inconceivable

to expect biologic adaptation to the arterialization in the chronic setting. Vein grafts in the macrocirculation have been shown to adapt adequately to arterialization, and it would be interesting to note microcirculatory venular adaptation to the same conditions in the chronic setting. In addition, biochemical conditions of hypoxia and lactic acidosis and hemodynamic conditions of venous hypertension, venular dilation and increased flow velocities are conditions known to induce neovascularization and angiogenesis, and these conditions are present in venous arterialization. These conditions may be responsible in the chronic setting for the improved clinical results following venous arterialization.

Conclusion

Venous arterialization may provide an improvement in microcirculatory red cell velocities but is accompanied by microcirculatory injury and dysfunction in the acute phase. These results suggest that mechanisms besides microcirculatory hemodynamics play a role in the overall picture of clinical effectivity of the procedure. Further investigations of the microcirculatory effects in the chronic phase and ancillary strategies to decrease microcirculatory injury and dysfunction are warranted before clinical application can be routinely recommended.

References

1. Mohl W. The relevance of coronary sinus interventions in cardiac surgery. Thorac Cardiovasc Surg 1991; 39:245-50.
2. D'Andrea V, Lepantalo M, Kantonen I, Biancari F, et al. Adjuvant arteriovenous fistula in infrapopliteal bypasses. G Chir 1998; 19: 117-9.
3. Jacobs MJ, Reul GJ, Gregoric ID, Ubbink DT, et al. Creation of a distal arteriovenous fistula improves microcirculatory hemodynamics of prosthetic graft bypass in secondary limb salvage procedures. J Vasc Surg 1993; 18: 1-8.
4. Chiu CJ, Mulder DS. New surgical approach to diffuse coronary occlusion: selective arterialization of the coronary veins. Adv Exp Med Biol 1977; 82: 340-1.
5. El-Sarky M, el-S. Local intravenous therapy in chronic inflammatory and vascular disorders of the foot. Int Surg 1997; 82: 175-81.
6. Lengua F. [Can distal vein arterialization be beneficial for the diabetic foot with necrosis?] Chirurgie 1994; 120: 143-52.
7. Dawson JM, Tyler KR, and Hudlicka O. A comparison of the microcirculation in rat fast glycolytic and slow oxidative muscles at rest and during contractions. Microvasc Res 1987; 33: 167-82.
8. Holtzman J, Caldwel M, Walvatne C, Kane R. Long-term functional status and quality of life after lower extremity revascularization. J Vasc Surg 1999; 29: 395-402.
9. Jacob S, Nassef A, Belli AM, Dormandy JA, Taylor RS. Vascular surgical society of great britain and ireland: distal venous arterialization for non-reconstructable arterial disease. Br J Surg 1999; 86(5): 694.
10. Ning F, Chen G, Li Q. [Arterialization of the great saphenous vein for treating severe ischemia of lower limbs.] Chung Kuo Hsiu Fu Chung Chien Wai Ko Tsa Chih 1998; 12(4): 215-7.
11. Lengua F, Cohen R, L'Huilleier B, and Buffet JM. Arteriovenous revascularization for lower limb salvage in unreconstructible arterial occlusive disease (long term outcome). Vasa 1995; 24(3): 261-9.
12. Hickey MJ, Hurley JV, and Morrison WA. Temporal and spatial relationship bewteen no-reflow phenomenon and postischemic necrosis in skeletal muscle. Am J Physiol 1996; 271(40): H1277-H1286.
13. Harris AG, Leiderer R, Peer F, and Messmer K. Skeletal muscle microvascular and tissue injury after varying durations of ischemia. Am J Physiol 1996; 271(40): H2388-H2398.
14. Harris K, Walker PM, Mickle AG, Harding R, et al. Metabolic response of skeletal muscle to ischemia. Am J Physiol 1986; 250(19): H213-H220.