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Skin capillary reperfusion after regional ischemia

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Abstract. Capillary reperfusion following four hours of regional ischemia was determined in the ear skin microvasculature of 10 homozygous hairless mice. Selective arteriolar ligation was used to produce flow stasis in capillaries within an ischemic risk zone (RSK) without direct compression of the microvasculature under study. Reperfusion capillary RBC velocities (CBV), diameters, and flow times in both RSK and nonischemic capillaries (NRSK) were measured and compared to baseline values after flow stasis was verified in the RSK capillaries. Baseline values of CBV, diameter and flow time did not differ between RSK and NRSK. In the RSK capillaries, the post-ischemic mean CBV was significantly less than baseline (120 ± 22 vs. $212 \pm 31\,\mu m/s$, $p < 0.01$), whereas in NRSK capillaries no differences were detected (195 ± 41 vs. $174 \pm 22\,\mu m/s$). The decrease in mean CBV in RSK capillaries was mainly due to an increase in the number of capillaries with low or zero reflow. The percentage of capillaries having CBV less than $100\,\mu m/s$ increased from 10% at baseline to 45% after ischemia. These results indicate that when capillary flow stasis is produced, durations of skin ischemia of four hours may subject the microvasculature to significant reperfusion deficits.

Introduction

The no-reflow phenomenon [1, 2] which may occur after ischemia is a critical factor in determining the final outcome or reversibility of tissue injury. The specific capillary cellular mechanisms causing the no-reflow are not fully clarified, but various features of the microvasculature after ischemia have been characterized in many tissue types. Evidence of no-reflow has been found in the brain [1], heart [6, 11], kidney [23], skeletal muscle [7, 8, 12, 18, 24, 25], skin [3, 19, 21, 27] and skin flaps [5, 14, 17].

Though the presence and extent of no-reflow is dependent on the tissue type, the duration of ischemia and possibly on the mode of ischemia induction, it is thought that the no-reflow phenomenon is mediated by changes within the capillaries which results in a subset of capillaries which have severely reduced or zero flow during reperfusion. Leukocyte trapping within affected capillary lumina has been suggested as one mechanism [2, 22]

among several alternative possibilities [12]. However, little is known co cerning the reperfusion blood flow in specific capillaries in which blood flo stasis during ischemia was verified [26]. This is an important consideratic because ischemia, produced by occlusion of supplying arteries or arteriol vessels, can result in a distribution of capillary flow patterns which rang from full stasis to unaffected. Judgements concerning the operant mechar isms of no-reflow are likely to be more useful if the precise capillary stat prior to reperfusion is verified. It was thus the purpose of the present stud to examine the reperfusion status of capillaries in which complete flow stasi was induced via arteriolar ligation.

Materials and methods

Experimental procedures

Ten male homozygous hairless mice (Charles River Laboratories) weighing between 35 to 40 g. were used in this study. The microscopic *in vivo* examination of the ear skin microcirculation was done under Nembutal anaesthesia (6 mg/100 g) administered IP with a maintenance dose (2 mg/100 g) every $1\frac{1}{2}$ to 2 hours via an indwelling intra-abdominal 25 gauge minicatheter (Abbott Laboratories). The animal is placed on an observation board which supports the animal's body with the ear positioned on an attached glass slide for microscopic viewing of the ear microcirculation. For high power microscopic study, a drop of paraffin oil, placed on the bottom of the surface of the ear is used to enhance visibility and to allow the ear to lay flat on the glass slide. A drop of oil is also placed on top of the surface of the ear to support an overlying cover slip. Temperatures are monitored using two thermistor probes (Bailey's Instruments Co.), one inserted rectally for monitoring core temperature, and another positioned on the ear surface. Temperatures are maintained using a heat lamp mounted on the microscope with the core temperature maintained between 30–34 °C [9]. Heart rate is monitored using a Medsonics Photopulse Adaptor (Model PA 13) with the cuff attached to the proximal portion of the tail and data recorded on a chart recorder (Grass RPS 7C 8A).

The methods for production regional ischemia in the mouse ear has been described in detail [15] and may be summarized as follows. Using a surgical microscope, arterioles and venules entering the base of the ear are noted; normally, there are three pairs. Distal zones affected by these vessels, as well interanastomosing accessory branches, are identified. Based on the vascular supply to the distal regions, they are classified as risk (RSK) or nonrisk

(NRSK) zones. The RSK are those tissue regions which will be affected by subsequent arterial ligations and the NRSK are those that will not be significantly affected. The observation board with the mouse secured, is then placed on the stage of the Laborlux microscope which has an attached low light level TV camera [MTI—65] and associated video recording system. Capillary loops within RSK and NRSK are identified using transillumination with a 420 nm narrow band filter [16]. The filter is interposed between the light source and the condenser to enhance RBC contrast. The loops are recorded for ten minutes each to obtain baseline data (effective magnification to the TV monitor is at 350 ×). Thereafter, with the aid of the surgical microscope, the central and one of the lateral entering arteries are ligated with a 7–0 vicryl suture. Additional primary interanastomosing branches are ligated as required. This procedure takes approximately 30 minutes.

The RSK and NRSK capillary loops are then relocated under high power magnification and the presence of capillary flow stasis in the RSK capillaries is verified. During the four hours of ischemia, RSK and NRSK loops are sequentially videorecorded for 10 minutes at 30–45 minute intervals. At the end of the four hour ischemia, the ligatures are removed and the RSK and NRSK capillary loops are videorecorded for two hours into reperfusion.

Data collection and statistical analysis

Capillary red blood cell velocity (CBV) was determined from the videorecordings of flow in the selected capillary branches of the RSK and NRSK loops. In this procedure, two cursors (upstream and downstream) are positioned in a cross-section fashion on the branch [IPM Video Photo Analyzer 204] and output signals are transmitted to a microcomputer. Using custom software, CBV is determined via cross-correlation. For each branch, crosschecks, using manually discerned delayed signals and frame-by-frame video analysis were randomly done.

Capillary diameters were estimated using the width of the erythrocyte column. This was done since the pre and post ischemia resolution was not consistently adequate to define the capillary wall-lumen interface and we wished to avoid potential confounding differential effects of a fluorescent plasma marker. Further, since the primary quantity of interest in this study was CBV, changes in capillary diameter were of greater interest than the absolute capillary diameter, we opted for the erythrocyte column method, recognizing that the diameters reported probably underestimate the true diameter [13]. Each capillary diameter was measured at three sites; at the site of each velocity cursor and midway between them, with the average being taken as the capillary diameter. As an index of flow intermittency, the

duration of nonzero flow in all capillaries was determined at baseline ar
after ischemia. This Flow Time parameter is expressed as the percentage
the observation interval during which the capillary had a nonzero flow.

Statistical comparisons between pre and post parameter values in tl
same capillaries were done using the Wilcoxon signed rank test. Grou
differences were tested using the Mann-Whitney test. The level considere
for significant difference is < 0.05.

Results

Data was obtained from 10 animals with CBV measured in four capillari
of each animal, two in the risk zone and two in the nonrisk zone. In eac
capillary, measurements were made at baseline (pre-ischemic) and durir
the reperfusion phase after the four hours of capillary flow stasis (pos
ischemic). Table 1 summarizes the measured overall mean data for CB
diameter, Flow Time, heart rate (HR), core temperature (Tc) and ear ski
temperature (Te). All post-ischemic values reported were obtained
60 ± 10 minutes into reperfusion. Pre-ischemic values of CBV, diamet
and flow time did not differ between RSK and NRSK capillaries, nor wa
there a significant pre- post-ischemic difference in HR, Tc or Te. In the RS
capillaries, the post-ischemic CBV was significantly less than the pre-ischen
ic value whereas no pre-post differences were detected in NRSK capillarie
The diameters in both RSK and NRSK capillaries appeared to be larger i
the post-ischemic phase. Since capillary diameter was estimated using tl
erythrocyte column, it is likely that this increase reflects a change in tl
erythrocyte concentration profile rather than an actual increase in diamet
[13] attributable to the ischemia. This may have been due to intravascul
dehydration and the subsequent hemoconcentration. The pre-ischemic Flo

Table 1. Parameters before and after four hours of regional ischemia

Parameters	RSK capillaries		NRSK capillaries	
	Pre-isch	Post-isch[a]	Pre-isch	Post-isch
CBV (um/s)	212 ± 31	120 ± 22*	174 ± 22	195 ± 41
Diameter (um)	4.5 ± 0.2	5.0 ± 0.3*	4.4 ± 0.3	4.9 ± 0.3
Flow time (%)	97.5 ± 1.2	77.5 ± 9*	98.6 ± 0.9	98.4 ± 1.1
HR (bpm)	270 ± 15	279 ± 12		
Tc (°C)	32.2 ± 0.6	32.7 ± 0.4		
Te (°C)	28.4 ± 0.5	29.6 ± 0.4		

Data are mean ± SEM from 10 animals with four capillary measurements per animal.
[a] Measured 60 ± 10 minutes into reperfusion, * p ± 0.05 as compared to pre-isch.

Time data indicates that there was practically no capillary flow intermittency. Flow times in the NRSK capillaries were not affected by the four hour ischemia whereas a significant decrease in the RSK capillaries was recorded.

Table 2 summarizes the CBV data in capillaries which are grouped according to whether the reperfusion velocity decreased or increased as compared with the pre-ischemic vlaues. Changes from pre-ischemic values of at least $\pm 20\%$ were required to classify a response as either a decrease or increase, otherwise it was classified as no change. In the RSK zone 12 of the 20 capillaries studied (60%) were found to have a decreased CBV whereas 9 of the 20 NRSK capillaries showed a decrease in CBV. In both RKS and NRSK zones some capillaries had a greater CBV during reperfusion compared with the baseline values. In both the RSK and NRSK zones the capillaries which responded with a decreased flow tended to be those which had greater pre-ischemic values. However, a statistically significant difference between pre-ischemic values was demonstrated only for the RSK zone capillaries (263 μm/s vs. 119 μm/s, p <) 0.01). In capillaries in which flow decreases from baseline were noted, the absolute value of the decrement in RSK capillaries was much greater than in the NRSK capillaries (210 μm/s vs. 85 μm/s, $p < 0.01$).

The way in which the ischemia affected the distribution of CBV is illustrated in Fig. 1. It may be noted from these frequency distributions that prior to ischemia, only 10% of the RSK capillaries had velocities $\leqslant 100$ μm/s, whereas following the four hours of ischemia this number jumped to 45%. Of this 45%, four capillaries (25%) had zero flow throughout the reperfusion phase.

Discussion

The main goal of this study was to follow individual capillaries and to characterize their reperfusion status under conditions in which zero capillary flow during ischemia was maintained and verified during the entire ischemic interval. Owing to adaptive responses and the presence of collateral pathways, arteriolar ligation by itself is no guarantee that regional capillary flow stasis will be produced or maintained. Thus, initial and sequential assessment of capillary status is required. This stringent requirement is less important when global ischemia or compressional methods of producing regional ischemia are employed. The use of the skin microvasculature of the ear of the homozygous hairless mouse allowed the necessary procedures to be done without extensive surgery and the associated complications of inflammatory

Table 2. Subgroup analysis of CBV changes

CBV response[a]	Number of capillaries		Pre-ischemic CBV (um/s)		CBV change[c] (um/s)		CBV change (%)	
	RSK	NRSK	RSK	NRSK	RSK	NRSK	RSK	NRSK
Decrease	12	9	263 ± 47[b]	202 ± 25	210 ± 54[d]	85 ± 12	74 ± 8	35 ± 6
Increase	5	9	119 ± 20	174 ± 39	126 ± 28	128 ± 48	113 ± 40	81 ± 34
No change	3	2	176 ± 40	72 ± 12				

[a] Grouped by directional change as compared with pre-ischemic value. Changes $< \pm 20\%$ are catagorized as No change.
[b] $p < 0.01$ as compared to capillaries that responded with an increase in CBV.
[c] Paired differences between pre- and post ischemic.
[d] $p < 0.01$ as compared to NRSK capillaries of the same subgroup.

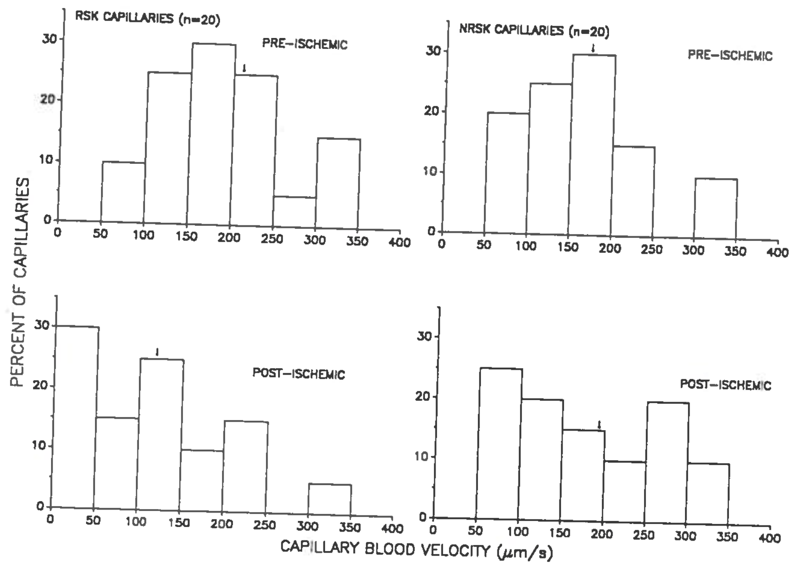

Fig. 1. Histogram showing the distributions of pre- and post ischemic CBV in the RSK and NRSK capillaries. Data is from 10 animals with four capillaries measured in each animal, two in the RSK and two NRSK zones. Arrows show mean of each distribution.

components. In addition by using selective arteriolar ligations it was possible to produce capillary flow stasis without direct compression of the microvasculature under study.

Because of the stringent requirements of the protocol it was not technically feasible to study large numbers of capillaries and we restricted our study to detailed measurements in four capillaries per animal. Thus, rather than sampling CBV for very brief intervals, each CBV was based on the mean of at least 10 minutes of continuous data. In this way, sampling bias which might be introduced by short term temporal variations are minimized, but at the cost of having a smaller sample size per animal. The identification of specific capillaries lying within the RSK and NRSK zones provided sufficient control for valid comparisons of red blood cell velocity.

Several considerations dictated the use of general anesthesia in the present protocol although Barker and co-workers [4] have described an elegant method for studying many features of the ear without the use of anesthesia. In studying skin microcirculation in awake animals (the bat wing) it was found (unpublished observations) that during the course of extended observations, as demanded by the present protocol, substantial changes in microvascular parameters (blood velocity, leukocyte rolling etc.) may occur. These unpredictable changes are related to variations in the arousal state of

the animal and associated physiological stress responses within the circulation. Since such changes vary with each animal and time, they would represent a significant confounding variable when attempting to compare the ischemic response. Though it is also true that the use of anesthesia has its own disadvantages, it at least allows control of the situation in a standardized manner. The second point concerns the need, in the present protocol, for manipulations to ligate and release the ligatures. It simply was not practical to have the animal awake during these procedures.

Though heart rate and core temperature were routinely monitored as indicies of the animals' physiological state, arterial blood pressure (BP) was not monitored. Though it has been shown that BP can be technically measured even in these small animals [4] several considerations guided us not to include this measurement as part of our protocol. BP monitoring would not only have substantially lengthened the already lengthy acute protocol, but would also produce an added surgical trauma to the animal. In preliminary studies we found that the remote surgical procedures associated with carotid artery cannulation could produce enhanced leukocyte activation which manifests itself as increased adherence within the microvasculature. As this could significantly influence the findings with regard to capillary velocity, these cannulation procedures were avoided. The absence of BP data could mean that the effect of long term trends in BP, if present, would not be directly determined. It is precisely for this reason that the protocol was designed to include capillary measurements in both risk and nonrisk regions of the same animal. Thus, if there were a BP change, each of these regions would be exposed to the same change.

The findings of the present study show that vascular occlusion which produces four hours of demonstrable flow stasis in the skin capillaries of the mouse ear results in a reduced reperfusion in these capillaries. The base line values of capillary blood velocity herein determined (mean $\pm$ sd) was $193 \pm 116\,\mu m/s$, ($n = 40$) which is somewhat higher than the figure of $60 \pm 30\,\mu m/s$, ($n = 27$) reported by Barker and co-workers for the same tissue [4]. The source of this difference in mean CBV is not known but may be related to the use of anesthesia in the present study or may simply be random variations consistent with the large standard deviations each group has found.

In the present study, as judged by the mean reduction of CBV across all RSK capillaries studied, the reperfusion was decreased by approximately 43%. The magnitude of this reperfusion deficit agrees well with the findings of Menger and co-workers [18], who demonstrated a 50% reduction in capillary velocity in skeletal muscle following four hours of compressional ischemia in the hamster skin fold preparation. Control mean CBV was

260 mm/s and mean reperfusion CBV was 130 μm/s. These authors also reported that approximately 65% of the capillaries measured had reperfusion velocities less than 100 μm/s. This may be compared with the 45% figure we have found with ligation induced ischemia. Marzella [17] also noted marked blood flow reduction after four hours of pressure ischemia in skin flaps. Sack [21] showed that after four hours of ischemia and fifteen minutes into reperfusion, the CBV was significantly reduced in over 50% of the capillaries. With two hours of ischemia, however, the CBV was unchanged. When, in the present study, the paired changes in individual capillaries are considered it is found that the reperfusion deficit is attributable to red blood cell velocity reductions in a subset of the capillaries. Thus, in 60% of the RSK capillaries the reperfusion CBV was less than the pre-ischemic values, whereas in the remainder, CBV increased or remained unchanged. Attempts to determine which morphometric features were characteristic of the capillary subset with reduced reperfusion flow, were not productive. After release of the ligatures the arteriolar vessels were microscopically observed for absence of flow return, presence of vasopasm and intravacular platelet aggregation. None of these were observed. There was a general tendency for capillaries with higher pre-ischemic CBV to have a reduced reperfusion flow.

The percentage of capillaries with reflow has been reported to be as low as 30% after four hours of pressure-induced ischemia in the hamster cheek pouch [19]. In the present study, absolute zero reperfusion flow was found in 20% of the RSK capillaries and these were confined to only two of the 10 mice studied. The corresponding NRSK capillary flow in these mice was not reduced during reperfusion suggesting that changes in central hemodynamics were not the primary cause of the no-reflow. Attempts to distinguish these no-reflow capillaries from others on morphometric grounds was not revealing. However, in these tissues, there was subjective evidence suggestive of significant tissue edema. In two animals with no-reflow, interstitial swelling surrounding the capillary loops under study was noted. Engorgement of the post-capillary venules was also observed but to some extent, this venous engorgement appeared to exist generally across all RSK capillaries during reflow. A tendency for capillary diameter changes to occur in response to ischemia has been previously reported [10]. Significant increases in the diameter of venules after four hours of pressure-induced ischemia were seen during reperfusion [18]. Our findings of an apparent tendency for capillary diameters to be larger in both RSK and NRSK at reperfusion may reflect changes in the erythrocyte radial profile resulting in a smaller peripheral plasma layer, rather than true increases in luminal diameter [13]. These changes should thus not be interpreted to be directly

related to the ischemia per se. However, our findings concerning red cell velocity indicate that when capillary flow stasis is produced, durations of skin ischemia of four hours may subject the microvasculature to significant reperfusion deficits.

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References

1. Ames A, Wright RL, Kowada M, Thurston JM, Majno G (1968) Cerebral ischemia II: The no-reflow phenomenon. Am J Path 52: 437–453
2. Bagge U, Amundson B, Lauritzen C (1980) White cell deformability and plugging of skeletal muscle capillaries in hemorrhagic shock. Acta Physiol Scand 108: 159–163
3. Barker J, Bartlett R, Funk W, Hammersen F, Messmer K (1987) The effect of Superoxide dismutase on the skin microcirculation after ischemia and reperfusion. Prog Appl Microcirc 12: 276–281 (Karger, Basel)
4. Barker JH, Hammersen F, Bondar I, *et al.* (1989) The hairless mouse ear for *in vivo* studies of skin microcirculation. Plast Reconst Surg 83(6): 948–959
5. Chait L, May J, O'Brien B, Hurley J (1978) The effects of the perfusion of various solutions on the no-reflow phenomenon in experimental free flaps. Plast Reconstr Surg 61: 421–430
6. Engler R, Dahlgren M, Morris D, Petterson M, Schmid-Schönbein G (1986) Role of leukocytes in response to acute myocardial ischemia and reflow in dogs. Am J Physiol 251: H314–22
7. Eriksson E, Anderson W, Replogle R (1974) The effects of prolonged ischemia on muscle microcirculation in the cat. Surg Forum 25: 254–255
8. Eriksson E, Replogle RL, Glagov S (1987) Reperfusion of skeletal muscle after warm ischemia. Ann Plast Surg 18(3): 224–9
9. Eriksson E, Boykin JV, Pittman RN (1980) Method for *in vivo* microscopy of the cutaneous microcirculation of the hairless mouse ear. Microvas Res 19: 374–379
10. Gidløf A, Lewis DH, Hammersen F (1987) The effect of prolonged total ischemia on the ultrastructure of human skeletal muscle capillaries. A morphometric analysis. Int J Microcirc: Clin Exp 7: 67–86
11. Kloner R, Ganote C, Jennings R (1974) The No-reflow phenomenon after temporary coronary occlusion in the dog. J Clin Invest 54: 1496–1508
12. Lewis D (1984) The response of the microvasculature in skeletal muscle to hemorrhage, trauma, and ischemia. In: Hammersen F, Messmer K (eds) Skeletal muscle microcirculation. Prog Appl Microcir 5: 127–138
13. Mahler F, Nagel G, Saner H, Kneubuhl F (1983) *In vivo* comparison of the nailfold capillary diameter as determined by using the erythrocyte column and FITC-labelled albumin. Int J Microcirc: Clin Exp 2: 147–156

14. May J, Chait L, O'Brien B, Hurley J (1978) The no-reflow phenomenon in experimental free flaps. Plast Reconstr Surg 61(2): 256–267
15. Mayrovitz HN, Sorrentino EA, Moore J. A method for producing regional ischemia in the skin microvasculature. Microvas Res (in press)
16. Mayrovitz HN, Moore J (1989) Hemodynamic and metric asymmetry of paired bifurcating capillaries. FASEB J 3: (4) A 1394 (Abstract)
17. Marzella L, Jesudass RR, Manson PN, Myers RA, Bulkley GB (1988) Functional and structural evaluation of the vasculature of skin flaps after ischemia and reperfusion. Plast Reconstr Surg 81: 742–50
18. Menger MD, Sack FU, Barker JH, Feifel G, Messmer K (1988) Quantitative analysis of microcirculatory disorders after prolonged ischemia in skeletal muscle. Res Esp Med 188: 151–165
19. Romanus M, Stenqvist, Haljamae H, Seifert F (1977) Pressure-induced Ischemia: An experimental model for intravital microscopic studies in hamster cheek pouch. Eur Surg Res 9: 444–459
20. Rosen HM, Slivjak MJ, McBrearty FX (1985) Preischemic flap washout and its effect on the no-reflow phenomenon. Plast Reconstr Surg 76: 737–47
21. Sack FW, Funk W, Hammersen F, Messmer K (1987) Microvascular injury of skeletal muscle and skin after different periods of pressure induced ischemia. Prog appl Microcirc 12: 282–288 (Karger, Basel)
22. Schmid-Schönbein GW (1987) Capillary plugging by granulocytes and the no-reflow phenomenon in microcirculation. FASEB Federation Proceedings 46: 2397–2401
23. Sheehan HL, Davis JC (1959) Renal ischemia with failed reflow. J Path Bact 78: 105–120
24. Strock PE, Majno G (1969) Microvascular changes in acutely ischematic rat muscle. Surg Gynecol Obstet 129: 1213–1224
25. Suval WD, Duran WN, Boric MP, Hobson RW 3rd, Berensen PB, Ritter AB (1987) Microvascular transport and endothelial cell alterations preceding skeletal muscle damage in ischemia and reperfusion injury. Am J Surg Aug 154(2): 211–218
26. Tyml K, Budreau CH (1988) Heterogeneity of microvascular response to ischemia in skeletal muscle. Int J Microcirc: Clin Exp 7: 205–221
27. Wilms-Kretchmer K and Majno G (1969) Ischemia of the skin: electronmicroscopy study of Vascular Injury. Am J Path 54: 327–353

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