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Age and site variability of skin blood perfusion in the hairless mouse ear determined by laser doppler flowmetry

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Abstract. Laser Doppler Flowmetry (LDF) was used to characterize skin blood perfusion variations present within the Homozygous Hairless mouse ear microvasculature as a function of 1) arteriolar level within the microvasculature, 2) animal age and 3) LDF probe distance above the tissue being studied. To this end blood perfusion measurements (arbitrary perfusion units) were made in the ear at vascular sites in the vicinity of primary vessels (site A), secondary vessels (site B), tertiary vessels (site C), and peripheral zones distant from larger vessels. These were done without any invasive procedures save anesthesia administration and were carried out at three different tissue-probe distances (0.5, 0.75 and 1.0 mm) on three groups of eight mice each with ages of three, five and eight weeks. Results show a significant gradient in perfusion from sites A to D in each age group and a significant increase in perfusion with increasing age. The effect of probe-tissue distance variations on perfusion values was found to be significant between 0.5 mm and 1.0 mm but overall differences were small. In addition to these specific findings the present results demonstrate the potential of LDF as a means of detecting, evaluating and correlating perfusion changes, either by itself or as a complementary method to detailed microvascular measurements using conventional in vivo microscopy.

Introduction

Laser Doppler flowmetry (LDF) has shown its utility in a wide variety applications dealing with the evaluation of blood perfusion in human skin [6, 8–10, 12–13, 15–18, 20–21]. When applied under these conditions data can be obtained from tissue up to about one millimeter below the surface. Since most experimental preparations currently used for *in vivo* microvascular study are less than this thickness it would appear that LDF may be useful to evaluate microcirculatory parameters and their change in appropriate tissues. Inroads in this direction have been made using the frog sartorius muscle [19] and more recently using the rat mesentery and cat sartorius muscle [5]. Another useful experimental model is the skin microvasculature of the homozygous hairless (H/H) mouse which has a thickness of about 0.2 to

0.3 mm. This model which can be utilized without surgical preparation [2, 7, 11] has proved to be quite useful in studying various aspects of skin microcirculation via direct microscopic observation and measurement [1, 3, 4, 14]. Of necessity the microscopic methods, which include measurements of individual microvessel diameters, blood velocity and other microcirculatory features have intrinsic sampling limitations in view of the large number of vessels present. The LDF method, which is capable of acquiring and integrating microvascular perfusion data over a surface area of approximately one mm^2 and a volume of about 1 mm^3 represents an improvement in the measurement sampling area though at the expense of some specificity. Thus LDF as applied to such experimental situations may be viewed as a method which may provide data to supplement that attainable with standard microscopic methods.

The application of the non-invasive LDF method to study blood microperfusion in the intact and untraumatized H/H mouse ear has the potential for providing new information relevant to a broad range of physiological/pathological problems. As initial steps in this direction the present research has sought to use LDF to characterize skin blood perfusion variations present within the H/H ear microvasculature as a function of 1) arteriolar level within the microvasculature, 2) animal age and 3) LDF probe height above the tissue being studied. To this end blood perfusion was measured in the vicinity of one primary arteriole, two secondary arterioles, three tertiary arterioles and in four peripheral zones of the ear. These were done without any invasive procedures save anesthesia administration and were carried out at three different tissue-probe distances on three groups of mice (n=8 each group) with ages of three, five and eight weeks.

Methods

Initial procedures

This investigation used 24 homozygous hairless (H/H) mice (SKH-1; Charles River Laboratories), divided into three groups at eight according to age; 3 wks, 5 wks and 8 wks. Following anesthesia induction (pentobarbital 6 mg/100 g i.p) each mouse was placed under a surgical microscope (Zeiss OPMI6-S) and a 25 Gauge Minicath (Abbott Laboratories) was inserted into the anterior intraperitoneal cavity for the administration of maintenance anesthesia. No further invasive procedures were used so as to minimize surgical or traumatic effects on normal circulation. The mouse was then wrapped in a 4″×4″ cotton gauze pad to minimize convective heat loss and

placed in the supine position on a temperature controlled heated board. The board supported the animal's body and had an attached copper plate on which the ear was placed for support. One end of the plate was wrapped with nichrome wire connected to a controlled current source used to generate heat which was conducted to the ear through the copper plate. The ear was carefully positioned with the dorsal surface gently flattened against the opposite end of the plate using paraffin oil on the dorsal surface of the ear. Ear skin temperature was monitored with a small thermistor and the ear temperature maintained at 31°C ± 1° by controlling the current to the nichrome wire. Core temperature was monitored using a thermistor inserted rectally and rectal temperature maintained between 33°C to 35°C by controlling the current level to the heating pad. Heart rate was 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 7C8A).

Blood perfusion data was obtained using the Periflux Laser Doppler Flow System (Perimed, Sweden PF 3, processing filter at 12 kHZ and output time constant at 0.2 s.) with the standard PF 308 probe held in a micromanipulator for precise positioning above the ear. The vertical distance of the

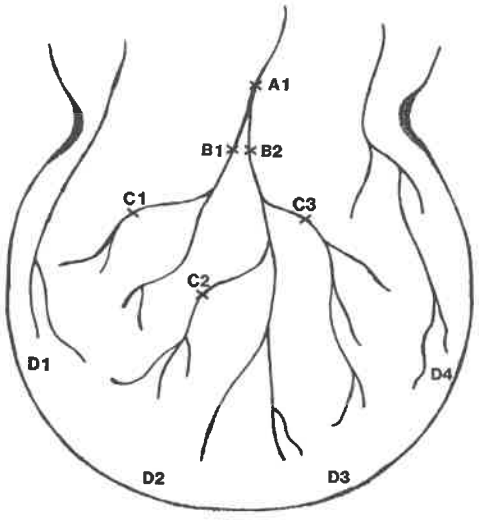

Fig. 1. Schematic drawing of part of the arteriolar distribution in the mouse ear showing the approximate locations of the 10 sites at which blood perfusion was determined. The venous – venular network (not illustrated) is usually paired with corresponding arteriolar vessels in regions A through C.

probe was set by first bringing the probe into contact with the copper plate and verifying the initiation of contact with a dissecting steromicroscope that was positioned at right angles to the probe. The probe was then raised 0.75 mm above the plate using the calibrated micrometer on the manipulator.

A total of 10 tissue sites were selected for measurement. Six of these included the perfusion of an ateriolar vessel and its accompanying paired venular vessel at different branching levels designated as A1, B1 and B2, and C1, C2 and C3 as shown in Fig. 1. To monitor the perfusion at these six sites the microscope stage was moved so that the target vessels were brought under the probe head. Registration of the detection of the perfusion in these vessels was indicated by a sharp increase in the perfusion signal. The stage was then moved back and forth slightly to obtain the maximum signal. Once this maximum was achieved the stage was not further moved in the horizontal plane until data acquisition at this site was completed. Four additional sites in peripheral zones of the ear with no larger microvessels present in the measuring area (D1, D2, D3, D4) were also measured. At each of the 10 sites the initial measurement was made for a period of three minutes and the data recorded on a chart recorder. Thereafter, the probe was moved vertically up a distance of 0.25 mm and the measurement process repeated. Finally the probe was moved vertically down a distance of 0.5 mm and measurements again were taken. This provided data on LDF perfusion at the same site for probe-tissue distances of 0.5, 0.75 and 1.0 mm above the posterior ear surface. When this sequence was completed a new site was measured and this process was continued until all 10 sites had been measured at the three different probe-tissue distances.

Statistical analysis of the LDF perfusion data was done initially using a single three factor model which included age, site, and z as factors in the three factor analysis of variance (ANOVA) procedure. Included in the model were the three levels of the age factor (3 wk, 5 wk and 8 wk), the four levels of the site factor (A, B, C, and D), and the three levels of the probe-tissue distances ($z=1$ mm, $z=0.75$ mm and $z=0.5$ mm). Since different numbers of measurements per animal were made at the various sites ($A=1$, $B=2$, $C=3$, and $D=4$), the values obtained at sites B, C, and D were averaged to produce a single characteristic per animal for each level of z. This resulted in four perfusion values for each animal at each of the three z levels which were subjected to ANOVA analysis. The results of the three factor ANOVA analysis were examined to determine if overall main effect significant differences were present in any of the three factors. If an overall difference was detected in a factor, it was subjected to post hoc comparisons (Scheffe test) to characterize in more detail the nature of the differences.

Table 1. LDF perfusion by age, site and z

		AGE (wks)		
SITE	z	3	5	8
A	1	65.1 (20.6)	76.5 (15.6)	101.8 (13.1)
	2	74.6 (32.3)	81.6 (17.6)	92.8 (20.5)
	3	74.4 (32.0)	87.0 (23.9)	97.9 (21.0)
B	1	47.8 (13.5)	59.5 (4.9)	85.0 (16.8)
	2	65.9 (24.0)	74.2 (12.5)	84.8 (19.6)
	3	78.0 (32.4)	86.2 (25.6)	83.1 (19.2)
C	1	39.6 (11.0)	47.6 (6.4)	74.1 (14.4)
	2	52.6 (15.1)	58.5 (14.4)	71.6 (13.2)
	3	74.1 (23.5)	65.3 (12.2)	66.8 (13.9)
D	1	8.8 (1.9)	12.0 (1.2)	17.2 (9.0)
	2	10.3 (2.6)	13.5 (1.9)	15.8 (8.5)
	3	12.9 (2.5)	15.5 (2.6)	15.9 (2.6)

Entries are group mean perfusion units in mv with standard deviations ( ). z is probe-tissue vertical distance with z = 1, 2, 3 respectively equal to 1 mm, 0.75 mm and 0.5 mm. N=8 mice at each age.

Results

Table 1 summarizes the group mean perfusion values obtained for each age, site and z level. ANOVA analysis showed significant differences in LDF perfusion with age, site and probe-tissue distance, z. The corresponding F and p values obtained were for age, F=23.5, $p<0.00001$; for site, F=235, $p<0.00001$; and for z, F=8.4, p=0.0005. As summarized in Fig. 2, the post

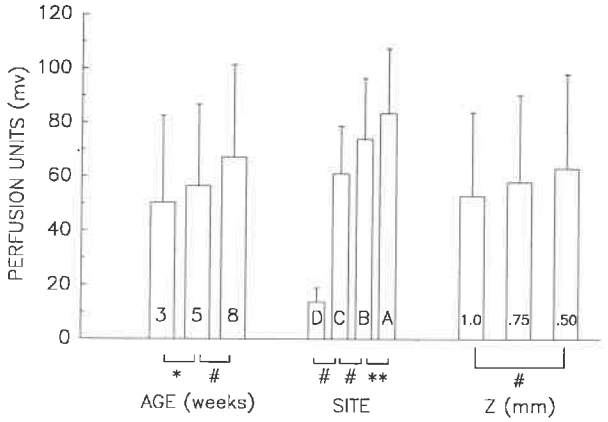

Fig. 2. Magnitude of perfusion by age (n=8 each age), probe-tissue distance, z and site (n=24). * denotes $p<0.05$, ** denotes $p<0.01$, # denotes $p<0.0001$.

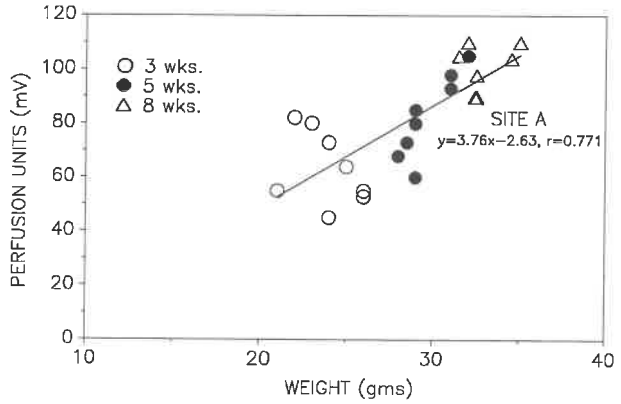

Fig. 3. Relationship between perfusion at site A and mouse weight. A highly significant positive correlation ($p<0.01$) was found.

hoc comparisons showed that there was a statistically significant monotonic increase in perfusion with age and a monotonic decrease in perfusion with site. Statistical differences in z were detected only between measurements at 0.5 and 1.0 mm with the value at 1.0 mm being 84% of that at 0.5 mm.

Further analysis of results (two-way ANOVA, age by site) using values obtained only at a particular z ($z=1$, 0.75 or 0.50) still reveals significant age ($p<0.001$) and site ($p<0.00001$) differences. However when perfusion is expressed relative to the value at site A it is found that this relative perfusion is independent of animal age although significant site differences remain. The individual mean perfusion values obtained for each animal at

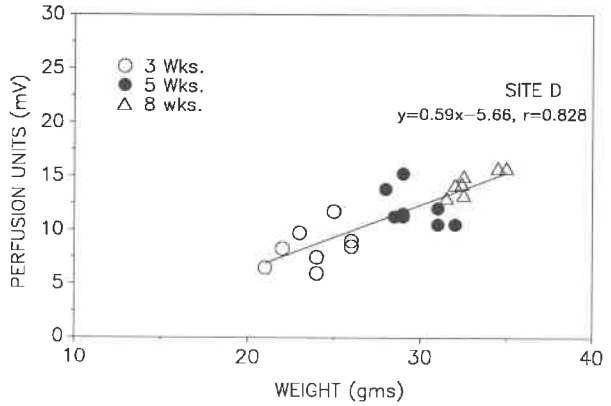

Fig. 4. Relationship between perfusion at site D and mouse weight. A highly significant positive correlation ($p<0.01$) was found.

z=0.75 mm at site A are shown in Fig. 3 and for site D in Fig. 4, each plotted as function individual animal weight. A significant correlation ($p<0.01$) between perfusion and weight is demonstrated for these sites as well as for sites B and C (not illustrated). The corresponding linear regression equations are shown in the figures. Weight data on one animal in the eight week age group was not available and is not included in the graphs.

Discussion

Methodological considerations

Laser Doppler flowmetry when used to evaluate human skin blood perfusion is most often accomplished with a self contained probe or a probe-thermal assembly [8–9] secured to and resting on the skin surface. In the case of the H/H mouse ear the thin skin tissue and the absence of significant underlying muscle support renders direct probe-tissue contact inappropriate. Indeed, the pressure from such direct contact can produce significant blood flow reductions. For these reasons all measurements in this study were done with a small air gap between the probe tip and the tissue surface. The overall thickness of the ear ranges from 0.2 to 0.3 mm [4] so that when the probe was placed at its nearest position of 0.5 mm from the dorsal surface an air gap of about 0.25 mm was present. At the furthest probe position of 1.0 mm the air gap increased to about 0.75 mm. The results show that over this range of distances there are overall differences in perfusion values obtained between 0.5 and 1.0 mm, with the greater distances tending to register smaller values of perfusion. However, even at the furthest distance the mean perfusion recorded was still 84% of that obtained at the closest position.

Perfusion variation with site

A significant monotonic decrease in blood perfusion from the proximal sites A to the distal sites D were clearly evident. This pattern undoubtedly reflects the overall decrease in blood flow as one traverses the vascular network from larger to smaller diameter vessels which respectively supply and drain greater and lesser amounts of tissue. Since at each measuring site (except sites D) the LDF sensing area included the most prominent vessels, the flow in these likely dominate the perfusion values obtained. From the multiple measurements obtained at corresponding D sites in each animal it was also of interest to estimate the spatial heterogeneity of capillary per-

fusion from the coefficient of variation (CV) of these measurements. For this purpose the CV of the four measurements on each animal was determined and the average CV for each age group determined. This parameter was found to be independent of animal age and averaged across all animals yielded a CV (mean ± sd) of 17.0 ± 5.2%. This value suggests the presence of a moderate spatial capillary perfusion heterogeneity in this experimental model.

Perfusion variation with age

A clear trend for perfusion to increase with increasing age was demonstrated. This increase correlated well with the growth of the animals and was linearly related to animal weight. The regressions showed no sign of leveling off at eight weeks of age, the oldest animal group studied, suggesting that perfusion may continue to increase beyond this age. Though there was a progressive increase in the magnitude of the perfusion at all levels, the fraction of the total perfusion at all levels remained essentially constant over the age range studied. Thus with growth there was a proportionate increase in measured blood perfusion that may be attributable to a variety of growth and adaptive processes including increases in ear tissue volume with age. The significance of this to the study of the microcirculation in this tissue lies in part on the need to control for these age related changes when using the model in either chronic studies or when making comparisons among different age animals. Since this model is well suited for repeated microscopic and perfusion studies it is anticipated that its use as a chronic model will yield important microvascular information in a variety of conditions. The present findings provide a baseline characterization of the perfusion changes and their variability from which to anticipate and plan for such chronic studies.

LDF vs detailed microvascular measurements

The present results demonstrate that LDF can be successfully used to study blood perfusion and its change in this model of skin circulation. The fact that the laser probe needs not directly contact the skin surface to obtain reliable and repeatable measurements is an important demonstration since such contact would affect the measured values in this thin tissue. The use of LDF as an investigate tool in this model offers some advantages as compared with other methods of detailed in vivo microscopy.

Using LDF allows for a greater tissue volume to be sampled with much less time expenditure and provides an intrinsic averaging of perfusion in multiple microvessels at a single instant in time. It therefore provides the

potential of obtaining a more reliable estimate of overall tissue perfusion by allowing a greater number of tissue perfusion samples to be taken as compared with the requirement for estimating blood velocity in large numbers of individual microvessels. The LDF method also has an advantage over detailed microvascular measurements in studies which require serial measurements in the same animal over extended periods of time. Location of the same blood vessel and the same site within that blood vessel in which baseline measurements were made hours, days or weeks later is difficult and sometimes impossible. However since the LDF measurement data represents an effective averaging over an area of about one square mm (one cubic mm volume) it makes repeated sequential measurements more feasible since small deviations in relocation are less significant. This is especially true when one is concerned with capillary network perfusion studies. A further advantage of LDF, even in animal tissues which permit individual microvessel blood velocity measurements, is the potential for LDF to provide more direct comparisons with data obtained on human skin [8–9, 15–16, 18, 20–21] in areas not amenable to direct vessel measurement. Finally it should be noted that LDF is not advocated as a replacement for detailed microscopic measurements in tissues in which these are possible and useful [1–4, 7, 11, 14], rather it is suggested that the use of LDF can serve as a complementary method for multiple purposes. The results of the present study show that the LDF method can be a useful and effective tool in this regard and has provided initial data on the perfusion characteristics of an important skin microvascular model.

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