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Stereological Analyses of Capillaries in Muscles of Dystrophic Mice

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Summary. Stereological analyses of the distribution of capillaries in skeletal muscles of congenitally dystrophic mice are described and reported. Two methods are used, each based on a different mathematical model of the 3-dimensional distribution of lines in space. For these analyses specimens of extensor digitorum longus muscle from clinically affected C57BL/6Jdy^{2J}/dy^{2J} dystrophic mice, and from non-littermate controls, were used. The analyses were carried out on transverse and longitudinally orientated semithin sections of these muscles. Although the two methods employed are based on different mathematical models and yield results relevant to each particular model, it is clear that there is a more extensive capillary network present per unit volume of the dystrophic muscle than in control muscles. These findings are relevant to the theories which involve a vascular aetiology for muscular dystrophy. It is apparent that, in order to explore the structure of the capillary network more fully, there is a need for the development of more sophisticated stereological techniques for analysis of capillaries in skeletal muscle.

Key words: Murine dystrophy – Capillaries – Stereology

Congenitally dystrophic animals have often been studied as comparative models of human dystrophies and also for their intrinsic interest. In particular, C57BL/6Jdy^{2J}/dy^{2J} dystrophic mice have been the subject of numerous physiological, histochemical, histological and electron microscopical investigations (Harris and Montgomery 1975; Silverman and Atwood 1980; Ontell and Feng 1981). Most attention has been directed to studies either of the muscle fibres in affected muscles, of their innervation, or of their muscle spindles (James and Meek 1975 and 1979). Little investigative work, however, has been carried out on the blood vessels of dystrophic muscles. Information on blood vessels is important because it has been hypothesised that

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murine dystrophy may have a vascular aetiology. Briefly, it is postulated that the pathological changes seen in dystrophic muscles are caused by areas of chronic anoxia within these muscles, this anoxia being precipitated by change in the vascular supply of the muscle. Such change is said to involve diminution in the effective extent of the capillary network, either by occlusion of some capillaries or by increased activity of arterio-venular shunts by-passing capillaries, though it may also (or alternatively) involve change in permeability of the capillary vessel wall. The theories involving a possible vascular aetiology have recently been summarised by Harris and Slater (1980).

In the present study, a quantitative analysis of the total mean capillary length per unit volume of skeletal muscle (L_V) has been carried out in normal and dystrophic mice to establish whether there are any significant differences in their capillary networks. Two methods have been used (Underwood 1970; Weibel 1980), each based on a different mathematical model of the two-dimensional distribution of lines in space. Both methods have previously been used to analyse capillaries in normal and hypertrophic skeletal muscles (James 1981).

Materials and Methods

Specimens of *extensor digitorum longus muscle* (EDL) were obtained from 6 clinically affected C57BL/6Jdy^{2J}/dy^{2J} dystrophic mice at three months of age, and also from six non-littermate controls. The specimens were prepared as for electron microscopy. Some of the qualitative features of these muscles (other than those concerning capillaries) have been recorded by James and Meek (1975).

The EDL muscles were initially fixed at their resting length *in situ* using 3% glutaraldehyde contained in 0.1 M phosphate buffer at pH 7.3. Six small portions were obtained from the central midline region of each muscle and fixed additionally for 4 h in fresh phosphate-buffered glutaraldehyde at 4 °C. They were then transferred to 0.1 M phosphate buffer at pH 7.3 to which sucrose had been added to a final concentration of 10%. The specimens were then placed in a 2% aqueous solution of osmium tetroxide for 1 h at 4° C, dehydrated in alcohol and embedded in Araldite.

Transverse and longitudinally orientated semithin (~ 1 μm) sections were prepared from each muscle specimen. The angle of sectioning was carefully controlled during the chuck mounting process and by re-orientating the chuck during the initial transverse sectioning for semithin sections.

For longitudinal sections each block was carefully reorientated by 90°. Care was taken to ensure that the longitudinal sections so far as possible contained muscle fibre profiles that possessed similar widths at all regions along their lengths. Each section was stained on a hot plate (at $\sim 90°$ C) using a 1% solution of toluidine blue contained in 1% aqueous borax (Pease 1964).

Sampling and Stereological Procedures. Transverse and longitudinal semithin sections were cut from each block for light microscopy. For each set of transverse and longitudinal sections of control and dystrophic muscle, 16 randomly distributed non-overlapping light microscopical fields (4 per block) were obtained at a magnification of 215×. Random sampling fields necessary for counting were obtained by setting the horizontal and vertical movement stage micrometers of a Wild M 20 Research Microscope with values from a table of random numbers (Lindley and Miller 1967). Each sampling field was estimated to be 4.26×10^{-2} mm^2. These transverse and longitudinal fields were used to count the numbers of capillaries per unit area of section [$(P_A)_\perp$ and $(P_A)_\parallel$ respectively]. The mean length of capillary per unit volume of muscle was

estimated for each specimen using techniques previously described (Underwood 1970; Weibel 1980) and also previously used for the analysis of skeletal muscles by James (1981). The terms $(P_A)_\perp$ and $(P_A)_\parallel$, as defined by Underwood (1970), are used in the present study for convenience; they are equivalent to the terms Q_{A1} and Q_{A2} defined by Weibel (1980).

The numbers of capillaries per unit area in transverse $(P_A)_\perp$ and longitudinal $(P_A)_\parallel$ planes were used to estimate the mean length of capillary per unit volume of muscle (L_V or J_V) using the relations:

$$L_V = (P_A)_\perp + (P_A)_\parallel \quad \text{(Underwood 1970)}$$

and

$$J_V = 2/3\,[(P_A)_\perp + 2(P_A)_\parallel] \quad \text{(Weibel 1980).}$$

Both L_V and J_V are measures of the length of capillaries per unit volume of muscle but, whereas the derivation of L_V is clearly described (Underwood 1970 page 61), the derivation of Weibel's term, J_V, is not given in his book (Weibel 1980) and it can only be assumed that these two terms are equivalent – although it is clear from these texts that the basis of measurement for $(P_A)_\perp$ and $(P_A)_\parallel$ is the same as for Q_{A1} and Q_{A2}.

Terms for the relative degree of orientation of the capillaries, $\Omega_{1,3}$ (Underwood 1970) and its equivalent (concentration) parameter, K (Weibel 1980) were also evaluated for each specimen from the relations:

$$\Omega_{1,3} = \frac{(P_A)_\perp - (P_A)_\parallel}{(P_A)_\perp + (P_A)_\parallel} \quad \text{(Underwood 1970),}$$

$$K = 2\left\{1 - \frac{(P_A)_\parallel}{(P_A)_\perp}\right\} \quad \text{(Weibel 1980).}$$

Two additional morphometric parameters were also calculated. The minimum intercapillary distances (ICD) were calculated as previously described (Bassingthwaithte et al. 1974; Gerdes et al. 1979; Odek-Ogunde 1982) from the relation:

$$\text{ICD} = \sqrt{\frac{2}{(P_A)_\perp \sqrt{3}}}.$$

The radii (R) of the diffusion cylinders surrounding capillaries, which can be approximated by the relation: $R = [\pi (P_A)_\perp]^{-\frac{1}{2}}$, were also calculated (Hoppeler et al. 1981). The diffusion cylinder represents (theoretically) that region of muscle which is supplied by diffusion from a single capillary (Krogh 1922).

Statistical Analysis. Differences in mean values of L_V, K and $\Omega_{1,3}$ between dystrophic and control mice were tested using the Welch test (Mack 1966). The two-tailed Welch test function (W) and a constant (h) were calculated by:

$$W = \sqrt{\frac{\bar{x} - \bar{x}'}{\frac{s^2}{n} + \frac{(s')^2}{n'}}} \quad \text{and} \quad h = \frac{\frac{s^2}{n}}{\frac{s^2}{n} + \frac{(s')^2}{n'}}$$

where $\bar{x}$, $\bar{x}'$ and s^2 and $(s')^2$ are the means and variances for control and dystrophic muscles respectively.

The mean diameter of capillaries was noted to be 4.8 μm. S_V was estimated by the relation $S_V = L_V \times P$ (or $S_V = J_V \times P$ for the Weibel data) where P was the equivalent diameter of a profile equal in radius to that of an average capillary.

Results

a) Qualitative. Each randomly selected field used for quantitative analysis was also examined to confirm the presence of the typical histological features of murine muscular dystrophy. The histological features were regarded as important, being of particular relevance to the understanding of the capillary network and its analysis. Muscle fibre sizes (diameters) were noted to be smaller and possess greater variability than in control mice. The characteristic features (consisting of 'split' fibres, centrally occurring vesicular nuclei and increased interstitial connective tissue) were noted in all microscopical fields subjected to quantitative analysis.

b) Quantitative. The experimental and calculated values obtained for normal and dystrophic mice are recorded in Tables 1 and 2. Significant statistical differences were found between the mean values for L_V in normal compared with dystrophic animals. Mean capillary length and the relative degree of orientation were increased in dystrophic mice compared with their non-littermate controls. An estimate of the external surface area of the capillaries per unit volume of tissue (surface density, S_V) in both tissues shows that the capillary surface area available for exchange is greater in dystrophic

Table 1. Capillary densities in mouse extensor digitorum longus (EDL) muscles. The terms $(P_A)_\perp$ and $(P_A)_\parallel$, defined by Underwood (1970) and used in the present study for convenience, are equivalent to the terms Q_{A1} and Q_{A2} defined by Weibel (1980).

Mouse No.	$(P_A)_\perp$ (mm^{-2})	$(P_A)_\parallel$ (mm^{-2})	L_V (mm/mm^{-3})	$\Omega_{1,3}$	J_V (mm/mm^{-3})	K (concentration coefficient)
Control mice						
1	579	365	945	0.23	873	0.74
2	655	390	1,045	0.25	957	0.81
3	882	378	1,259	0.40	1,093	1.14
4	757	466	1,222	0.24	1,127	0.77
5	624	323	947	0.32	847	0.96
6	741	409	1,150	0.29	1,040	0.90
Mean:	706	389	1,095	0.29	990	0.90
Standard Error:	±45	±20	±56		±47	
Dystrophic mice						
1	919	441	1,360	0.35	1,201	1.04
2	831	390	1,222	0.36	1,075	1.06
3	453	453	906	0.00	906	0.00
4	831	441	1,272	0.31	1,143	0.94
5	1,134	340	1,474	0.54	1,210	1.40
6	1,070	341	1,411	0.52	1,171	1.36
Mean:	869	401	1,274	0.37	1,118	1.08
Standard Error:	±96	±21	±82		±47	

Table 2

	$(P_A)_\perp$	Minimum[a] intercapillary distance (I.C.D.) μm	Capillary surface density (S_v) $mm^2 \cdot mm^{-3}$ (using L_V)	(using J_V)	Krogh's[b] Tissue radius Rμm (using L_V)	Krogh's[c] Tissue radius Rμm (using J_V)
Control mice						
1	579	44.7	14.3	13.2	18.4	19.1
2	655	42.0	15.8	14.4	17.5	18.2
3	882	36.2	19.0	16.5	15.9	17.1
4	757	39.1	18.4	17.0	16.1	16.8
5	624	43.0	14.3	12.8	18.3	19.4
6	741	39.5	17.3	15.7	16.6	17.5
Mean	706	40.8	16.5	14.9	17.0	17.9
Dystrophic mice						
1	919	35.4	20.5	18.1	15.3	16.3
2	831	37.3	18.4	16.2	16.1	17.2
3	453	50.5	13.7	13.7	18.7	18.7
4	831	37.3	19.2	17.2	15.8	16.7
5	1,134	31.9	22.2	18.2	14.7	16.2
6	1,074	32.8	21.3	17.7	15.0	16.5
Mean	874	37.5	19.2	16.9	15.6	16.8

[a] Calculated from I.C.D. $= \sqrt{\frac{2}{(P_A)_\perp \cdot \sqrt{3}}}$

[b] $R = (\pi \cdot L_V)^{-\frac{1}{2}}$

[c] $R = (\pi \cdot J_V)^{-\frac{1}{2}}$

animals than in controls. No significant difference in capillary diameters between control and dystrophic animals was noted.

Discussion

a) Technical. The techniques used to measure L_V are each based upon a different mathematical model of the structure of partially-orientated systems of lines in space, in which part of the total length of the lines is orientated in a specific direction while the remaining segments may have essentially a random orientation. The model used by Underwood (1970) is based upon an earlier derivation clearly given by Saltykov (1954), whilst that given by Weibel (1980) is based on the Fisher distribution. The exact derivation of the latter formula is not given in Weibel's book and is not otherwise available. The derivation of the Underwood formula, however, is quite simply summarised in his book (Underwood 1970 page 61). The two sets of results generated are relevant to each model and, although each theoretical model has previously been used as the basis for analyses of the capillary network in skeletal muscles (James 1981; Mathieu et al.

1981), until further information is available it is not clear which is the more appropriate model. However, both techniques clearly demonstrate the same general increase in the parameters estimated, and the use of either one of the models and its associated methods does not invalidate or contradict the biological results reported in the present study.

The finding that capillaries in dystrophic and normal muscles differ in their relative degree of orientation (as measured by $\Omega_{1,3}$) and in their concentration parameter (K), has important implications for the many other techniques that have been used in analysing capillaries. For example, techniques involving the assumption of a *constant* degree of orientation (Mathieu et al. 1981) would not be applicable.

In general, with the available techniques for quantitative measurement, the estimation of L_V is difficult. Independent agreement with an alternative method for determining L_V would not necessarily validate the methods used in the present study. For example, measuring L_V by using the relation $L_V = V_V/\bar{a}$ (where $\bar{a}$ is the mean capillary area) may yield unreliable results because $\bar{a}$ could well be biased by the variation in the proportions of oblique profiles between adjacent tissue sections; any over-estimation by such method may be greater with larger numbers of obliquely cut capillaries, so a good correlation might not necessarily be a validation. Thus the use of $\bar{a}$, or of P (as in the present study), may be no more than an approximation, of value for comparative purposes only. Clearly, more sophisticated techniques are needed to measure capillary properties accurately.

b) Biological. Many workers have argued that murine dystrophy has a vascular aetiology – their views are mentioned in our Introduction and are summarised by Harris and Slater (1980). If this is true, we should have expected to find some evidence from our stereological analyses that the capillary network in dystrophic muscles would be *decreased* in its extent, relative to that in normal muscles, sufficient to result in areas of local anoxia within the now dystrophic muscles. However, the findings reported in the present study would seem to suggest that, since capillary surface area and length are actually *increased* in dystrophic muscle, the anatomical site of the causative lesions must either be within the endothelial cells themselves or else be in a non-capillary site. Our results show that there is more capillary tissue present per unit volume of dystrophic muscle than in controls and, allowing for the smaller size of the dystrophic muscle fibres, the dystrophic muscle possesses a higher ratio of capillaries to fibres than does normal muscle. However, estimation of the classical fibre to capillary ratios in dystrophic muscle cannot have the same meaning as in normal muscle because of the extremely frequent occurrence of "split" fibres which do not extend along the entire length of the intact fibres. Other quantitative measures would, therefore, seem to be more appropriate.

When comparing normal with dystrophic muscles, the estimation of L_V can be seen to be superior to measurement of either the fibre to capillary ratio or the mean number of capillary profiles per unit area of cross section, which is still the most commonly used quantitative method (James 1981;

Aquin and Banchero 1981). Measurement of the fibre to capillary ratio would be affected by the occurrence of the supposedly 'split' fibres in dystrophic muscle – without providing data on the exchange surface area or total amount of capillary tissue present, which are likely to be better indicators of potential metabolic activity. The counting of the number of capillary profiles in transverse sections alone would underestimate the absolute amount of capillary tissue present, as it does not allow for any increased length of capillaries resulting from tortuosity, nor for the presence of capillary cross linkages now known to exist within skeletal muscle. Therefore, no attempt was made to use such techniques to quantify the capillary network.

The minimum intercapillary distance was found to be significantly reduced in dystrophic muscle; however, this finding would be entirely consistent with the occurrence of smaller fibres in dystrophic muscle. On the other hand, the significant reduction in the radius of the Krogh cylinder found in dystrophic muscle is consistent with the increased oxidative capacity of dystrophic fibres previously reported (Silverman and Atwood 1980). The increase found by these workers in the mitochondrial volume fraction within dystrophic fibres may result from a reduction in the size of muscle fibres or from an adaptive response to increased muscular activity. Both mechanisms would clearly necessitate a reduction in the radius of the Krogh cylinder if regions of tissue anoxia were to be avoided. Our finding that the Krogh cylinder radius is, in fact, reduced in dystrophic muscle would appear, therefore, to argue against the occurrence of tissue anoxia in these fibres. Thus, if our findings are supported, tissue anoxia resulting from a decrease in the size of the capillary network supplying a given volume of muscle fibres is unlikely to be involved in the aetiology of dystrophic changes.

No evidence exists in human dystrophy for damage to small vessels of dystrophic muscles, and capillary occlusion has (apparently) not been reported. The results of investigations designed to determine whether the blood flow in dystrophic muscle is abnormal have not proved conclusive (Bradley 1977). However, it is well established experimentally that induction of micro-emboli (Sephadex beads) and use of vasoactive agents (for example, 5-hydroxytryptamine and noradrenaline: Engel 1975) are capable of inducing dystrophic-like changes in experimental animals. The ambiguities of other experimental work (and the finding that L_V and S_V are increased) would seem to suggest that, even if the vascular hypothesis is subsequently verified, then a reduction in size of the capillary network is not involved. It is clear that further study is required in order to establish what, if any, is the involvement of vascular change in the aetiology of murine muscular dystrophy.

References

Aquin L, Banchero N (1981) The cytoarchitecture and capillary supply in the skeletal muscle of growing dogs. J Anat 132:341–356

Bassingthwaithte JB, Yipintsoi T, Harvey RB (1974) Microvasculature of the dog left ventricular myocardium. Microvasc Res 7:229–235

Bradley WG (1977) The peripheral circulation in Duchenne dystrophy. In: Rowland LP (ed) Pathogenesis of human muscular dystrophies. International Congress Series no 404. Excerpta Medica, Amsterdam, pp 672–677

Engel WK (1975) Workshop on the aetiology of Duchenne muscular dystrophy: The vascular hypothesis. In: Bradley WG, Gardner-Medwin D, Walton JN (eds) Recent advances in myology. International Congress Series no 360. Excerpta Medica, Amsterdam, pp 163–173

Gerdes MA, Callas G, Frederick KH (1979) Differences in regional capillary distribution and myocyte sizes in normal and hypertrophied rat hearts. Am J Anat 156:523–531

Harris JB, Montgomery A (1975) Some mechanical and electrical properties of distal hind limb muscles of genetically dystrophic mice (C57BL/6Jdy^{2J}/dy^{2J}). Exp Neurol 48:569–585

Harris JB, Slater CR (1980) Animal models: what is their relevance to the pathogenesis of human muscular dystrophy? Br Med Bull 36:193–197

Hoppeler H, Mathieu O, Weibel ER, Krauer R, Lindstedt SL, Taylor CR (1981) Design of the mammalian respiratory system. VIII. Capillaries in skeletal muscles. Respir Physiol 44:129–150

James NT (1981) A stereological analysis of capillaries in normal and hypertrophic muscle. J Morphol 168:43–49

James NT, Meek GA (1975) Ultrastructure of muscle spindles in dystrophic mice. Nature 254:612–613

James NT, Meek GA (1979) Ultrastructure of muscle spindles in C57BL/6Jdy^{2J}/dy^{2J} dystrophic mice. Experientia 35:108–109

Krogh A (1922) The anatomy and physiology of capillaries. Yale University Press, New Haven, p 276

Lindley J, Miller JVP (1967) Cambridge statistical tables. Cambridge University Press, Cambridge

Mack C (1966) Essentials of statistics for scientists and technologists. Heinemann, London, p 174

Mathieu O, Hoppeler H, Cruz-Orive LM, Weibel ER (1981) Stereological estimation of the length density of anisotropic structures: capillaries in skeletal muscle. Stereologica Iugoslavika 3, Suppl I: 643–647

Odek Ogunde M (1982) Myocardial capillary density in hypertensive rats. Lab Invest 46:54–60

Ontell M, Feng KC (1981) The three-dimensional cytoarchitecture and pattern of motor innervation of branched striated muscle. Anat Rec 200:11–31

Pease DC (1964) Histological techniques for electron microscopy. Academic Press, London, p 381

Saltykov SA (1954). *loc cit* Underwood (1970)

Silverman H, Atwood HL (1980) Increase in oxidative capacity of muscle fibers in dystrophic mice and correlation with overactivity in these fibers. Exp Neurol 68:97–113

Underwood EE (1970) Quantitative stereology. Addison-Wesley, Cambridge Massachusetts, chapter 3

Weibel ER (1980) Stereological methods, vol 2. Academic Press, London, chapter 10

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