

Greater capillary-fiber interface per fiber mitochondrial volume in skeletal muscles of old rats

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Running head: aging and muscle capillary supply

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ABSTRACT

The objective was to examine whether muscle structural capacity for O₂ flux (i.e. capillary-to-fiber surface ratio) relative to fiber mitochondrial volume deteriorates with the muscle atrophy of aging in predominantly slow- (soleus, S) and fast-twitch (extensor digitorum longus, EDL) muscles of old (24-month) and very old (35-month) F344BN rats compared to adult (12-month old). Wet muscle mass decreased 29% (196±4 to 139±5 mg) in S and 22% (192±3 to 150±3 mg) in EDL between 12- and 35-month of age, without decline in body mass. Capillary density increased 65% (1387±54 to 2291±238 mm⁻²) in S and 130 % (964±95 to 2216±311 mm⁻²) in EDL, due to the muscle fiber atrophy while capillary per fiber number remained unchanged. Altered capillary geometry, i.e. lesser contribution of tortuosity and branching to capillary length, was found in S at 35- compared to 12- and 24-month, and not in EDL. Accounting for capillary geometry revealed 55% (1776±78 to 2750±271 mm⁻²) and 113% (1194±112 to 2540±343 mm⁻²) increases in capillary length / fiber volume between 12- and 35-month of age in S and EDL, respectively. Fiber mitochondrial volume density was unchanged over the same period, causing mitochondrial volume / μm fiber length to decrease in proportion to the fiber atrophy in both muscles. As a result of the smaller fiber mitochondrial volume in the face of the unchanged capillary / fiber number, capillary-to-fiber surface ratio relative to fiber mitochondrial volume not only did not deteriorate, but in fact increased 2-fold in both muscles between 12- and 35-month of age, independent of their different fiber type.

INTRODUCTION

The reduction in muscle maximal capacity for aerobic work with aging is well known, and previous studies have shown alterations in both convective and diffusive components of O₂ transport in muscles. For example, reduced leg blood flow, and increased leg O₂ extraction, were found in older (63 ± 2 years old) compared to younger (27 ± 1 years old) endurance-trained men during submaximal exercise (42), and blood flow redistribution from highly oxidative to highly glycolytic muscles was recently demonstrated in the hindlimb of old (27-29 month old) compared to young (6-8 months old) F344BN rats during submaximal exercise (37). Using an *in situ* pump-perfused hindlimb preparation to match convective O₂ delivery and eliminate age-related decline in blood flow, Hepple et al (15) found a 22% decrease in the mass-specific O₂ uptake of the distal hindlimb in late middle aged (28-39 months) compared to young adult (8 months) F344BN rats. In contrast, structural data on muscle capacity for O₂ transfer for capillary to fiber mitochondria have been contradictory. Human and/or animal studies have shown decreased (5, 12, 14, 38), unchanged (3, 4, 36) or even increased (8, 19, 36) capillary-to-fiber number ratios (C:F ratio) in skeletal muscles with aging. Part of the discrepancies have been attributed to the fact that differences in activity levels among subjects can offset age related effects (4), and a greater percentage of aerobic fibers (as IIB fibers are lost) can also explain greater capillarities in specific muscles with aging (36). Reductions in muscle mitochondrial volume density and function are also found with age (6), but maintenance (9) or tendency towards increase mitochondrial densities (50) have also been reported, and differential mitochondrial and glycolytic enzymatic changes are known to exist among muscles and fiber types (17).

Besides the effect of fiber type composition on muscle capillarity and mitochondrial density, little is known of the effect of aging on muscle capillary-to-fiber structure relative to fiber size and mitochondrial volume. The well known relationships between muscle capillary length and mitochondrial volume (18, 25) have not been tested in the context of age, and the impact of aging on capillary-to-fiber surface ratio per fiber mitochondrial volume has not been previously investigated. Such information is important for understanding the contribution of age-related alterations in muscle capacity from O₂ flux from capillary to fiber mitochondria in the declined muscle aerobic performance with aging. In particular, the notion of a major role of the capillary-fiber interface in determining maximal O₂ flux in muscle (13) implies that muscle structural capacity for O₂ flux from capillary to muscle fiber mitochondria needs to be assessed in terms of capillary surface per fiber surface (rev. 29). In order to account for differences in fiber size, fiber capillarization also needs to be assessed in relation to the volume of mitochondria to be supplied in the muscle fibers (i.e. the product of mitochondrial volume density and fiber cross-sectional area), rather than fiber capillary number alone, or mitochondrial density. Thus, in this study, we investigated the fate of the size of the capillary-fiber interface relative to fiber mitochondrial volume as skeletal muscle fibers atrophy with age, i.e. whether or not muscle structural capacity for O₂ flux from capillary to fiber mitochondria deteriorates with aging. Obviously, capillary rarefaction in the face of fiber atrophy may not necessarily reduce the size of the capillary-fiber interface in a muscle relative to its fiber mitochondrial volume, if the capillary or mitochondria losses are disproportional to the fiber atrophy, i.e. for example, lesser capillary or greater mitochondrial losses occur than the reduction in fiber size. Alternatively, an unchanged capillary-to-fiber surface ratio, because of proportional losses of capillary and fiber surface areas, may result in a greater capillary-to-fiber interface per fiber

mitochondrial volume, if the volume density of mitochondria is either maintained or reduced during fiber atrophy.

Our previous morphological data supported the concept of an important role of the size of the capillary-fiber interface in determining maximal O₂ flux in muscle, and showed that capillary-to-fiber surface ratio and fiber mitochondrial volume increased in proportion to one another in various muscles in response to different perturbations such as endurance training, chronic electrical stimulation, and adaptation to altitude (rev. 26). Interestingly, different alterations in capillary density, geometry and fiber mitochondrial volume occurred among conditions, but yet, capillary-to-fiber surface ratio per fiber mitochondrial volume was maintained. These findings supported our earlier observation that the size of the capillary-fiber interface may be regulated in direct proportion to fiber mitochondrial volume or maximal O₂ demand in skeletal muscles, independent of their fiber type composition, level of aerobic capacity, degree of capillarization and capillary geometry (39). They also suggested remarkable integration of signaling pathways from O₂ sensing to vascular and mitochondrial growth in response to increased activity and hypoxia (26). Based on these previous findings, we hypothesized that the size of the capillary-fiber interface would also be regulated in direct proportion to fiber mitochondrial volume, i.e. capillary-to-fiber surface ratio per fiber mitochondrial volume would remain unchanged, and this independent of the muscle fiber type, as muscle atrophies with aging.

MATERIALS AND METHODS

Male Fisher 344 × Brown Norway (F344BN) F1 hybrid rats were obtained at 12-month (adult, n=7), 24-month (old, n=12) and 35-month of age (very old, n=6) from the NIA colony at

Harlan Industry. They were housed (2-3 of same age per cage) in the same room and on 12:12 day/night cycle in a pathogen-free facility and fed standard rat chow (Harlan Teklad 8604) and untreated tap-water *ad libitum*. Within approximately 1 to 3 weeks, and in random order with regard to age, they were anesthetized with sodium pentobarbital (30-90mg/kg), and muscle vascular perfusion-fixation *in situ* was performed as described previously (24). Briefly, the chest was cut open, and the entire vasculature was perfused with saline (11.06 g/l NaCl; 350 mosM; 20000 USP heparin/l), via a cannula inserted directly into the left ventricle and the right atrium cut open to secure outflow. Perfusion fixation followed with a 6.25% solution of glutaraldehyde in 0.1 M sodium cacodylate buffer (total osmolarity of fixative, 1,100 mosM, pH 7.4). All perfusions were carried-out at non pulsatile pressure of 100-120 mmHg. Soleus (S) and extensor digitorum longus (EDL) muscles were excised and stored in the glutaraldehyde fixative for 2-6 months. A tissue sample (entire midbelly cross-sectional area \times ~ 3 mm) was completely cut into thin longitudinal strips, which were further cut into small blocks and processed for electron microscopy as described previously (24).

From each sample, 8 blocks were cut into 1 μ m-thick sections (four transverse and four longitudinal) using an LKB Ultratome III. They were stained with 0.1% aqueous Toluidine Blue solution, and sarcomere length, l_o , was measured in each longitudinal section (24). Briefly, the position of the specimen holder relative to the knife edge was controlled for each section by mean of a scale available on the specimen holder of the Ultratome. At least three longitudinal sections were taken at three consecutive angles with respect to the muscle fiber axis from each block, by systematically varying the sectioning angle by steps of 1 scale unit. Sarcomere length was measured in each section (average of 10 measurements of 10 consecutive sarcomeres, systematically sampled over the entire section area), and the process was continued until the

longitudinal section, i.e. the section with the shortest sarcomere length was obtained. A section was considered longitudinal when changes of the specimen holder by +1 and -1 scale units gave sections with longer averaged sarcomere length. Ultrathin sections (50-70 nm) were cut transversely to the muscle fiber axis from the same four blocks used to obtain the four transverse 1 μm -thick sections, and immediately adjacent to these 1 μm -thick sections, in each sample; they were contrasted with uranyl acetate and bismuth subnitrate (44), and electron micrographs for morphometry were taken on 70 mm films using a Zeiss 10 electron microscope.

Capillary numbers per fiber cross-sectional area and longitudinal section area, $Q_A(0)$ and $Q_A(\pi/2)$, respectively, were measured by point-counting using a 100-point eyepiece square-grid test system on 1 μm -thick sections examined at a magnification of 400 \times with a light microscope. On average, 6.9 ± 0.4 (SE) and 6.2 ± 0.6 fields were examined per sample on transverse sections of S and EDL, respectively, yielding 164 ± 11 (S) and 266 ± 35 (EDL) fiber profiles in each sample, and 1149 ± 150 (S) and 1598 ± 206 (EDL) fiber profiles per age group. The number of fields examined per sample in longitudinal sections averaged 17 ± 1 (S) and 13 ± 1 (EDL). As in previous studies (24), capillary density estimates were related to the muscle fibers as a reference space (rather than to muscle volume) in all samples, to avoid variations due to the unreliable preservation of the intercellular spaces by the preparation procedures. In tissues similarly prepared, we have previously shown that fiber cross-sectional area did not statistically differ in portions of the same section with large differences in intercellular spacing, i.e. that there was no evidence of differential shrinkage of the muscle fibers (32). Thus, an unreliable preservation of the intercellular spaces by the preparation procedure should not affect our results, since all measurements of capillarity and mitochondrial volume (see below) collected in this study were related to fiber (and not tissue) area or volume.

The size of the capillary-to-fiber interface, i.e. capillary surface per fiber surface was obtained from capillary-to-fiber perimeter ratio, $B_B(0)$, measured by intersection-counting using the same 100-point eyepiece square-grid test system on the same transverse section fields used to estimate $Q_A(0)$. Mean fiber cross-sectional area, $\bar{a}(f)$, was also obtained by point-counting, capillary-to-fiber number ratio was calculated from capillary and fiber counts in the same fields, and capillary number around each fiber, N_{CAF} , was also counted in the same fields. Capillary geometry, i.e. the anisotropy coefficient $c(K,0)$, and capillary length per volume of muscle fiber, $J_V(c,f)$, i.e. the product of $c(K,0)$ and $Q_A(0)$, were estimated via a model-based method as described previously (33). Briefly, the ratio between $Q_A(0)$ and $Q_A(\pi/2)$ is used to calculate, via a table or graph of known coefficients, 1) the orientation concentration parameter K of capillaries with respect to the axis of the muscle fibers and 2) the anisotropy coefficient $c(K,0)$. For non-branched capillaries all oriented strictly parallel to the axis of muscle fibers, $K = \infty$, and $c(K,0) = 1$, whereas for capillaries with no preferred orientation (i.e. isotropic), $K = 0$, and $c(K,0) = 2$. Therefore, the additional capillary length provided by tortuosity and branching is given by $[c(K,0)-1] \times Q_A(0)$.

The volume density of mitochondria, myofibrils and lipid droplets per volume of muscle fiber was estimated by point-counting at a final magnification of 47,000 \times on up to 20 fields obtained by systematic random sampling on one ultrathin transverse section from each of four blocks from each muscle (total 73 ± 3 fields/sample in S, and 71 ± 3 in EDL). Mitochondrial volume per μm fiber length was calculated as the product of mitochondrial volume per volume of fiber, $V_V(mt,f)$, and fiber cross-sectional area, $\bar{a}(f) \times 1 \mu\text{m}$.

Analysis of S was done first, and subsequent sectioning of EDL revealed that in a few cases, the perfusion fixation had been complete in S but not EDL of the same animal, likely because of the large differences in resting blood flows between the two muscles. Musch et al (2004) recently reported 5 and 11 fold greater resting muscle blood flows in S than EDL of old (27-29 months) and young (6-8 months) F344BN rats, respectively (calculated from 37). The collapsed capillary profiles in unperfused portions of EDL precluded subsequent analysis of capillary geometry and capillary surface per fiber surface ratio in those samples, and we used successfully perfused EDL muscles from another animal to complete our analysis. Overall, we analyzed both EDL and S muscles from the same hindlimbs in 4 out of 6 rats in the 24 month group, and 5 out of 6 rats in each of 12- and 35-month age groups. Further, we increased the sample size from 6 to 10 in the 24-month S group, to confirm the lack of significant fiber atrophy in that muscle at that age, in contrast to the significant decline seen in EDL compared to 12-month (see Results), and one S sample could not be analyzed in the 35-month group. Thus, sample sizes for all data reported in this study were n=6 per group for EDL at each age, and n=6, 10 and 5 for S at 12-, 24- and 35-month of age, respectively.

Statistical analyses. Data are expressed as mean \pm SE. Group means were compared by analysis of variance (ANOVA), i.e. one-way ANOVA to compare body mass between age groups (12, 24 and 35-month), and two-way ANOVA to test for differences among age groups and muscles (S and EDL), and for interaction between group and muscle, with Holm-Sidak multiple comparison *post hoc* tests (Sigmastat 3.0, SPSS Inc.). Differences were taken as significant for $P < 0.05$.

RESULTS

Figure 1 illustrates the substantial decrease in fiber size and increased capillarity density in both S and EDL with aging. Fiber atrophy occurred without decline in body mass (group means, 511 ± 15 , 501 ± 19 and 532 ± 10 g at 12-, 24- and 35-month, respectively). At the hindlimbs' position at perfusion fixation, EDL samples were fixed in generally more extended position (l_0 mean, 2.38 ± 0.02 μm ; range, 2.23 – 2.69 μm) than S (2.11 ± 0.04 μm ; 1.84 - 2.34 μm). Accounting for those differences, i.e. normalizing data to a similar sarcomere length of 2.1 μm , revealed significantly smaller fiber size in EDL than S across age groups ($P=0.002$) and significant fiber atrophy with aging ($P<0.001$; Fig. 2A). Fiber cross-sectional area decreased 42% (2214 ± 129 to 1277 ± 136 μm^2) in S and 48 % (1852 ± 189 to 959 ± 188 μm^2) in EDL, from 12- to 35 month of age, with significant atrophy already detected at 24-month in EDL, but not S (Fig. 2A). Over the same period, capillary density increased 65 % (1387 ± 54 to 2291 ± 238 mm^{-2}) in S and 130 % (964 ± 95 to 2216 ± 311 mm^{-2}) in EDL (Fig. 2B) while capillary-to-fiber number ratio and average number of capillary around a fiber remained unchanged (Table 1), indicating that the increased capillary density with age was entirely due to the fiber atrophy. There was no significant difference in capillary density between 12- and 24-month in either muscle.

At the sarcomere length at which the muscles were examined, the anisotropy coefficient $c(k,0)$ ranged from 1.01 to 1.17 in EDL, and from 1.13 to 1.34 in S, indicating that capillary length per fiber volume was 1-17 and 13-34% greater in EDL and S, respectively, than a simple count of capillary density in transverse sections, $Q_A(0)$, would indicate (see Methods). Since capillary tortuosity increases (i.e. anisotropy decreases) with muscle fiber shortening (24), it is important to account for sarcomere length when comparing capillary geometry between muscles and age groups. Figs. 3A and 3B show the plots of the ratio of capillary density in transverse

and longitudinal sections [$R = Q_A(0) / Q_A(\pi/2)$; used to estimate capillary geometry (33)] and sarcomere length in S and EDL, respectively, and previous data in rat soleus and EDL muscles for comparison. We used the linear regression of R against sarcomere length in S of the 12- and 24-month old rats of this study [$n = 16$; $R = (1.76 \times \text{sarcomere length}) - 1.04$; $r = 0.72$] to normalize capillary geometry data in S to a similar sarcomere length. The value of R at 2.1 μm was significantly greater in S at 35-mo (group mean, 3.31 ± 0.08) compared to 12- and 24-month (2.63 ± 0.13 and 2.65 ± 0.08 , respectively; $P < 0.001$). Consequently, the capillary anisotropy coefficient $c(K,0)$ at 2.1 μm sarcomere length was significantly smaller (group mean, 1.15 ± 0.01) in S at 35- compared to 12- and 24-month of age (1.24 ± 0.02 and 1.23 ± 0.01 , respectively; $P = 0.004$). In other words, tortuosity and branching contributed significantly less to capillary length in S of the very old rats, i.e. 15% compared to 23-24% at 12-24 month; Fig. 4). In EDL, we found that capillary anisotropy was greater (i.e. $Q_A(0) / Q_A(\pi/2)$ was greater) and more variable than in S, and that there was no age-related difference in capillary geometry (Fig. 3B). Because of the lack of correlation between R and sarcomere length (Fig. 3B), capillary anisotropy data in EDL (Fig. 4) were not normalized to sarcomere length. Therefore, the lower group means of capillary anisotropy data at each age compared to S (Fig. 4) are partly due to the greater sarcomere length at which EDL samples were examined (Fig. 3). However, a greater capillary anisotropy (i.e. lesser contribution of tortuosity and branching to capillary length) was also observed previously in rat EDL (27). We show here that this pattern was not significantly altered with age (Figs. 3B and 4). Accounting for capillary geometry in the muscles revealed 55 and 113% increases in capillary length per fiber volume from 12- to 35-month of age in S and EDL ($P < 0.001$), respectively, and no significant difference between 12- and 24-month of age in either muscle (Table 1). Following the greater relative increase with age in EDL, the smaller

capillary length per fiber volume observed in EDL at 12-month of age compared to S was no longer significant in the 24- and 35 month-old rats.

Because the capillary orientation coefficient $c(K,0)$ was < 1.53 in all samples, the anisotropy coefficient of capillary surface was 1, and therefore capillary-to-fiber perimeter ratio in transverse sections was a direct estimate of capillary surface per fiber surface ratio in each muscle (see Ref. 28). Capillary surface per fiber surface was significantly greater in S than EDL in each age group ($P<0.001$), and it tended to increase with age in both muscles (Table 1), but the effect of age did not reach significance ($P=0.077$). On average, capillary surface area represented 32-35% and 21-26% of fiber surface in S and EDL, respectively.

The volume density of mitochondria per volume of fiber averaged 7 to 9% among groups, and it was smaller at 24- than 12- or 35-month of age in EDL ($P=0.05$; Table 1). Because of the substantial fiber atrophy with age (Fig. 2A), mitochondrial volume per unit fiber length (i.e. the product mitochondrial volume density \times fiber cross-sectional area \times 1 μm) also declined with age in both muscles. Normalization of data to sarcomere length revealed 44 and 45% losses of mitochondrial volume per unit fiber length in EDL and S, respectively, from 12- to 35-month of age, and significant declines (EDL, 39%, S, 24%) already observed at 24-month in both muscles (Table 1).

The increased capillary length per fiber volume in the face of modest changes in mitochondrial volume density (Table 1) resulted in 57 and 87% increases in capillary length per ml mitochondria from 12- to 35-month of age in S (23 ± 2 to 36 ± 3 km/ml) and EDL (15 ± 1 to 28 ± 3 km/ml) muscles, respectively (Fig. 5A). Examination of the size of the capillary-fiber interface relative to fiber mitochondrial volume, i.e. the ratio of capillary surface per fiber surface and fiber mitochondrial volume per unit fiber length, revealed similar values in S and

EDL, and 1.90- and 2.28-fold increases from 12- to 35-month of age in S (0.0021 ± 0.0003 to $0.0040 \pm 0.0004 \mu\text{m}^{-3}$) and EDL (0.0018 ± 0.0003 to $0.0041 \pm 0.0008 \mu\text{m}^{-3}$) muscles, respectively (Fig. 5B).

DISCUSSION

Contrary to our hypothesis, we found that the size of the capillary-fiber interface relative to fiber mitochondrial volume not only did not deteriorate, but in fact increased in both muscles, with aging, as a result of the substantial fiber atrophy with age, in the face of unchanged capillary per fiber number and modest or no alteration in fiber mitochondrial volume density. This was observed in both predominantly slow- (S) and fast-twitch (EDL) muscles, suggesting that the altered regulation of the size of the capillary-fiber interface relative to fiber mitochondrial volume with aging occurred independent of muscle fiber type and, as discussed below, oxidative vs glycolytic fiber proportions in the muscles.

Animal and muscle models

We chose to use the F344BN rat strain because of its longer life span and slower occurrences of major pathologies with age compared to other rat strains (22). The weight-bearing S and non weight-bearing EDL muscles were selected to allow us to assess the impact of aging on capillary-fiber structure in muscles of homogeneous, predominantly slow- (S), compared to fast-twitch (EDL) fiber type composition. In a comparative histochemical study of capillary and fiber type distributions in calf and anterior leg muscles of rats of the same strain and age groups, we found ~ 90% and <5% relative sectional area of slow-twitch fibers in S and EDL, respectively, and no change in slow- or fast-twitch oxidative fiber type proportions with age (7). Specifically, the relative sectional area of type I (slow-twitch oxidative), IIA (fast-

twitch oxidative glycolytic) and IIB (fast-twitch glycolytic) fibers in S were $91 \pm 2\%$ (I), $3 \pm 1\%$ (IIA) and $6 \pm 1\%$ (IIB) at 12 months of age, compared $86 \pm 2\%$ (I), $6 \pm 3\%$ (IIA) and $8 \pm 1\%$ (IIB) at 24 months, and $88 \pm 2\%$ (I), $3 \pm 1\%$ (IIA) and $9 \pm 1\%$ (IIB) at 35 months. In EDL, the values were $1 \pm 0\%$ (I), $14 \pm 1\%$ (IIA) and $85 \pm 1\%$ (IIB) at 12 months, $1 \pm 0\%$ (I), $17 \pm 2\%$ (IIA) and $83 \pm 2\%$ (IIB) at 24 months, and $4 \pm 2\%$ (I), $20 \pm 3\%$ (IIA) and $75 \pm 4\%$ (IIB) at 35 months of age. The vascular perfusion-fixation necessary to assess capillary geometry and surface per fiber surface ratio in the muscles also precluded measurement of muscle wet mass in our rats. Our most complete data set from other studies of F344BN rats at similar ages (7, 23) indicates 29% (196 ± 4 to 139 ± 5 mg) and 22 % (192 ± 3 to 150 ± 3 mg) declines in S and EDL muscle wet mass, respectively, between 12- and 35-month ($P < 0.001$; $n=17-21$ rats/ group), with no mass loss of either muscle at 24- compared to 12-month, consistent with previous findings of a delayed onset of muscle mass loss in F344BN rats until after 28 month of age compared to other rat strains (2).

Muscle fiber atrophy

Whereas there have been numerous reports on muscle atrophy in rat with advanced age, specific data on muscle weight and fiber cross-sectional area have been highly variable, partly because of the differences in life span, and therefore relative age, of different strains between studies. In limb muscles of F344BN rats, Brown and Hasser (2) reported 6% (EDL) and 12% (S) greater age-related declines in fiber cross-sectional area than muscle mass (S, -18%; EDL, -16%) between 6 and 36-month of age. We also found greater fiber atrophy (S, -42%; EDL, -48%) than muscle mass decline (S, -29%; EDL, -22 %) between 12 and 35-month, and we already observed a significant fiber size decline at 24-month in EDL, but not S (Fig. 2A) in the same strain. The reason for the greater muscle and fiber atrophies in our rats compared to the

previous report (2) is unclear. It was not related to tissue preparation or procedure [e.g. chemical-fixation and examination of thin (1 μ m) sections of small subsampled plastic-embedded tissue blocks (allowing more precise transverse sectioning) in this study, vs tissue freezing and histochemistry of thick (10 μ m) cross-sections of whole muscle midbelly in the previous study], since we found as large decreases in composite fiber sectional area between 12-and 35-month (S, 37%; EDL, 48%) in the same strain (calculated from 7) after tissue freezing and histochemistry. It is also of note that in that study, fiber size was measured from entire midbelly sections of the muscles. Thus, the close (S) or similar (EDL) % declines in fiber cross-sectional area as those observed in the present study rule out a sampling effect and indicate that our data are representative of muscle fiber atrophy across the entire midbelly in each muscle. Others have also found comparable or greater age-related atrophy in S and EDL muscles in F344BN rats than reported here (11, 35, 48). The greater fiber atrophy than muscle mass decline we and others observed (2, 48) could be related to the concomitant increase in intramuscular fat and fibrous connective tissue found in oldest (36-month old) rats (2). Greater fat and connective tissue intramuscular contents could also explain why a decreased fiber number in S muscle at 35-month of age (35) did not result in further decrease in muscle mass relative to fiber cross-sectional area in the oldest rats.

Muscle fiber capillarization

Data on the impact of aging on skeletal muscle capillarization have also been variable (rev, 10), depending on species and muscles studied, as well as methodology (e.g. 12). Age-related decrease (plantaris, 12), maintenance (S and EDL, 3), or increase (S and plantaris, 19) of capillary-to-fiber number ratios have been reported in Wistar rats, whereas Mitchell et al (36) found unchanged (S) or increased (deep region of EDL) capillary-to-fiber number ratios in

Fisher 344 rats with aging. Hepple et al (16) recently reported unchanged number of capillary around a fiber and individual capillary-to-fiber ratio in S and red (GR) and white (GW) portions of gastrocnemius muscles of late middle aged (28-30 month) compared to young adult (8 month) F344BN rats. To our knowledge, there has been no previous report of skeletal muscle capillarization in F344BN rats beyond 28-30 month of age. Interestingly, the unchanged capillary per fiber number previously observed at late middle age in the F344BN rat strain occurred in the face of unchanged (S), decreased (GW) or increased (GR) fiber cross-sectional area compared to young adult (16). We show here that fiber atrophy in S and EDL muscles at more advanced age (35-month) is still not accompanied by capillary loss.

Capillary geometry

We are aware of only one previous study of the impact of aging on capillary geometry in skeletal muscle. Using intravital microscopy, Russell et al (47) found a similar degree of tortuosity and branching in spinotrapezius muscle of F344BN rats at 26-28 month of age, compared to 6-8 month. The greater capillary anisotropy (i.e. lesser degree of tortuosity and branching) we found in S with aging was only detected at 35-month (Fig. 4A), and therefore it is possible that age-related alteration in capillary geometry may also occur in spinotrapezius muscle at later age in this strain. However, we found no alteration in capillary anisotropy with age in EDL. The reason for the differential effect of age on capillary geometry in S vs EDL in the face of fiber atrophy in both muscles is not clear. The finding of a greater capillary anisotropy in EDL is consistent with previous stereological findings by similar (27) or confocal (21) morphometric analysis in adult rats, and the difference with S remained with aging.

Mitochondrial volume

Like capillarization, data on muscle oxidative capacity and aging have also been variable, with studies in rat reporting decreased or unchanged mitochondrial volume densities, oxidative enzyme activities and respiratory rates, depending on the strains and muscles examined (e.g. 20 and references therein). We are not aware of previous morphometric data on the effect of aging on mitochondrial volume density in S or EDL muscles of F344BN rats. The apparent decrease in the volume density of mitochondria per volume of muscle fiber we found in EDL at 24- vs 12- and 35-month could be due to differential atrophy of fiber compartments with aging, since earlier study showed greater relative loss of myofibrils in S and EDL muscles of old (21-25 month) and very old (27 month) Wistar rats, compared to 5-6 month old (1). However, we found no significant change in the volume density of myofibrils per volume of fiber with aging in either S or EDL muscle of F344BN rats in this study (data not shown).

Fiber capillarization and mitochondrial volume

With the fiber atrophy of aging, substantial increases in both capillary length per ml mitochondria and the size of the capillary-fiber interface relative to fiber mitochondrial volume occurred in both S and EDL. For example, the group mean values of 28 ± 3 (EDL) and 36 ± 3 (S) km capillary/ml mitochondria in the 35-month old rats were as high as those found in some of the most intensely aerobic muscles known in adult animals, such as the diaphragm of shrew (the smallest mammal, 30) and the flight muscles of small birds and bats (25). Similarly, the ratios of capillary surface per fiber surface and fiber mitochondrial volume per μm fiber length found in S ($0.0040 \pm 0.0004 \mu\text{m}^{-3}$) and EDL ($0.0041 \pm 0.0008 \mu\text{m}^{-3}$) at 35 month were not significantly different from those in shrew diaphragm (30) and flight muscle of hummingbird and small bats (25). They were ~ 2-fold greater than in S and EDL muscles of 12-month old

F344BN rats (this study) and various muscles (S, EDL, plantaris, tibialis anterior) of adult Sprague-Dawley rats (26).

The increased size of the capillary-fiber interface relative to fiber mitochondrial volume found in S and EDL with aging is in contrast to muscle response to other perturbations such as endurance training, chronic electrical stimulation and adaptation to cold and altitude, which we have previously shown to all result in maintained capillary-to-fiber surface ratio per fiber mitochondrial volume (rev. 26). Interestingly, the greater capillary-fiber interface per fiber mitochondrial volume with aging occurred in both S and EDL muscles, with opposite slow- vs fast-twitch fiber type composition, and different capillary per fiber number and capillary geometry. Further, histochemical analysis of fiber type size in S and EDL muscles of rats of the same strain and age groups (7) revealed differential atrophy of oxidative (I and IIA) vs glycolytic (IIB) fibers with aging in the two muscles, as previously reported in this rat strain (2). Yet, capillary surface per fiber surface relative to fiber mitochondrial volume increased to a similar extent (2-fold) in S and EDL with aging, suggesting that the alteration in the regulation of the size of the capillary-fiber interface relative to fiber mitochondrial volume in the muscles compared to younger rats also occurred not only independent of their fiber type, capillary per fiber number and capillary geometry, but also their aerobic vs glycolytic fiber proportions and relative atrophies.

Interestingly, a greater size of the capillary-fiber interface relative to fiber oxidative capacity was also recently reported in S and in white portion of gastrocnemius (GW), but not in red portion of gastrocnemius muscles (GR) of late middle-aged (28-30 month old) compared to young adults (8 month old) in the same rat strain (16). In that study, the size of the capillary-fiber interface was assessed via the capillary-to-fiber perimeter exchange (CFPE) index, i.e. the

quotient of capillary per fiber number ratio (on individual fiber basis) and fiber perimeter in histochemical sections, and fiber oxidative capacity was evaluated by biochemical assay of the specific activity of the complex I-III pathway relative to total muscle protein in muscle homogenates. It is also interesting that the similar outcome of a greater size of the capillary-fiber interface relative to fiber oxidative capacity with age was achieved via different alterations in fiber size, capillary density and oxidative capacity in S compared to GW at the same late middle-age point of 28-30 month (16), or S and EDL at later, very old age (this study). At 28-30 month, Hepple and Vogell (16) found fiber atrophy in GW but not S, whereas increased fiber size, interpreted as postural-induced compensatory hypertrophy, was observed in GR, compared to 8 month of age. As capillary per fiber number ratio remained unchanged in the muscles, capillary density changed in inverse proportion to fiber size, and fiber oxidative capacity decreased in S, but not in GW or GR (16).

Conley et al (6) recently showed that a reduced oxidative capacity per mitochondrial volume was superimposed to the loss of mitochondrial volume itself in muscle of elderly subjects compared to adults. This, and the finding of lower flux through electron transport chain complexes I-III in S and GR muscle homogenates at 28 to 30-month compared to 8-month F344BN rats (16), also suggests that fiber mitochondrial volume (i.e. the product of mitochondrial density and fiber cross-sectional area) may overestimate actual oxidative capacity, and therefore underestimate the size of the capillary-fiber interface relative to fiber oxidative capacity, in S and EDL muscles of the very old rats in our study. On the other hand, the significant decline in mitochondrial volume density we observed in EDL of 24- compared to 12 and 35-month rats, if maintained at 28-30 month, could be consistent with the biochemical data on specific activity of the complex I-III pathway. Clearly, further study is needed to clarify the

issue. However, the notion of an increased size of the capillary-fiber interface relative to fiber oxidative capacity in S and EDL muscles of late middle-aged to very old rats compared to adults remains.

Functional implications

At a given muscle mitochondrial electron transport capacity (34, 45), muscle O_2 extraction depends on the interaction between muscle convective and diffusive properties for O_2 transport, according to the equation $\%O_2 \text{ extraction} = 1 - e^{-DO_2/\beta Q}$, where DO_2 is muscle O_2 diffusive capacity, β is the slope of the O_2 dissociation curve in the physiological range, and Q is muscle blood flow (46). To our knowledge, only two studies have evaluated the impact of aging on the microcirculation hemodynamics. Increased red blood cell (RBC) velocity, V_{RBC} , and altered patterns of microvascular flow were found in resting EDL of old (28 months) compared to mid-aged (12 months) Fisher 344 rats, in the face of unchanged density of perfused capillaries with the muscle atrophy of aging (49). In the mixed fiber type spinotrapezius muscle also at rest, Russell et al (47) measured increased V_{RBC} , unchanged capillary hematocrit and decreased lineal density of perfused capillaries in the absence of muscle fiber atrophy in late middle-age (26-28 months) compared to young (6-8 months) F344BN rats. The increased structural capacity for O_2 flux, i.e. greater capillary surface per fiber surface ratio relative to fiber mitochondrial volume, we found in this investigation in S and EDL with fiber atrophy is expected to improve muscle O_2 extraction in the face of the increased V_{RBC} with aging (by increasing the ratio of $DO_2/\beta V_{RBC}$) and be synergic to the lesser flow increase found in the old with exercise (43). In this respect, however, a recent comprehensive study of regional blood flow responses to exercise in various hindlimb muscles of old (27-29 months) compared to young (6-8 months) F344BN rats revealed a lesser flow increase in S, but not EDL, and substantial redistributions of flows from highly

oxidative to highly glycolytic muscles or muscle regions in the old rats during submaximal exercise (37). Further, peak muscle O₂ extraction measured at VO₂ max in an *in situ* pump-perfused hindlimb preparation tended to be lower ($P=0.10$), not higher, in late middle-aged (28-30 months) compared to young adult (8 months) F344BN rats (15). Thus, the increased capacity for O₂ flux relative to fiber mitochondrial volume in aged muscles appears to mitigate the effect of increased V_{RBC} (47, 49) and decreased mitochondrial electron transport capacity (15) on muscle O₂ extraction, in the face of the age-related blood flow redistributions (37) during exercise.

In conclusion, the size of the capillary-fiber interface relative to fiber mitochondrial volume increased in S and EDL muscles of F344BN rats with aging, as capillary per fiber number was preserved in the face of substantial fiber atrophy and modestly altered or unchanged fiber mitochondrial volume density. The disproportion between capillary surface / fiber surface and fiber mitochondrial volume in aged muscles compared to young is in contrast to muscle response to other perturbations such as endurance training, chronic electrical stimulation and adaptations to altitude, which we have previously found to all result in proportional increases in capillary-to-fiber surface ratio and fiber mitochondrial volume. It suggests altered regulation of vascular structure relative to fiber mitochondrial volume in aging in both muscles, independent of their fiber type. Together with the lesser blood flow increase known to occur in S with age during exercise, the greater structural capacity for O₂ flux relative to the volume of mitochondria in the muscle fibers is expected to mitigate the effect of age-related alterations in vascular hemodynamics and mitochondrial function on muscle O₂ extraction in S and EDL during exercise.

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Table 1. Morphometric data on fiber capillarization and ultrastructure in soleus and EDL muscles of F344BN rats at 12-, 24- and 35 months of age

	Muscle	12-mo	24-mo	35-mo
Capillary-to-fiber number ratio	S	3.05 ± 0.11	2.89 ± 0.10	2.81 ± 0.19
	EDL	1.71 ± 0.07 ^ϕ	1.79 ± 0.06 ^ϕ	1.86 ± 0.12 ^ϕ
Capillary number around a fiber	S	7.35 ± 0.15	6.81 ± 0.20	6.57 ± 0.31
	EDL	4.68 ± 0.14 ^ϕ	4.89 ± 0.13 ^ϕ	5.12 ± 0.19 ^ϕ
Capillary length / fiber volume, mm ⁻²	S	1776 ± 78	1858 ± 78	2750 ± 271 [*]
	EDL	1194 ± 112 ^ϕ	1629 ± 165	2540 ± 343 [*]
Capillary surface / fiber surface	S	0.32 ± 0.02	0.32 ± 0.02	0.35 ± 0.02
	EDL	0.21 ± 0.01 ^ϕ	0.23 ± 0.01 ^ϕ	0.26 ± 0.01 ^ϕ
Mitochondrial volume density, %	S	7.9 ± 0.4	7.1 ± 0.4	7.6 ± 0.3
	EDL	8.3 ± 0.4	6.8 ± 0.2 [*]	9.0 ± 0.4
Mitochondrial volume / μm fiber ^a , μm ³	S	175 ± 15 [*]	133 ± 8	96 ± 7
	EDL	154 ± 21 [*]	95 ± 9 ^ϕ	86 ± 17

Mitochondrial volume density = volume/ fiber volume; mitochondrial volume / μm fiber = product of mitochondrial density and fiber cross-sectional area × 1μm; ^a, normalized to 2.1 μm sarcomere length; significant differences: ^{*}, compared to other age groups; ^ϕ, compared to soleus in same age group.

FIGURE LEGENDS

- Figure 1: Light micrographs of portions of muscle bundles in transverse sections, showing the substantial fiber atrophy and greater capillary density in soleus (B) and EDL (D) muscles of F344BN rats at 35- compared to 12-month of age (soleus, A; EDL, C). Capillaries are empty after the vascular perfusion fixation.
- Figure 2: Fiber atrophy (A) and increased capillary density (B) in soleus and EDL muscles of F344BN rats with aging. Values are group mean \pm SE. Mean fiber cross-sectional area (A) and capillary density (i.e. number/ fiber mm^2) are normalized to 2.1 μm sarcomere length. Significant differences: *, compared to other ages; **, compared to 12- and 24-months; ϕ , compared to soleus in same age group.
- Figure 3: Plots of the ratios of capillary density in transverse and longitudinal sections [$R = Q_A(0) / Q_A(\pi/2$; see Results] and sarcomere length in soleus (A) and EDL (B) muscles of F344BN rats. Lines, linear relationship $R = (1.45 \times \text{sarcomere length}) - 0.54$ in soleus muscle of young to adult Sprague Dawley and Wistar rats ($n = 34$, data from Refs. 24, 31, 40, 41); \times , data in control EDL of adult Sprague Dawley rats (from Ref. 27).
- Figure 4: Effect of aging on capillary anisotropy coefficient $c(k,0)$ in soleus and EDL muscles. Values are group mean \pm SE, and $c(K,0)$ value in soleus (A) is normalized to 2.1 μm sarcomere length. Significant differences: **, compared to 12- and 24- months; ϕ , compared to soleus in same age group.
- Figure 5: Increases in capillary length / ml mitochondria (i.e. ratio of capillary length per fiber volume and mitochondrial volume density; A) and the size of the capillary-fiber interface relative to fiber mitochondrial volume (i.e. ratio of capillary surface /fiber

surface and mitochondrial volume per μm fiber length; B) in soleus and EDL muscles with aging. Values are group mean \pm SE. Significant differences: *, compared to 24- and 35-months; **, compared to 12- and 24-months; ϕ , compared to soleus in same age group.

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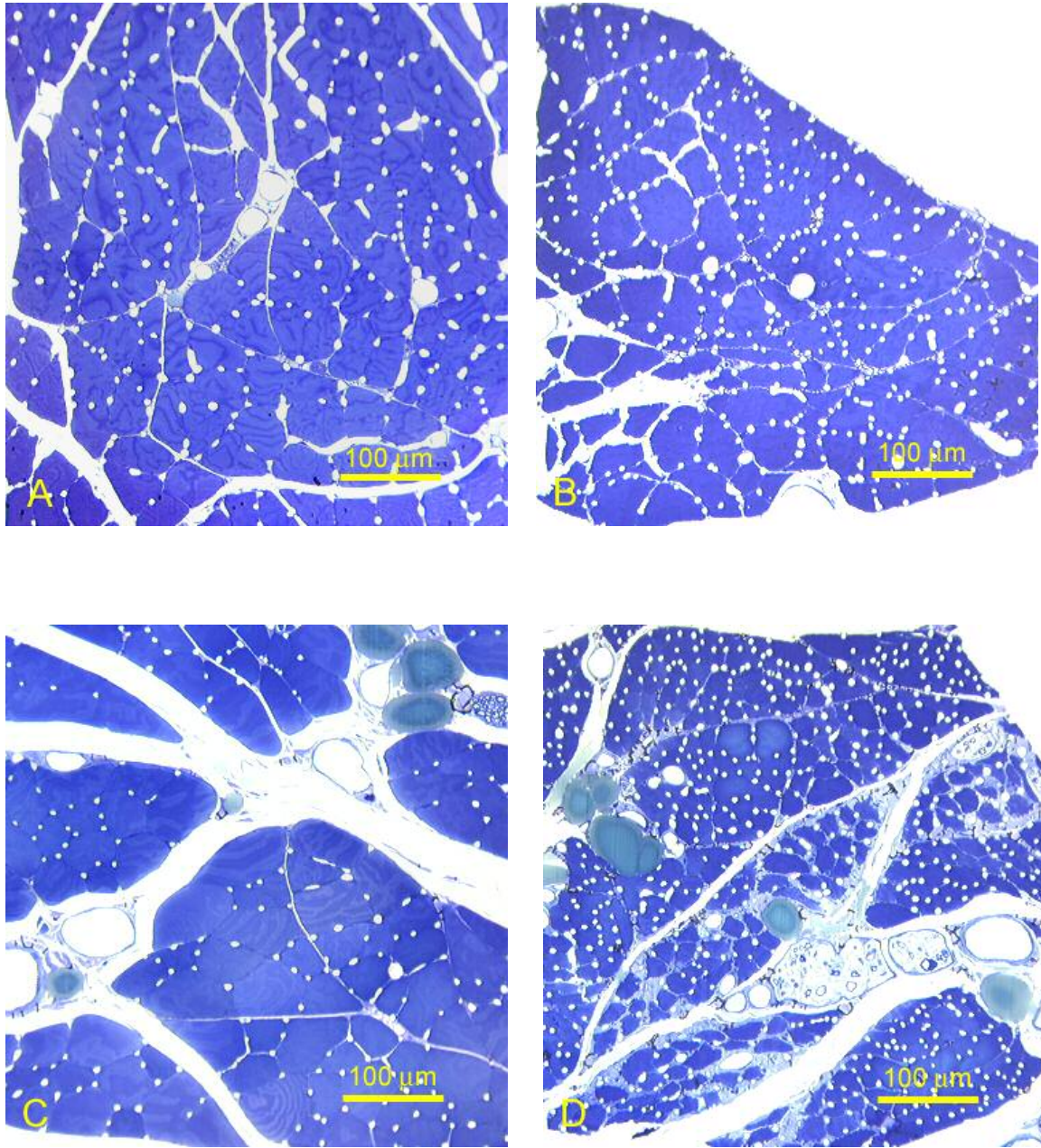


Figure 1, Mathieu-Costello et al

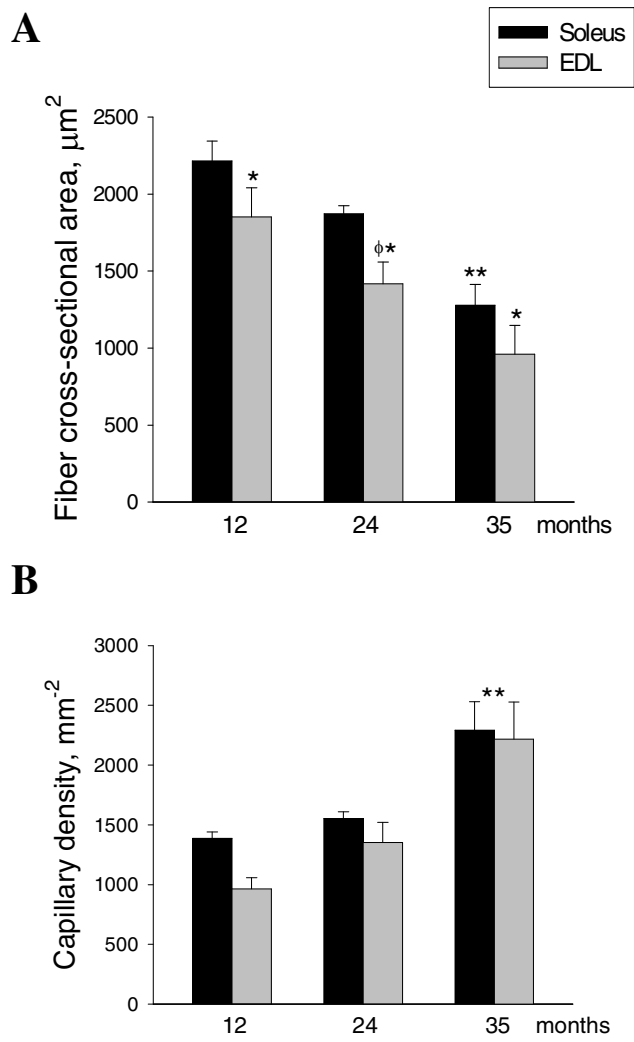


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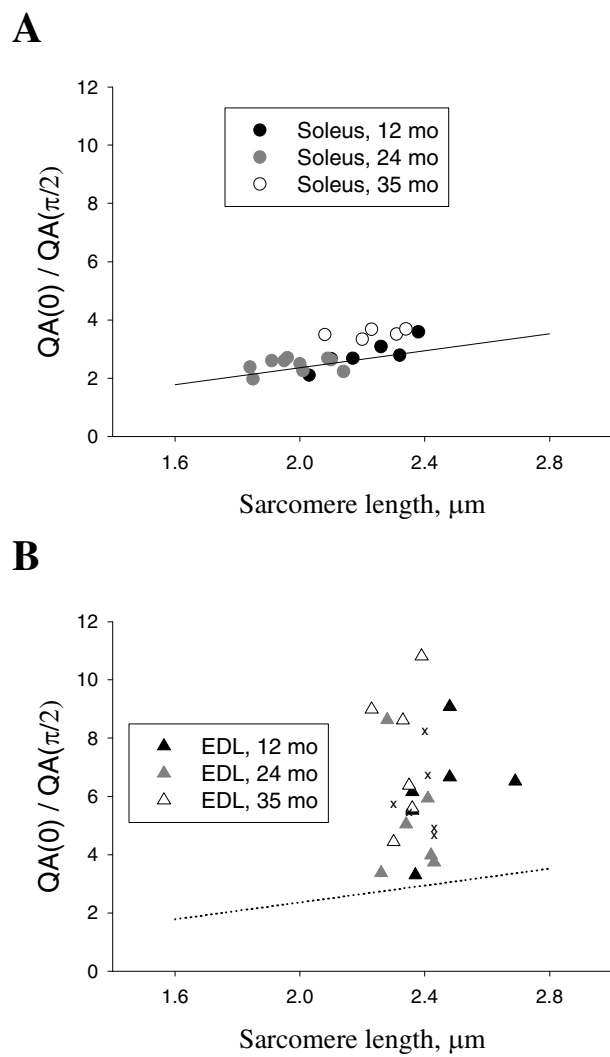


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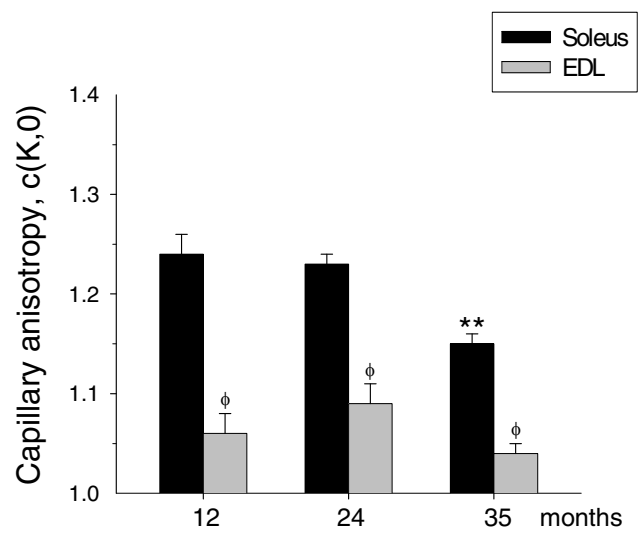


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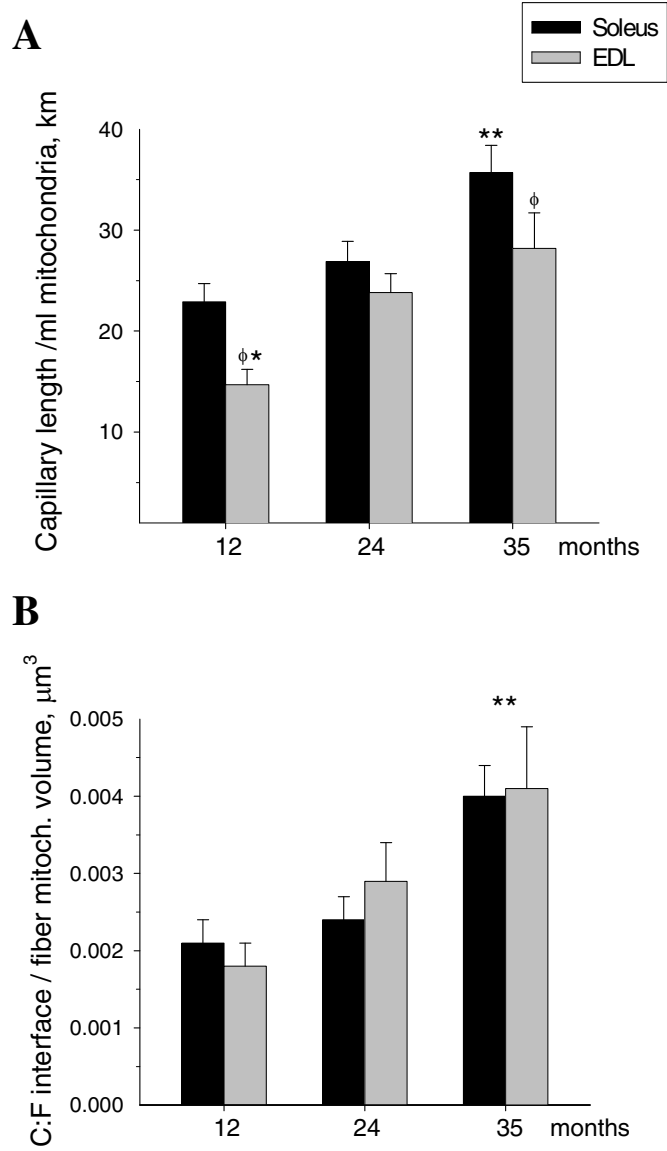


Figure 5, Mathieu-Costello et al