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Abbreviations	
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Abstract
<p>We have previously shown by 3D study that 2 weeks after nerve injury there was no change in the length of capillaries per muscle fibre length in rat extensor digitorum longus muscle (EDL). The primary goal of the present 2D study was to determine the capillarity of rat EDL 4 weeks after various modes of nerve injury. Additionally, we wished to calculate the same capillary/fibre parameters that were used in our 3D stereological study. EDL muscles derived from denervated (4 weeks after nerve injury), re-innervated (4 weeks after two successive nerve crushes) and age-matched controls from the beginning (CON-1) and the end (CON-2) of the experiment were analyzed in two ways. Global indices of capillarity, such as capillary density (CD) and capillary/fibre (C/F) ratio, were determined by automatic analysis, local indices as the number (CAF) and the length of capillaries around individual muscle fibres (Lcap) in relation to muscle fibre size were estimated manually by tracing the muscle fibre outlines and the transversally and longitudinally cut segments of capillaries seen in 5-µm-thin muscle cross sections. Four weeks after both types of nerve injury, CD increased in comparison to the CON-2 group ($p < 0.001$) due to atrophied muscle fibres in denervated muscles and probably proliferation of capillaries in re-innervated ones. Higher C/F, CAF (both $p < 0.001$) and Lcap ($p < 0.01$) in re-innervated than denervated EDL confirmed this assumption. Calculated C/F ratios were comparable to our previous 3D study, which strengthens the practical value to the adapted 2D method used in this study.</p>

Introduction

Peripheral nerve injury induces extensive morphological and physiological changes in skeletal muscles [Midrio, 2006]. A number of studies have examined the manifold effects of injury on skeletal muscles, but only few of them focused on the morphological changes in capillaries [Chernukh and Alekseeva, 1975; Carpenter and Karpati, 1982; Large and Tyler, 1985; Tyml et al., 1999; Borisov et al., 2000; Dedkov et al., 2002; Wagatsuma et al., 2005; Cebasek et al., 2006, 2007; Janáček et al., 2009].

In animal [Borisov et al., 2000; Dedkov et al., 2002] and human muscles [Carpenter and Karpati, 1982], it was shown that long-term denervation is accompanied by capillary loss. However, in short-term denervated muscles, the morphological changes in capillaries are more controversial and depend on the duration of the denervation time and on the type of skeletal muscle. For example, after induction of denervation of the rat sciatic nerve by tetrodotoxin treatment for 2 weeks, it was shown that the capillary/fibre ratio (C/F) and the number of capillaries around a muscle fibre (CAF) were decreased in the gastrocnemius muscle but not in the extensor digitorum longus muscle (EDL), where CAF remained unchanged and C/F was even slightly higher [Tyml et al., 1999]. Similar diverse responses of rat muscles capillaries were also found in our study, where a 2-week sciatic nerve injury decreased the length of capillaries adjacent to an individual muscle fibre in a 5- μ m-thick tissue section (L_{cap})/muscle fibre length (L_{cap}/L_{fib}) only in the soleus muscle but not in EDL [Cebasek et al., 2006]. We used the custom-made stereological *slicer* method for the estimation of capillary length, where digital triplets of test probes have been applied onto series of confocal images, which represented 3D images of 40- μ m-thick muscle tissue slices [Kubínová et al., 2001; Tomori, 2006]. Thus, our capillary measurements were done in 3D mode, while those in the study by Tyml et al. [1999] were in 2D. Due to different methodical approaches, the results of those studies cannot be directly compared, but there was a main common finding: the rat EDL capillaries demonstrated specific unresponsiveness to 2 weeks of denervation, which was in contrast to other rat muscles, where diminishing of capillaries was found during the same period of denervation.

Skeletal muscle capillaries run in the endomysium mostly parallel to the longitudinal axis of muscle fibres, but together with anastomosing capillaries that run in oblique and

transverse directions, they form a delicate oblong 3D mesh. Therefore, it seems logical that 3D measurement methods can better estimate the anatomical size of muscle capillaries than 2D methods. Indeed, theoretically, using relevant mathematical formulas, 3D analysis of capillaries was proven to be a more precise approach to evaluate muscle capillarity than 2D analysis with the classic 2D parameter CAF [Čebašek et al., 2010]. This study clearly shows that the CAF parameter (which counts capillaries running longitudinally to the long axis of muscle fibres, i.e. those that are transversally cut in muscle cross sections) can lead to an incorrect estimation of the length of capillaries, especially if (a) the capillary mesh is very complex, (b) the changes in capillary length between investigated groups are small and (c) the degree of capillary tortuosity is diverse. Also, other studies confirmed that the CAF parameter could properly not establish small changes in capillary length and that *the capillary tortuosity index* (which considers the 3D nature of capillary meshes) shows better sensitivity to small changes in capillary length [Sullivan and Pittman, 1987; Charifi et al., 2004; Ichinose et al., 2008; Huntsman et al., 2013].

For the quantification of capillaries in skeletal muscles, an appropriate morphometric parameter is of great importance [Lexell, 1997]. Global parameters, as the number of capillaries per muscle cross section (CD) or the number of capillaries/number of muscle fibres (C/F), can assess robust changes that occur homogeneously through the muscle tissue. Local parameters, such as CAF or the local C/F ratio, calculated as a sum of fractions of the capillary domains that actually overlap the muscle fibre [Degens et al., 1993; Al-Shammari et al., 2014], can better detect more subtle local changes in capillaries. Local parameters that also estimate the size of the capillary to fibre exchange area, i.e. the capillary/fibre perimeter exchange index [Hepple and Mathieu-Costello, 2001], index of tortuosity [Charifi et al., 2004], originally called capillary-fibre contact length [Sullivan and Pittman, 1987] or $L_{cap}/\text{muscle fibre surface area}$ (L_{cap}/S_{fib}) and $L_{cap}/\text{muscle fibre volume}$ (L_{cap}/V_{fib}) [Kubínová et al., 2001], are recommendable when muscle structural capacity for O_2 uptake are studied [Mathieu-Costello, 1994; Mathieu-Costello and Hepple, 2002]. The latter group of local parameters may become even more relevant, especially if we consider the recently published observation that local capillarization is primarily dictated by muscle fibre size [Bosutti et al., 2015].

Published data on anatomical adaptations of skeletal muscle capillaries to denervation are scarce and poorly comparable, in part due to the different parameters used. Thus, the present study has two aims: (1) to determine the capillarity of muscle fibres in rat EDL

muscle 4 weeks after two different types of nerve injury (after neurotmesis and after two successive axonotmesis of the sciatic nerve) to provide new data on the capillarity of this special muscle (regarding its specific response to short-term denervation) using classic and adapted 2D measurement techniques, which measure also the length of longitudinally cut segments of capillaries, and (2) to calculate the same capillary/fibre parameters (L_{cap}/L_{fib} , L_{cap}/S_{fib} and L_{cap}/V_{fib}) that have been used in our previous stereological 3D study. We presumed that the calculated values will be comparable to the values of our previous study, and using the same parameters enables a better comparison of the results of 2D and 3D methods.

Materials and Methods

Animals

Sixteen male Wistar rats (weighing 193--233 g) were randomly divided into four groups: denervated (nerve cut; $n = 4$), re-innervated (two nerve crushes; $n = 4$), CON-1 ($n = 4$) age-matched control at the start of the experiment and CON-2 ($n = 4$) age-matched control at the end of the experiment. Surgery was performed under general anaesthesia with Rompun® (2% solution; Bayer AG, Germany) and Ketanest® (10 mg/ml; Parke-Davis GmbH, Germany). In the denervated group, the left sciatic nerve was cut on day 14, and 4 weeks later the animals were sacrificed and the complete left EDL was excised. In the re-innervated group, the left sciatic nerve was crushed for 1 min with a serrated haemostat on days 1 and 14, and 4 weeks after the second nerve crush animals were sacrificed. The proximal part of the nerve cut or nerve crush lesion was approximately 5 mm distal to the femoral trochanter. A 2-cm segment was excised from the distal part of the cut sciatic nerve in the denervated group. Excised muscles were weighed and frozen in liquid nitrogen until analyzed. The experimental protocols were approved by the Veterinary Administration of the Ministry for Agriculture, Forestry and Food, Republic of Slovenia (permit 34401-19/2009/10).

Tissue Sections

Five- μ m-thick transverse sections of EDL muscles were cut at -22°C using a Leica CM1850 (Leica Microsystems, Germany) microtome. Two to 3 tissue sections from each animal group were mounted on the same glass slide to assure identical staining conditions for all muscle groups. Two different staining techniques were applied for

quantitative analysis of capillaries and muscle fibres: one for *automatic* counting and one for *manual* analysis.

Two-colour fluorescent staining (to visualize capillaries and muscle fibres by two different fluorochromes) was performed for automatic counting of capillaries and muscle fibres. After fixation in cold acetone (for 2 min) and rinsing in Triton X-100 (PBS-T), sections were incubated with pre-immune goat serum (1:5) for 30 min. Monoclonal mouse anti-human laminin (1:1,000; MAB 1920; Chemicon), diluted in PBS-T and 3% pre-immune rat serum, was added overnight. After copious rinsing, goat anti-mouse Alexa Fluor 546 (1:350; A11003; Molecular Probes) was applied to the sections for 2 h for visualization of the basal lamina of muscle fibres (and the basal lamina of capillaries). After rinsing the sections in PBS-T, fluorescein-labelled *Griffonia* (Bandeira) *simplicifolia* lectin (GSL I; 1:300; FL 1101; Vector Laboratories) was added for 2 h for visualization of capillaries. After a final rinsing, the muscle sections were mounted in Elvanol.

Multicolour fluorescent staining of myosin heavy chains with double fluorescent staining of capillaries was used for manual analysis of capillaries around individual muscle fibres, the outlines of which were only visualized by phase-contrast microscopy [Ribarič and Čebašek, 2013]. Briefly, after fixation (for 2 min in cold acetone) and rinsing [for 20 min in PBS (0.01 M, pH 7.4) with 0.2% PBS-T], fluorescein-labelled GSL I (1:300; FL 1101) and rhodamine-labelled Griffonia GSL I (1:100; RL 1102; Vector Laboratories) were applied for 2 h at 4°C. Subsequently, a cocktail of primary antibodies against myosin heavy chains [BA-D5 (1:100), SC-71 (1:100) and BF-F3 (1:20) -- all from the Deutsche Sammlung von Microorganism und Zellkulturen] was added for 1 h. After rinsing in PBS-T, a cocktail of secondary antibodies [Alexa Fluor 350 – A21140 (1:350), Alexa Fluor 546 – A21123 (1:350) and Alexa Fluor 488 – A21042 (1:350) -- all purchased from Invitrogen, Carlsbad, Calif., USA] was applied for 30 min. After a final rinsing, the tissue sections were mounted in Elvanol.

Microscopy

Microscopic analysis was done with a Nikon Eclipse E800 microscope (Nikon Corporation, Tokyo, Japan) equipped with a DXM1200F (Nikon Corporation) digital camera.

Single-channel images were acquired by appropriate optical filter blocks using Nikon Plan-Fluor Ph1, x20 objectives (see details in Ribarič and Čebašek [2013]). Images of red (R) and green (G) channels were captured in dark light; during acquisition

of the blue (B) channel image, phase-contrast microscopy (white light) was simultaneously applied. Merged images of red and green channels were stored as single merged RG-TIFF images; merged images of red, green and blue channels were stored as single merged RGB-TIFF images both with x-y dimension of 1,632 × 680 pixels (680 µm × 283 µm).

Morphometry

Two non-overlapping fields were randomly selected per 5-µm-thick cross section of EDL muscles for morphometric analysis. Merged RG-TIFF or RGB-TIFF images were analyzed with the Ellipse program (ViDiTo, Kosice, Slovakia) [Tomori, 2006]. Global indices of capillarity, such as the muscle fibre density and the capillary density (CD), were assessed automatically by applying the threshold on single red and green channel images, respectively. Manual analysis was used for the determination of local indices of capillarity. It was performed by interactive tracing of capillaries (double staining with lectin) and muscle fibre outlines (visualized only by applying the phase-contrast microscopy) by a computer mouse. Muscle fibre area (in µm²), muscle fibre perimeter (FP; in µm) and the length of longitudinally cut capillaries adjacent to an individual muscle fibre were assessed by the Ellipse program. The number of transversally cut CAF was counted separately and saved in Microsoft Office Excel. Lcap in EDL muscle transverse sections was calculated as: CAF × 5 µm (thickness of muscle tissue samples) + the length of longitudinally cut capillaries adjacent to individual muscle fibre, similar to the *tortuosity index* that was used to study vascular growth in skeletal muscles in response to eccentric exercise [Huntsman et al., 2013]. Lcap/muscle fibre length (Lcap/Lfib), Lcap/Sfib (mm⁻¹) and Lcap/Vfib (mm⁻²) were calculated similarly to the calculations made in our previous studies [Cebasek et al., 2006, 2007], where morphometric data were obtained from 40-µm-thick transverse tissue sections.

Statistics

Statistical analysis was performed with the IBM SPSS statistics version 22. All data are presented as means ± SEM. Differences in mean values were tested with ANOVA, applying Bonferroni correction. Results were considered to be statistically significant when $p < 0.05$.

Results

Animal and Muscle Weight

The effects of nerve injuries on body and muscle weights are presented in [table 1](#). During the experimental time, the body weight of animals increased: in the CON-2 group 51% ($p < 0.01$), in the denervated group 46% ($p < 0.01$) and in the re-innervated group 45% ($p < 0.05$) compared to the CON-1 group. The wet weight of the denervated EDL was smaller than of the CON-2 group ($p < 0.001$), but the muscle/body weight ratio of the denervated group was smaller compared to all other groups ($p < 0.001$ vs. the CON-1 and CON-2 groups and $p < 0.01$ vs. the re-innervated group, where the mass of EDL muscles already approached the values of the control groups).

Muscle Fibre Morphometry and Capillary Characteristics

Four weeks after the nerve cut, the denervated muscle fibres of EDL muscles were markedly atrophied according to both manual and automatic analyses. Four weeks after the nerve crushes, the re-innervated EDL muscle fibres recovered, reached the mean fibre size of the CON-1 group and approached the mean size of muscle fibres of the CON-2 group (fig. 1). *Manual analyses* of capillaries and muscle fibres were done on merged RGB-TIFF images, where double-stained capillaries were very clearly seen also because the muscle fibre outlines were unstained. Results of the manual analysis are presented in table 2. The muscle fibre area and FP of the denervated group were smaller than in the CON-1, CON-2 and re-innervated groups (all $p < 0.001$). CAF of denervated muscles was smaller than in the CON-1 ($p < 0.005$) and re-innervated groups ($p < 0.001$). Lcap of the denervated group was smaller than that of the re-innervated group ($p < 0.01$). All calculated C/F ratios, except Lcap/Vfib, showed a higher capillarization of the re-innervated muscle fibres than the denervated and CON-2 muscle fibres (table 2). The Lcap/Lfib values of the control and re-innervated groups were about 10% higher than values of the CAF parameter, while in the denervated group this difference between those two parameters was only about 4%. Differences in the values of the two parameters indicated lower tortuosity of capillaries in the denervated EDL muscles. *The automatic analysis* of capillaries and muscle fibres was performed on separate green and red channel images (fig. 2). The results are shown in table 3. CD was higher in the denervated and re-innervated groups compared to the CON-2 group ($p < 0.001$). C/F

increased only in re-innervated muscles, and was much higher than in the denervated ($p < 0.001$) and CON-2 groups ($p < 0.01$; table 3).

Discussion

The main finding of the present 2D study is that the capillary network of rat EDL muscles has been modified by both types of nerve injury. Several parameters revealed that more capillaries are present around re-innervated than around denervated muscle fibres, i.e. higher CAF (4.81 ± 0.19 vs. 2.98 ± 0.17 ; $p < 0.001$), Lcap (26.79 ± 1.94 vs. $16.83 \pm 1.94 \mu\text{m}$; $p < 0.01$) and Lcap/Lfib (5.36 ± 0.24 vs. 3.10 ± 0.19 ; $p < 0.001$). A higher Lcap/Lfib parameter of the re-innervated group compared with the CON-2 group (5.36 ± 0.24 vs. 4.06 ± 0.18 ; $p < 0.05$) also indicated proliferation of capillaries in re-innervated muscles. This finding is in contrast with our previous studies, where no anatomical changes in EDL capillaries have been found after 2 weeks of denervation and re-innervation [Cebasek et al., 2006, 2007]. Together, these two findings (changed and unchanged capillary networks) are in agreement with a similar diverse response in the study of Tyml et al. [1999], who reported changed capillarity in EDL muscles 8 weeks after sciatic injury with tetrodotoxin, but not after a period of 2 weeks.

It seems that a delayed regression of capillaries in early denervation is characteristic only for rat EDL muscles, because other rat skeletal muscles, e.g. the gastrocnemius [Chernukh and Alekseeva, 1975; Tyml et al., 1999] and soleus muscles [Cebasek et al., 2006; Degens et al., 2008], show regression of capillaries already in the early denervation period. Tyml et al. [1999] speculated that such a specific postponed decrease in EDL capillaries to early denervation could be a consequence of the specific high susceptibility of EDL muscles to sympathectomy, because they have observed an increased blood flow after tetrodotoxin denervation (that could induce angiogenesis or prevent rarefaction of capillaries) only in the EDL but not in gastrocnemius muscles. This assumption is in agreement with a recent finding that the influence of the sympathetic nervous system on vascular resistance is more marked in muscles comprised predominately of muscle type IIB (like the EDL muscle) than in muscles with more fibre type I (like the soleus muscle) [Behnke et al., 2011].

In the present study, we assessed the global parameter EDL capillarity by *automatic analysis* thin tissue sections subjected to two-colour fluorescent staining. Automatic delineation of capillaries was made from single green and red channel images of the

same merged RG-TIFF image, thus counting of capillaries and muscle fibres was performed from exactly the same field of view (fig. 2). The calculated C/F value of 4-week denervated EDL was 1.10 ± 0.11 , i.e. comparable to 117 ± 5 capillaries per 100 muscle fibres, a coefficient of capillarization which was found in 1-month denervated EDL muscles [Borisov et al., 2000]. Unfortunately, a direct comparison can only be made for the C/F parameter, because we did not find any other global parameter for denervated EDL muscles in the literature. Local parameters were determined by *manual analysis* of 5- μ m-thin tissue sections, where capillaries were double stained with fluorescein and rhodamine-labelled lectin, and muscle fibre outlines were unstained. Although staining of the basal lamina or the sarcolemma of muscle fibres with anti-fibronectin, anti-laminin or anti-desmin antibodies can be very useful for image analysis [Hantai et al., 1984; Kubínová et al., 2001; Cebasek et al., 2004; Janáček et al., 2009; Meunier et al., 2010; Janáček et al., 2011; Talmadge et al., 2014], applying only the phase-contrast microscopy for visualization of muscle fibre outlines improves visibility of capillaries and facilitates manual tracing of capillaries.

We used an adapted 2D measurement method to estimate capillary length, where besides counting of transversely cut capillaries (which run parallel -- longitudinal to the long axis of muscle fibres), also the length of the longitudinally cut segments of capillaries were measured (which have an oblique or transverse course to the long axis of muscle fibres). In principle, similar measurement techniques have already been used in some other studies [Sullivan and Pittman, 1987; Charifi et al., 2004; Ichinose et al., 2008; Huntsman et al., 2013], but we used thinner tissue section (5 μ m is an average thickness of rat capillaries) in order to avoid (or to minimize) underestimations of the length of obliquely running capillaries. Underestimations can occur if thicker tissue sections are used for analysis, because of the difference that can appear between the actual length of obliquely run capillaries and the length of their projection seen from the surface of the tissue section.

Morphometric data of capillaries and muscle fibres obtained from 5- μ m-thin tissue sections were also used for the calculation of the capillary/fibre ratios (L_{cap}/L_{fib} , L_{cap}/S_{fib} and L_{cap}/V_{fib}) for the same parameters that have been previously calculated from 40- μ m-thick tissue sections. Values of all calculated ratios were in the range of calculated ratios obtained from 40- μ m-thick tissue sections [Cebasek et al., 2006, 2007]. Although 3D stereological techniques are superior to 2D techniques in many cases of morphometric estimations of geometric characteristics of different microscopic structures [Kubínová and

Janáček, 2015], it seems that the adapted 2D analysis of rat EDL capillaries, used in this study, is equal in precision to the 3D method and requires less effort. Using 2D methods, one can avoid problems associated with the preparation of thick tissue samples, i.e. variable visualization of capillaries and muscle fibres in thick tissue sections, difficulties with the visualization of myosin heavy chain muscle fibre types and tissue shrinkage [Janacek et al., 2012].

Different methods are used for quantitative analysis of skeletal muscle capillaries and it is still under debate, which one is the best. But, it is clear, also from the present study, that no single parameter can replace information obtained from several different parameters of capillarization, especially if they are combined with morphometric data of muscle fibres. Although CD values of denervated and re-innervated muscles were nearly the same and were both higher than in control muscles ($p < 0.001$; [table 2, 3](#)), it is obvious that the major reason for the observed over-capillarization in denervated muscles was severe atrophy of the denervated muscle fibres (the mean muscle fibre area of the denervated group was approximately 50% lower than in the CON-1 and re-innervated groups and even 67% lower than in the CON-2 group (all $p < 0.001$). However, over-capillarization in the re-innervated muscles was probably due to angiogenesis because the muscle fibre area was similar in both groups ([table 2](#)). If we also consider the lower CAF of the denervated group in comparison to the CON-1 group ($p < 0.005$) and the higher C/F ($p < 0.001$), CAF/FP ($p < 0.001$), Lcap/FP ($p < 0.05$) and Lcap/Lfib ($p < 0.001$) of the re-innervated muscles in comparison to the denervated muscles, these results suggest that proliferation of capillaries occurs in the re-innervated and regression in the denervated EDL.

We were not able to determine the main trigger for remodelling of the capillary network after nerve injuries, because we did only analyze the capillaries in relation to muscle fibre size in this study, but in one of our previous studies we found that the capillarity in EDL is scaled by muscle fibre size and not by the oxidative potential of muscle fibres [Janáček et al., 2009]. Regression of capillaries during denervation when muscle fibres are subject to atrophy and proliferation of capillaries during re-innervation processes when muscle fibres regain their original size are in agreement with this finding and also with recently published observations that local capillary supply is not determined by local oxidative capacity, but rather by factors such as muscle fibre size [Bosutti et al., 2015].

In conclusion, our results clearly demonstrate that a 4-week nerve injury is long enough to provoke changes in capillaries in rat EDL muscles. Comparable calculated

values of capillary/fibre parameters indicate that these parameters can be used as 'a bridge' for easier and more direct comparisons of the results of studies where different methods are used. Being less time consuming and cheaper, the adapted 2D measurement method used in this study has proven to be a good compromise for 3D methods, at least for measurements of capillaries in rat EDL muscle.

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Appendix after References (Editorial Comments)

Legend(s)

Fig. 1. Inverted images of merged RGB-TIFF fluorescent images of transverse sections of EDL muscles of the CON-1 (**a**), CON-2 (**b**), re-innervated (**c**) and denervated (**d**) groups. Capillaries (black spots and lines) are visualized in green and red channels by staining with fluorescein- and rhodamine-labelled lectin GSL I; unstained muscle fibre outlines (watery coloured light grey lines) are visualized by applying phase-contrast light microscopy on acquisition of the blue channel image.

Fig. 2. Representative transverse sections of re-innervated EDL muscle stained for capillaries and muscle fibres in two different colours. In the merged RG-TIFF image (**a**), the capillaries are yellow due to co-localization of fluorescein-labelled lectin GSL I and rhodamine-labelled anti-laminin, and the muscle fibres outlines are red because of rhodamine-labelled anti-laminin. Single green (**b**) and red (**c**) channel images are used for automatic thresholding and delineating of capillaries (**d, f**) and muscle fibres (**e, g**), respectively.

Table(s)

Footnote(s)

Abbreviations used in this paper

CAF	number of capillaries around a muscle fibre
CAF/FP	number of capillaries around an individual muscle fibre/muscle fibre perimeter
C/F	capillary number/muscle fibre number
CD	capillary density expressed as number of capillaries/mm ²
EDL	extensor digitorum longus muscle
FP	muscle fibre perimeter
Lcap	length of capillaries adjacent to an individual muscle fibre in a 5- μ m-thick tissue section
Lcap/FP	Lcap/muscle fibre perimeter
Lcap/Lfib	Lcap/muscle fibre length
Lcap/Sfib	Lcap/muscle fibre surface area
Lcap/Vfib	Lcap/muscle fibre volume

Table 1. Data on body weight, muscle weight and muscle/body weight ratio (n = 4 for each group)

	CON-1	CON-2	Denervated	Re-innervated
Body weight, g	219±6 ^a	332±14	320±18	318±27
EDL wet weight, mg	87±2	130±15 ^b	62±5	100±5
EDL/body weight, %	3.97±0.08	3.96±0.35	1.94±0.09 ^c	3.18±0.18

Values are means ± SEM. Significant differences between groups are indicated by letters. ^aCON-1 < CON-2 (p < 0.01), denervated (p < 0.01), re-innervated (p < 0.05); ^bCON-2 > CON-1 (p < 0.05), denervated (p < 0.001); ^cdenervated < CON-1 (p < 0.001), CON-2 (p < 0.001), re-innervated (p < 0.01).

Table 2. Data on muscle fibres and capillaries of control and experimental EDL muscles obtained by manual analysis of RGB images

	CON-1	CON-2	Denervated	Re-innervated
Fibre area, μm^2	1,024 $\pm$ 25	1,599 $\pm$ 63	527 $\pm$ 36 ^a	1,085 $\pm$ 60
FP, μm	126 $\pm$ 2	158 $\pm$ 3	93 $\pm$ 3 ^a	133 $\pm$ 3
CAF	3.99 $\pm$ 0.16	3.51 $\pm$ 0.11	2.98 $\pm$ 0.17 ^b	4.81 $\pm$ 0.19 ^c
Lcap, μm	24.18 $\pm$ 2.44	20.30 $\pm$ 0.92	16.83 $\pm$ 1.94	26.80 $\pm$ 1.18 ^d
Lcap/Lfib	4.45 $\pm$ 0.31	4.06 $\pm$ 0.18	3.10 $\pm$ 0.19 ^e	5.36 $\pm$ 0.24 ^f
Lcap/Sfib, mm^{-1}	37.12 $\pm$ 1.98	27.17 $\pm$ 0.99	33.68 $\pm$ 1.81	41.15 $\pm$ 1.37 ^g
Lcap/Vfib, mm^{-2}	5,204 $\pm$ 244	3,180 $\pm$ 75	6,619 $\pm$ 510 ^h	5,502 $\pm$ 181
CAF/FP, $\mu\text{m}^{-1} \times 10^{-3}$	3.16 $\pm$ 1.98	2.22 $\pm$ 0.05 ⁱ	3.20 $\pm$ 0.17	3.62 $\pm$ 0.08
Lcap/FP $\times 10^{-2}$	19.11 $\pm$ 1.81	12.81 $\pm$ 0.45 ^j	18.02 $\pm$ 1.87	20.22 $\pm$ 0.75

Lcap and Lcap/Lfib, Lcap/Sfib and Lcap/Vfib in 5- μm -thick tissue section were calculated for each individual muscle fibre using Microsoft Excel program macros (257 $\pm$ 47 muscle fibres per muscle). Values are means $\pm$ SEM. Significant differences between groups are indicated by letters. ^a Denervated < CON-1, CON-2, re-innervated ($p < 0.001$); ^b denervated < CON-1 ($p < 0.005$), re-innervated ($p < 0.001$); ^c re-innervated > CON-1 ($p < 0.05$), CON-2 ($p < 0.001$), denervated ($p < 0.001$); ^d re-innervated > denervated ($p < 0.01$); ^e denervated < CON-1 ($p < 0.01$), re-innervated ($p < 0.001$); ^f re-innervated > CON-2 ($p < 0.05$), denervated ($p < 0.001$); ^g re-innervated > CON-2 ($p < 0.001$), denervated ($p < 0.05$); ^h denervated < CON-1 ($p < 0.05$), CON-2 ($p < 0.001$); ⁱ CON-2 < CON-1 ($p < 0.001$), denervated ($p < 0.001$), re-innervated ($p < 0.001$); ^j CON-2 < CON-1 ($p < 0.05$), re-innervated ($p < 0.05$). RGB image: merged image of red, green and blue channels.

Table 3. CD and muscle fibre density of EDL muscles obtained by automatic analysis of single-channel images (495 ± 60 muscle fibres/muscle)

	CON-1	CON-2 ^a	Denervated	Re-innervated
CD	1,779 $\pm$ 80	1,073 $\pm$ 105 ^a	2,164 $\pm$ 234	2,330 $\pm$ 75
FD	1,263 $\pm$ 91	862 $\pm$ 53	1,988 $\pm$ 197 ^b	1,259 $\pm$ 76
C/F	1.43 $\pm$ 0.14	1.24 $\pm$ 0.05	1.10 $\pm$ 0.11	1.87 $\pm$ 0.092 ^c

Values are means $\pm$ SEM. Significant differences between groups are indicated by letters. ^aCON-2 < CON-1 ($p < 0.05$), denervated ($p < 0.001$), re-innervated ($p < 0.001$); ^bdenervated > CON-1 ($p < 0.001$), CON-2 ($p < 0.01$), re-innervated ($p < 0.01$); ^cre-innervated > CON-2 ($p < 0.01$), denervated ($p < 0.001$). FD = Muscle fibre density expressed as the number of muscle fibres/mm².