*Article

Vascularized peripheral nerve grafting promotes myelination of regrowing optic nerve

Seiji **Komatsu**[a], Taketoshi **Wakabayashi**[c], Kiyoshi **Yamada**[a], Kumiko **Matsumoto**[a], Yoshihiro **Kimata**[a] and Jun **Kosaka**[b, *]

[a]Department of Plastic and Reconstructive Surgery, and [b]Department of Cytology and Histology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan.

[c]Department of Anatomy and Cell Science, Kansai Medical University,
2-5-1 Shin-machi, Hirakata 573-1010, Japan.

Corresponding author*: Jun Kosaka, MD, DSci.
Department of Cytology and Histology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences,
2-5-1 Shikata-cho, Kita-ku, Okayama 700-8558, Japan.
Fax. +81-86-235-7079, Email: junksk@md.okayama-u.ac.jp

Conflict of Interest: "None declared"

Running Head: Vascularized PN graft to CNS

Author Contributions:

S.K., J.K., T.W. and Y.K. designed the research and wrote the paper.

S.K., J.K., K.M., K.Y. and T.W. performed the experiments.

S.K., T.W. and J.K. analyzed the data.

List of Abbreviations:

CNS: central nervous system

PN: peripheral nerve

PNS: peripheral nervous system

RGC: retinal ganglion cell

ON: optic nerve

GB: granular blue

ABSTRACT

We investigated whether the use of vascularized peripheral nerve grafts to the optic nerve stump enhances axonal regeneration of retinal ganglion cells compared to isolated non-vascularized grafts. The rat median nerve was microsurgically sutured with its supplying artery and vein to the optic nerve stump. The number of retinal ganglion cells with regenerating axons was evaluated by retrograde labeling into the grafted peripheral nerve, and the myelination of the regenerating axon fibers was examined by electron microscopy. The number of retinal ganglion cells with regenerating axons was significantly higher in the vascularized graft than in the non-vascularized graft. The ratio of myelinated axon fibers was also increased in vascularized grafts. Thus, grafting with their supplying arteries and veins to an injured nerve stump represents a promising strategy to accelerate axonal regeneration from neurons of the central nervous system.

Key Words:

Retinal ganglion cells; Schwann cells; Microsurgery; Graft; Optic nerve; Vascularized nerve

INTRODUCTION

Damage and injury to axonal fibers in the central nervous system (CNS) results in degeneration of the somata, and surviving neurons never exhibit axonal regrowth in mature mammals [1]. Autologous peripheral nerve (PN) grafts enhance axonal regeneration and survival of damaged CNS neurons [2]. It is widely believed that PN grafts supply CNS neurons with neurotrophic factors and a permissive extracellular environment that supports their ability to survive and regenerate axons, mimicking the developmental stage [3, 4].

Retinal ganglion cells (RGCs) and their axons, which make up the optic nerve (ON), have provided a good model for studying axonal regeneration and functional recovery from CNS injury since the pioneering work of So and Aguayo, who showed that axotomized RGCs can regrow axons through a grafted PN [5]. Although RGCs in mature mammals can survive axotomy and exhibit axonal regrowth after PN grafting, the number of intact RGCs that regenerate axons is too small and myelination of regenerating ON remains immature or unmyelinated, when the graft consists of a free PN isolated completely from its supplying artery and vein [6-9]. One possible cause of the poor regenerating activity is the low viability and activity of Schwann cells in the grafted PN. Several attempts have been made to introduce surviving Schwann cells in

classical PN grafting, including an artificial tube containing cultured Schwann cells [10]. New strategies that enhance the number and quality of regenerating ON axons should be established to bring this approach nearer to clinical application [8].

In this study, we established a new surgical method to suture a PN along with its supplying artery and vein (the vascularized graft) to the transected ON stump to promote the survival of Schwann cells within the graft. We then evaluated the number and extent of myelination of regrowing axons 1 month after the operation, comparing the effect of vascularized and classical non-vascularized grafts. Our findings suggest the potential for a new microsurgical strategy for treating CNS injuries and damage.

METHODS

Surgical Procedures

Twelve adult male Wistar rats (SLC, Shizuoka, Japan) weighing 250-300g were used in this study. All animal experiments were conducted in strict accordance with institutional and NIH guidelines for “Using Animals in Intramural Research” and all experimental protocols were approved by the Animal Research Committee of Okayama University, Japan (No. OKU-2011304, 2012394).

Vascularized and non-vascularized grafting procedures were modified from published methods [7, 8]. After establishing deep anesthesia by intraperitoneal injection of sodium pentobarbital, we opened the skin from the right forearm to the ON and then transected the ON just behind the sclera, taking care to avoid damage to the ophthalmic artery.

The vascularized PN graft procedure was developed based on published methods with minor modifications [11, 12]. The trunk of the right median nerve was clearly and carefully exposed from the head of the humerus to near the palm on the flexor side of the forearm, avoiding damage to the supplying blood vessels [11]. To ensure complete blood supply from the brachial artery to the supplying artery of the median nerve, peripheral branches were ligated using a bipolar coagulator (Janus Electrosurgical Unit, J-45, Keisei-Ika, Tokyo, Japan). We carefully transected the median nerve with its blood supply from a site near the palm to the head of humerus, yielding a segment at least 5 cm in length. The median nerve was then rotated and the distal end sutured to the ON stump using 10-0 nylon sutures (Keisei Medical, Tokyo, Japan). The proximal end of the median nerve was transected at the brachial plexus and sutured to the temporal muscle. This operation method is illustrated in Figure 1.

For non-vascularized PN grafting, at least a 5 cm segment of the right median

nerve was excised from the forelimb without any artery or vein, according to our strategy [7, 8]. The distal end of the freely isolated nerve was sutured to the ON stump with 10-0 nylon sutures. The other end of the graft was labeled with red thread and sutured to the temporal muscle. The animals in both groups underwent a mid-humerus-level amputation to avoid severe necrosis due to removal of the blood supply in the vascularized graft group. Following recovery, all rats were caged individually with *ad libitum* access to food and water until further analysis.

Retrograde labeling and counting of RGCs

Axonal regeneration from RGCs was detected by retrograde labeling of the grafted PN [7, 8]. One month (30-31 days) after grafting, rats were anesthetized with sodium pentobarbital, and the operation site was opened to expose the graft. A small piece of gelatin sponge (Spongel; Astellas, Tokyo, Japan) soaked with p-amidinophenyl *p*-(6-amidino-2-indolyl) phenyl ester (Granular Blue, GB, Sigma, St. Louis, MO) was implanted in the nerve graft at a site 10 mm peripheral to the PN/ON suture site. Three days later, the rats were anesthetized deeply with sodium pentobarbital and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). For flatmount preparations of the retina, the isolated neural retina was spread onto a

1-mm-grid glass slide (Matsunami Glass, Osaka, Japan) and viewed from the vitreous side. In the vascularized graft group, three samples with severe intraocular hemorrhage and ischemic changes were excluded from the analysis. Specimens were observed under a fluorescence microscope with a UV filter (Axiopran, Carl Zeiss, Heidelberg, Germany). The number of GB-fluorescent RGCs was counted manually using the grid, and images were recorded using a charge-coupled device camera (AxioCam; Carl Zeiss).

Transmission electron microscopic study

Myelination of regenerating axons was evaluated by transmission electron microscopy [13]. A piece of median nerve around ~5 mm peripheral to the PN/ON suture site was isolated after transcardial perfusion (above) and postfixed with 2% paraformaldehyde/2% glutaraldehyde overnight and then with 1% osmium tetroxide (Merck, Darmstadt, Germany) at 4°C for 1 hr. After dehydration with an ethanol series (50-100%), specimens were embedded in Epon 812 (Oken Shoji, Tokyo, Japan) and polymerized at 60°C for 3 days. Ultrathin vertical sections of PN were cut using a diamond knife and stained with 5% aqueous uranyl acetate and lead citrate, and observed by transmission electron microscopy (H-7650 at 80V; Hitachi, Tokyo, Japan).

We categorized the degree of myelination in three stage types. A myelin sheath, which was thicker than half the axon diameter, was considered “mature” and a myelin sheath that was thinner than half the axon diameter was considered “immature”. For this purpose, the axon diameter was defined as the mean of the maximum diameter and the diameter orthogonal to it. From their fine structure and typical low electron density, histologically defined unmyelinated axon fibers were regarded as “unmyelinated”. At least 400 axon fibers were categorized in each sample using electron micrographs from four rats, two following non-vascularized grafting (a, b) and two following vascularized grafting (c, d).

Statistical analysis

Statistical comparisons of the number of RGCs with regenerating axons between vascularized grafts (n = 3) and non-vascularized grafts (n = 6) were made by Student’s unpaired *t*-test. The significance cutoff was set at $P < 0.05$. All numerical data are presented as the mean ± SD. Chi-square test independence $p = 0.05$ and residual analysis were used to examine significant differences with three categorical variables; unmyelinated, immature and mature myelinated axon fibers, and the comparison of non-vascularized (a + b) and vascularized (c + d) grafting.

RESULTS

Number of RGCs with regenerating axons following vascularized grafting

GB-positive cells in vascularized and non-vascularized-PN grafted rats were localized from the central (Fig. 2A–C) to peripheral retina. The number of GB-positive cells (i.e., RGCs with regenerating axons) following vascularized PN grafts was significantly greater than the number following non-vascularized grafts (compare Fig. 2A with 2B). The average number of GB-positive cells in the vascularized group was 1,200 ± 208 (n = 3), which represents 1.2% of the total number of RGCs in an intact rat retina (~98,000) [14]. In the non-vascularized group, there were 691 ± 213 (n = 6) GB-positive RGCs, representing 0.7% of the total RGCs in an intact rat retina. The use of vascularized PN grafts resulted in a statistically significant increase (~1.7-fold; $P<0.05$) in axonal regeneration (Fig. 2D). The morphology of GB-positive RGCs in the vascularized group (Fig. 2C) was not different from that in the non-vascularized group or that in a previous study [7].

Myelination of regenerating axon fibers in the vascularized grafts

Regenerating retinal axon fibers in vascularized and non-vascularized PN grafts

were observed by transmission electron microscopy to evaluate the extent of myelination. Non-vascularized grafts contained unmyelinated axon fibers and a small number of thinly myelinated axon fibers (Fig. 3A). The axon fibers were loosely distributed, with many spaces between them. At higher magnification, unmyelinated axon fibers were in contact with Schwann cells (arrowheads in Fig. 3B). Axon fibers with a thin myelin sheath were categorized here as immature myelinated axon fibers (c.f. Fig. 4). Initial stages of myelination were also detectable in the non-vascularized grafts (arrows and asterisks in Fig. 3B). These observations were consistent with earlier findings for non-vascularized sciatic nerve grafts in both rats and cats [9, 15]. In contrast, many myelinated axon fibers were observed in vascularized grafts at low magnification (Fig. 3C). At higher magnification, regenerating retinal axon fibers were observed as bundles in the vascularized PN, and each myelinated axon was covered with Schwann cell cytoplasm (indicated by arrowheads in Fig. 3D). Tight collagen fibrils were also observed around the myelinated fibers.

At lower magnification, the number of myelinated axon fibers seemed to be greater in the vascularized PN than the non-vascularized PN (compare Fig. 3A with 3C). Thus, we quantified the degree of myelination in both graft groups using a categorization of unmyelinated, immature, and mature myelinated axon fibers (Fig. 4,

upper panels). In non-vascularized grafts, ~70% of regenerating axon fibers were unmyelinated 1 month after the operation, and only ~10% had mature myelination. In contrast, more than 35% of regenerating axons had mature myelination in vascularized grafts, and less than 50% were unmyelinated. This semi-quantitative analysis indicated that myelination of regenerating axon fibers was significantly enhanced by the use of vascularized PN grafts (p <0.05, Chi-square test + residual analysis) (Fig. 4).

DISCUSSION

We demonstrated that the number of RGCs with regenerating axons after vascularized grafts was significantly higher than after non-vascularized grafts, although the number still remained small. One possible explanation for the small number of RGCs with regenerating axons in the vascularized group is that some inhibitory factors were up-regulated in the vascularized PN graft. Inhibitory factors such as myelin-associated glycoprotein and chondroitin sulfate proteoglycan have been reported in the PN [16, 17]. Contrary to our expectation, it is possible that both inhibitory and accelerative factors were activated by Schwann cells in vascularized PN grafts. Molecular approaches that block such inhibitory signals may be effective at increasing the number of regenerating retinal axons.

Another possibility is that the median nerve has a smaller diameter than the sciatic nerve, which was used in previous non-vascularized PN grafts and has the largest diameter among PNs. The ON stump was completely covered with the freely moving sciatic nerve previously [7-8], whereas in this study the dissected optic nerve and median nerve were sutured, stump to stump. We speculate that regenerating axons from the ON stump penetrated more easily into the grafted sciatic nerve with a larger diameter than the end-sutured median nerve. To increase the number of RGCs with regenerating axons, it will be useful for future studies to examine the effect of grafting other vascular PNs with larger diameters.

The enhanced myelination of axon fibers and increased number of Schwann cells in vascularized PN grafts compared with non-vascularized grafts has already elucidated using the research system of axonal regrowth of PN/PN grafts [18, 19]. Watanabe et al. have clearly demonstrated that axon fibers in a non-vascularized sciatic nerve graft were regrowing ON fibers originating from anterograde labeled RGCs [9]. Retrograde labeled RGCs in the flatmounted retina (Fig. 2) strongly suggest that the axon fibers observed by electron microscopy were regrowing retinal axons originating from RGCs (i.e., ON fibers) in the vascularized graft. Taken together with earlier results of the regrowth and re-myelination of PN axons, these observations strongly suggest that

enhanced myelination of regenerating retinal axon fibers resulted from an increased number of Schwann cells in the vascularized graft [18, 19]. Further studies could clearly show that axonal regeneration and myelination of RGC are promoted by increased numbers and activities of Schwann cells in the vascularized grafts. It is reasonable to suggest that some factors secreted by an increased number of Schwann cells enhanced axonal regeneration of RGCs in the vascularized grafts. Several factors synthesized by Schwann cells are candidates for supporting axonal regeneration of RGCs, including ciliary neurotrophic factor, brain-derived neurotrophic factor and osteonectin [3-4, 17, 20]. Additionally, our results demonstrate that myelination of retinal axon fibers had developed to a mature stage within 1 month in the vascularized graft. Further analysis could reveal the molecular cascade by which an increase in Schwann cells establishes permissive conditions for retinal axon regrowth and progressive re-myelination of ON fibers within the PN graft.

CONCLUSIONS

The use of vascularized PN grafts enhanced axonal regeneration of RGCs and profoundly affected the myelination of regenerating axon fibers. Maturation of myelinated axon fibers following a vascularized PN graft can quickly accelerate the

functional recovery of the ON after injury and damage. This microsurgical strategy may lead to a breakthrough in the challenges of reconstructing synaptic pathways in the CNS.

ACKNOWLEDGEMENTS

The authors thank Ms. M. Furutani and E. Naomura (Central Research Laboratory, Okayama University Medical School) for their excellent technical assistance in the electron microscopic study. This study was supported by Grants-in-Aids for Scientific Research from the Ministry of Education, Culture, Sports, Sciences and Technology of Japan.

References

[1] Cajal SR. Cajal's degeneration and regeneration of the nervous system (translated by R.M. May). New York: Oxford Univ. Press; 1991.

[2] David S, Aguayo AJ. Axonal elongation into peripheral nervous system "bridges" after central nervous system injury in adult rats. Science 1981; 214: 931–933.

[3] Meyer M, Matsuoka I, Wetmore C, Olson L, Thoenen H. Enhanced synthesis of brain-derived neurotrophic factor in the lesioned peripheral nerve: different mechanisms are responsible for the regulation of BDNF and NGF mRNA. J Cell Biol. 1992; 119: 45–54.

[4] Dezawa M, Adachi-Usami E. Role of Schwann cells in retinal ganglion cell axon regeneration. Prog Retin Eye Res. 2000; 19: 171–204.

[5] So KF, Aguayo AJ. Lengthy regrowth of cut axons from ganglion cells after peripheral nerve transplantation into the retina of adult rats. Brain Res. 1985; 328: 349–354.

[6] Villegas-Pérez MP, Vidal-Sanz M, Bray GM, Aguayo AJ. Influences of peripheral nerve grafts on the survival and regrowth of axotomized retinal ganglion cells in adults rats. J Neurosci. 1988; 8: 265–280.

[7] Wakabayashi T, Fukuda Y, Kosaka J. Monoclonal antibody C38 labels surviving retinal ganglion cells after peripheral nerve graft in axotomized rat retina. Brain Res. 1996; 725: 121–124.

[8] Quan MZ, Kosaka J, Watanabe M, Wakabayashi T, Fukuda Y. Survival of axotomized retinal ganglion cells in peripheral nerve-grafted ferrets, Invest. Ophthalmol. Vis. Sci. 1999; 40: 2360–2366.

[9] Watanabe M, Sawai H, Fukuda Y. Myelination of regenerated optic fibers in peripheral nerve graft of adult cats. Exp Brain Res. 1994; 98: 39–43.

[10] Negishi H, Dezawa M, Oshitari T, Adachi-Usami E. Optic nerve regeneration within artificial Schwann cell graft in the adult rat. Brain Res Bull. 2011; 55: 409–419.

[11] Bertelli JA, Saadi MTA, Mira JC, Pecot-Dechavassine M. The rat brachial plexus and its terminal branches: an experimental model for the study of peripheral nerve regeneration. Microsurg. 1995; 16: 77–85.

[12] Kimata Y, Sakuraba M, Hishinuma S, Ebihara S, Hayashi R, Asakage T. Free vascularized nerve grafting for immediate facial nerve reconstruction. Laryngoscope. 2005; 115: 331–336.

[13] Morizane Y, Mohri S, Kosaka J, Toné S, Kiyooka T, Miyasaka T, et al. Iris movement mediates vascular apoptosis during rat pupillary membrane regression. Am J Physiol Regul Integr Comp Physiol. 2006; 290: 819–825.

[14] McCall MJ, Robinson SR, Dreher B. Differential retinal growth appears to be the primary factor producing the ganglion cell density gradient in the rat. Neurosci Lett. 1987; 79: 78–84.

[15] Dezawa M, Kawata K, Adachi-Usami E. The role of Schwann cells during retinal

ganglion cell regeneration induced by peripheral nerve transplantation. Invest Ophthalmol Vis Sci. 1997; 38:1401–1410.

[16] Bähr M, Przyrembel C. Myelin from peripheral and central nervous system is a nonpermissive substrate for retinal ganglion cell axons. Exp Neurol. 1995; 134: 87–93.

[17] Harvey AR, Hu Y, Leaver SG, Mellough CB, Park K, Verhaagen J, et al. Gene therapy and transplantation in CNS repair: the visual system. Prog Retin Eye Res. 2006; 25: 449–489.

[18] Kawai H, Baudrimont M, Travers V, Sedel L. A comparative experimental study of vascularized and nonvascularized nerve grafts. J Reconstr Microsurg. 1990; 6: 255–259.

[19] Tark KC, Roh TS. Morphometric study of regeneration through vascularized nerve graft in a rabbit sciatic nerve model. J Reconstr Microsurg. 2001; 17: 109–114.

[20] Sawai H, Clarke DB, Kittlerova P, Bray GM, Aguayo AJ. Brain-derived neurotrophic factor and neurotrophin-4/5 stimulate growth of axonal branches from

regenerating retinal ganglion cells. J Neurosci. 1996; 16: 3887-3894.

Figure Legends

Fig. 1. Schematic representation of the vascularized PN graft model in the rat. The right median nerve **(yellow)** was carefully exposed with its supplying artery (red) and vein **(blue)** from the head of humerus to the palm. The distal end of the nerve was turned and sutured to the right ON stump **(arrow)**. The proximal side was transected from the brachial plexus **(dotted line)**.

Fig. 2. GB-positive RGCs with regenerating axons in the central retina following non-vascularized and vascularized grafts. (A, B) Representative images of flatmount preparations show GB-fluorescence in the central retina at lower magnification in rats with non-vascularized **(A)** and vascularized **(B)** grafts. Scale bar = 500 μm. **(C)** Typical RGCs labeled with GB in a rat following a vascularized PN graft. RGCs with their axon and dendrite can be observed. Scale bar = 50 μm. **(D)** Number of RGCs with regenerating axons following "Non-vascularized" (n = 6) and "Vascularized" (n = 3) PN grafts. Values are the mean ± SD; **P* <0.05.

Fig. 3. Electron microscopy of regenerating axon fibers following

non-vascularized and vascularized PN grafts. (A) Unmyelinated axon fibers at low magnification in a non-vascularized graft. **(B)** Higher magnification reveals axon fibers with thin myelination (asterisks) or no myelination (arrowheads). Thinly myelinated axon fibers and unmyelinated fibers are in contact with Schwann cells (**S**). One Schwann cell contains three atypical structures with similar low electron density to the axon fibers, which are covered by an electron-dense surface, representing thin myelin (arrows). **(C)** In a vascularized graft, myelinated axon fibers are observed at low magnification. **(D)** Higher magnification reveals tightly packed myelinated axon fibers (arrowheads), each individually surrounded by Schwann cell cytoplasm. Two additional Schwann cells contain the intracellular laminated structure of myelin (arrows). Scale bar = 10 μm **(A, C)**, 2.0 μm **(B, D)**.

Fig. 4. Extent of myelination in grafted PNs following non-vascularized and vascularized grafts. Top: Electron micrographs demonstrating categories used to assess myelination based on thickness (see Material and Methods). Bottom: Quantification of axon fibers in each category: unmyelinated **(white)**, immature myelinated **(grey)**, and mature myelinated axon fibers **(black)**. Analysis performed on 4 rats, 2 following non-vascularized grafting **(a, b)** and 2 following vascularized grafting **(c, d)**. The ratio

of myelination in the vascularized graft was significantly more enhanced than that in the non-vascularized graft. *p <0.05, Chi-square test and residual analysis.

Figure-1-CMYK(new)
Click here to download high resolution image

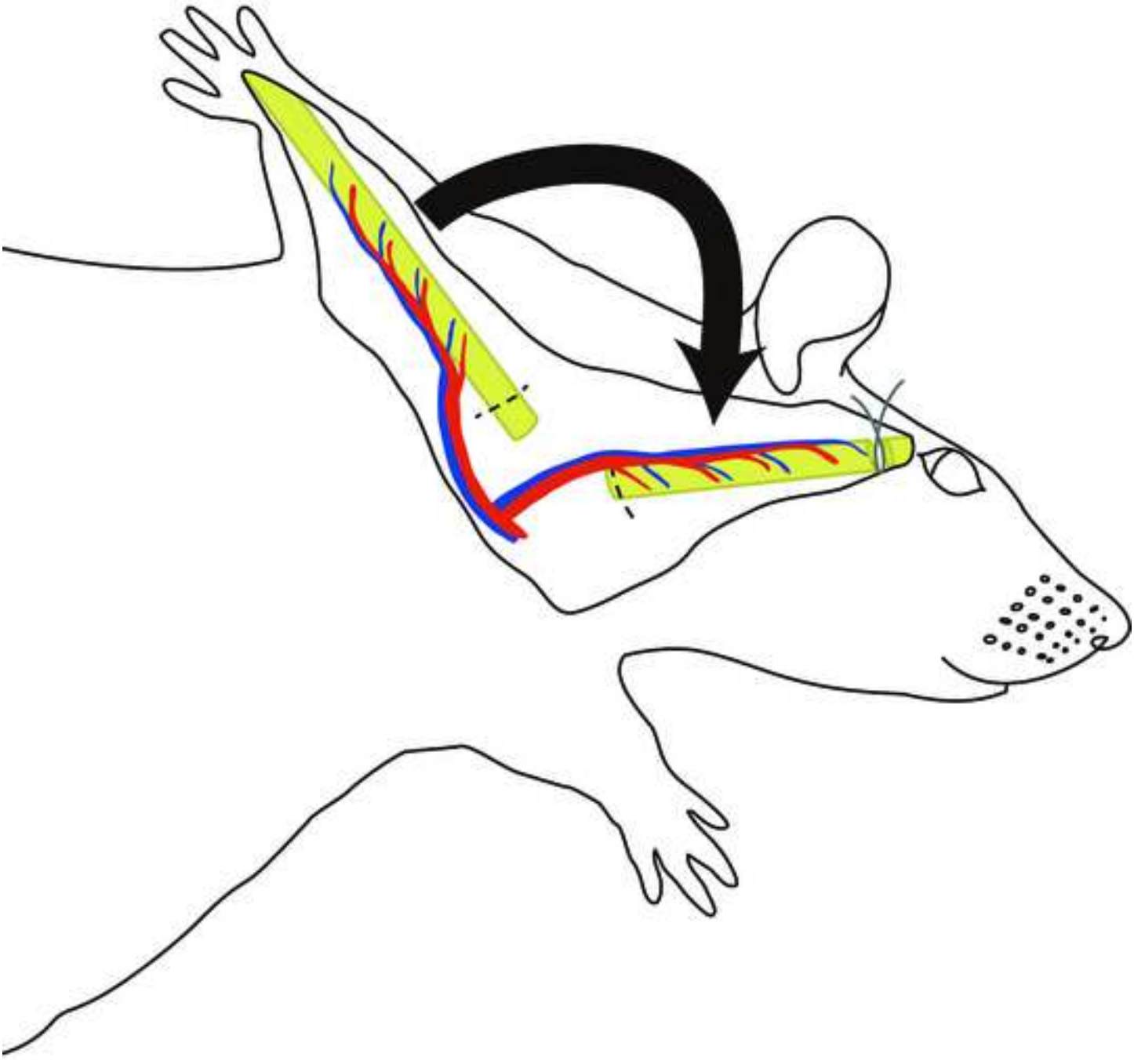

Figure-2-CMYK-tif
Click here to download high resolution image

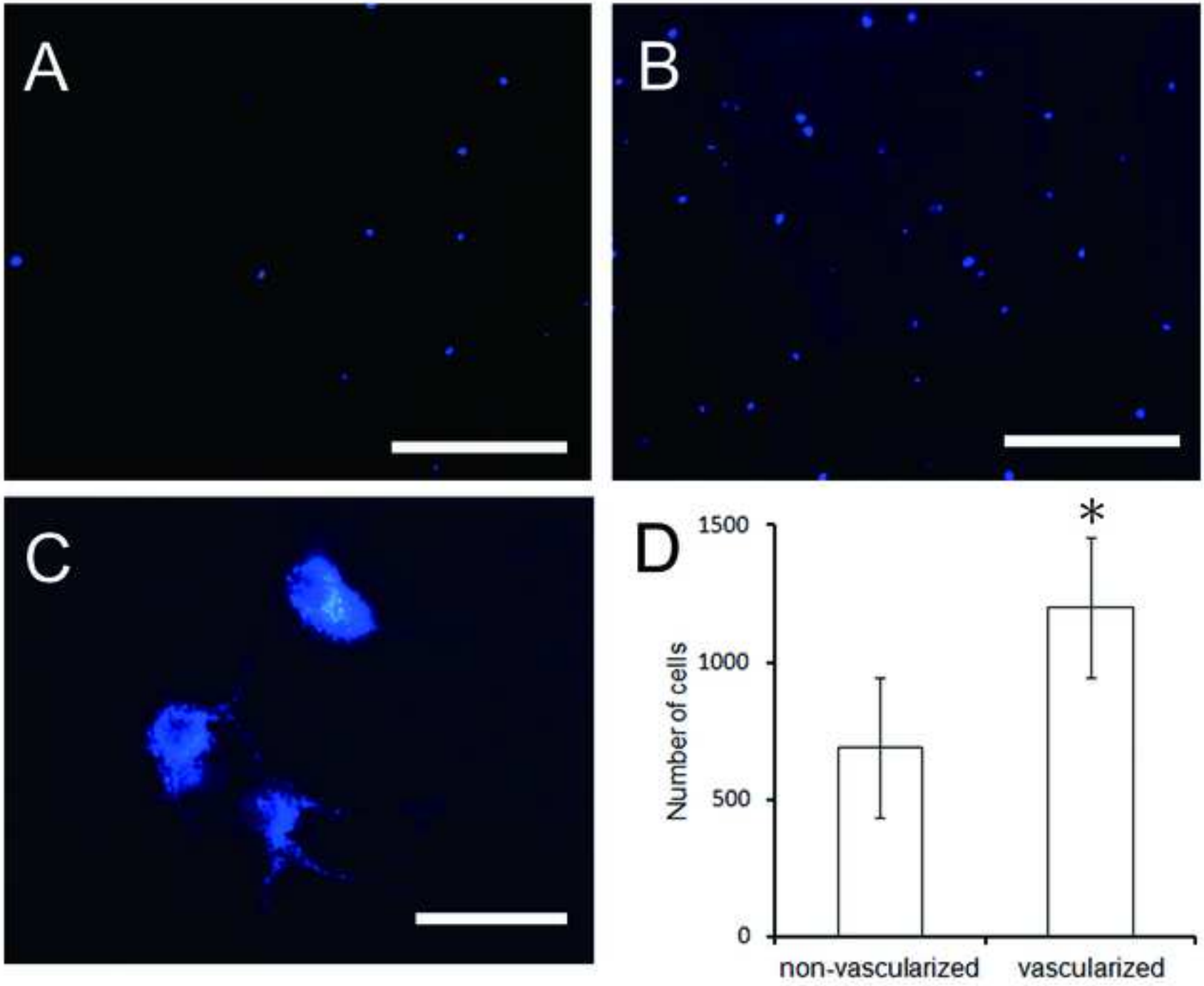

Figure-3-BW

Click here to download high resolution image

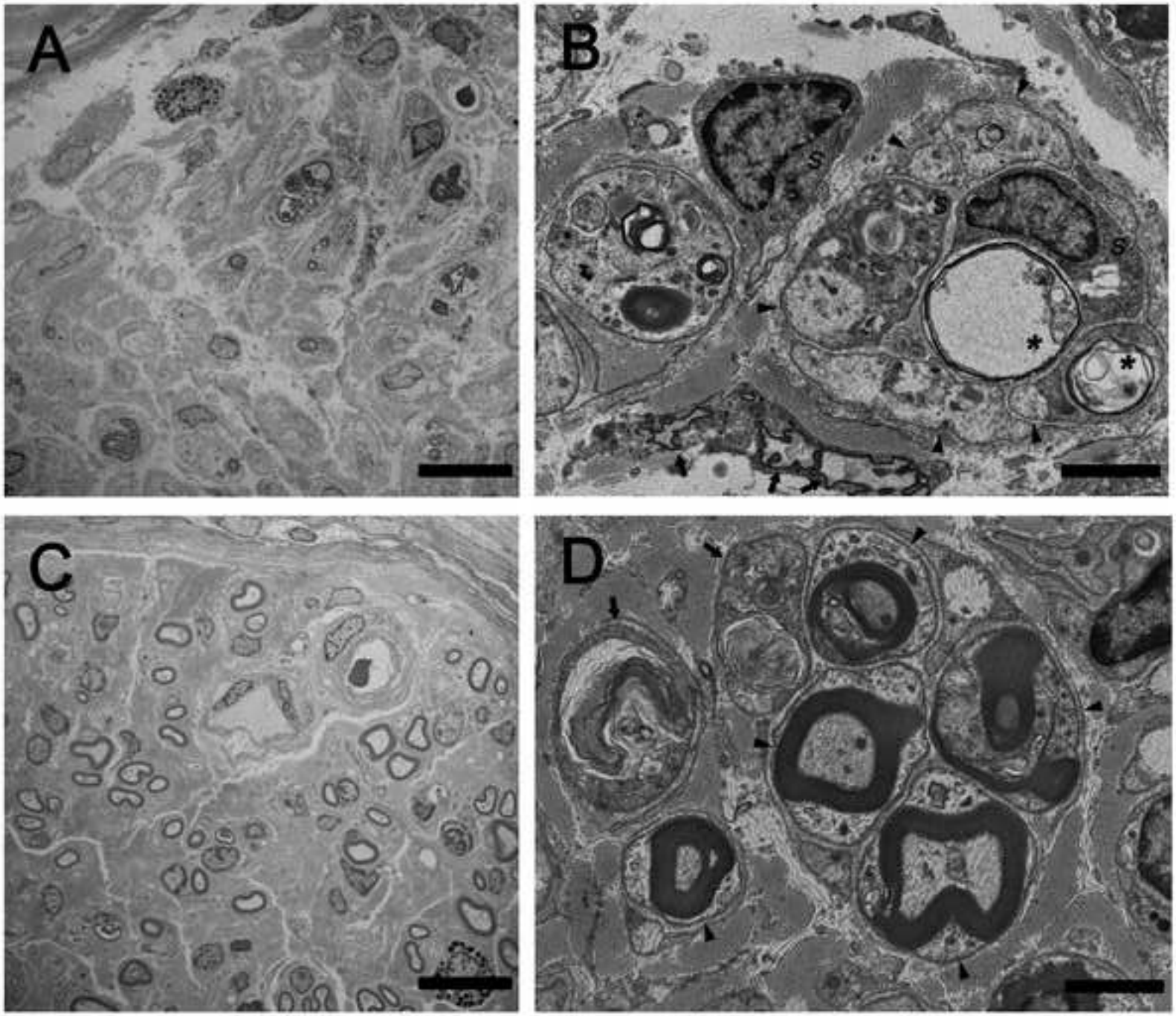

Figure-4-BW-final
Click here to download high resolution image