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Citation	Journal of Neuroscience Methods, 205(2), 246-251 <a href="https://doi.org/10.1016/j.jneumeth.2011.12.026">https://doi.org/10.1016/j.jneumeth.2011.12.026</a>
Issue Date	2012-04-15
Doc URL	<a href="http://hdl.handle.net/2115/49138">http://hdl.handle.net/2115/49138</a>
Type	article (author version)
File Information	JNM205-2_246-251.pdf



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Title:

Dental pulp can be a good candidate for nerve grafting in a xeno-graft model.

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## Abstract

Dental pulp is discarded after extirpation of dental pulp and after tooth extraction. However, it contains nerve tissue abundantly and could be used more effectively. This study was designed to examine whether a dental pulp could be a candidate of donor for nerve grafting in xenografting model. The dental pulp was obtained from a human vital extracted tooth for orthodontic treatment, and treated with freezing and thawing method for reducing antigenicity. The treated sample was inserted into chitosan mesh tube for easy suturing, and then the complex was implanted into transected sciatic nerve in Sprague-Dawley (SD) rats (dental pulp group). As controls, chitosan tubes with and without sciatic nerve harvested from another SD rats were implanted (isograft group and tube group, respectively).

As early as four weeks after grafting, regenerating axons accompanied by host Schwann cells were found to grow out through basal laminae by electron microscopy. The intact structure of basal laminae at this period suggested that they were derived from the original structure of donor graft.

Twelve weeks after grafting, sporadic axonal regeneration was confirmed by light microscopy in the dental pulp group. Thirty-two weeks after implantation, aggregation of axons was observed in this group and matched that in isograft group. The average diameter of axons in dental pulp group was comparable to that in isograft group, whereas number of minifascicles and axon proportion were smaller. It was suggested that some delay occurred in dental pulp group because of the phagocytosis and absorption of tissue debris components remained after the freezing and thawing treatment.

These findings clearly demonstrate that even dental pulp can act as conduits for regenerating axons.

#### Key words

Nerve grafting, Nerve regeneration, Dental pulp, Xenograft, Scaffold

## 1. Introduction

Sural nerve is often selected for a source of nerve grafting due to its characteristic of serving as noncritical function. Autologous nerve graft, however, has some problems such as donor site scarring, numbness, and occasionally amputation neuroma pain. In order to avoid these problems, bioabsorbable materials have been developed. We have developed a chitosan nano/micro fiber mesh tube as one of effective nerve conduits (Itoh et al., 2003; Itoh et al., 2005; Wang et al., 2009). One of the primary targets of nerve grafting research is how to enhance Schwann cell activities to form Schwann cell column resulting in enhanced axonal elongation. Development of artificial substitute is also a topic of interest. For this purpose we propound the use of dental pulp that is discarded after its extirpation treatment or tooth extraction. The pulp is a complex of peripheral nerves and blood vessels. Thick nerve bundles enter the pulp canal through the apical foramen with blood vessels and ascend centrally along the longitudinal axis of the tooth up to the odontoblast layer (Maeda et al., 1987). This anatomical structure may

be applicable for nerve conduit. Besides, dental pulp contains affluent growth factors as well as mesenchymal progenitor cells (Nosrat et al., 2001), and dental pulp stem cell is one of the topics in the regenerative medicine (Zhang et al, 2006). However, there is no trial thus far to use the dental pulp as a nerve scaffold. The aim of the present study was to examine whether basal laminae of Schwann cells in the human dental pulp may be a reliable conduit for bridging peripheral nerve defect.

## 2. Materials and Methods

In this study, sampling of human dental pulp and its application were approved by the Ethics Committee of the Graduate School of Dental Medicine, Hokkaido University. Informed consent was obtained in agreement with the Declaration of Helsinki after being given a complete description of the study. All animal procedures were approved by the Institutional Animal Care and Use Committee in Hokkaido University (approval No. 08-0094) and

performed in accordance with the provisions of the NIH Guide for the Care and Use of Laboratory Animals.

## 2. 1. Preparation of dental pulp

Dental pulp was obtained from the vital extracted tooth (Fig. 1a). The tooth was divided into 2 segments by chisel in a longitudinal direction (Fig. 1b), and the dental pulp was harvested in a whole structure (Fig. 1c). To lessen the antigenicity, the cryo-treatment of the nerve was performed prior to grafting using a previously described method (Osawa et al, 1987) with a minor modification. Briefly, the nerve was put into the liquid nitrogen several seconds for freezing and then into physiologic saline at room temperature another several seconds for thawing. This cycle was repeated five times. Radicular part of the dental pulp was selected and cut into 8 mm long for graft since it had an adequate diameter and larger number of myelinated axons than chamber part.

## 2. 2. Implantation of prepared nerves

The prepared nerve was inserted into the chitosan tube (10 mm in length) with a deacetylation rate of 93% (Hokkaido soda Co., Ltd., Hokkaido, Japan) (Itoh et al., 2003) to prevent dental pulp deterioration (Fig. 1d). Male Sprague-Dawley (SD) rats weighing 180-200 g were anesthetized with an intraperitoneal injection of sodium pentobarbital (50mg/kg body weight, Dainippon Sumitomo Pharma, Osaka, Japan), their right sciatic nerves were exposed and 3mm-sections were excised at the center of the thigh. Pulp contained tubes were implanted into the nerve defects with 2 stitches of 8-0 monofilament nylon thread at each stump. The following combinations of chitosan tubes were used for implantation.

### 1. Isograft group.

Chitosan tube containing a sciatic nerve harvested from other SD rat (8 mm of length). (N=12).

### 2. Dental pulp group.

Chitosan tube containing a human dental pulp nerve treated with freezing and thawing. (N=12)

### 3. Tube group.

Chitosan tube alone. (N=4)

These tubes were anastomosed to the nerve stumps by end-to-end suturing with 8-0 mono-filament nylon under surgical loupe, with the pulp cavity side facing proximally.

### 2. 3. Histomorphological evaluation

Samples were taken from the middle third of the grafted tube in each isograft and dental pulp group 4, 12 and 32 weeks after grafting. Samples from the tube group were taken at 12 weeks, because chitosan tube alone had been already proven to be very effective scaffold (Itoh et al., 2003; Itoh et al., 2005; Wang et al., 2009) and was used as positive control. They were embedded in Epon 812 resin after fixation with 2.5v/v% glutaraldehyde in 0.1

M phosphate buffered saline (PBS) followed by postfixation of 1% OsO<sub>4</sub> buffered with 0.1 M PBS.

Thin transverse sections of 1 µm thickness sampling from 12 and 32 weeks after grafting were stained with toluidine blue for light microscopy.

Immunohistochemical evaluation was also performed by confocal microscopy (AX80TR, Olympus, Tokyo, Japan) using the sample above 12 weeks after grafting. Four µm thick paraffin sections were incubated overnight at 4 °C with the following primary antibody solutions, monoclonal mouse anti-NF160 (1:100, clone NN18, Sigma, St Louis, MO) and rabbit anti-S-100 (1:100, Sigma, St Louis, MO) to identify axons and Schwann cells, respectively. Secondary antibodies were diluted 1:300 in 0.5% triton in PBS of goat anti-mouse IgG FITC for NF160 and goat anti-rabbit IgG TRITC for S-100. These sections were mounted on glass microscope slides with mounting medium and allowed to cure for 1 hour prior to analysis. In addition, three rats in both isograft and dental pulp group 4 weeks after

grafting were prepared for transmission electron microscope observation (TEM; Hitachi H-600; Hitachi Co., Tokyo, Japan).

Photographs of whole transverse sections of the distal nerves 32 weeks after grafting were taken to measure the diameter and density of myelinated axons and to determine the percentage of axon area. Analysis was carried out with the use of Scion Image software (Scion Co., Frederick, MD). The axon area percentage was calculated with the use of the following formula: (total area of myelinated axons / area of distal nerve) x 100%.

Statistical significance was evaluated using the Mann-Whitney's U test (Level of significance was  $P < 0.05$ ).

### 3. Results

#### 3. 1. Histological findings

In a normal dental pulp, myelinated axons aggregated and made up bundles (Fig. 2).

Twelve weeks after grafting, regenerated nerve tissue comprising aggregates of minifascicles was observed throughout the whole tube in the isograft group (Fig.3a). Minifascicles consisting of abundant small and large axons were also observed in the dental pulp group (Fig. 3b). The number of minifascicles in this group was, however, relatively small and they were found localized. The degraded myelin sheaths were still present, and inflammatory cells were seen, suggesting mixture of Wallerian degeneration and nerve regeneration. In the tube group, regenerated axons aggregated numerously as shown in the isograft group (Fig. 3c). Thirty-two weeks after implantation matured axons with thick myelin were found and the number of inflammatory cells was little in the dental pulp group, showing matched nerve maturation to the isograft group (Fig. 4a, 4b). We next carried out immunohistological investigation. In the isograft group, regenerated axons stained green with anti-neurofilament antibody were accompanied by Schwann cells stained red with Anti S-100 antibody 12 weeks after grafting (Fig. 5a). In the dental pulp group, similar regenerating pattern was found,

however, the number of this fundamental unit was few compared to the isograft group (Fig. 5b).

### 3. 2. Histological analysis

At 32 weeks after grafting, the average axon diameter in the dental pulp group was comparable to that in the isograft group (Table 1). However, the total axonal area and axonal proportion in the dental pulp group were smaller than those in the isograft group.

### 3. 3. Electron microscopic findings

In the dental pulp group, myelinated axons were already present accompanying the Schwann cells as early as 4 weeks after grafting. The structure of basal laminae nearby was intact and apparent, suggesting that it came from donor nerve treated by freezing and thawing. The thickness of myelin sheath of axons found in these basal laminae was, however, very thin, suggesting that they are immature regenerating axons extending from the

proximal stump through the basal lamina tube (Fig.6a, 6b).

#### 4. Discussion

Dental pulp is the complex of various components in the connective tissue, such as nerves, blood vessels, lymphatic ducts, and some potentialized cells. It also involves many Schwann cells as well as multi-potential cells (Torneck et al., 1985), and contains a large amount of potentiated neurotrophic factor (Nosrat et al., 2001). These dental pulp stem cells are recently popularized in the regenerative medical field (Yen et al., 2008) and they also had attracted the interests of researchers. Nosrat et al. reported that dental pulp-derived cell grafting promotes the survival of injured motor neurons in the spinal cord injury (Nosrat., 2001). Nowadays, cell or tissue engineering technique is getting popular and progressing. As a result, application of autologous other organs as a substitute for donor of nerve has been commanded. We conducted a review of the use of Schwann cell basal laminae structure of dental pulp as a possible scaffold for nerve regeneration

in a xenografting experimental model. To circumvent immunoreactive rejection, repeated freezing and thawing treatment of dental pulp was adopted. This method effectively kills all the vital cells in the graft (Osawa et al., 1987), however, endoneurial collagen fibrils and Schwann cell basal laminae remain in situ (Ide, 1996). This unique characteristics of nerve also accelerated our ambition to make use of Schwann cell basal laminae of dental nerve as a scaffold.

Meanwhile, the chitosan mesh tube is reported as an effective scaffold because of neuronal tissue affinity, appropriate permeability and mechanical strength (Ide et al., 1996; Itoh et al., 2003). Utilization of this chitosan tube as a holder of dental pulp nerve enables an easy suture and uniformity is secured because of less invasive anastomosis, and its efficacy to bridge nerve gap was confirmed in this study.

The TEM observation that myelinated axons accompanying Schwann cells were present through the remained basal laminae as early as 4 weeks after grafting indicates that it was derived from donor nerve and served as

pathways for regenerating axons. This is the first report to suggest that basal laminae of Schwann cells act as an effective guide for nerve regeneration in the dental pulp.

Newly regenerated axons were found in each group 12 weeks after grafting, however, degraded myelin sheaths were also present in some region in the dental pulp group only, suggesting that Wallerian degeneration and nerve regeneration process are co-existing. These histological findings of delayed nerve regeneration in the dental pulp group correspond to the results of histological analysis; the volume and number of regenerating nerves were a little small in the dental pulp group compared with those in the isograft group, but the axon diameter in the former matched that in the latter. This is presumably because dental pulp contains a large amount of tissue debris formed after freezing and thawing treatment; they should be phagocytised and removed before the axon begins sprouting out through the empty Schwann cell basal lamina column, resulting in delayed nerve regeneration. Moreover, various kinds of genomic DNA (double-stranded), nucleotides

(ATP) and nucleosides (adenosine) released from necrotic cells exert immunostimulatory effects on macrophages (Ishii et al., 2001; Krysko et al., 2005; Skoberne et al., 2004), followed by severe and long lasting inflammatory occurs, resulting in delayed sprouting out. Meanwhile, in the isograft group, removal of debris of degenerated nerve progressed rapidly because non-neuronal tissue is very limited, and the axon elongating from proximal stump can easily contact the conduit in donor sciatic nerve.

In conclusion, the dental pulp nerve can provide basal lamina column that acts as a scaffold for regenerating axon in xenograft model, however, other treatment method for reducing antigenicity instead of freezing and thawing, is needed to enhance the process of axons sprouting into residual basal lamina. The benefit to use dental pulp is an effective and affirmative utilization of peripheral nerve that is usually thrown away. The dental pulp could be defined as an applicable and effective organ for nerve grafting.

Acknowledgement

This work was funded by the Grant-in-Aid for Young Scientists (B: 19791477) and Scientific Research (C: 22592251) from the Ministry of Education, Culture, Sports, Science and Technology. The chitosan nano mesh tubes were kindly provided by Hokkaido soda Co., Hokkaido, Japan.

Conflict of interest: None

Contributors: None

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cryopreservation. *Tissue Eng* 2006; 12: 2813-23.

## Figure Legends

Fig. 1. Preparation of dental pulp nerve inserted in chitosan tube.

- a. Upper first premolar is extracted for orthodontic treatment to improve crowding of the dental arch.
- b. Dental pulp nerve (arrow) is harvested from the pulp cavity by a forceps.
- c. Dental pulp nerve (upper) and chitosan tube (lower).
- d. The harvested and trimmed dental pulp nerve (arrow) is inserted into the chitosan tube.

Fig. 2. Histology of the dental pulp. Toluidine blue stain.

Myelinated axons are obviously found in the dental pulp, consisting the bundles.

Fig.3 Histology of the regenerated nerve tissue at 12 weeks after grafting.

Toluidine blue stain.

- a. Isograft group (Chitosan tube containing a sciatic nerve).

Many large and small myelinated axons have matured to form a large

fascicle.

b. Dental pulp group (Chitosan tube containing a dental pulp nerve).

Matured fascicles are also present, but their number is small. Some degraded myelin sheaths (arrows) and inflammatory cells (arrow heads) are seen as well.

c. Tube group (Chitosan tube alone).

Many large and small myelinated axons are aggregated.

Fig. 4 Histological views at 32 weeks after grafting. Toluidine blue stain.

a. Isograft group.

b. Dental pulp group.

Minifascicles are abundantly seen with maturation in both groups. Nerve regeneration in the dental pulp group matches that of the isograft group.

Fig. 5 Immunofluorescence staining at 12 weeks after grafting.

Axons are stained green and Schwann cells are stained red. Co-localization

of them appears yellow.

a. Isograft group.

Many regenerated axons stained in green are accompanied by Schwann cells stained in red, indicating good nerve regeneration.

b. Dental pulp group.

Similar regenerating pattern is found, however, the number of this fundamental unit is small.

Fig. 6 Electron micrograph of the sample taken from dental pulp group at 4 weeks after grafting.

a: Schwann cells encircle the axons and myelination has already proceeded.

The basal laminae of the Schwann cells are found and they are separated into small compartment. (X 2,500)

b: High-power view. Myelinated axon accompanying Schwann cell on the basal laminae (allow) are seen. The basal lamina is left intact. (X 20,000)

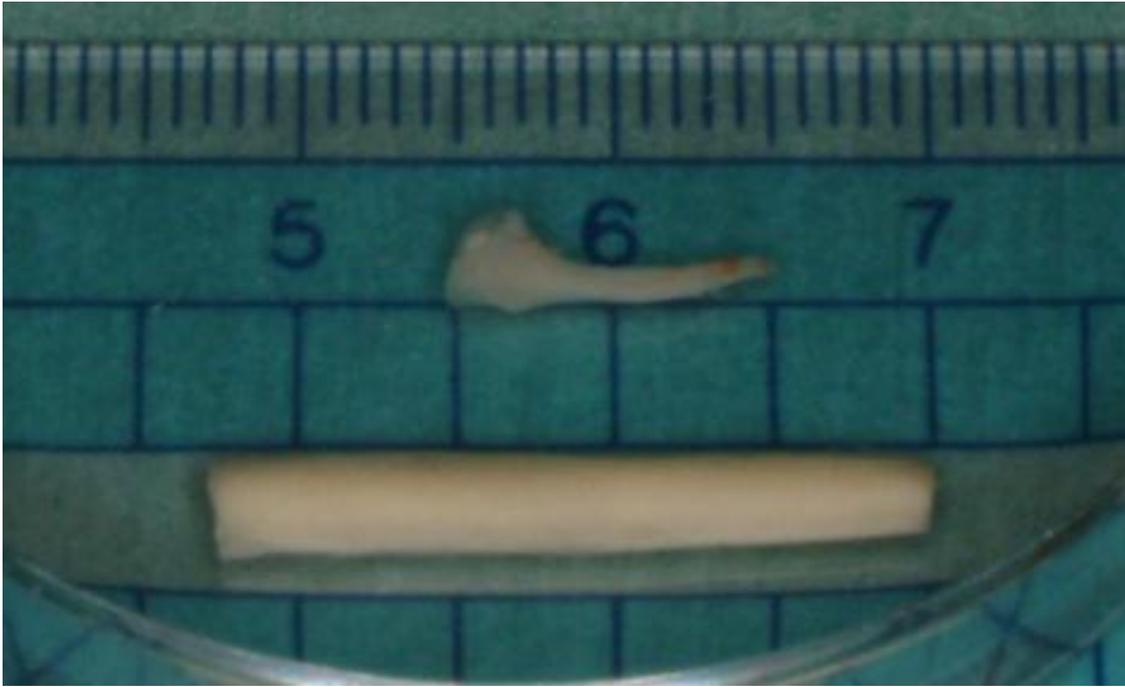


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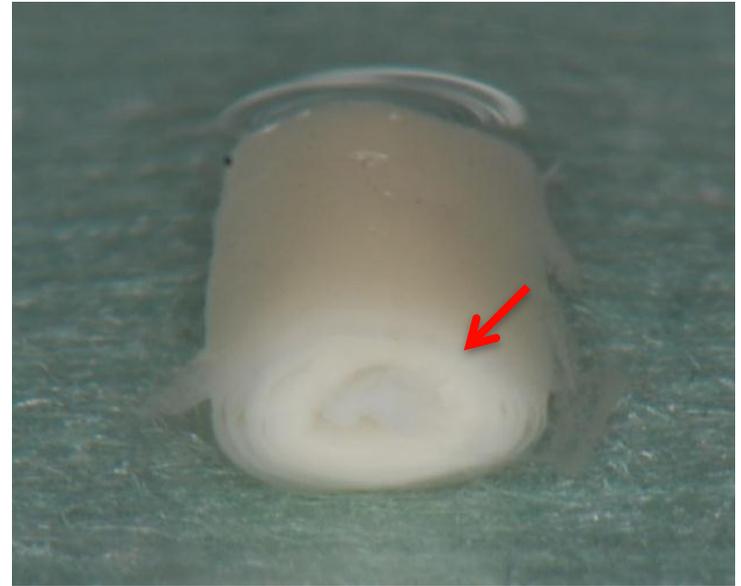


b

Fig. 1



c



d

Fig. 1

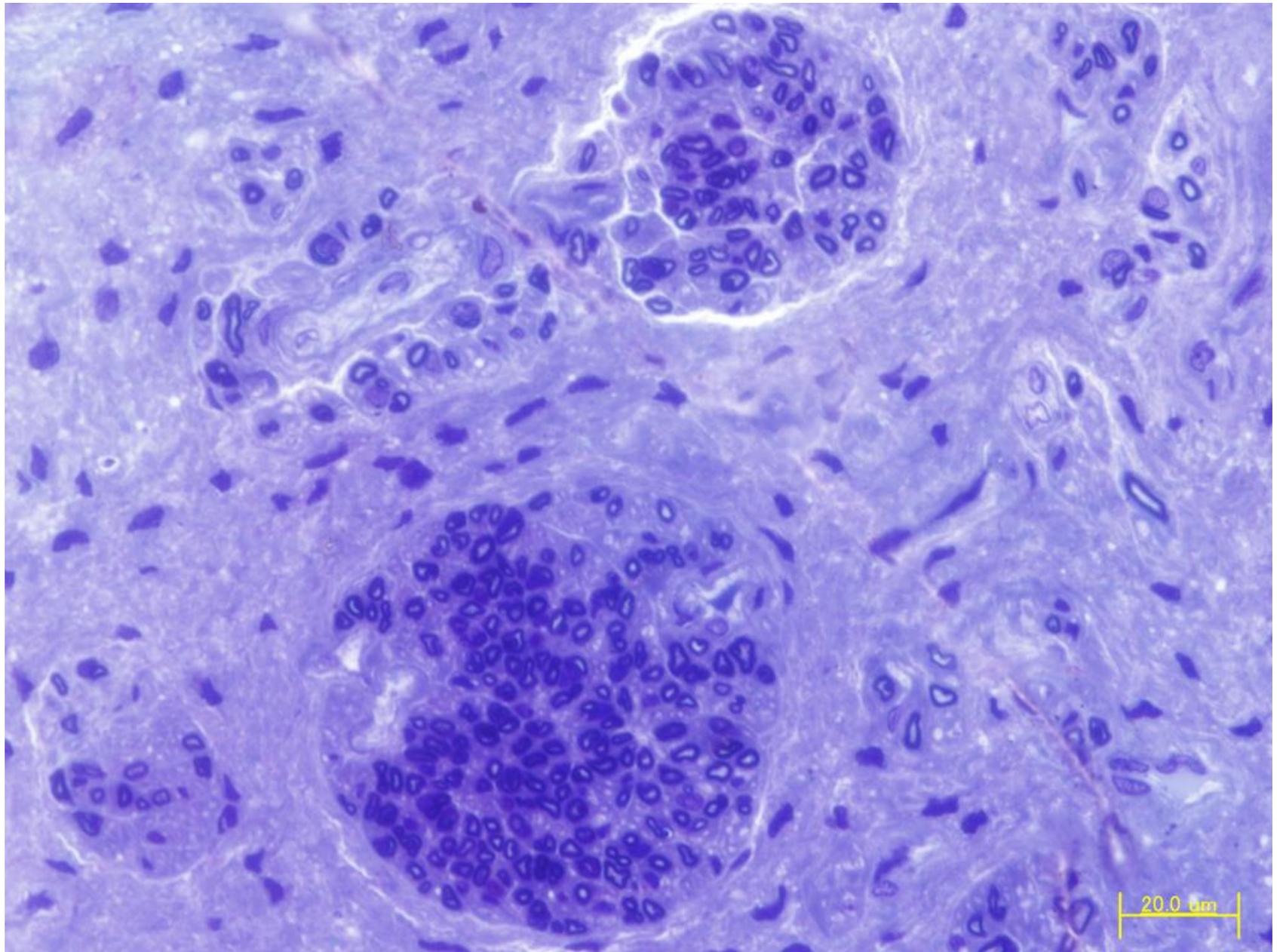


Fig. 2

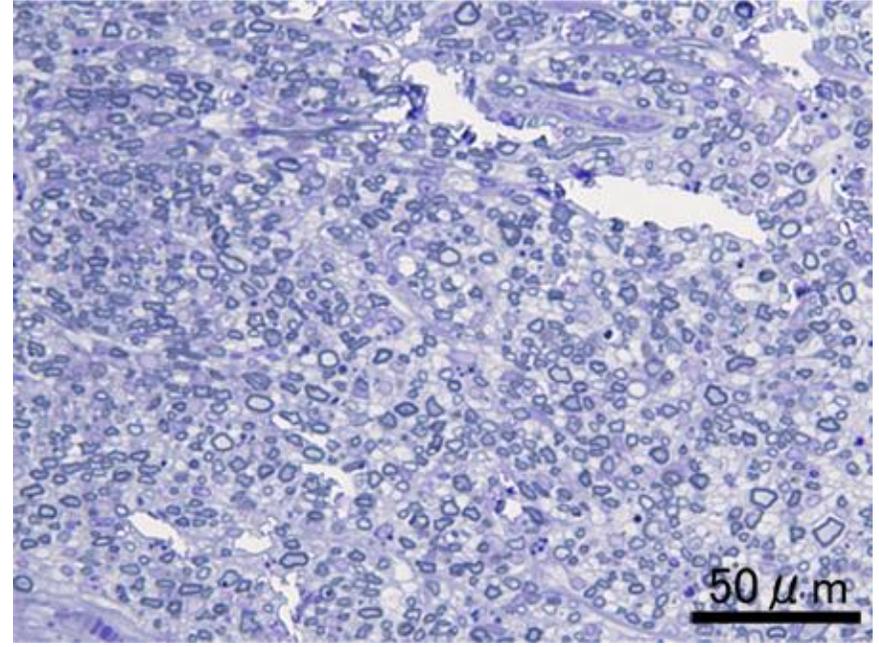
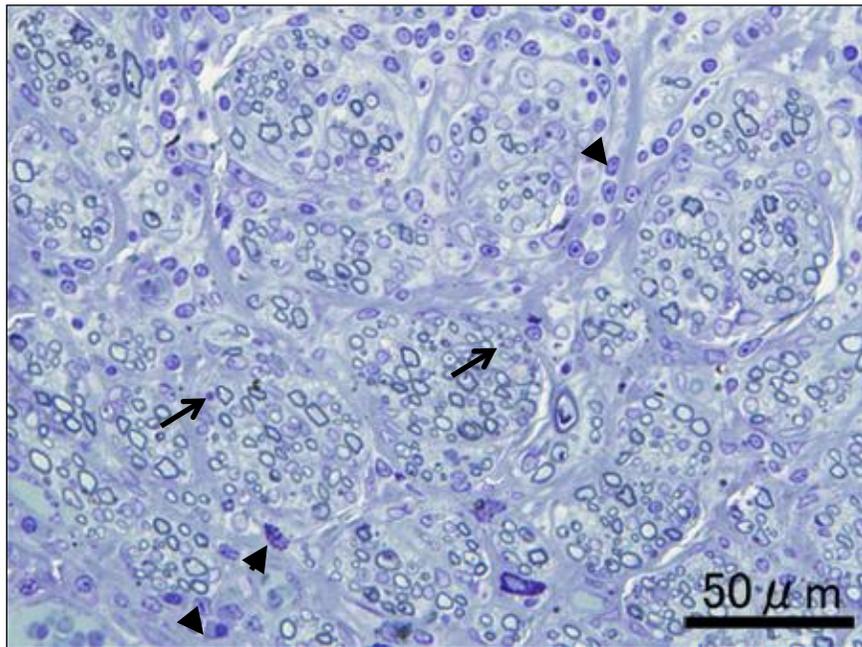
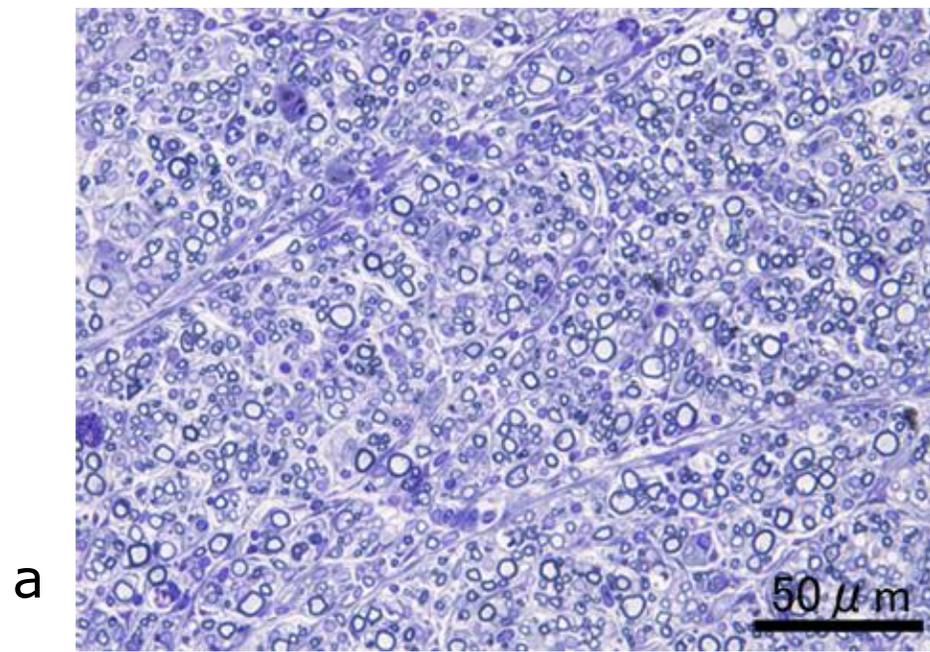
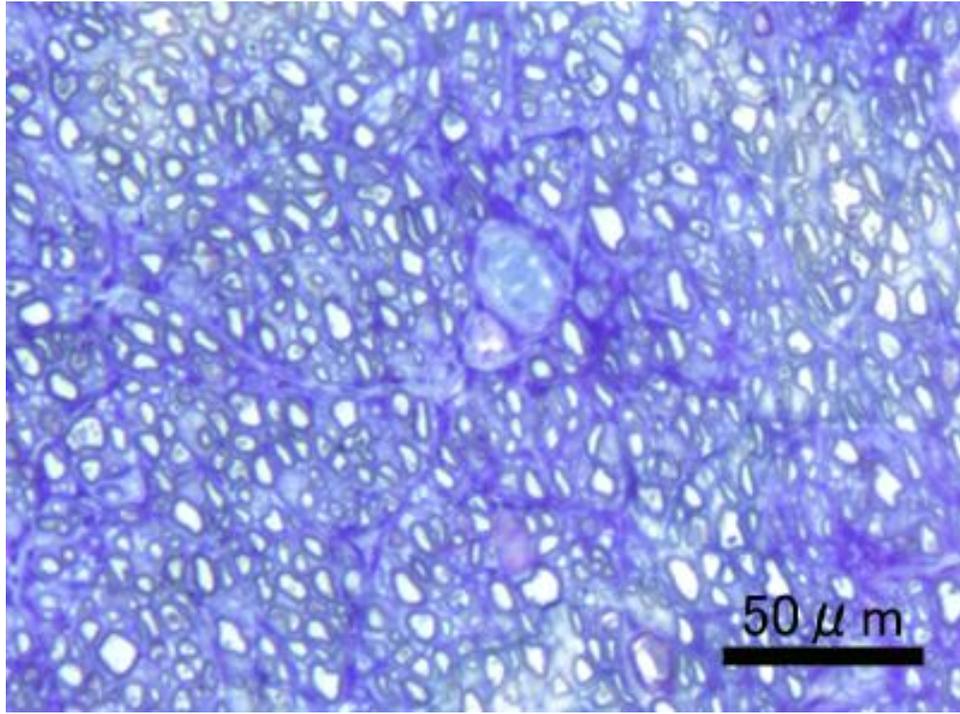
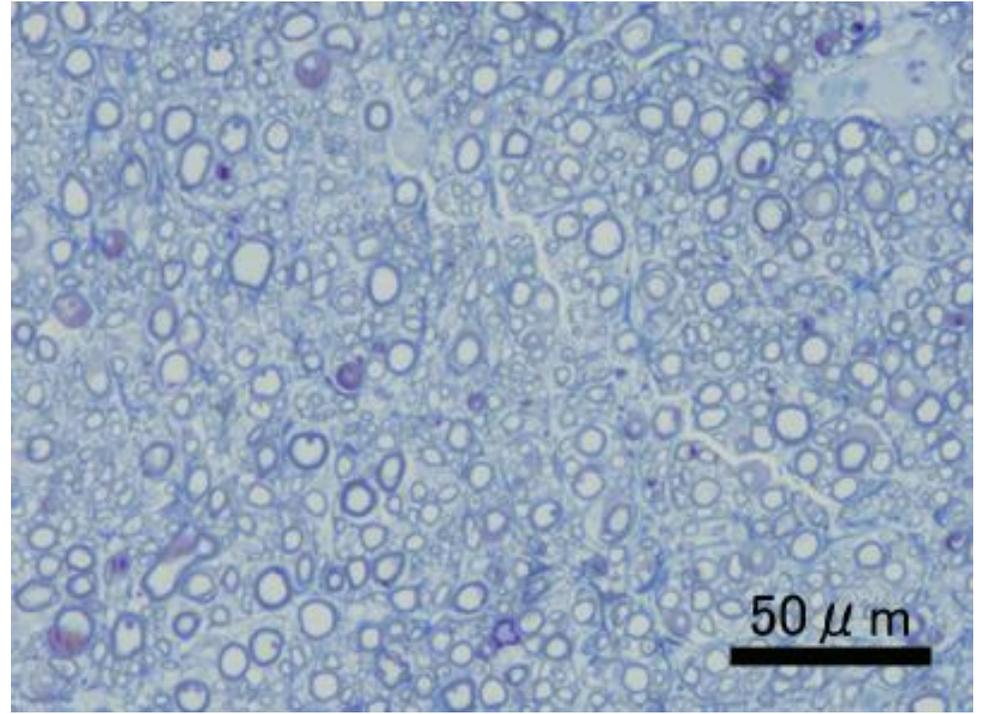


Fig. 3

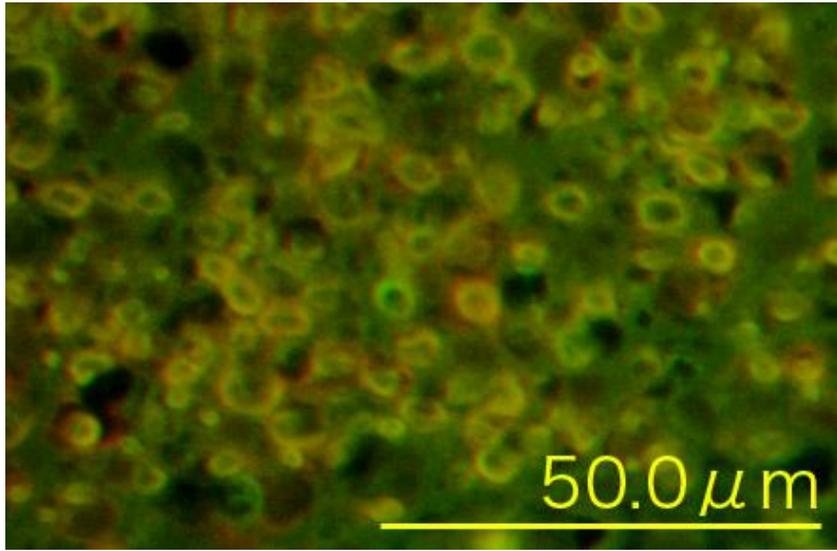


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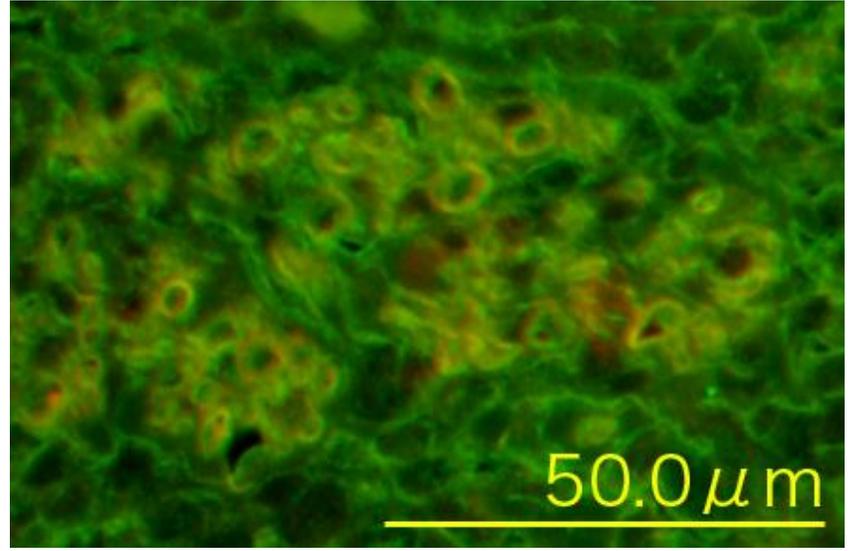


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Fig. 4

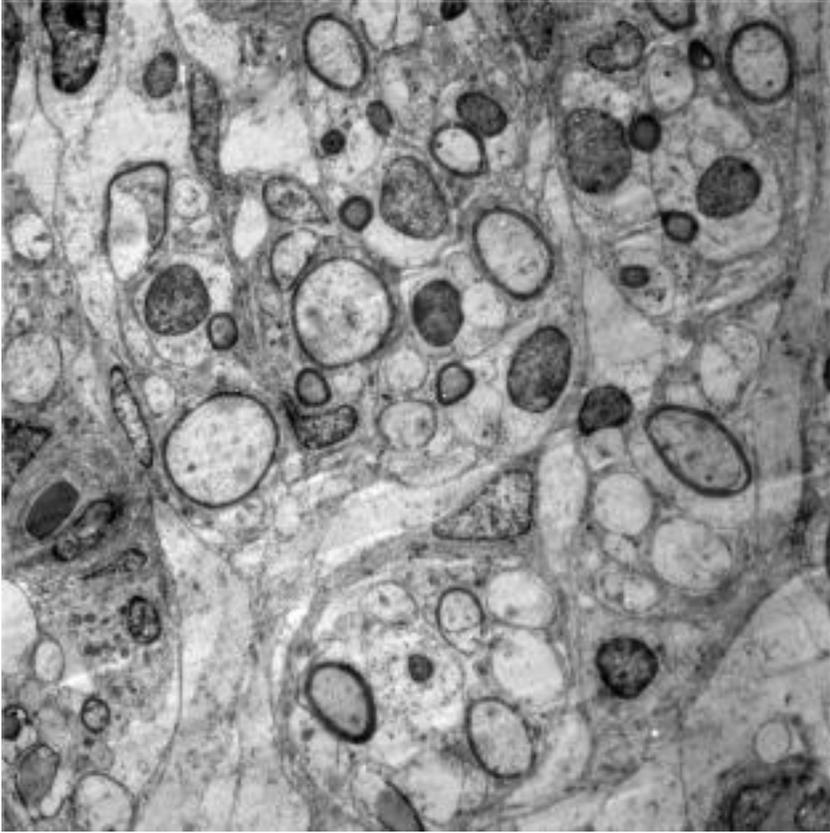


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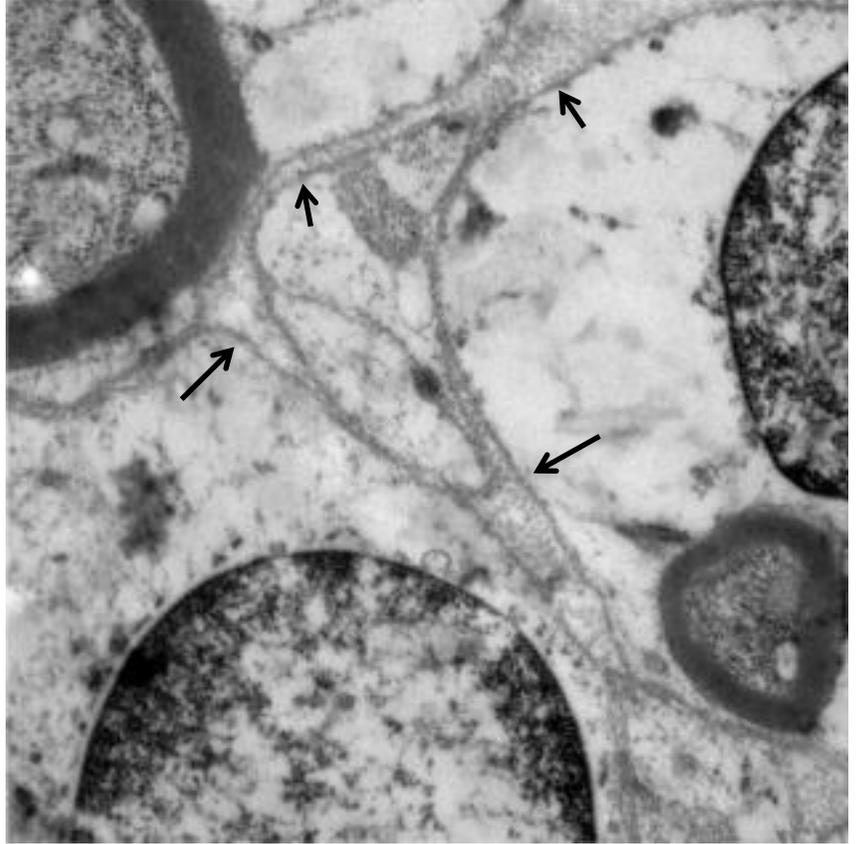


b

Fig. 5



a



b

Fig. 6

Histological analysis of the grafted tubes (means±SD)

	Dental pulp Group	Isograft Group
Number	N=4	N=5
Axon diameter (μm)	2.43±0.17	2.09± 0.37
Axon density (X10 <sup>3</sup> /mm <sup>2</sup> )	20.10±3.14*	46.37±11.15*
Axon area (%)	20.60±1.94*	33.79± 6.82*

There is no significant differences in axon diameter between 2 groups.  
 The axon density and axon area in experimental group are significantly smaller than those in isograft (\*p<0.05).

Table 1