CHAPTER 3

MATERIALS AND METHODS

This chapter involves protocol of axotomy only, axotomy with ligation, and treatment of LIF on median and ulnar nerve in young adult rat. Sensory and motor neurons were counted. The location of median and ulnar motor neurons in spinal cord was identified. This localization was confirmed in this study using the retrograde transport of Fluorogold. All detailed methodologies are described in a step-wise manner as follows;

The Experimental Animals

Experiments were carried out on 140 adult male Wistar rats (age 6 weeks) weight between 100-110 g. Animals were housed in individual cage under controlled environmental conditions (12:12 light-dark cycle, room temperature 37^{o}c). Animals were given food and tap water ad libitum.

Animal Preparations and Measurement

Animals were anesthetized by intraperitoneal injection of 4% chloral hydrate at the initial dose of 1ml/100g bodyweight, then, they were maintained at anaesthetic condition by observing foot withdrawal reflex to assess the level of anesthesia.

Experimental Protocol

Animals were divided into three groups of similar age.

1 Axotomy only group
2 Axotomy with ligation group
3 Treatment of Leukemia inhibitory factor (LIF) group

1 Axotomy only group

Surgical Procedures

The median and ulnar nerve of adult rats were axotomised with the protocol described by Cheema SS. et al. [32] Surgery was performed using aseptic techniques under 4% chloral hydrate (1ml/100g ip). They were placed on the surgical table in supine position, the right median and ulnar nerves were exposed at axillary brachial plexus level and was axotomised using a pair of iridectomy scissors. A 5-0 Ethicon silk suture was used to close the skin incision. Upon recovery to consciousness, the lesioned animals displayed an obvious dorsiflexion of the right forepaw. The contralateral median and ulnar nerves serve as the intact control side (Figure 5).

2. Axotomy with ligation group

Surgical Procedures

The median and ulnar nerves of adult rats were axotomised with ligation the protocol described by Vesterggaard S. et al.[36] Surgery was performed using aseptic techniques under 4% chloral hydrate (1ml/100g ip). They were placed on the surgical table in supine position, the right median and ulnar nerve were exposed at axillary brachial plexus level, were ligated at two positions (approximately 2 and 4 mm. elbow to the ganglia), and were transected between the two ligatures. The skin incision was close by silk. Upon recovery to consciousness, the lesioned animals displayed an obvious dorsiflexion of the right forepaw. The contralateral median and ulnar nerves serve as the intact control side (Figure 6).

Tissue Preparation

At 1,2,4,6,8,16 and 32 weeks after axotomy or axotomy with ligation, the animals were anesthetized with 4% chloral hydrate (2ml/100g ip) and killed by perfusion of fixative through the aorta ascendens. The perfusion was initiated by prerinsing with MTPBS (Mount Tonicity Phosphate Buffer Saline) for 10 seconds followed by a 10 minute

perfusion with Bouin's solution (Figure 7A). After the perfusion, the right and left C7, C8 and T1 dorsal root ganglia and spinal cord were removed (Figure 7B) and postfixes for a further 24 hour in the same fixative. The cord segments were dehydrated and embedding in the paraffin (Figure 8).

Histological Techniques

Serial cord sections of 10 μm thickness were cut by microtome. (Figure 9) The sections were collected in gelatin and were then picked up onto microscopic glass slides. Identification of the cord structures was facilities by staining the sections with 0.1% cresyl violet (Sigma). After the staining, the sections were dehydrated through graded series of ethyl alcohol and xylene and mounted under a cover-glass.

Counts of neuron

- Dorsal root ganglion (DRG) cells counting

Neurons displaying a prominent nucleolus were counted at a final magnification of 500X. Counts were performed on every fifth cord section.

The proportion of neurons lost was calculated as a percentage loss as follows

$$\frac{\text{neurons in the intact C7,C8 or T1 DRG} - \text{neurons in the axotomised C7,C8 or T1 DRG}}{\text{neurons in the intact C7,C8 or T1 DRG}} \times 100$$

To ensure an accurate estimation of neuronal loss, two steps were taken. First the section thickness exceeded the greatest nucleolar height by a factor of about 4.

Secondly, contralateral controls were used throughout all animals were based on proportional rather than absolute values.

The proportion of neurons survival was calculated as a percentage survival as follow

$$\frac{\text{neurons in the axotomised C7,C8 or T1 DRG}}{\text{neurons in the intact C7,C8 or T1 DRG}} \times 100$$

- Motoneuron counting

The location of median and ulnar motor neurons in spinal cord were identified. This localization was confirmed in this study using the retrograde transport of Fluorogold. The protocol described by Cheema SS. et al. [33]

Retrograde axoplasmic transport of fluorogold

The three adult male Wistar rats (age 6 weeks) were anesthetized by intraperitoneal injection of 4% chloral hydrate at the initial dose of 1ml/100g bodyweight, then, they were maintained at anaesthetic condition by observing foot withdrawal reflex to assess the level of anesthesia. They were placed on the surgical table in supine position, the right median and ulnar nerve were exposed at axillary brachial plexus level and were transected without ligation.

The proximal stump of the median and ulnar nerves was wrapped with a 2-mm^3 piece of gelfoam soaked in 1% Fluorogold(Fluorochrome) solution for 48 hrs (Figure 10). The skin incision was close. The contralateral median and ulnar nerve serve as the intact control side.

Tissue Preparation

After 48 hrs animals were deeply anesthetized with 4%chloral hydrate (2 ml/100g injected ip) and exsanguinated by transcardial perfusion. The perfusion was initiated by prerinsing with MTPBS (200 ml) followed by a 4% paraformaldehyde in 0.2 M phosphate buffer at pH 7.4. Immediately after perfusion, the vertebrae overlying the right and left C7,C8 and T1 dorsal root ganglia were removed and postfixes for a further 24 hour in the same fixative. The cord segments were dehydrated and embedding in the paraffin.

Histology

Serial cord sections of 10 μm thickness were cut by microtome. The section were collected in gelatin and were then picked up on to gelatinized slides once sections are dry they can be viewed under a fluorescent microscope with a UV filter.

Neurons displaying a prominent nucleolus were counted and were calculated by same as dorsal root ganglia cells counting.

Statistical Analysis

Data obtained from the experiments, using One-way Analysis of Variance (ANOVA) compared the mean and standard error of mean (S.E.M) for each week. Significant different results in the ANOVA were further analyzed by post-hoc testing using Duncan-multiple range test at p-value of ≤ 0.05.

3. Treatment of Leukemia inhibitory factor (LIF)

Surgical Procedure

The median and ulnar nerve of adult rats were axotomised and treatment of LIF with the protocol described by Cheema SS. et al.[32,33] Surgery was performed using aseptic techniques under 4% chloral hydrate (1ml/100g ip). They were placed on the

surgical table in supine position, the right median and ulnar nerves were exposed at axillary brachial plexus level and was axotomised using a pair of iridectomy scissors.

Treatment

The proximal stump of the median and ulnar nerve was wrapped with a 2-mm^3 piece of gelfoam (Upjohn) containing either 20 μl phosphate-buffered saline, or 20 μg of recombinant murine LIF (AMRAD Corporation, Melbourne, Australia) in 20 μl phosphate buffered saline. (Figure 11) The skin incision was close. The contralateral median and ulnar nerves serve as the intact control side.

Tissue Preparation

After survival periods of 1,2, and 4 weeks, the adult rat were anesthetized same as axotomy group and killed by perfusion. The perfusion was initiated by prerinsing with MTPBS for 10 seconds followed by a 4% paraformaldehyde in 0.2 M phosphate buffer at pH 7.4. Immediately after perfusion, the vertebrae overlying the right and left C7,C8 and T1 dorsal root ganglia were removed and postfixes for a further 24 hour in the same fixative. The cord segments were dehydrated and embedding in the paraffin.

Histological Techniques

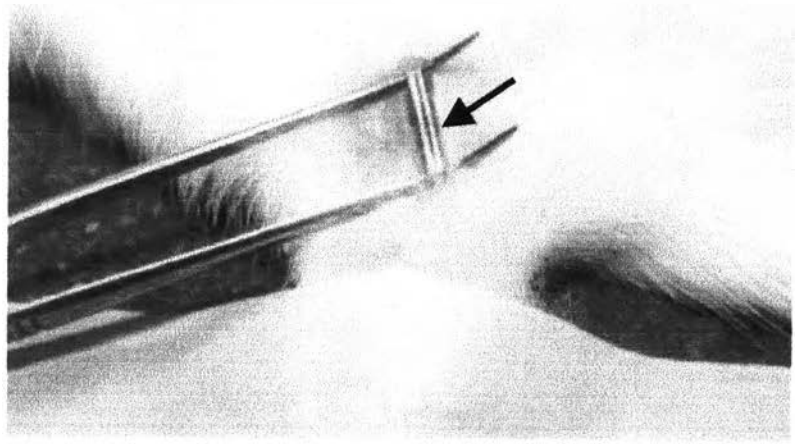
Serial cord sections of 10 μm thickness were cut by microtome. The sections were collected in gelatin and were then picked up onto microscopic glass slides. Identification of the cord structures was facilities by staining the sections with 0.1% cresyl violet (Sigma). After the staining, the sections were dehydrated through graded series of ethyl alcohol and xylene and mounted under a cover-glass.

Counts of neuron

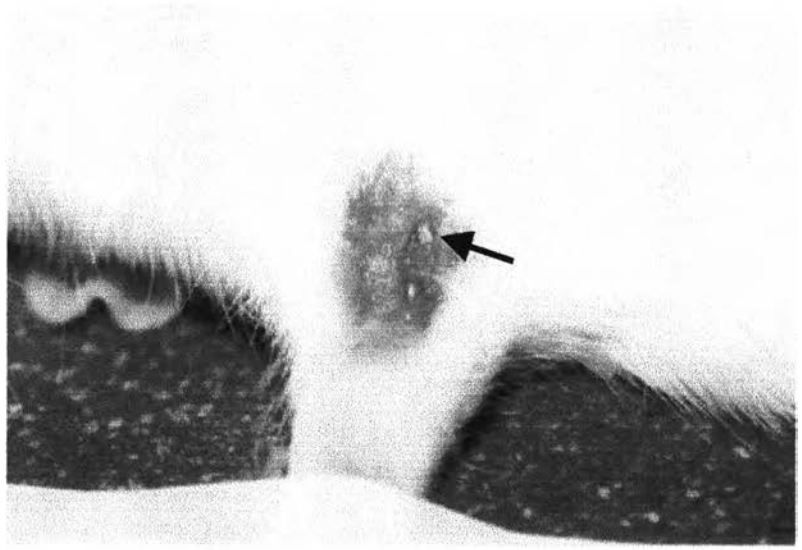
Sensory and motor neuron were counted and calculated by same as axotomy only and axotomy with ligation group.

Statistical Analysis

Comparison of data between the control and the treated rat were made calculated using Independent pair t-test at p value $\leq$ 0.05 and were considered as significant difference. Data present as mean $\pm$standard error of mean (S.E.M).

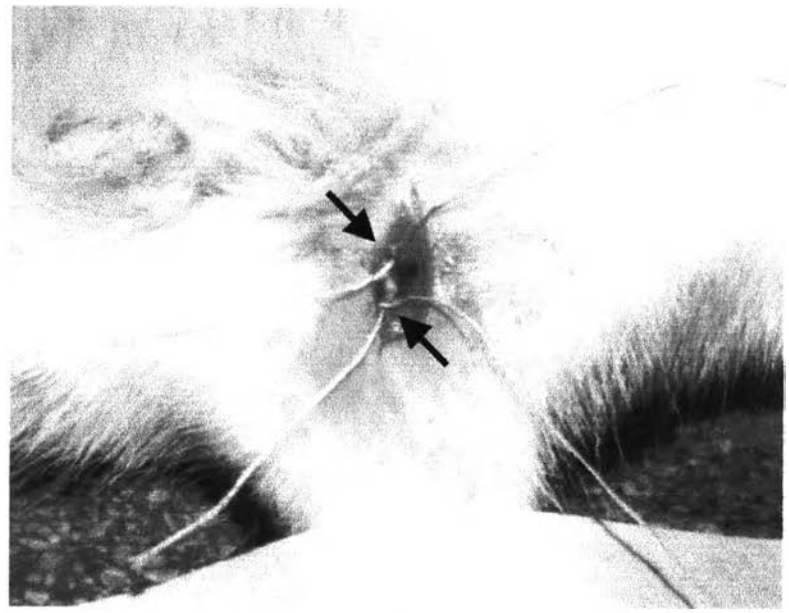


(A)

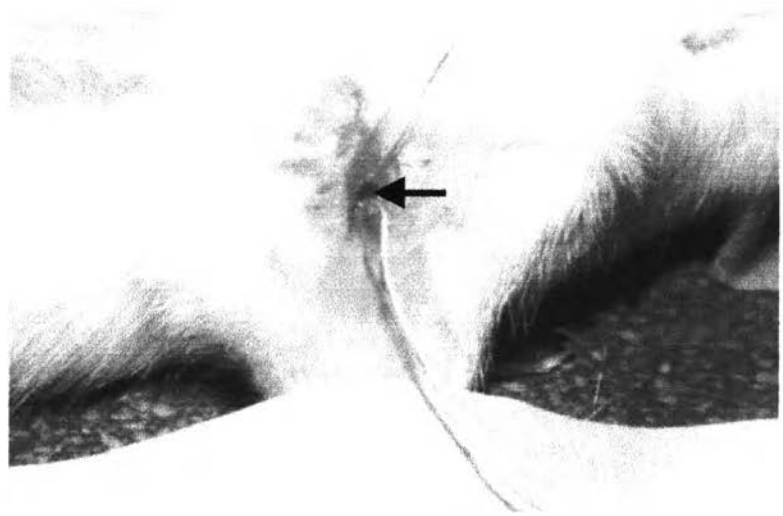


(B)

Figure 5 *(A)*, The right median and ulnar nerve were exposed at axillary brachial plexus level (arrow) and, *(B)* axotomised using a pair of iridectomy scissors. (arrow)

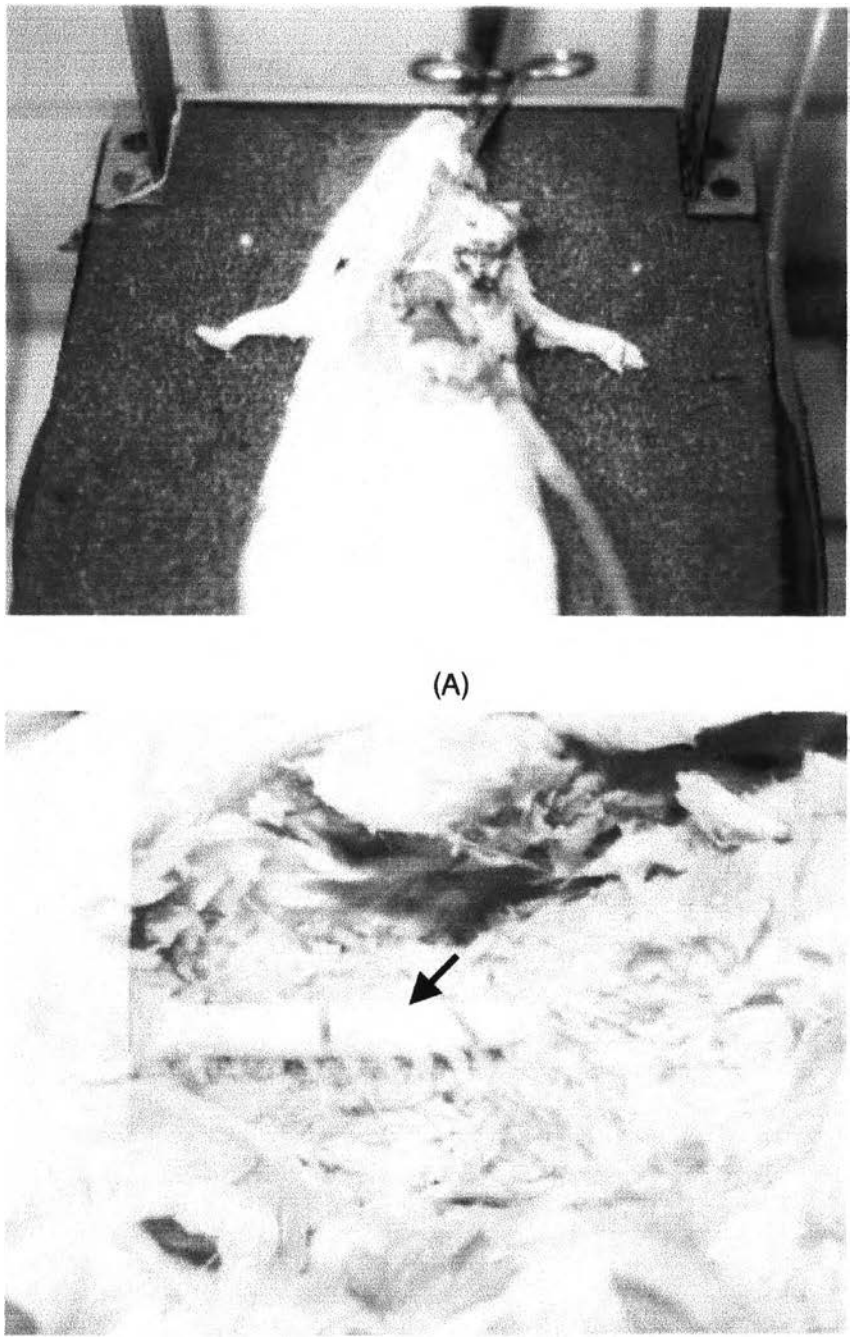


(A)



(B)

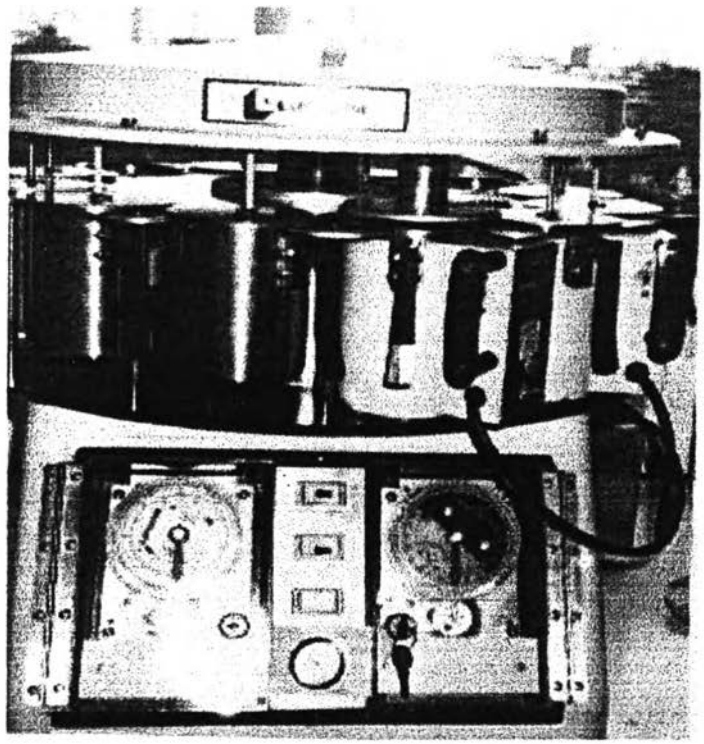
Figure 6 The right median and ulnar nerve were exposed at axillary brachial plexus level, ligated at two positions (approximately 2 and 4 mm. elbow to the ganglion)(arrow) *(A)*, and transected between the two ligatures (arrow) *(B)*.



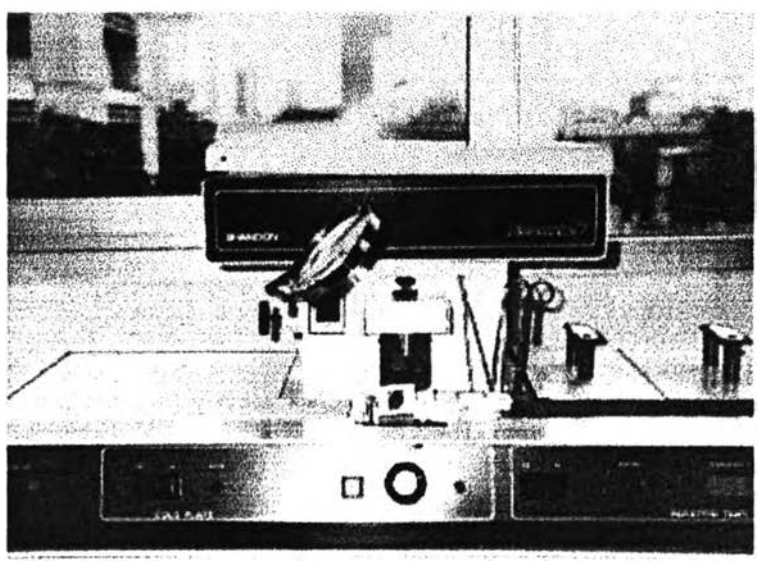
(A)

(B)

Figure 7 *(A)*, The perfusion of fixative through the aorta ascendens with MTPBS for 10 seconds followed by a 10 minute perfusion with Bouin's solution. *(B)* The spinal cord levels C7-T1 (arrow) were removed after perfusion.



(A)



(B)

Figure 8 *(A)*, The tissue processor and (B), tissue embedding center were used to process the cord segment

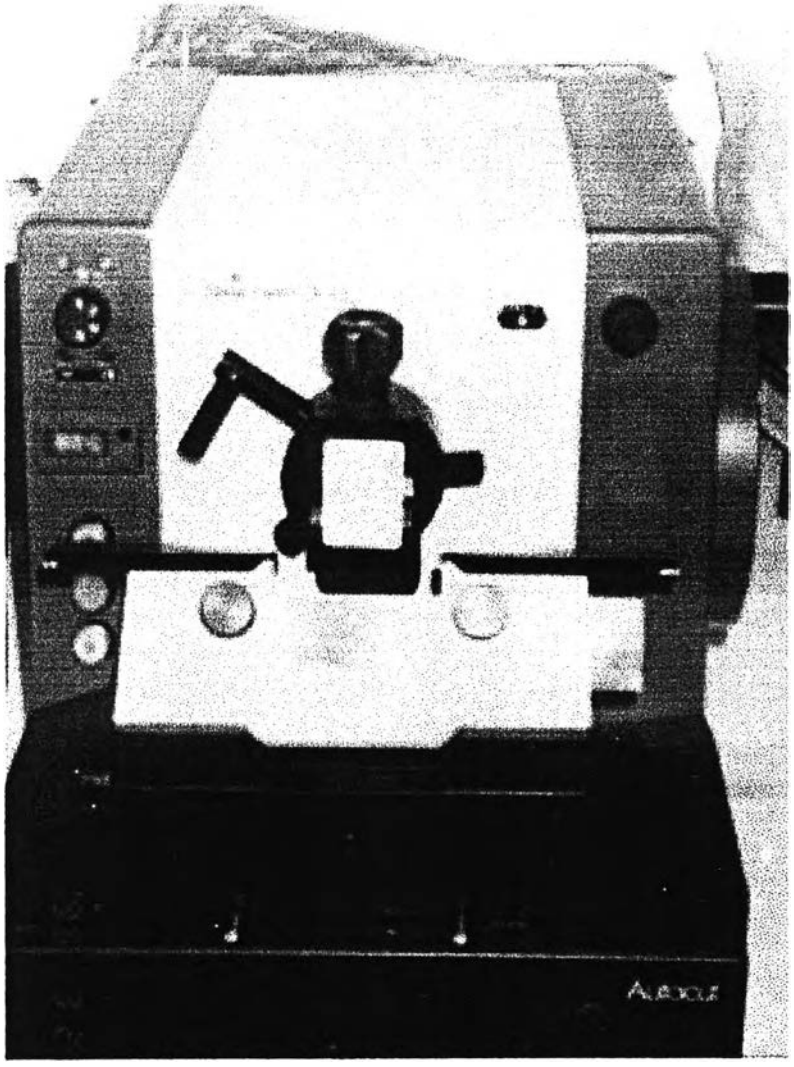


Figure 9 Microtome was used to cut the cord segment.

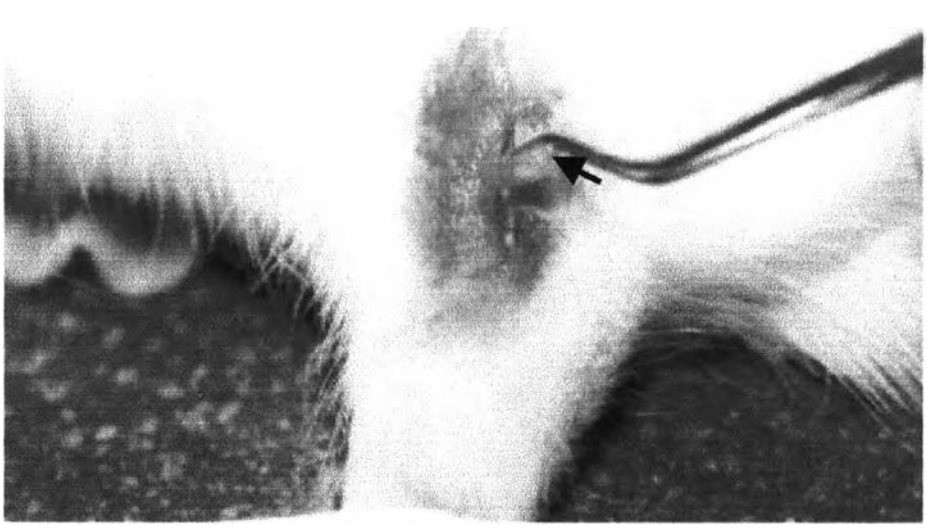


Figure 10 Fluorogold labeling technique. The right median and ulnar nerve were exposed at axillary brachial plexus level and transected without ligation. The proximal stump of the median and ulnar nerve were wrapped with a 2-mm^3 piece of gelfoam soaked in 1% Fluorogold. (arrow)

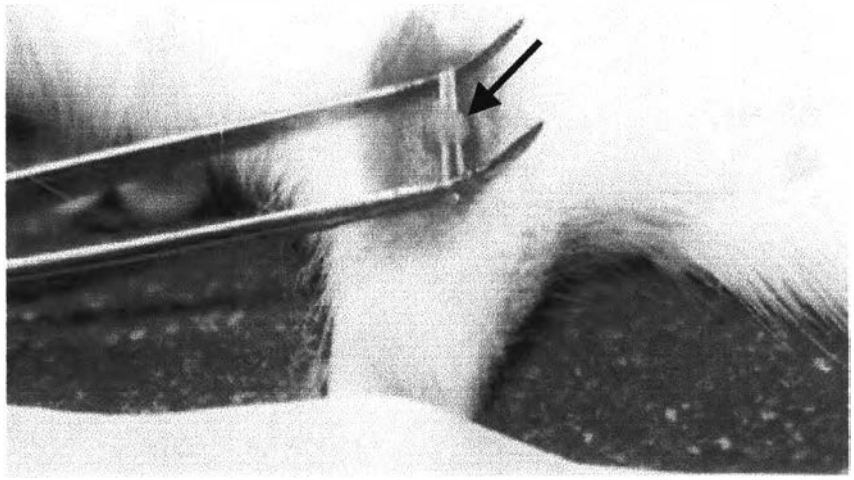


Figure 11 A method employed to treat injured nerves with PBS or LIF. The proximal stump of the median and ulnar nerve was wrapped with a 2-mm^3 piece of gelfoam containing either 20 μl phosphate-buffered saline saline, or 20 μg of LIF in 20 μl phosphate buffered saline. (arrow)