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Short Communication

COMPARISON OF HEMATOXYLIN AND EOSIN STAINING WITH AND WITHOUT PRE TREATMENT WITH MARCHI'S SOLUTION ON NERVE SAMPLES FOR NERVE DEGENERATION AND REGENERATION STUDIES

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ABSTRACT: The study was conducted on four healthy guinea pigs (*Cavia porcellus*) of either sex in which the nerve was identified and subjected to crush injury with the tip (3mm) of a curved hemostatic forceps. 30 days after the injury nerve samples were collected and subjected to Hematoxylin and Eosin staining with or without pretreatment with Marchi's solution. The routine Hematoxylin and Eosin (H&E) stained all neural elements in various intensities of pink and in purple and the degenerative changes were seen as vacuoles ranging from vacuolated foci- containing eosinophilic material and associated with a distorted cell nucleus to larger, multilocular, linear array of compartmentalized digestion chambers supposed to contain myelin debris .The myelin on the other hand appeared as empty zones in H&E staining. Combining Marchi's and H & E procedures revealed the presence black aggregates/ deposits in the vacuoles and digestion chambers. This method confirmed the presence of degenerated myelin inside the vacuoles and digestion chambers and thus may allow better analysis of nerve damage and regeneration.

Key words: Nerve tissue, Staining, Osmium Tetroxide pretreatment, H & E staining.

The study of peripheral nerve injuries and diseases is attracting more and more interest among both basic and clinical researchers with the goal of enhancing nerve healing. Assessment of histopathological changes of sciatic nerve by light microscopy is a pillar of the investigation of nerve damage and regeneration (Algora *et al.* 1996, Park *et al.* 2011, Tan *et al.* 2013). Hematoxylin and Eosin (H&E) are the most used staining agents for light microscopy in pathology and research. H&E staining is commonly non-specific for many tissue elements due to its electropolar nature (Carriel *et al.* 2011a, b). Using this method, it is not possible to observe the myelin and the ECM is nonspecifically stained. Therefore, H&E is not an ideal method for accurate analysis of the nerve regeneration process (Di Scipio *et al.* 2008, Raimondo *et al.* 2009, Carriel *et al.* 2011a).

Regarding the myelin, it is composed by several types of lipids and lipoproteins. Unfortunately, a significant amount of these elements are dissolved by organic solvents during tissue processing, but some of them are preserved (associated to structural proteins), thus allowing their identification. Interestingly, pre-embedding myelin sheath staining with Os O_4 (used in Marchi method) is a useful alternative to identify myelin with high accuracy in light microscopy (Di Scipio *et al.* 2008, Li *et al.* 2013). This method can be combined with other histochemical methods including standard H&E and Masson's method or immune-histochemical procedures. In the original March technique (Marchi *et al.* 1986, Marchi 1992), pieces of tissue are immersed for 8-10 days in an aqueous solution of potassium dichromate and osmium tetroxide.. In more recent years Marchi's fluid has been replaced with a solution containing formaldehyde, osmium tetroxide and potassium chlorate (Swank and Davenport 1934), which is usually used on blocks of formaldehyde-fixed tissue. The hydrophilic oxidizing agent (dichromate or chlorate ion) prevents the reduction of osmium bound as esters to unsaturated sites in the relatively hydrophilic myelin (Bayliss 1984, Adams 1965, Adams *et al.* 1967). The purpose of this study was to observe and compare the histopathological changes, after sciatic nerve injury, by standard HE and pre-embedding treatment by Marchi's staining protocol.

The study

Four healthy guinea pigs (*Cavia porcellus*) of either sex were used in this study. Prior to the study, all the animals were provided with the standard diet, *ad libitum*

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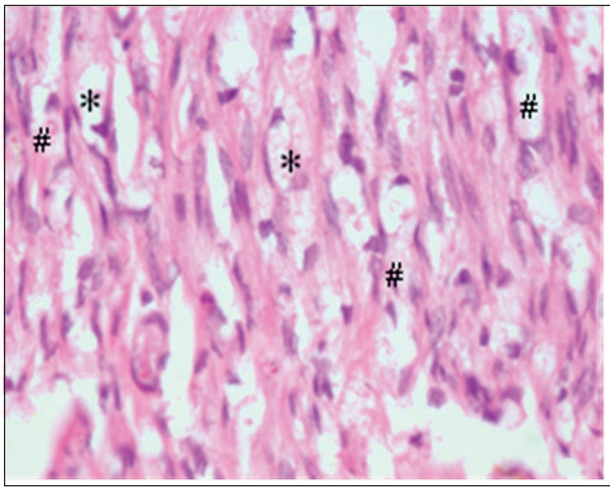

Fig. 1. Photomicrographs of crushed nerve in longitudinal section in Guinea pigs.
[*Shows vacuolated foci- larger, multilocular, containing less intense Eosin ophilic material and associated with a distorted cell nucleus. # shows digestion chambers as larger, multilocular, linear array of compartmentalized myelin debris. (Hematoxylin and Eosin, 40X)].

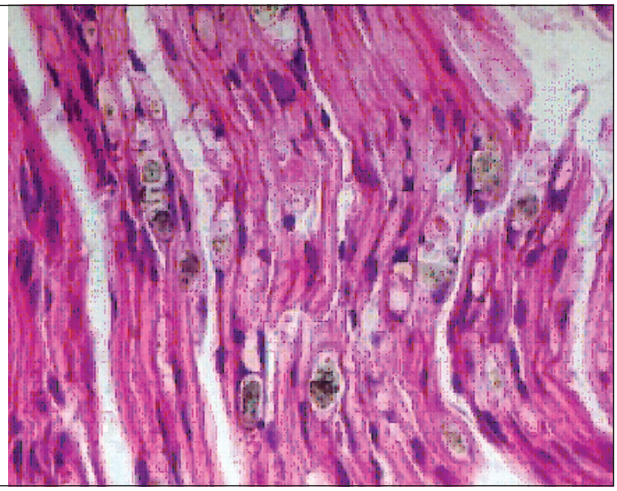

Fig. 2. Photomicrographs of crushed nerve in longitudinal section in Guinea pigs.
[Digestion chambers filled with dark stained degenerated myelin can be seen (Combined Marchi's and H&E, 40 X)].

water and allowed to acclimatize for approaching, handling and animal house conditions for a period of 10 days. The study was approved by animal ethics of the institute.

The animals were anaesthetized by intramuscular injections of xylazine and ketamine in the thigh muscles as per standard protocol. Left thigh region was prepared for aseptic surgery. The animals were secured in right lateral recumbency. A linear skin incision of 3-4 cm length was made at lateral surface of the animal's left thigh and blunt dissection was performed to separate the biceps femoris and semitendinosus muscle to expose the sciatic nerve. Few drops of local anaesthetic (Lignocaine 2%) were instilled over the nerve to provide local analgesia. Five minutes later, the nerve was identified and subjected to crush injury with the tip (3 mm) of a curved hemostatic forceps. The site of the crush injury was the intermediate region of the sciatic nerve in its course down the thigh region before bifurcation into the tibial and peroneal nerves. The strength used for compression was standardized at the second locking position of the hemostatic forceps for 60 seconds. The muscle and skin were sutured in standard manner. Postoperatively, all the animals were housed in individual boxes and administered with broad spectrum antibiotic enrofloxacin for 5 consecutive days and analgesic meloxicam for three days, intramuscularly. The dressing of the skin wound was done using povidone iodine and skin sutures were removed on 10th postoperative day.

Animals were euthanized on 30th day by intracardiac injection of over dosage of thiopental sodium. Crushed nerve samples were collected and stored in 10 % formalin. Two of the nerve samples were processed for paraffin embedding and cut into 4-5µ thick sections and stained with Hematoxylin and Eosin and other two nerve samples were teased with needle and subjected to pre-embedding treatment with Marchi's solution. For this purpose the formalin fixed specimen were kept for five days in 3% potassium dichromate and then transferred to Marchi's solution (Table 1) for 15 days. Thereafter the specimen was processed for paraffin embedding and cut into 4-5µ thick slice and stained Hematoxylin and Eosin.

Observation

After sciatic nerve injury, Wallerian degeneration occurs in the distal stump consisting of a series of processes, including axonal degeneration, myelin degeneration and disintegration, Schwann cell proliferation, infiltration of macrophages and mast cells, and axonal and myelin debris clearance (Dubovy 2011).

Table 1. Composition of marchi' solution used for pre-embedding treatment to the nerve samples.

Marchi's Solution	
4% Osmium tetroxide	1.5 ml 1%
Potassium chlorate	15 ml
37% Formaldehyde	3 ml
Glacial acetic acid	0.3 ml
Distilled water	80 ml

The routine Hematoxylin and Eosin (H&E) stained all neural elements in various intensities of pink and in purple, the cell nuclei (Carriel *et al*. 2014). The degenerative changes were seen as vacuoles ranging from vacuolated foci- containing Eosin ophilic material and associated with a distorted cell nucleus to larger, multi-locular, linear array of compartmentalized digestion chambers supposed to contain myelin debris (Fig. 1). The myelin on the other hand appeared as empty zones in H&E section. It was thus difficult to state whether the vacuoles are empty or contain myelin after simple H&E staining. Peripheral nerve regeneration is the process when degenerated axons and myelin are cleared with the establishment of a regeneration friendly microenvironment, after which nascent buds grow from axons proximal to the injury and extend along the regenerative channel to target organs and contact with them to achieve re-innervation of the target organs (Gu *et al.* 2011).

However, the products of degeneration of myelin can be selectively stained by the Marchi method. Marchi's staining procedure stains degenerated myelin stained black whereas normal myelin takes light brown stain (Gupta *et al.* 2013). As a strong oxidizing agent OsO_4 reacts with a wide range of organic compounds and is itself reduced to a black lower oxide, OsO_2 (Griffith 1967, Schroder 1980, Behrman 1984). The osmium dioxide adheres to structures that come in contact with OsO_4, thus imparting dark color. Further reduction and deposition occur when OsO_4 reacts with ethanol or methanol used for dehydrating the tissue. It is however necessary to that specimens be less than 1 mm thick and shredded with fine needle as OsO_4 penetrates tissues slowly (Kiernan 1999). Combining Marchi's and H & E procedures revealed the presence of black aggregates/ deposits in the vacuoles and digestion chambers (Fig. 2). This method confirmed the presence of degenerated myelin inside the vacuoles and digestion chambers and thus may allow better analysis of nerve damage and regeneration.

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