

Evaluation of changes in the locomotion and histology of sciatic nerve following experimental autoimmune encephalomyelitis

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Abstract

Introduction: Involvement of peripheral nerves in the experimental model of multiple sclerosis (MS) is rarely observed. The objective of this study was to investigate the changes in the locomotion in a mouse model of experimental autoimmune encephalomyelitis (EAE) and correlate with histological changes, if any, in the sections of sciatic nerve and lumbar part of spinal cord. **Material and Methods:** C57BL/6 mice (10 weeks, n = 8) were immunized with single subcutaneous injection of 300 µg of MOG35-55 and 200 µL of complete Freund's adjuvant (CFA) to produce EAE models. Limp tail with weakness of hindlimb was observed on day 10 and improvement in the weakness was observed on day 20 onwards. Footprint analysis was done to evaluate the impairment in the locomotion on day 0, 5, 10, 15 and 20 of the experiment. **Results:** One way repeated measure ANOVA found significant reduction in the mean hindlimb stride length on day 10 and 15 (left) and on day 15 and day 20 (right) when compared to mean stride length in day 0 ($p < 0.05$). Histological analysis showed evidence of macrophage infiltration around the dilated blood vessels in the epineurium of sciatic nerve and evidence of damage in the myelinated white matter of lateral funiculus of the lumbar sections of the spinal cord in EAE mice. **Conclusion:** It is concluded that in mouse model of EAE, the impairment of locomotion due to damage in the lumbar part of spinal cord can be associated with inflammatory changes in the sciatic nerve.

Keywords: experimental autoimmune encephalomyelitis, footprint analysis, histology, sciatic nerve, spinal cord.

1 Introduction

Multiple sclerosis (MS) is an immune mediated inflammatory disease associated with immune attack on myelin, which leads to axon and myelin degeneration. A commonly used MS animal model is experimental autoimmune encephalomyelitis (EAE). EAE does not occur spontaneously but it does mimic some of the pathological and histological hallmarks of MS. Myelin-specific T cells become activated and migrate into the central nervous system (CNS). This autoimmune inflammation results in CNS infiltration of CD4+, CD8+ T cells and B cells (BHASIN, WU and TSIRKA, 2007). Perivascular infiltration of immune cells across the blood-brain barrier was observed as one of the pathological characteristics of MS (JAVED and REDER, 2006). However, the coexistence of multiple sclerosis and peripheral neuropathy with infiltration of immune cells in the peripheral nerves has been reported occasionally. A Norwegian family with adult-onset demyelinating disease affecting both the peripheral and central nervous system was described in 1998 (HAGEN, MELLGREN and BOVIM, 1998). In the histopathological studies of inflammation and demyelination associated with MS, immune cells including T cells, B cells, activated macrophages and microglia have been found to be involved (HEMMER, ARCHELOS and HARTUNG, 2002). Many of the therapeutic modalities related to the clinical studies of MS were evolved out of the mechanisms observed in the studies involving regulation of EAE in inbred rat and mice model (SWANBORG, 1995). Myelin oligodendrocyte glycoprotein (MOG) is a myelin

protein that has been found in the outermost turn of the myelin membrane. During myelin sheath formation by oligodendrocytes, MOG has been proposed to play a role in transducing signals through the membrane of the myelin to the cytoskeleton elements (DYER, 1993). A previous study has reported that peripheral blood lymphocytes from MS patients responded predominantly to MOG compared to myelin basic protein (MBP), suggesting its importance in the pathogenesis of MS (ROSBO, HOFFMAN, MENDEL et al., 1997).

EAE can be induced, as a chronic disease, in C57BL/6 mouse by immunization with MOG (HARTUNG, KIESEIER and HEMMER, 2005) and it manifests morphological changes of demyelination, inflammation and axonal loss, similar to human MS (BRUCK, 2005). The existence of few cases of peripheral neuropathy in MS patients suggested that an etiological link might exist between the two conditions. In this study, an animal model of EAE was produced in C57BL/6 mouse by immunisation with MOG. Estimation of locomotion was done by footprint analysis and histological changes were observed in the sections of sciatic nerve and was correlated with changes in the sections of lumbar part of the spinal cord.

2 Materials and Methods

Female C57BL/6 mice (8-10 weeks old) were procured from and maintained in the animal holding facility of Brain Research Institute of Monash University Sunway Campus Malaysia.

MOG peptide, corresponding to 21 mouse MOG amino acid residues, was synthesized by ProspecBio (Israel). On day 0 (zero), mice (n=8) received subcutaneous injections of 300 µg of the MOG35-55 peptide mixed with 200 µL of complete Freund's adjuvant (CFA) containing 4 mg/ml heat-killed H37Ra strain of *Mycobacterium tuberculosis* (Chondrex Inc.) over the tail pleat, followed by intra-peritoneal administration of 400 ng of pertussis toxin (PT) (Sigma-Aldrich). The second post-immunization dose of PT was also administered IP on day 2. Using a protocol adapted from the University of Pennsylvania's Institutional Animal Care and Use Committee guideline (IACUC guideline experimental autoimmune encephalomyelitis rodent models), the mice were examined and scored daily for clinical signs of EAE. The standard clinical score used were as follows: 0 = no signs, 1 = limp tail, 2 = limp tail and mild paralysis of hind limbs, 3 = paralysis of hind limbs or with urinary incontinence, 4 = hind limb paralysis with weakness of forelimbs or with atonic bladder, and 5 = quadriplegia. Sham-treated mice (n=8), that were injected with CFA followed by PT were used as controls. The animal protocol was approved by the animal ethics committee of the Monash University.

The footprint analysis done on day 0 (pre-experiment), day 5, day 10, day 15 and day 20 was used to compare the gait of EAE mice with that of sham-treated mice. The forepaws and hindpaws of the mouse were stained with red and blue organic colours by dipping over the stamp-pad. The mouse was then allowed to run through a 50-cm-long and 10-cm-wide paper-lined plastic tunnel to a goal-box, creating consecutive footprints of forelimb and hindlimb with red and blue colors. At least nine steps were measured for each mouse. Three steps from the middle portion of each run were measured for the data on stride and stance lengths. Three readings were taken with a gap of 30 minutes each to prevent stress to the mouse. A fresh sheet of white paper was placed on the floor of the runway for each run. A piece of wet paper towel was placed at the opposite end of the tunnel, to wipe the ink on the paws. Measurements of stride length, fore-base width, and hind-base width gave an indication of gait. Stride length was measured for forelimb and hindlimb as the average distance of forward movement between each stride. The mean values for each set of three values of stride length, fore-base width and hind-base width were used in subsequent analysis. Statistical analysis using one way repeated measure ANOVA was carried out to determine any significant difference ($p < 0.05$) between the values of footprint analysis on day 0, day 5, day 10, day 15 and day 20.

The mice in each group were perfused transcardially with normal saline through the left cardiac ventricle, followed by fixation with cold 4% paraformaldehyde (PFA) on 22nd day of the MOG immunisation. Spinal cords and sciatic nerves were carefully dissected out, post-fixed in 4% PFA for 24 hours, and processed for paraffin embedding. The lumbar segments of spinal cord were identified by the hindlimb enlargement and sudden reduction in the diameter in sacral segments. The identification was corroborated by presence of prominent motor neurons in the anterior horn of the sections. Coronal Sections were cut at 8 µm on a Leica semi-automatic microtome and stained for histologic examination under an Olympus Provis AX70 optical microscope. Serial sections of the spinal cord were screened by the observers from the histology laboratory who were unbiased

and were not related to the research study. Haematoxylin and eosin (H&E) staining was used to reveal perivascular or sub-pial inflammatory infiltrates, and Luxol Fast Blue (LFB) staining, was used for the screening of demyelination.

The results were expressed as mean \pm standard error of mean. Statistical test employed to find differences between the mean values of footprint analysis data in different days of observation, was one-way repeated measure ANOVA followed by the post-hoc test of Bonferroni. Two way repeated measure ANOVA was employed to find out inter-group differences in the footprint analysis. In all analyses, differences between mean values of the data were considered to be significant when $p < 0.05$.

3 Results

The mean clinical score of EAE mice treated with MOG showed appearance of limp tail (score 1) at day 8 with progressive increase in manifestation of symptoms until day 10 when paralysis of hindlimb was observed in addition to limp tail (mean score 1.81) (Figure 1). After day 16, the mean score decreased gradually reaching a score of 1.0 at day 20.

Observation of footprint analysis could not find any recognisable change in the mean fore-base width, hind-base width and forelimb stride length in foot print analysis between the two groups of mice. Sham-treated control mice group did not show any significant change in the mean hindlimb stride length in one way repeated measure ANOVA analysis between pre-experiment day 0 data and any of the post-experiment data (day 5, 10, 15, 20). Mean value for left hindlimb stride length in MOG-treated EAE mice group showed significant reduction on day 10 and 15 compared to day 0 mean value for pre-experiment stride length [Wilk's lambda = 0.334, $F(4,20) = 9.9$, $p = 0.000$, Repeated Measure ANOVA] (Figure 2A). Mean value for right hindlimb stride length in MOG-treated EAE mice group showed significant

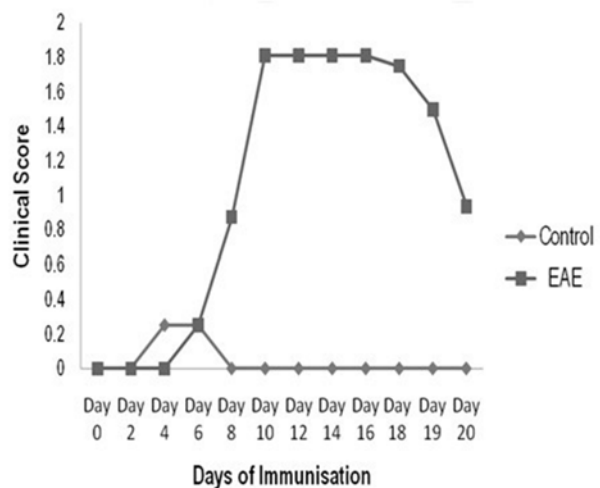


Figure 1. Mean clinical score observed in MOG-treated EAE mice group compared to sham-treated control mice group from day 0 to day 20. MOG- myelin oligodendrocyte glycoprotein; EAE- experimental autoimmune encephalomyelitis.

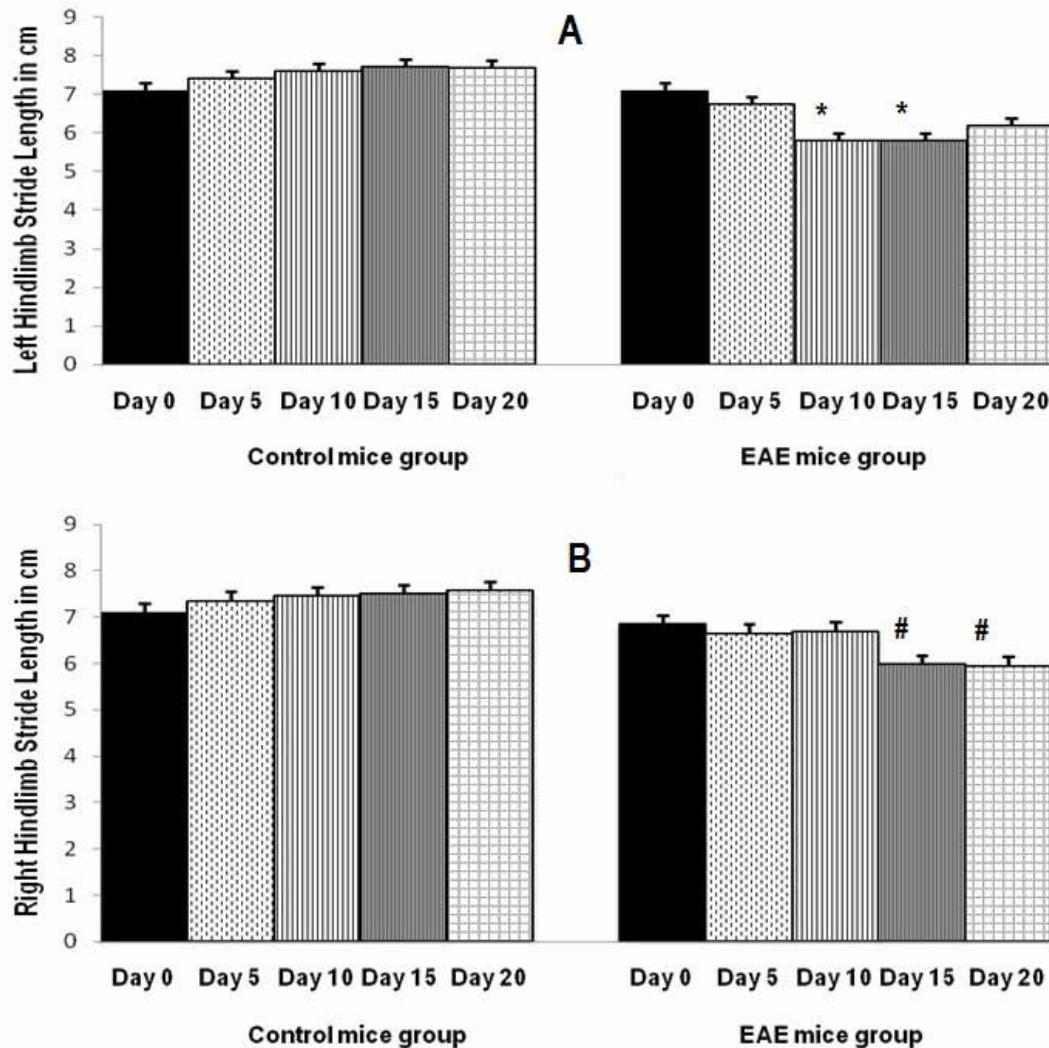


Figure 2. Mean left hindlimb stride length (cm) (\pm S.E.) (A) and mean right hindlimb stride length (cm) (\pm S.E.) (B) in sham-treated control mice group and MOG-treated EAE mice group respectively. MOG- myelin oligodendrocyte glycoprotein; EAE- experimental autoimmune encephalomyelitis. In EAE mice group ($n=8$) significant reduction in hindlimb stride length on day 10 and day 15 ($*p<0.05$) (left) and day 15 and day 20 ($\#p<0.05$) (right) compared to pre-experiment day 0. One way repeated measure ANOVA with post-hoc Bonferroni.

reduction on day 15 and 20 compared to day 0 mean value for stride length [Wilk's lambda = 0.54, $F(4,20) = 4.23$, $p = 0.012$, Repeated Measure ANOVA (Figure 2B). Two Way repeated measure ANOVA showed significant group-wise difference in the stride length of left hindlimb between sham-treated control group and MOG- treated EAE group of mice ($p<0.05$).

Under LFB staining, compared to the sham-treated control group of mice, showing normal bluish-stained myelin (Figure 3A), the MOG-treated EAE group of mice showed evidence of damage in the myelin sheath of the lateral white funiculus of the lumbar spinal cord sections (Figure 3B). Sections of all the lumbar spinal cord segments showed similar changes. The damaged myelin in the lateral white funiculus showed vacuoles with few coarse LFB-stained myelin in spinal cord sections of MOG-treated EAE mice. Low-power photomicrograph of lumbar spinal cord sections stained with

LFB stain (Figure 3C) showed area of white-matter funiculus, which failed to take up the LFB stain. Under H & E staining, the sham-treated control group of mice showed a rim of epineurium enclosing bundles of axons in section of sciatic nerve (Figure 4A). Within the bundle, the circular darkly stained axons surrounded by pink-colored myelin were clearly observed. The nuclei of Schwann cells were also seen in between the sections of the axons. The MOG-treated EAE group of mice showed accumulation of macrophages (arrows) in the space beneath the epineurium of the left sciatic nerve. The macrophages were particularly observed around the dilated blood vessels located within the epineurium. There was also evidence of an apparent increase in the size of the nerve bundle in the section of sciatic nerve of the EAE group of mice. These changes were only observed in the sections of left sciatic nerve (Figure 4B).

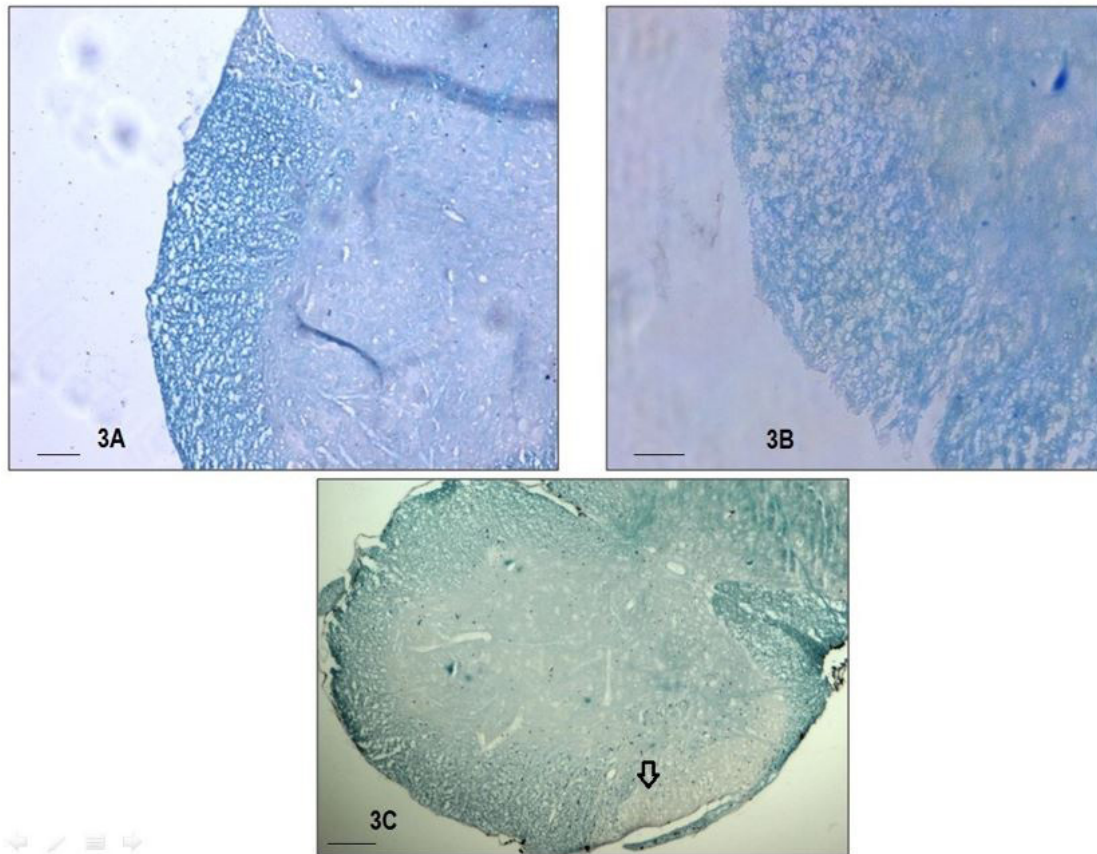


Figure 3. Photomicrograph showing the white matter of lateral funiculi of lumbar part of spinal cord in sham-treated control group of mice (3A) and MOG-treated EAE group of mice (3B) stained with luxol fast blue (8 μ m, 3A, 3B 100X, 3C 40X, 50 μ m bar). Well stained myelin in the white matter in control group (3A). Pale blue stained coarse myelin with vacuolation in the white matter in MOG-treated EAE group (3B). Low-power photomicrograph of spinal cord section in EAE group showing area of damaged white matter failed to take up LFB stain (arrow) (3C). MOG- myelin oligodendrocyte glycoprotein; EAE- experimental autoimmune encephalomyelitis.

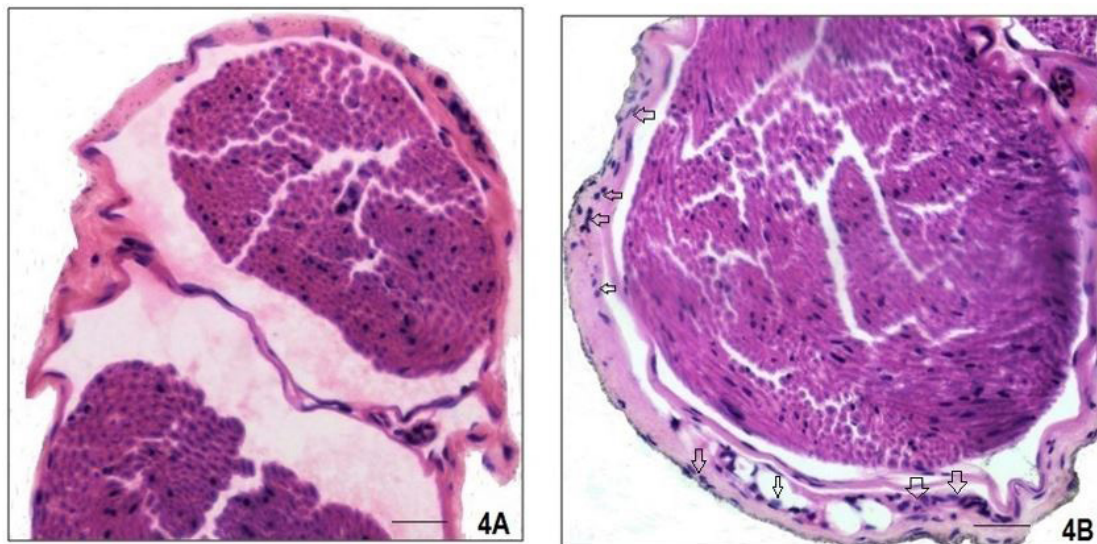


Figure 4. Photomicrograph showing the transverse sections of left sciatic nerve from sham-treated control group of mice (4A) and MOG-treated EAE group of mice (4B) stained with H & E (8 μ m, 200X, 50 μ m bar). Within the outer rim of epineurium, bundles of darkly stained axons surrounded by eosin-stained myelin with interposition of haematoxylin-stained Schwann cell nuclei are observed. In the sections of sciatic nerve from EAE group of mice (4B), bundle of the nerve is swollen with wide spaces appearing between the axons. The epineurium shows dilatation of blood vessels, surrounded by the collection of macrophages (arrows). MOG- myelin oligodendrocyte glycoprotein; EAE- experimental autoimmune encephalomyelitis.

4 Discussion

In the present study, the EAE mice reached a mean clinical score of 1.8, on the 10th day of the experiment following immunisation with MOG. The motor impairment was seen in the hindlimbs, as evidenced in the study of locomotion by the footprint analysis. The statistical analysis of the stride length showed that the impairment of the locomotion appeared earlier and was more severe in the left hindlimb than the right hindlimb. Qualitative histological analysis of the spinal cord sections in the EAE mice showed demyelination in the lumbar sections of the spinal cord. The motor neurons in the anterior horn of the lumbar spinal cord sections showed changes suggestive of damage (pyknosis and loss of nucleolus). In the peripheral nervous system (PNS), EAE mice showed evidence of inflammatory changes in the histological study of the sciatic nerve in the form of accumulation of macrophages in the epineurium. The sciatic nerve of left side showed inflammatory changes corresponding to the left hindlimb being more affected in the EAE mice. Prior studies using the EAE model have used the behavioural tests to assess the impairment of motor functions. The contact area of each hind limb footprint was used in the walking track test for motor evaluation in a study evaluating pregabalin treatment on EAE model (SILVA, PRADELLA, MORAES et al., 2014). Grid walk test was used to test the locomotor function in an EAE model using Lewis rats. In another study, moderate impairment of hindlimb locomotor function was detected on the third day after MOG immunisation and the animals recovered by day 14 (KERSCHENSTEINER, STADELMANN, BUDDEBERG et al., 2004). In this study, the stride length of foot print analysis was used to evaluate the locomotor function in EAE mice following MOG immunisation. Use of stride length in footprint analysis was used successfully to compare locomotor differences between normal dogs and dogs with spinal cord injury (SONG, OLDACH, BASSO et al., 2016).

Compared to the sham-treated control mice group, where no significant change was observed in the mean value of the hindlimb stride length in the post-experiment days, in the MOG-treated EAE mice group, significant reduction ($p < 0.05$) in the mean values of the left (day 10 onwards) and right hind limb stride length (day 15 onwards) was observed compared to day 0 (pre-experiment observation). The impairment of locomotion in the hindlimb observed in foot print analysis correlated with evidence of demyelination in the lateral funiculi of LFB-stained lumbar spinal cord sections of EAE mice.

In this study, observation of histological features of the sciatic nerve of the side of the body similar to the side of the hindlimb having more severe impairment of locomotion was a significant finding. It helped to correlate changes in the PNS in the mice model of EAE. Investigation into the involvement of peripheral nerves in EAE model is important as several studies have already proven the involvement of PNS in multiple sclerosis (POGORZELSKI, BANIUKIEWICZ and DROZDOWSKI, 2004; COURATIER, BOUKHRIS, MAGY et al., 2004; SAROVA-PINHAS, ACHIRON, GILAD et al., 1995). The sciatic nerve was selected based on the symptoms of motor impairment and that it was easier to harvest. Results of histological analysis showed infiltration of macrophages into the epineurium of the sciatic nerve compared to the nerve from the control group. The involvement of macrophages in the pathophysiology of EAE has been established, as the

modulations of these cells appear to affect the disease course (BHASIN, WU and TSIRKA, 2007). Nerve conduction abnormalities with slow nerve conduction was found in a small proportion of large diameter myelinated nerve fibres in between sciatic nerve and dorsal nerve root entry zone of L4 and L5 spinal segments in EAE rats with hindlimb weakness (PENDER and SEARS, 1986).

5 Conclusion

The present study was able to produce motor function loss restricted to the hindlimb of C57BL/6 mouse model of EAE with corresponding damage to the myelinated axons in lateral funiculus of lumbar segment of spinal cord by immunisation with MOG. Presence of inflammatory cells in the sciatic nerve indicated inflammatory changes in the PNS. The lack of immunohistochemical conformation of the inflammatory cells in the sections of the sciatic nerve remained a limitation of the study.

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