**Original Article**

**Auditory function and inner ear pathology in guinea pigs with peripheral nerve demyelination**

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**Abstract:** The audiological or physiological data showed that auditory neuropathy was due to the demyelination disease in peripheral nerve, especially in auditory nerve. The aim of this study was to explore the changes of auditory function and auditory nerve pathology through establishing a guinea pig model with demyelinated peripheral nerve. The guinea pig model was established by immunizing guinea pigs with heterologous peripheral nerve myelin. Auditory brainstem response (ABR) and compound action potential (CAP) were tested weekly before and after immunization for a period of 7 weeks. Serum anti MBP-IgG levels, sciatic nerve conduction velocity (NCV), the immunization associated lesions in the inner ear, and pathological changes in the auditory nerve, sciatic nerve and cochlea were tested. In comparison with the control group, the experimental group showed the existence of demyelination in sciatic nerve and auditory nerve, had a significant increase in serum anti MBP-IgG levels ($P<0.01$), ABR, CAP thresholds and the latencies of wave I, III, V and N1 ($P<0.01$) with no noticeable changes in the inter-peak latencies of I-III, III-V ($P>0.05$), while had a marked decrease in sciatic NCV ($P<0.01$). Immunohistochemistry revealed that the antibodies to inner ear from the MBP-sensitized animals distributed in cochlear nerve, nerve fiber of inner ear, and spiral ganglion cells. Cochlear scanning electron microscopy indicated the pathological changes of stereociliary disorder and cytoplasmic protrusions in the inner hair cell. In the present experiment, the EAN animal model which has been proved a reliable model with peripheral nerve demyelination demonstrated auditory nerve demyelination and auditory malfunction. The results suggest that this model can be an effective one for the study of the auditory dysfunction due to demyelination disorders.

**Keywords:** Myelin basic protein, auditory neuropathy, demyelination, inner ear

**Introduction**

Auditory neuropathy is a recently recognized sensorineural hearing loss with special pattern of electrophysiological findings [1]. The etiologies and pathogenesis of this disorder are still unclear. From the most widely accepted view, one possibility of this disorder results from an impaired function of the auditory nerve due to demyelination [2, 3]. Experimental autoimmune neuritis (EAN) is an acute demyelinating inflammatory polyradiculoneuropathy. In this experiment, myelin basic protein (MBP) from bovine sciatic nerve was adopted to develop a guinea pig model of EAN with a well-defined immunization [4, 5]. The purpose of this study was to observe the pathological changes in the auditory nerve, and to determine the auditory electrophysiological alterations.

**Materials and methods**

**Experimental animals**

Healthy adult hartley guinea pigs weighing 250 to 350 g, with a normal Preyer’s reflex, supplied by the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology, were used in this study. For all experimental procedures, the animals were anesthetized by intramuscular injection of ketamine (30 mg/kg body weight) and chlorpromazine (15 mg/kg).

**Antigen preparation**

MBP was isolated and purified from the fresh bovine sciatic nerve by the method of London [6]. The MBP was determined by Lowry assay with a concentration of 1.8 mg/ml.
Animal immunization

All animal treatments were strictly in accordance with international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals, and the experiments were carried out with the approval of the Animal Experimentation Ethics Committee of Zhongnan Hospital, Wuhan University. Forty-eight guinea pigs were randomly divided into an experimental group (n=36) and a control group (n=12). The EAN was induced by the modified protocol of Hall [4]. The guinea pigs were subjected to a subcutaneous injection of 300 µg MBP with equal volume of complete Freund's adjuvant (Sigma, USA) on the back. The animals in the control group received the same amount of the mixture of Freund's adjuvant and 0.9% saline.

Immunooassay

Sera were collected from the heart before animals were sacrificed, and the levels of anti-MBP antibody were determined by the indirect enzyme-linked immunosorbent assay (ELISA).

Auditory brainstem response

The auditory brainstem response (ABR) was determined before and after immunization weekly for a period of 6 weeks, and recorded using a Nicolet Spirit™ evoked potential system. The response signals were recorded using subcutaneous needle electrodes. The active electrode was inserted subcutaneously into the vertex, and the reference electrode was placed postauricularly and the ground placed on the nose. The sound stimulus consisted of 10-ms clicks at a repetition rate of 11.1/s. The potentials were bandpass-filtered between 20 and 1500 Hz, and averaged for 1000 stimuli. The hearing thresholds were determined as the lowest intensity at which a wave N1 was observable. And the peak latencies, interpeak latencies of N1 and N2, and the amplitude of N2 were measured at a stimulus level of 100 dB peSPL.

Auditory nerve compound action potential

The auditory nerve compound action potential (CAP) was recorded at the same time points and by the same system as the ABR. The active electrode was inserted subcutaneously into the post-superior corner of the external auditory meatus for about 4 mm with a direction vertical to the sagittal plane of the head, and stopped when the resistance was evident, and the reference electrode was placed postauricularly and the ground placed on the nose. The sound stimulus consisted of 10-ms clicks at a repetition rate of 11.1/s. The potentials were bandpass-filtered between 20 and 1500 Hz, and averaged for 1000 stimuli. The hearing thresholds were determined as the lowest intensity at which a wave N1 was observable. And the peak latencies, interpeak latencies of N1 and N2, and the amplitude of N2 were measured at a stimulus level of 100 dB peSPL.

Preparation of the specimens for transmission electron microscopy

The deeply anesthetized animals were perfused transcardially with a fixative of 2.5% glutaraldehyde in 0.1 M phosphate buffered solution (pH 7.4) and decapitated. The labyrinths were rapidly dissected, and the intact auditory nerves were immersed in the same fixative above. The sciatic nerves from the hind legs were also immersed in the same fixative. These specimens were kept at 4°C for 4 h, and then were washed in phosphate buffer (pH 7.4) and post-fixed in 1% osmium tetroxide. Following osmification, the specimens were prepared for transmission electron microscopy. A Opton 10C scanning electron microscope (Germany) was used to examine the specimens.

Scanning electron microscopy

After the animals were sacrificed, the cochleas were prepared for the observation under Scanning Electron Microscopy (SEM). Cochleas were quickly removed and perfused with phosphate buffered 2% glutaraldehyde, immersed in it for more than 4 h at 4°C, post-fixed with 1% OsO4, and dissected in 70% alcohol. After dissection, the specimens were prepared for SEM. A Opton 10C scanning electron microscope (Germany) was used to examine the specimens.

Immunohistochemistry

10 albino guinea pigs were sacrificed for immunohistochemistry using the strepavidin-biotin peroxidase complex (SABC) method. The cochlea was gently perfused with 10% formalin phosphate-buffered solution, fixed in this solu-
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Section of tissue overnight at 4°C, and then decalcified in 10% EDTA-Na₂ for 2 weeks, dehydrated in a graded series of alcohols and embedded in paraffin. The sections were cut at a thickness of 7 µm. These sections were deparaffinized, treated with 3% H₂O₂ for 10 min to block intrinsic peroxidase activity, digested with compound digestion solution to retrieve antigen and then treated with normal goat serum for 20 min. They were incubated overnight at 4°C with 1:50 sera from the animals of experimental group and from control group separately. Subsequent steps were performed at room temperature. The rinsed sections were incubated with 1:1000 goat polyclonal antibodies to guinea pig IgG (Sigma, American) for 20 min, followed by incubation with SABC (Boster, China) for 20 min. They were developed with 3,3'-diaminobenzidine for 10 min, dehydrated and mounted.

Statistical analysis

All data were expressed as mean ± standard deviation and were analyzed by analysis of variance or Student's t-test, as appropriate. A P value of less than 0.05 was accepted as significant.

Results

Comparison of ABR and CAP thresholds between experimental group and control group

According to our findings of the auditory stem response, MBP-immunized guinea pigs were divided into 2 subgroups: hearing loss group (group A), which demonstrated an increased threshold of more than 10 dB in 26 ears from 16 MBP-immunized guinea pigs (in 16 of 26 ears, the increase was more than 20 dB), and non-hearing loss group (group B), which did not show significant changes. In addition, 24 ears from 12 animals served as control group (group C, Table 1). The threshold of CAP in group A was also elevated (Table 1). As shown in Table 1, the elevation of the threshold of ABR and CAP started at 2 weeks postimmunization and lasted till the end of the study. However, the threshold of ABR and CAP in groups B and C did not display any change.

<p>| Table 1. Threshold of ABR and CAP (dB peSPL) |
|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Weeks after immunization</th>
<th>Group A</th>
<th>Group C</th>
<th>Group A</th>
<th>Group C</th>
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</thead>
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<tr>
<td>0</td>
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<td>43.21±5.04</td>
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<tr>
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<td>48.08±5.96</td>
<td>44.17±4.05</td>
<td>53.57±12.20</td>
<td>43.10±5.83</td>
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<td>43.13±5.04</td>
<td>57.14±9.89</td>
<td>41.36±6.76</td>
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<tr>
<td>4</td>
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<td>43.65±3.88</td>
<td>57.91±12.51*</td>
<td>42.96±5.24</td>
</tr>
<tr>
<td>5</td>
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<td>43.57±5.73</td>
<td>64.29±12.77*</td>
<td>41.81±5.69</td>
</tr>
<tr>
<td>6</td>
<td>61.00±10.56*</td>
<td>43.90±3.95</td>
<td>61.39±11.98*</td>
<td>43.24±7.49*</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SD. *P<0.01 compared with group C (Student-Newman-Keuls test).

Comparison of peak latency and interpeak latency of ABR and CAP between control group and experimental groups

The animals in group A demonstrated prolonged peak latencies of wave I, III, and V in a period of 1 week before immunization and 6 weeks after that. The interpeak latencies seemed to be normal as observed in control group. There were 4 animals (8 ears) in group B which had prolonged peak latencies, but the threshold of ABR and CAP was not elevated. Therefore, these animals served as group A1. The remaining animals in group B served as group B1. As shown in Figure 1, wave I latency was prolonged first at 2 weeks postimmunization, followed by changes in the wave III, and V at 3rd and 4th week respectively (all P<0.01, Student-Newman-Keuls test). No obvious prolongations were observed in interpeak latencies of I-III, and III-V in groups A1, B1, or C. The peak latencies of waves N₁ and N₂ were also prolonged at 2nd week postimmunization, but at last two weeks some slowly decrease were observed (all P<0.01, Student-Newman-Keuls test). The interpeak latency of N₁-N₂ showed a little increase at 4th, 5th, and 6th week postimmunization, but the difference was not significant (all P>0.05, analysis of variance).

Transmission electron microscopic findings of the sciatic and cochlear nerves

In both nerves, demyelination was observed. The myelin sheath displayed malformation with splitting and vacuolization of consecutive lamellae, and the axoplasm seemed fine, but
Changes of wave latency and interpeak latency in ABR and CAP. Wave I latency was prolonged first at 2 weeks postimmunization, followed by changes in the wave III and V at 3rd and 4th week respectively ($P < 0.01$). The peak latencies of waves N1 and N2 were also prolonged at 2nd week postimmunization ($P < 0.01$). The interpeak latency of N1-N2 showed a little increase at 4th, 5th, and 6th week postimmunization, but the difference was not significant ($P > 0.05$).
the dilation of the submyelinal space was observed especially in cochlear nerve (Figure 2).

**Scanning electron microscopic findings of the hair cells**

The scanning electron microscopy (SEM) of the organ of Corti showed the damage predominantly on the inner hair cells (IHC). The IHC stereocilia disruption and swelling, and cytoplasmic protrusions were observed. The outer hair cells appeared normal. Generally, the extent of stereocilia damage within each cochlea progressed from apical to basal turns (Figure 3).

**Immunohistochemical staining of anti-MBP antibody**

The serum antibodies in experimental group bound mainly to cochlear nerves, spiral ganglion cells and the nerve fibers in the osseous spiral lamina. No evident positive staining in the organ of Corti, stria vascularis and spiral ligament were detected. There was no immunostaining in sections incubated with sera from animals of the control group (Figure 4).

**Discussion**

In 1996, Starr et al [1] reported 10 patients with auditory neuropathy. These investigators found evidence of peripheral neuropathy in 8 patients. Three of them were diagnosed as having Charcot-Marie-Tooth. Because demyelination is a feature of this disease, they concluded that, in these cases at least, the abnormal evoked potential findings were most likely the result of myelin damage. The exact pathology of auditory neuropathy was not known with the lack of a biopsy of the affected nerves. From the point of view at present, this disorder was most likely due to the segmental demyelination of auditory nerve fibers [1-3]. Varying degrees of demyelination among fibers of the same dimension would affect neural synchrony of this population. Loss of discharge synchrony might account for the failure to detect the evoked auditory brainstem responses and the speech recognition deficits out of proportion to the puretone hearing loss [7, 8]. Some patients with auditory neuropathy were accompanied with limb peripheral nerve demyelinating impairment, such as/ slowed or blocked motor conduction velocity (MCV), sensory conduction velocity (SCV) and abnormal deep tendon reflexes. Such demyelinating disease as Guillain-Barre syndrome, HSMN type I (Charcot-Marie-Tooth disease) and primary demyelinating disorder in central nervous system (multiple sclerosis) might involve the auditory nerve resulting in the manifestation of auditory neuropathy [1, 3, 9]. The diagnosis of an “auditory neuropathy” is mainly based on the/audiological or physiological data. The clinical-pathologic correlation was not yet convincing as there was few autopsy studies carried out in auditory neuropathy patients diagnosed in life.

At present, there was no such animal model as the demyelination was restricted to the auditory nerve for audiological study. Matsuoka [10] induced hearing loss of mice by peripheral nerve myelin protein P0 immunization. Peak latencies of wave I, III, and V and the interpeak latency I-III were prolonged in the BAEP study. Histopathologic findings showed the mononuclear cell infiltration in the cochlear nerve region, and a reduced number of spiral ganglion cells detected in the inner ear of the mice with hearing loss. This indicated that cochlear nerve was involved in this animal model with inflammatory demyelination in the peripheral nerve. Naito et al [11, 12] reported abnormal auditory evoked potential in mutant hamster ‘black tremor (bt)’ with significantly prolonged...

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**Figure 2.** Transmission electron microscopy of nerves in experimental animals with significant hearing loss. There are splitting and vacuolization of consecutive lamellae in myelin sheath, and the dilation in the submyelinal space of the axoplasm (Original magnification ×5000). A. Sciatic nerve; B. Cochlear nerve (†myelin sheath; ※axoplasm).
wave latencies or auditory brainstem responses and prolonged N1 latencies of compound action potentials. Morphological observation revealed a myelin deficiency in the cochlear nerve. Experimental allergic neuritis (EAN) is an accepted animal model for human autoimmune demyelinating disease of the peripheral nervous system (PNS), Guillain-Barre syndrome (GBS) [13]. EAN can be induced by immunization with myelin basic protein (MBP) or passive transfer of MBP-reactive T cell lines and clones. We established the guinea pig model of experimental allergic neuritis by immunization with whole bovine MBP. The animal demonstrated hunched posture, poor coat, loss of body weight, and abnormal walking with a waddling gait. The serum levels of antibody against MBP were increased and the peripheral nerve displayed demyelination with electrophysiologic change of slowed motor conduction velocity. At the same time, the involvement of cochlear nerves was observed under transmission electron microscopy and immunohistochemistry. To establish the dysmyelination model in this animal for hearing research, it is important to assess the physiological status of the peripheral and brainstem auditory system in the guinea pigs. In this study, some similar findings as the mutant hamsters were observed [11, 12].

In myelinated nerve fibers, the ionic processes accounting for the generation of the action potential are restricted to the nodes of Ranvier, the junction points between adjacent myelin glial cells. The nodal membrane is rich in Na+ channels essential for the generation of the nerve action potential, whereas paranodal and internodal membrane sites have few Na+ channels but are rich in K+ channels. Thus, the generation of Na+ current contributing to the development of the action potential in myelinated nerve is not continuous along the nerve but is restricted to the nodes of Ranvier. The restriction of the action potential to the nodes of Ranvier results in a discontinuity of conduction from node to node known as salutatory conduction and accounts in part for the rapid conduction velocity of myelinated fibers. Demyelination results in an increased membrane capacitance and a decrease in membrane resistance, leading to a delayed excitation, a reduction on the velocity of action potential propagation, and an
increase in conduction vulnerability. The slowed internodal conduction velocity in a segmentally demyelinated axon varies with the extent of demyelination [14, 15]. So the slowed sciatic nerve conduction velocity was examined, and altered auditory function was found with the prolonged wave latencies of ABR and CAP in the guinea pigs of EAN we established. Wave I of the ABR generated in the Schwann cell-myelinated part of the VIII nerve in the cochlea, whereas wave II generated more proximally in the myelin of oligodendroglial origin, and wave III in the brainstem where it is unequivocally oligodendroglial. Demyelination due to Schwann cell pathology will, therefore, prolong the wave I latency or I-III interval. In contrast, it is the wave III-V interval that will be prolonged by demyelination due to oligodendroglial pathology affecting brain stem tracts [3]. Both N1 of the ECochG and wave I of the ABR arise from the distal portion of the cochlear nerve [3, 11]. Therefore, the electrophysiological findings of abnormal wave latency with relatively normal wave interval in these Guinea pigs indicate the damage to the distal cochlear nerves. Partial or complete loss of myelin has profound effects on the generation and propagation of action potentials within auditory nerve fibers, resulting in the reductions in the temporal synchrony of the nerve fibers. The presence of ABR depends on the ability of auditory neurons to respond synchronously to an auditory stimulus. The findings of relatively normal wave intervals might indicate that the lesion in the animal was not severe enough to destroy more centrally auditory pathway, or the impairment in those guinea pigs was only Schwann cell origin. The deficits appear to involve the firing of the auditory nerve in the peripheral portions of the auditory pathway while sparing the signal conduction on the more central portions of the auditory pathway. The latencies of wave III and wave V might be secondary to the affect of wave I.

ABR waveform disappears or shows obvious anomalies, which indicates that lesions begin in the distal end of auditory nerve. The normal appearance of otoacoustic emission in patients indicates that the lesions begin in the inner hair cells, inner hair cells and auditory nerve fiber tip, ganglion cells and nerve fibers [1]. As stated above, the auditory dysfunction of auditory neuropathy might be caused by a demyelination of auditory nerve. We have demonstrated an animal model of auditory nerve pathology due to demyelination, and discovered IHC stereocilia disruption and cytoplasmic swelling under SEM. We assumed that the disorders of IHC are subsequent to the primary injury of the VIII nerve. The abnormal electrophysiological findings in the peripheral component of ABR and CAP appeared to be consistent with the pathology. It appeared that the lesion may be at the site of ‘post-outer hair cells’ as the outer hair cells seems to be intact. More sufficient audiological data and further histology should be pursued in this animal model to clarify the underlying histological abnormalities and their relationship to altered auditory function. This animal model seems to be a promising model of auditory neuropathy due to demyelination disorders.

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Disclosure of conflict of interest
None.

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