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To Study the Diagnostic Utility of a Revised Protocol Comprising of Routine Histology, Myelin Stain, Anti-Neurofilament Immunostain and Electron Microscopy in Evaluation of Nerve Biopsies from Patients with Peripheral Neuropathies

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Abstract

Background: Although major progresses have been made in the diagnostic approaches of peripheral neuropathy in past ten years, 25 to 40% of patients remain undiagnosed. Recently, with use of immunohistochemical (IHC) and ultrastructural techniques, the diagnostic yield has enhanced. The aim of this study was to study the diagnostic utility of incorporating myelin stain, anti-Neurofilament (NF) immunostain and ultrastructural evaluation in comparison with routine histological stains, to establish an optimal diagnostic protocol for workup of neuropathies. *Methods:* Thirty four nerve biopsies received from patients with neuropathies were included with relevant clinical and electrophysiological information. In all the cases, nerve biopsy tissue submitted for evaluation was processed for light microscopic (LM), histochemistry using Luxol-fast blue (LFB) myelin stain, and IHC using anti-NF antibody. In 15 cases, an additional bit was received in saline/3% glutaraldehyde for electron microscopic (EM) examination. Statistical analysis was performed using Epi Info 3.5.3 software (CDC, Atlanta, 2008) for definitive correlation. *Results:* Overall the diagnostic yield was 46.7% with morphology alone but when combined with LFB stain combined was 73.3% ($p<0.05$). Combined diagnostic yield of H&E with NF was 17 cases (56.7%) ($p>0.05$). On evaluating the combined efficacy of LFB, NF-IHC and EM in comparison to routine staining using hematoxylin and eosin, it was observed that 21 biopsies (70%) were abnormal of which 10 were missed on routine examination ($p<0.05$). *Conclusion:* This study highlights that large number of cases and the spectrum of nerve pathologies that were otherwise missed owing to evaluation by routine histology alone can be diagnosed using a protocol which combines myelin stain, IHC with NF, and at least assessing semithin sections, for optimal workup of nerve biopsies. Since proper evaluation and accurate diagnosis have direct therapeutic and prognostic connotations, it is imperative that diagnostic centres evolve diagnostic protocol combining the above methodologies.

Keywords: Diagnostic Yield; Immunohistochemistry; Nerve Biopsy; Ultrastructure.

Introduction

Neuropathies are defined as dysfunction of peripheral nerves, which may show motor, sensory and autonomic symptoms. Although most neuropathies are symmetric, it is important to distinguish a polyneuropathy from a mononeuropathy, a multiple mononeuropathy or a polyradiculoneuropathy [1].

Major progresses have been made in the ten past years in the management and diagnostic approaches of peripheral neuropathy. The history and the physical examination followed by electrodiagnostic studies and then laboratory tests is the diagnostic approach of peripheral neuropathies. However, even after a careful work-up of a patient with neuropathy, 25 to 40% of patients with polyneuropathies remain undiagnosed [1-3].

One of the major limiting factors is that most laboratories use routine histological stains, and few use only myelin stain to detect demyelinating pathologies. Recently, morphological examination has

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greatly benefited from the contribution of new immunohistochemical and ultrastructural techniques, which can often be used together to enhance the diagnostic yield, especially in cases undiagnosed on routine histology having direct therapeutic and prognostic connotation [4,5].

In the current scenario, study of peripheral nerve diseases is evoking substantial interest among both basic and clinical researchers with the goal of improving diagnosis subsequent treatment of the patients [6-9]. In this regards, assessment of peripheral nerve histomorphology is the mainstay of the investigation of nerve damage and regeneration [10]. The main issues that need to be addressed in evaluating a nerve biopsy includes confirming the existence of a neuropathy; if it is axonal or demyelination or both; is it acute, chronic or polyphasic ? ; type of fascicular involvement : focal or diffuse; if the changes are age-related, like atheroscelrotic change, perineurial calcification and if there is a specific diagnosis, like :

- *Inflammatory pathology:* granulomatous inflammation, vasculitis, Hansen's disease, chronic inflammatory demyelinating polyneuropathy (CIDP) or chronic inflammatory demyelinating polyneuropathy (AIDP)
- Association with a neoplasm or paraprotein, like amyloidosis or lymphomatoid granulomatosis.
- Genetically determined disease like hereditary motor sensory neuropathy (HMSN) types 1,2,3; hereditary neuropathy with pressure palsies (HNPP); giant axonal neuropathy; neuroaxonal dystrophy; polyglucosan body disease; hereditary sensory autonomic neuropathy (HSAN); or storage diseases.
- Metabolic/toxic neuropathy secondary to diabetes or amiodarone therapy.

Since assessment of nerve histology using routine staining methods is poor, the gold standard in peripheral nerve studies is toluidine blue staining of resin-embedded semithin sections. However, this also has limitations that it is expensive, time consuming and requires ultramicrotome, which can be found only in specialized electron microscope laboratories only and performing immunohistochemistry (IHC) examination on these resin-embedded sections is very difficult and depends on complex etching protocols to remove antigen masking (especially due to glutaraldehyde and the resin) [11].

An analysis of the existing peer-reviewed literature in MEDLINE, EMBASE, Science Scientific Index and Current Contents by England et al recommended a protocol comprising of autonomic testing, nerve biopsy and skin biopsy for optimal evaluation of patients with peripheral polyneuropathy [12]. They observed that nerve biopsy is generally of use in cases of inflammatory neuropathies such as vasculitis, CIDP, sarcoidosis and leprosy, and also in infiltrative neuropathies like amyloidosis and tumours [13-18].

The aim of this study was to study the diagnostic utility of a incorporating myelin stain, anti-Neurofilament immunostain and ultrastructural evaluation in comparison with routine histological stains, to establish an optimal diagnostic protocol for workup of patients with peripheral neuropathies. The objectives we wish to ascertain was to study the morphological changes evident on routine haematoxylin and eosin (H&E) stain in nerve biopsies, to study the changes evident on myelin, anti-Neurofilament immunostain and electron microscopic (EM)/ultrastructural changes in nerve biopsies, to compare the diagnostic utility of adding myelin, anti-neurofilament (NF) immunostain and EM to routine H&E stain, to study the clinical spectrum and electrophysiological pattern of neuromuscular disease in patients undergoing nerve biopsies in a tertiary care hospital and to identify the spectrum of peripheral neuropathies that will benefit by this revised diagnostic protocol comprising of routine histology, myelin stain, anti-NF immunostain and EM.

Material and Methods

A total of 34 nerve biopsies (retrospective and prospective) received from patients with peripheral neuropathies were included in the study. Relevant clinical and electrophysiological information were collected.

In all the cases nerve biopsy tissue submitted for evaluation were processed for light microscopic (LM), histochemistry using Luxol-fast blue (LFB) myelin stain, and IHC using anti-NF antibody. In 15 cases, an additional bit was received in saline/3% glutaraldehyde for EM examination.

One bit was collected in 4% buffered neutral formalin (pH 7.2 – 7.4) for LM examination. Five–seven micron serial sections of formalin fixed paraffin embedded (FFPE) tissue were evaluated by LM after being stained by H&E, LFB which helps in analyzing the status of myelin and demyelination and Masson trichrome stain to evaluate fibrosis.

For IHC, representative formalin-fixed paraffin-embedded sections of four - five micron from the nerve biopsy were stained immunohistochemically using

labeled streptavidin biotin (LSAB) technique. After deparaffinisation and rehydration, the sections were autoclaved in 0.01 M citrate buffer (pH 6.0) at 121°C for 10 min. Then, the sections were cooled at room temperature for 60 min, immersed in 3% hydrogen peroxidase for 10 min to block endogenous peroxidase activity, and then washed in phosphate-buffered saline (PBS) for 5 min. To detect axonal status, mouse anti-human neurofilament (NF) mAb (Dako Cytomation, Denmark) were used. The sections were incubated with the antibody (diluted 1: 100) for 01 h at 4°C in a moist chamber. After washing three times with phosphate buffer saline (PBS) for 5 min, the sections were reacted with the secondary antibody (biotinylated anti-mouse antibody) for 30 min at room temperature. Then the sections were washed again three times with PBS for 5 min after which they were reacted with peroxidase-conjugated streptavidin for 30 min at room temperature. Finally, the sections were washed three times with PBS for 5 min and then reacted with a solution containing 0.06mM 3,30-diaminobenzidine and 2mM hydrogen peroxide in 0.05% Tris–HCl buffered at pH 7.6 for 10 min. They were then counterstained with haematoxylin for 30 seconds. After dehydrating with 60–100% isopropyl alcohol, penetrating, and mounting, the sections were observed under light microscope.

For EM, the bit was transported in normal saline/ 3% glutaraldehyde. After 2 hours fixation, tissue was thoroughly washed by phosphate buffer solution (pH 7.2) and post-fixed in 2% osmium tetroxide. The sections were then dehydrated by graded alcohol and then embedded in epoxy resin: EPON-812 : basic resin; DDSA (dodecenyl succinic anhydride): hardener; MNA (methyl nadiac anhydride): hardener; DMP-30 (2,4,6 tridimethylaminomethyl phenol): accelerator.

Semi-thin sections (500 nm) obtained using ultramicrotome with glass knife, were stained by 1% toluidine blue and evaluated under LM at x 100 oil immersion at x 1000 magnification.

In cases where the diagnosis was equivocal on LM, the section was processed further for EM examination. Semi-thin sections that were adequate (i.e. having adequate numbers of fibrils) were selected for ultra-thin sections (70 nm) for electron microscopy. These sections were taken on 3mm diameter copper grid (400-mesh) and stained by uranyl acetate and lead citrate.

Detailed morphological examination was done as per Table 1. Histologic diagnosis was offered as per the classification to determine axonopathy or demyelination.

Histochemistry detected presence or absence of demyelination by evaluating sections stained by LFB stain for myelin status. This was graded semiquantitatively (mild, moderate, severe) evaluated the extent of loss.

Immunohistochemistry detected presence or absence of axonal loss by evaluating sections stained by anti-neurofilament (NF) monoclonal antibody which semiquantitatively evaluated the presence of axons :

- + : 0-25%
- ++ : 26-50%
- +++ : 51-75%
- ++++ : 76-100%

EM evaluated the ultrastructural alterations which in relevant cases, were noted.

Correlation of Results

Relevant clinical and electrophysiological data were correlated with morphological and ultrastructural changes. Statistical analysis was performed using Epi Info 3.5.3 software (CDC, Atlanta, 2008) for definitive correlation.

Results

A total of at least 33 nerve biopsies (retrospective and prospective) received from patients with peripheral neuropathies were included in the study. The study population consisted of 25 males and 9 females (M:F :: 2.7 : 1). The age of the study population ranged between 4 and 85 years with a mean of 39.5 years (Table 2).

The average duration between onset of symptoms and biopsy was 4.9 months (range, 2-9 months). Nerve conduction studies in all the cases showed conduction velocity below 30m/sec in 18 patients, as compared to 15 with mild reduction.

Biopsy Profile: (Figure 1-2)

In 3 cases the biopsy was fragmented and tiny, hence was considered inadequate for evaluation. The average number of fascicles present in the remaining 30 biopsies was 4.5 (range, 3-6). Fascicular involvement was seen in 14 biopsies, where it was diffuse in 10, while in the remaining 4 there was focal involvement. Chronic changes and fibrosis were noted in 4 and 2 cases, respectively. Evidence of axonal loss /axonolysis was noted in 3 cases, while

demyelination, evident as "*myelin digestion chambers*" was evident in 11, of which 4 were secondary to inflammatory pathology. Vessels were adequate in 24 cases, while 1 case showed features of vasculitis and 5 displayed perivascular inflammation. The salient histopathological features are summarized in Table 3.

The changes that were evident solely on morphology included axonolysis/axonal degeneration, demyelinating process, and demyelination secondary to chronic inflammatory or vasculopathy in 3, 7 and 4 cases, respectively. In 16 cases there were no specific changes noted, while in 3 cases no opinion could be offered due to inadequate biopsy, which were excluded from the study. Overall the diagnostic yield was 46.7%. Details of the histological opinion offered on histology are summarized in Table 4.

Histochemical Examination

A total of 19 nerve biopsies showed demyelination on staining with LFB, of which 8 were apparently normal on routine H&E stain. On analysis the difference was statistically significant. So, the diagnostic efficacy of H&E and LFB stain combined was 73.3% (22 cases). Comparison between H&E and LFB stain are summarized in Table 5.

Immunohistochemical Examination

Out of the 30 nerve biopsies, a total of 6 showed axonal loss of varying degrees, on immunostaining with NF, as compared to 2 on routine H&E evaluation, which did not show any significant difference on statistical analysis. Interestingly, one case which appeared to have axonolysis on routine evaluation, revealed normal axonal count on IHC. Thus the combined diagnostic yield of H&E with NF was 17 cases (56.7%). Comparison between H&E and IHC stain are summarized in Table 6.

Ultrastructural Examination : (Figure 3)

Electron microscopy was performed on 15 cases, of which one showed evidence of fibrosis only, and hence excluded from the study. Of the 12 cases that revealed normal histology on H&E, 11 showed normal ultrastructural morphology. Interestingly, semithin sections stained by Toluidine blue that were prepared for all the 15 cases and viewed under the LM, prior to cutting ultra thin sections for EM, showed similar results. Comparision between H&E and EM studies are summarized in Table 7.

On evaluating the combined efficacy of LFB, NF-IHC and EM in comparison to routine staining using hematoxylin and eosin, it was observed that 21 biopsies (70%) were abnormal of which 10 were missed on routine examination. However, of the 9 cases that showed normal features on LFB, NF and EM 8 were normal on routine evaluation.

The variation of one case between combined H&E with LFB (22 cases) and combined H&E with LFB, NF, EM (21 cases) was due to the solitary case, which showed features of axonal loss on H&E stain but IHC with NF showed otherwise. Comparison between combined efficacy of LFB, NF and EM with H&E alone are summarized in Table 8.

Of the 18 cases with velocity <30m/sec, diagnostic efficacy of routine H&E was 55.6% (10 cases) while combined H&E with LFB, NF and EM was 88.9% (16 cases). In contrast, the diagnostic yield in the remaining 15 cases with mild reduction in NCV was 26.7% (4 cases) and 40% (6 cases), respectively.

Statistical analysis using unpaired t test showed significant difference (p=0.02) between the group using combination of methods as compared to the one reporting on routine H&E stain in the <30m/s population, while it was not so in the e 30m/s cluster (p=0.07). Correlation of nerve conduction velocity with diagnostic efficacy of combined LFB, NF, EM and H&E are summarized in Table 9.

The onset biopsy interval (mean 4.9; range 2-9 months) was correlated with diagnostic efficacy. It was noted that cases with an onset-biopsy interval of less than 6 months had better diagnostic yield in comparison to the ones where the onset-biopsy interval was more than 6 months.

Statistical analysis using unpaired t test showed significant difference (p,0.05) between the group using combination of methods as compared to the one reporting on routine H&E stain in the <6months population, while it was not so in the e 6 months cluster (p=0.36). Correlating onset-biopsy interval with diagnostic efficacy of combined LFB, NF, EM and routine H&E examination are summarized in Table 10.

Table 1: Systemic analysis of nerve biopsy

Adequacy of sample
Number of fascicles
Morphology :
Fascicular involvement : Focal or diffuse
Acute or chronic involvement
Any specific diagnosis
Axonal loss
Demyelination : *"Myelin degradation chambers"*
"Onion bulb"
Fibrosis
Inflammation / granuloma
Status of vessels :
Adequacy
Vasculitis
Arteriosclerosis

Table 2: Age distribution of cases

Age Group (years)	No. of patients
≤ 15	4
15-30	7
30-45	5
45-60	7
> 60	10

Table 3: Salient histopathological features (N=33)

Histological features	*No. of cases*
Adequacy of sample	30 *(90.9%)*
Mean no. of fascicles (Range)	4.5 *(3-6)*
Morphology :	
• Fascicular involvement :	
➢ Focal	4 *(13.3%)*
➢ Diffuse	10 *(33.3%)*
➢ No involvement	16 *(53.4%)*
• Chronic involvement	4 *(13.3%)*
• Any specific diagnosis :	
➢ Vasculitis	1*(3.3%)*
• Axonal loss	3 *(10%)*
• Demyelination : *"Myelin degradation chambers"*	
➢ Total	11 *(36.7%)*
➢ Secondary effect	4*(13.3%)*
• *"Onion bulb"*	0
Fibrosis	2 *(6.7%)*
Inflammation	5 *(16.7%)*
Granuloma	0
Status of vessels :	
• Adequacy	24 *(80%)*
• Vasculitis	1 *(3.3%)*
• Perivascular inflammation	5 *(16.7%)*

Table 4: Diagnosis based on histopathology only (N=33)

Histological subtypes	No. of cases
Axonolysis / axonal degeneration	3
Demyelinating process	7
Demyelination secondary to chronic inflammatory or vasculopathy	4
Normal histomorphology	16
No opinion due to inadequate biopsy	3

Table 5: Evaluating efficacy of detecting demyelination : comparison between H&E and LFB stains (N=30)

	Demyelination on LFB	No demyelination on LFB	
Demyelination on H&E	11	0	11
No demyelination on H&E	8	11	19
	19	11	

Chi square test with Yates correction 4.01 p : 0.045

Table 6: Evaluating efficacy of detecting axonal loss: comparison between H&E and NF-IHC stains (N=30)

	Axonal loss on NF -IHC	*No axonal loss on NF -IHC*	
Axonal loss on H&E	2	1	3
No axonal loss on H&E	4	23	27
	6	24	

Chi square test with Yates correction 1.88, p : 0.17

Table 7: Evaluating efficacy of ultrastructural examination : comparison between H&E and EM studies (N=14)

	Abnormal on EM	Normal on EM	
Abnormal on H&E	2	0	2
Normal on H&E	1	11	12
	3	11	

Chi square test with Yates correction 3.98, p: 0.*046*

Table 8: Evaluating combined efficacy of LFB, NF, EM in comparison to routine H&E examination (N=30)

	Abnormal on LFB, NF-IHC, EM	Normal on LFB, NF-IHC, EM	
Abnormal on H&E	11	1	12
Normal on H&E	10	8	18
	21	9	

Chi square test with Yates correction 2.92, p : 0.08

Table 9: Correlating nerve conduction velocity with diagnostic efficacy of combined LFB, NF, EM and routine H&E examination (N=30)

	NCV		
	<30m/s (n=18)	≥30m/s (n=15)	
Routine H&E	10	4	14
Combined H&E, LFB, NF, EM	16	5	21

Table 10: Correlating onset-biopsy interval with diagnostic efficacy of combined LFB, NF, EM and routine H&E examination (N=30)

	Onset-biopsy interval		
	<6 months (n=20)	≥6 months (10)	
Routine H&E	9	5	14
Combined H&E, LFB, NF, EM	15	7	22

Fig. 1:

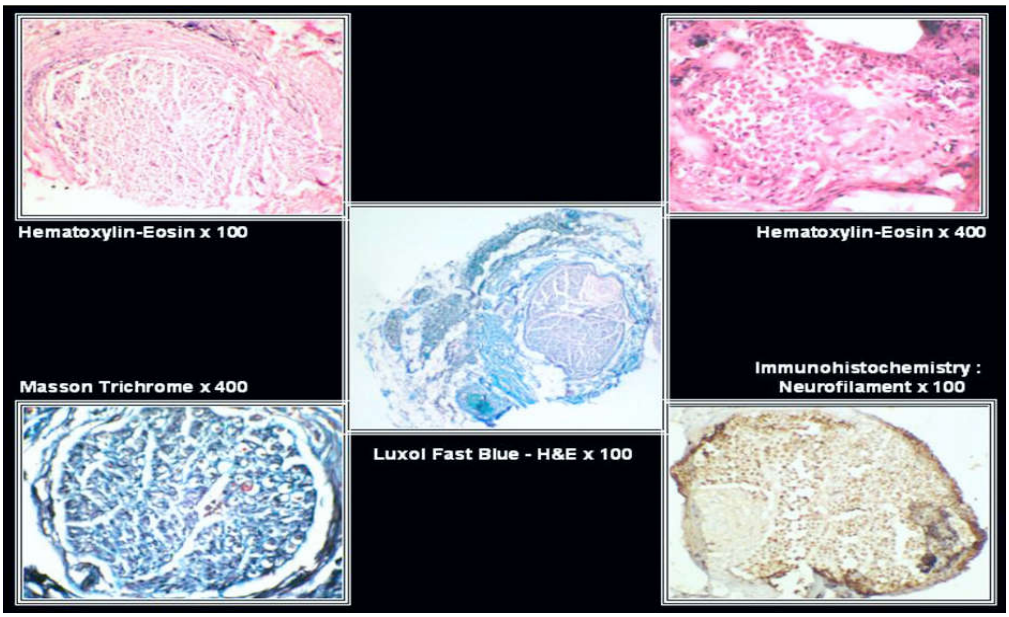
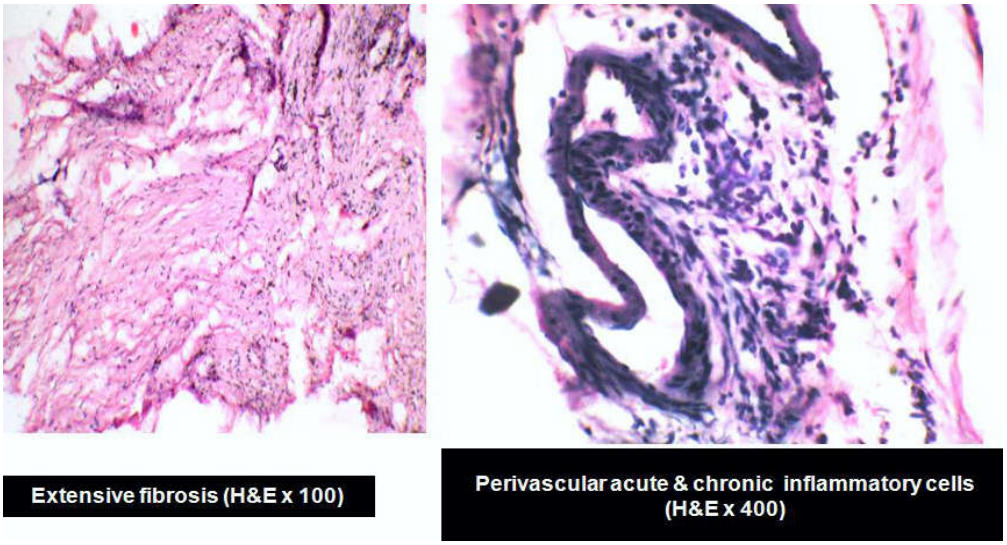


Fig. 2:



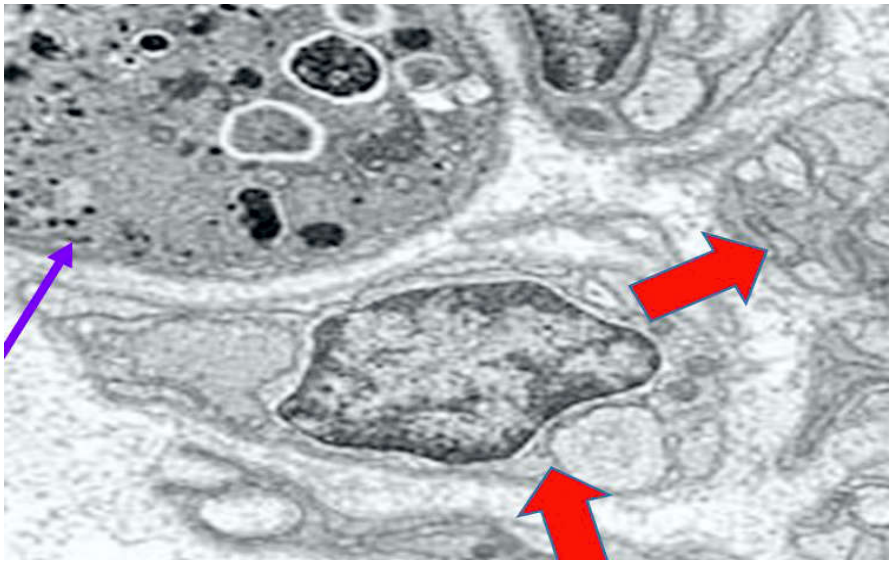


Fig. 3: Degenerating axon (Red) adjacent to intact unmyelinated fibers (Blue)

Discussion

Peripheral neuropathy is a common condition, which finds favour in the elderly population [16]. The present study included cases of all age groups in order to obviate any bias related to age. Thus the mean age was 39.5 (range 4-85) years. The clinical spectrum of cases included AIDP, CIDP, CIAP, HMSN, diabetes, drug induced polyneuropathies, and vasculitis. Demyelinating pathologies with 36.7% biopsies constituted the most frequent change on routine examination.

Of the 33 cases, three were excluded due to inadequate biopsies received. The diagnostic yield of the remaining 30 cases was 46.7% on routine histology. This increased significantly to 73.3% on combining H&E stain with LFB for evaluating the myelin status. Owing to paucity of cases with axonal loss, addition of neurofilament IHC stain with a yield of 56.7% did not significantly enhance the diagnostic efficacy. Similarly, electron microscopy performed on 15 cases (one of which was excluded from evaluation due to presence of fibrosis only) helped to identify only a solitary case that was missed on H&E. Overall the combined efficacy of H&E with LFB, IHC for NF and EM did not offer any better yield as compared to H&E with LFB, thus confirming that demyelinating disorders are the most common affliction in peripheral neuropathies. This observation was synchronous with that of the available literature [12-18].

The diagnostic yield noted by various studies ranged between 35.5 to 47.3% [14,17,18], while a solitary study involving above 65 years documented 91% [16]. Our results of the combined efficacy of all special histochemical and immunohistochemical stains with a yield of 73.35 are comparable.

As observed by Deprez et al [18], the diagnostic efficacy of a nerve biopsy is dependant on multiple factors, which include various clinical parameters viz. (a) the presumptive diagnosis at time of referral for biopsy; (b) the distribution of clinical symptoms; and (c) the interval between disease onset of the symptoms and biopsy. They noted that a higher yield was associated with clinically suspected vasculitis, inflammatory demyelinating neuropathy or hereditary sensorimotor neuropathies. Contributive findings were more often reported with multifocal or asymmetrical presentations, which were also observed in the present study. It also varied with the onset-to-biopsy interval with higher diagnostic yield in cases that were less than 6 months. The current study had 20 cases where the onset-to-biopsy interval was less than 6 months, and the diagnostic yield was comparatively better. The contribution of nerve biopsy varied according to neuropathological techniques, which has been aptly demonstrated in this study.

Argov et al [14] studied 53 sural nerve biopsies from 120 patients. They noted that patients with motor conduction velocity below 30 m/s, sural nerve histology was helpful in 65% of biopsies. In patients with milder reduction in conduction biopsy contributed in only 11%. They concluded that though in general neurological practice, nerve biopsy is of limited value as a routine diagnostic procedure. However, in patients with marked slowing of conduction velocity, in whom the diagnosis is not immediately apparent, sural nerve biopsy will be helpful. In the present study it was observed that of the 18 cases with velocity <30m/sec, diagnostic

efficacy of routine H&E was 55.6% (10 cases) while combined H&E with LFB, NF and EM was 88.9% (16 cases). In contrast, the diagnostic yield in the remaining 15 cases with mild reduction in NCS was 26.7% (4 cases) and 40% (6 cases), respectively, which was significantly different.

Overall, the present study also reiterates the observations made by other workers [12-18] that nerve biopsy alone, and that too evaluating using only routine hematoxylin and eosin stain, may be of limited value, but when used in conjunction with detailed clinical history, electrophysiological studies, and performing a detailed evaluation using myelin stain, axonal stain and electron microscopy can make a significant contribution in enhancing the diagnostic efficacy. Centers that lack the availability of electron microscope may incorporate semithin sections stained by toluidine blue in their protocol, since it has equally comparable efficacy without increased cost factor [11].

Conclusion

Routine light microscopic examination of nerve biopsy is often fraught with problems of missing subtle findings of demyelination and/or axonal loss. The efficacy is dependent on multiple factors, most important of which are the experience in the field of nerve biopsies of the reporting pathologist, as well as the quality and staining of sections.

This has led to the introduction of various myelin stains, of which LFB finds favour due to its easy processing protocols; and also IHC for NF to assess the axonal status as part of the complete evaluation of nerve biopsies for peripheral neuropathies. Further, utility of EM in nerve biopsy is well established. However owing to the non-availability of this facility, most centers reporting nerve biopsies rely only on LM supplemented by myelin and axonal stains.

The present study has evaluated the diagnostic utility of incorporating myelin stains and anti-NF immunostain in conjunction with ultrastructural studies in establishing an optimal diagnostic protocol for nerve biopsies from patients with peripheral neuropathies undergoing management in a tertiary care hospital. The current study has also observed that the diagnostic utility of LM evaluation of toluidine blue-stained semithin sections of nerve biopsies is comparable to that of assessing ultra thin sections using electron microscope, and recommends its usage in specialized neuropathology centers. It has highlighted the large number of cases and the spectrum of nerve pathologies that were otherwise missed owing to evaluation by routine histology. Since proper evaluation and accurate diagnosis have direct therapeutic and prognostic connotations, it is imperative to evolve a diagnostic protocol using myelin stain, IHC with NF, and at least assessing semithin sections, for optimal workup of nerve biopsies.

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