Optimization of Immunohistochemical Staining with Anti Protein Gene Product 9,5 (PGP 9,5) Antibodies to Detecting Intraepidermal Nerve Fiber

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Keywords: Intraepidermal Nerve Fiber, PGP 9.5, Immunohistochemistry

Abstract: The intraepidermal nerve fibers (INF) are ending branches of the skin sensory nerves. These fibers can be conceived due to the protein gene product 9.5 (PGP 9.5) as a marker recognized by immunohistochemistry. Various studies have visualized these INF with anti-PGP 9.5 antibodies. However, this study differs in immunohistochemical (IHC) staining methods based on the tissue thickness, the antigen retrieval process, and the antibody product used. This study aimed to find an IHC staining optimizer with an anti-PGP 9.5 antibody to detect INF from paraffin blocks. The INF was determined by mice skin biopsy stained with IHC anti-PGP 9.5 antibodies. This procedure was altered in the dilution, duration, incubation temperature of primary antibodies, the tissue's thickness, and the antigen retrieval temperature. We quantitatively analyzed the staining results. Optimization of IHC stain entail of 1:2000 dilution of the primary antibody, the thickness of the tissues were 4 µm, overnight incubation, and low temperature of antigen retrieval. However, the results were inconsistent. The contributing factors that enhance the IHC staining method are thinness of the tissue, low-temperature antigen retrieval, the ratio of antibodies dilution (1: 2000), and incubation overnight at 21°C.

1 INTRODUCTION

The intraepidermal nerve fibers are the ending branches of the skin's sensory nerves (Malik *et al*., 2011). This fiber is one of the diagnostic parameters of neuropathy (Chen et al., 2015). Neuropathy is a clinical problem in the form of unpleasant sensations caused by peripheral nervous system damage. Neuropathy is assessed by decreasing the density of intraepidermal nerve fibers, which can be identified by immunohistochemical staining.

The data is obtained through the skin tissue section. Therefore intraepidermal nerve fibers must be visualized by immunohistochemical staining to recognize the markers expressed by the nerve fibers, one of which is protein gene product 9.5 (PGP 9.5) (Sun et al., 2014). Various studies have visualized these intraepidermal nerve fibers with anti-PGP 9.5 antibodies. However, the study differs from the method of immunohistochemical staining, the thickness section of the tissue, the antigen retrieval process, and the antibody product used.

Detection of intraepidermal nerve fibers can use tissue from paraffin blocks with thin sections (<20 µm). The thin section's advantage is the penetration of antibodies that are used faster and better and do not require cutting tools and special microscopes. The HRP label using to have the advantage of longer tissue structure to observed than the fluorescent label.

A precise staining technique is needed to obtain a quantification of intraepidermal nerve fibers. In this study, we will optimize immunohistochemical staining with PGP 9.5 antibody (Abcam ab8189) was performed to detect intraepidermal nerve fibers in a simple laboratory using tissue from paraffin blocks.

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Pakaya, D., Ari Sumiwi, Y., Herwiyanti, S. and Susilowati, R.
Optimization of Immunohistochemical Staining with Anti Protein Gene Product 9,5 (PGP 9,5) Antibodies to Detecting Intraepidermal Nerve Fiber.
DOI: 10.5220/0010487400430046
In *Proceedings of the 1st Jenderal Soedirman International Medical Conference in conjunction with the 5th Annual Scientific Meeting (Temilnas) Consortium of Biomedical Science Indonesia (JIMC 2020)*, pages 43-46
ISBN: 978-989-758-499-2

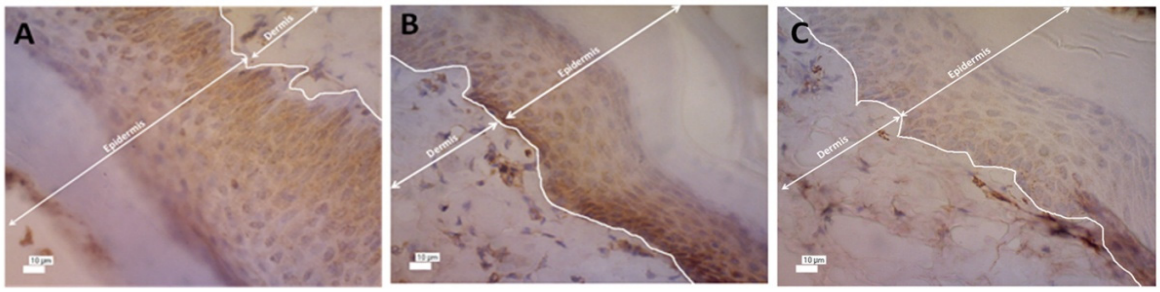

Figure 1: Results of phase I optimization, immuno-histochemical staining feature with PGP 9.5 primary antibody on 4 µm tissue sliced thickness. (A). 1:500 dilution. (B). 1:1000 dilution (C). 1:2000 dilution. 400x magnification.

2 MATERIAL AND METHODS

2.1 Animal Samples

This study type is a quasi-experimental study with a cross-sectional design. It utilized two male Balb/cJ mice strain, 8-weeks old, 20-40 g body weight. It utilized two male Balb/cJ mice strain, 8-weeks old, 20-40 g body weight, using two mice to obtain the skin samples by applying the principle for animal laboratory used. This particular study has received its permit from the ethical commission of integrated research and development bureau (LPPT) UGM with its certified number: 00111/04/LPPT/II/2017.

2.2 Immunohistochemical Staining

Mice were terminated, and their right foot skin was necropted using skin punch biopsy (Premier®, PMU9033505, Premier Medical, India) at 5 mm of diameter. The resultant necropsies were grown in 5% agarose gel and incubated overnight in paraformaldehyde 4%; then paraffin blocks were made. The paraffin blocks section was using Microtome Leica RM 2235, at 4 and 15 µm thickness with a fraction of 1/20. Immunohistochemical staining was performed using anti-PGP 9.5 (Abcam® ab8189, Abcam, USA. MA) antibodies. The staining was performed within several phases and modifications upon the primary antibody (comparison of dilution, temperature, and duration of incubation), tissue thickness, and antigen retrieval.

2.3 Statistical Analysis

The results of the staining were analyzed qualitatively for the intensity of the nerve fiber color.

3 RESULTS

3.1 Optimizing Immunohistochemical Staining with Anti PGP 9.5 Antibodies

The first stage optimization is performed to obtain the best primary antibody dilution. In the thickness of the 4 and 15 µm tissue slices using 1:500 and 1:1000 primary 1-cell antibody dilutions, the resulting 1:2000 dilution of the color with a balanced intensity and good, but not specific. The immunohistochemical brown color is seen in most skin epithels so that intraepidermal nerve fibers cannot be found (Figure 1).

Phase II is carried out at 4 and 15 µ thickness and lower retrieval antigen temperature. There was one folded slide with 15 µm thickness. The staining results were a picture of nerve fibers with good color intensity, as in Figure 2. In phase III, trials were conducted on three blocks of samples with 15 µm thickness using the same phase II method. After the retrieval antigen process, there were six loose slides and six folded slides. The results of the staining look like a picture of less good nerve fibers. In phase, IV modification is done without antigen retrieval, a primary antibody with 1: 2000 dilution. The results appeared in overnight incubation as the medium-intensity brown color, and no nerve fibers were found. At incubation of 2 and 3 nights, the results appear in brown with a concentrated intensity and are non-specific.

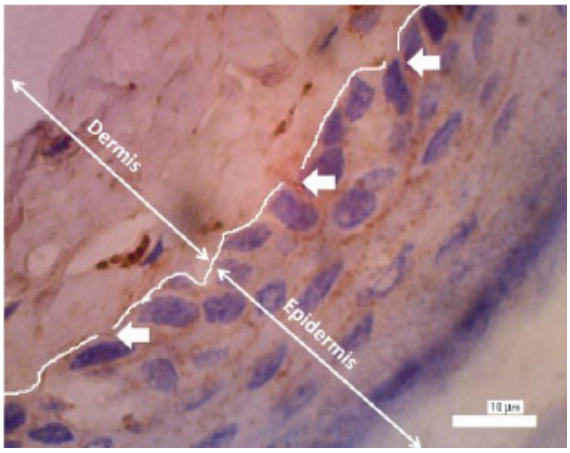

Figure 2: Intraepidermal nerve fibers with anti-PGP 9.5 antibodies are shown as brown perpendicular lines that pass through the basal membrane (indicated by arrows) with 1000x magnification.

Phase V was performed at 15 µm thickness, dilution of primary antibody at 1: 2000 with modification of the antigen retrieval temperature and the primary antibody's incubation temperature. The result showed several loose or folded slides, and the staining results appear as a brown stain with moderate intensity, and no nerve fibers are found. Stage VI was performed at a thickness of 4 µm, with 1: 2000 dilution of primary antibody and the modification of lower retrieval antigen temperature. The result showed no slide folding or losing, and there was a picture of nerve fibers.

4 DISCUSSION

Optimization is important to make a good quality of tissue staining. We will optimize immunohistochemical staining with PGP 9.5 antibody to detect intraepidermal nerve fibers using skin tissue from paraffin blocks to diagnose neuropathy. The immunohistochemical optimizing at phase I, a 1:2000 dilution was obtained with its best stain production and balanced and good intensity. In phase II, the nerve fibers appear well at 15 µm thickness. In another study, nerve fibers appeared well on 5 µm slice thickness of paraffin blocks (Thomsen et al., 2009; Ventura et al., 2011), 50-100 µm thicknesses with frozen section (Stavniichuk et al., 2011) and 2 µm thickness with a confocal microscope (Periquet et al., 1999). To quantify the number of nerve fibers with specific antibody markers, it's is recommended to use thin slices (Beiswenger et al., 2008). In phase III, the same thickness and method do not produce a consistent picture. This phenomenon is influenced by other processes such as antigen retrieval temperature and the incubation of primary antibodies.

Good nerve fibers appear after antigen retrieval was conducted with low temperatures. However, these low-temperature modifications remain inconsistent, just like the results in phase V. In the case of axon injury, the image of nerve fibers is also obtained after low-temperature antigen retrieval (Stone et al., 2009). This study's obstacles were the thickness of the skin foot sample (15 µm) with the cornification at risk of loose or folded after antigen retrieval. In phase IV, the retrieval antigen process is not performed, but the results are not specific. The phase VI optimization was carried out at 4 µm thickness with the same retrieval antigen temperature as stage V, obtained a picture of nerve fibers with balanced color intensity. To get a good picture of the nerve fibers needs to be preceded by the retrieval antigen at low temperatures. The open epitope will be very good to bind to the incubated antibody. A good description of the nerve fibers was obtained in the second stage of optimization with 15 µm slice thickness and stage VI with 4 µm slice thickness. The thickness of 4 µm slices is the most likely to be done because at 15 µm, the thickness is risky to lose or fold after retrieval antigen.

To quantitate the intraepidermal nerve fibers density, the stereology principle was used. The samples were randomly and systematically obtained from the paraffin block slices that have the same thickness. With this method, we will get some slices to be quantification according to the specified fraction. The entire cross-section must look good to be quantified, so when using a 15 µm thickness, it will have difficulty reporting the results. Skin tissue is not isotropic, i.e., uniformity in parameter values in all directions, so it takes orientation or determination of the direction of network cutting to avoid bias (Witgen et al., 2006). However, manual quantification still possesses the possibility of bias, so that software usage is recommended. The software will generate randomly oriented virtual isotropic fields in a 3D virtual field containing parallel lines so that any nerve fibers tangent to the line can be quantified (Karlsson et al., 2013; Karlsson et al., 2016). Inconsistent optimization results and the shortcoming of manual quantification may cause difficulty in conducting the study.

5 CONCLUSION

The immunohistochemical staining method with the most optimal anti-PGP 9.5 antibodies was performed on thin, with low-temperature antigen retrieval and 1: 2000 antibody dilution incubated overnight at 21°C.

The results of this study can be directed to become a diagnostic method of neuropathy.

ACKNOWLEDGEMENTS

The author would like to express gratitude to BPPDN Dikti 2015 (906.II/E4.4/2015). Mrs. Wiwiet Setyowati, Histology, and Cell Biology Laboratory of UGM Medical School.

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