ORIGINAL ARTICLE

EFFICACY OF FORMALIN FIXATION IN DETECTING GFAP AND MAP2 IN THE SPINAL CORD VIA IMMUNOFLUORESCENCE AND IMMUNOHISTOCHEMISTRY

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ABSTRACT

Objective: To compare immunofluorescence and immunohistochemistry methods in assessing the effectiveness of formalin fixation for preserving spinal cord tissue, focusing on detecting astrocytes and neurons through Glial Fibrillary Acidic Protein (GFAP) and Microtubule-Associated Protein 2 (MAP2) expression.

Materials and Methods: This experimental study was conducted at the Institute of Basic Medical Sciences, Khyber Medical University, Peshawar, from February 2021 to March 2023. Thirty male Sprague Dawley rats were used to examine GFAP in astrocytes and MAP2 expression in neuronal cell bodies within the spinal cord. Rats were anesthetized with isoflurane, and cardiac perfusion was performed using normal saline, followed by 4% paraformaldehyde for tissue fixation. Spinal cord segments corresponding to T8-T10 vertebrae were dissected, suspended in 4% buffered formalin, and fixed for 24 hours. Immunofluorescence and immunohistochemistry were performed using primary antibodies for GFAP and MAP2, followed by fluorescent-conjugated or enzyme-linked secondary antibodies. DAPI was used as a nuclear stain during immunofluorescence.

Results: The immunofluorescence analysis did not show any specific signs of GFAP or MAP2. Immunohistochemical staining using the same primary antibodies clearly indicated both GFAP and MAP2 in the spinal cord sections.

Conclusion: The findings suggest that formalin fixation of central nervous system tissues is suboptimal for immunofluorescence applications; however, it remains effective for immunohistochemical analyses.

Keywords: Formalin Fixation, Immunofluorescence, Immunohistochemistry, MAP2, GFAP

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INTRODUCTION

Histological studies of the spinal cord are essential for addressing the increasing need for effective treatments in neurological disorders because it serves as the main pathway connecting the brain to the body and controlling motor function and sensory feedback. [1, 2] Understanding its structure and function is vital for uncovering how neural communication works. This research is important for detecting spinal cord injuries and pathological changes, as well as for developing successful therapeutic interventions. By studying animal models and human tissue, scientists also gain insights into neurodegenerative diseases such as Parkinson's, Alzheimer's, and spinal atrophy at the cellular level. [3]

Astrocytes are the key regulators that maintain homeostasis. They provide metabolic support and control synaptic activity in the spinal cord. Glial Fibrillary Acidic Protein (GFAP) remained a key marker for identifying their activity. [4] Neurons are responsible for signal transmission that relies on Microtubule-Associated Protein 2 (MAP2) for structural stability as well as synaptic plasticity[5]. As-

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trocytes are closely related to neurons, which help support their function and survival. [6] The detection of GFAP and MAP2 helps us to assess the underlying pathological changes. It also supports evaluating the effect of a given therapy for a related disease. [7]

The most widely employed technique for preserving tissue samples is Formalin fixation. This technique is intended for use in immunofluorescence and immunohistochemistry. [8] This method is a prime choice in many laboratories due to its multiple advantages. This method also has some limitations when compared to alternative fixation techniques. [9] A key advantage of formalin fixation lies in its widespread acceptance and the availability of standardized protocols, which enhance consistency and reproducibility across various studies. [10] Formalin fixation ensures the effective preservation of tissue morphology, which is essential for accurate histological evaluation and maintaining structural integrity in microscopic analysis. [11] Moreover, formalin fixation preserves the antigenicity of many proteins, allowing continued accessibility for antibody binding. This preservation makes it a versatile fixative suitable for various immunostaining techniques. [12]

Formalin fixation also has some disadvantages. One main disadvantage is the cross-linking of proteins that occurs during fixation, which can mask specific epitopes. [13] This masking effect can lead to reduced sensitivity in detecting specific antigens. This process potentially compromises the effectiveness of immunostaining. [14] This process often requires extended fixation times that can alter the antigenicity of the tissues. This also affects the outcomes of subsequent analyses in some cases. [15] Additionally, formalin can also contribute to higher background staining due to residual formaldehyde. This contribution, in some cases, can interrupt the required results. [16] The current study was conducted to assess and compare the effectiveness of the formalin fixation method. The assessment of immunofluorescence and immunohistochemistry techniques is also undertaken to detect the expression of glial fibrillary acidic protein (GFAP) and microtubule-associated protein 2 (MAP2) in spinal cord tissue.

MATERIALS AND METHODS

The study was carried out at the Institute of Basic Medical Sciences, Khyber Medical University, Peshawar, Pakistan. The study was conducted from February 2021 to March 2023, serving as a pilot investigation for a doctoral research project. The sample size was calculated using the “Resource Equation”. Thirty healthy male Sprague-Dawley rats were obtained from the National Institutes of Health (NIH). The study received approval from both the Institutional Advance Study Research Board and the KMU Ethics Committee with approval No DIR/KMU-AS&RB/RP/001153. We adhered to the guidelines set by the Institutional Animal Care and Use Committee (IACUC) throughout the experiment. [17] This pilot study served as a preliminary investigation preceding an experimental study aimed at evaluating the therapeutic efficacy of Pirfenidone in the treatment of compression spinal cord injury. For euthanasia, intraperitoneal injection of 200 mg/kg pentobarbitone sodium was administered, following the guidelines set by the American Veterinary Medical Association. [18] After careful removal of the entire vertebral column along with the spinal cord, it was placed in 10% neutral buffered formalin for fixation and slight stiffening. After 24 hours, undamaged 1.5 cm segments of the spinal cord were extracted from T7 to T8. [19] These segments were processed and embedded in paraffin wax for sectioning. [10] Using a microtome, thin longitudinal tissue sections were created, measuring 5 μm, and cut from the dorsal to the ventral direction of the spinal cord.

From each spinal cord, slides were prepared, having two sections arranged in serial order. Standard procedure and protocols were followed for immunofluorescence and immunohistochemical staining[20-21]. To detect astrocytes through immunofluorescence, we employed the GFAP primary monoclonal antibody, paired with Alexa Fluor 594 (anti-mouse red, Invitrogen, Thermo Fisher) as the secondary antibody. For neuron detection using the immunofluorescence technique, the MAP2 monoclonal primary antibody was employed, along with Alexa Fluor 488 (anti-mouse green, Invitrogen, Thermo Fisher) as the secondary antibody. DAPI (405 blue, Invitrogen, Thermo Fisher) served as the nuclear counterstain. To enhance the effectiveness of immunofluorescence staining, the protocol was systematically optimized through a series of strategic adjustments.

For immunofluorescence, slides were subjected to three 5-minute xylene washes, followed by two 10-minute washes each in 100%, 95%, 70%, and 50% ethanol, then two 5-minute washes in deionized water. Antigen retrieval involved boiling in 10 mM sodium citrate buffer (pH 6.0) for 10 minutes, followed by 30 minutes of cooling at room temperature and a 5-minute wash in distilled water. Permeabilization and blocking included treatment with 3% hydrogen peroxide in methanol for 15 minutes, followed by two 5-minute washes with distilled water and two 10-minute washes with PBS-T (0.4% Triton X-100, 1% serum). Non-specific binding was blocked with 5% serum in PBS-T for 30 minutes. The primary antibody was applied in 1% serum PBS-T for 1–2 hours at room temperature and then incubated overnight at 4°C. After two 10-minute PBS-T washes, the secondary antibody in 1% serum PBS-T was applied for 1–2 hours, followed by additional PBS-T washes. Nuclear counterstaining was performed using DAPI.

For immunohistochemistry, the same primary antibodies were used, with DAB (3,3'-diaminobenzidine) as the secondary reagent. Antibody optimization involved testing dilutions of 1:200, 1:150, 1:100, and 1:50; GFAP showed optimal results at 1:100, and MAP2 at 1:50. Tissue

sections were deparaffinized with xylene (twice for 10 minutes), rehydrated through graded alcohols (from 100% to 70%, 5 minutes each), and rinsed with distilled water. After 10 minutes in wash buffer, antigen retrieval was performed using a 1:50 dilution of target retrieval solution at 95°C for 35 minutes, followed by 20 minutes of cooling. Slides were rinsed again, and a hydrophobic barrier was applied with a PAP pen. Endogenous peroxidase was blocked with 3% H_2O_2 for 15 minutes. Non-specific binding was minimized using a serum blocking reagent for 15 minutes. Primary antibodies were applied for 1 hour at room temperature. After three 5-minute washes, slides were incubated with HRP for 30 minutes, washed again, and stained with DAB for 10 minutes. Sections were rinsed, dried, mounted with DPX, and covered with glass coverslips without counterstaining. Imaging and analysis were performed using a Nikon Eclipse 80i microscope.

RESULTS

The immunofluorescence analysis on the spinal cord samples showed negative results for the target proteins GFAP (glial fibrillary acidic protein) and MAP2 (microtubule-associated protein 2), with no specific staining visible under the microscope. As shown in Figure 01, there were no noticeable fluorescence signals for either GFAP or MAP2, indicating these proteins were not detected or localized in the tissue sections examined. In contrast, a strong positive fluorescence signal was seen only for DAPI (4',6-diamidino-2-phenylindole), a nuclear stain that binds selectively to DNA, marking the nuclei of cells in the samples. This, also shown in Figure 01, suggests that nuclear staining was successful. Still, the neuronal and glial markers (GFAP and MAP2) did not exhibit specific immunofluorescence, supporting the conclusion that this technique was unable to detect these proteins.

In comparison, immunohistochemical analysis using the same primary antibodies for GFAP and MAP2 yielded successful and specific staining, as depicted in Figure 2. The positive immunohistochemical staining confirmed the presence of both glial and neuronal markers within the tissue sections, indicating that the formalin fixation protocol preserved the antigenicity of these proteins.

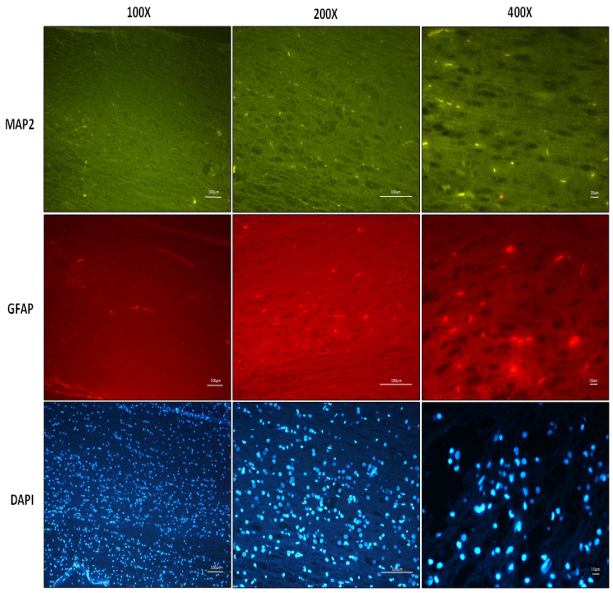

Fig 1: Photomicrograph of a 5 µm thick longitudinal section of the rat spinal cord showing immunofluorescence staining for MAP2, GFAP, and DAPI at 100X, 200X, and 400X magnifications. The images show non-specific, diffuse fluorescence with no clear target labeling in both MAP2- and GFAP-stained sections. In contrast, the DAPI-stained sections display bright blue fluorescence, clearly highlighting the cell nuclei and accurately localizing nuclear structures.

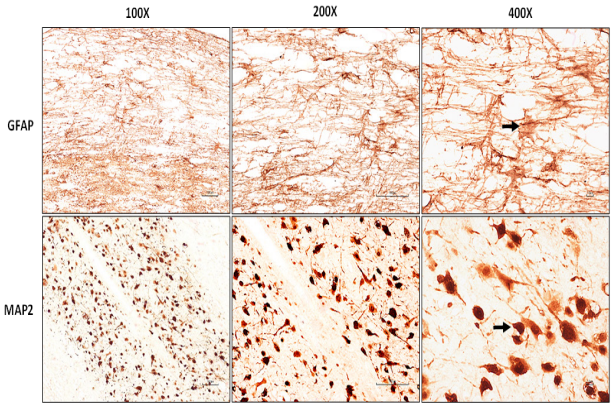

Fig 2: Photomicrograph of a 5 µm thick longitudinal section of the rat spinal cord, showing immunohistochemical staining for GFAP and MAP2 at magnifications of 100X, 200X, and 400X. GFAP expression is clearly visible, outlining astrocytes, as indicated by the arrowhead. MAP2 expression is also evident, highlighting neuronal structures, marked by an arrowhead.

DISCUSSION

The study findings reveal the detection of GFAP and MAP2 using immunofluorescence and immunohistochemistry techniques. The results show varying efficacy of formalin fixation in preserving antigen properties for these detection methodologies. Immunofluorescence analysis yielded negative results for both GFAP and MAP2. No specific staining was detected for these proteins. The absence of apparent fluorescence signals indicates a lack of detectable protein expression or localization within the examined tissue sections. This could be due to the reduced antigenicity caused by formalin fixation. The reduced antigen results in reduced sensitivity of detection, leading to the absence of these proteins in the analysed tissues. Our study results align with a study in which researchers have reported auto-fluorescence in detecting ß-catenin in formalin-fixed spinal cord tissues. [22] Our study results are also supported by a study that reveals loss of protein antigenicity during formalin tissue fixation, resulting in intrinsic

auto-fluorescence in detecting NeuN in spinal cord samples. [23] DAPI is a DNA-binding agent that marks cell nuclei, showing prominent staining, which confirms the presence of intact cells within the sample. On the other hand, the inability to detect the target proteins suggests a limitation of immunofluorescence in formalin-fixed tissues.

In contrast, immunohistochemistry revealed the presence of both GFAP and MAP2, with specific staining of glial and neuronal tissues. This positive result demonstrates that formalin fixation effectively preserves antigenicity, allowing for successful immunohistochemical detection. The clear distinction between glial and neuronal cells within the spinal cord tissue highlights the effectiveness of the IHC protocol. This capability is useful for maintaining protein integrity and improving epitope accessibility. The differences seen between these two techniques are likely due to their distinct detection mechanisms and sensitivities. Results from this study are consistent with those of a study where immunohistochemical markers for glial cells, such as GFAP and MAP2, were accurately quantified in formalin-fixed human neocortex. [24] The immunofluorescence will need a higher level of epitope exposure compared to immunohistochemistry.

This comparison assesses the effectiveness of protein detection protocols. Formalin fixation may hinder immunofluorescence by causing protein cross-linking, which can mask epitopes crucial for antibody binding. In contrast, allowing for clear visualization of targeted proteins preserves antigenicity for immunohistochemical staining more effectively. These results suggest that improved antigen retrieval methods may be necessary to optimize immunofluorescence for detecting proteins such as GFAP and MAP2. Nonetheless, formalin remains a standard fixative.

When choosing detection methods, different results were observed between immunofluorescence and immunohistochemistry. These results underscore the importance of carefully considering the limitations associated with fixation. Immunofluorescence may benefit from adjustments in fixation protocols, provided that accessible epitopes are available. Additionally, enhancing antigen retrieval processes could improve detection sensitivity in immunofluorescence. Conversely, the reliability of immunohistochemistry was also demonstrated in this study, reinforcing its usefulness in identifying cellular markers in formalin-fixed tissues. Future research should focus on refining fixation and staining protocols, which are essential for optimizing both methods and achieving comprehensive protein expression analysis. Overall, this comparative analysis offers valuable guidance for experimental design in neuroanatomical research, particularly in studies aimed at characterizing glial and neuronal markers within the spinal cord.

CONCLUSION

This study compares formalin fixation for immunofluorescence and immunohistochemistry. The results show that, while immunofluorescence failed to detect GFAP and MAP2—possibly due to poor antigen preservation or technical issues—immunohistochemistry successfully identified both markers. Therefore, formalin fixation may not be ideal for the immunofluorescence detection of specific proteins in spinal cord tissue; however, it remains effective for immunohistochemical analysis. Further research on alternative fixation methods is recommended to improve the performance of immunofluorescence.

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Authors Contribution:

Following authors have made substantial contributions to the manuscript as under

Authors	Conceived & designed the analysis	Collected the data	Contributed data or analysis tools	Performed the analysis	Wrote the paper	Other contribution
Wazir NU	✓	×	✓	×	×	✓
Ahmad M	✓	✓	×	✓	✓	×
Humayun A	✓	×	✓	×	×	✓
Shehzad K	✓	✓	×	×	✓	×
Faisal MS	✓	×	✓	×	×	✓
Ahmed N	✓	✓	×	✓	✓	×

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethical Approval:

This Manuscript was approved by the Ethical Review Board of Khyber Medical University, Peshawar. Vide No. Dir/KMU-EB/RP/000768. Dated: 30 06 2021

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