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Original Article

Comparison of different fixatives effects in histochemical stainings of peripheral nerve tissue

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Abstract

A pathological condition in the peripheral nerve tissue, which provides the connection between the organism and the external environment, negatively affects the standard of living. The nerve tissue histotechnology is of serious importance both for scientific studies and for clinical diagnosis. The fixation, which is one of the leading procedures for histological examination of tissues, aims to preserve tissue morphology. Another essential part of the histological examination is staining process. This study, it was aimed to determine the fixative that provides optimal histological appearance in peripheral nerve tissue. Therefore, various histochemical stainings of tissues fixed with some fixatives used in practice were compared. Sciatic nerves from each rat (n=7) used in the study were fixed with different fixatives and histochemical staining was performed. In histological examination, cellular (nucleus-cytoplasm) and intercellular morphological details, staining intensity and distribution were evaluated. At the end of the study, formaldehyde was found to be the most ideal fixing agent for all stains. Although Bouin and Carnoy fixatives differed according to the staining type, their fixation quality was similar in general. Glutaraldehyde did not give as good results as other fixatives in all stainings. This study is an important technical reference for clinical and experimental studies.

Keywords: Fixation, Histochemical staining, Histotechnology, Nerve tissue, Sciatic nerve

1. Introduction

The peripheral nervous tissue damage is an important subject of scientific study, since a pathology that may occur in the peripheral nervous tissue, which provides transmission to the central nervous system with autonomic and motoric nerve fibres, adversely affects the life of living beings. The histopathological examination is frequently used to microscopically observe histological changes (pathophysiology) in peripheral nervous tissue. The histopathological examination technique is a diagnostic method that allows the observation of possible morphological changes in tissues at the end of a clinical and experimental process. This diagnostic method consists of a series of critical steps [1-3].

The fixation is the most important and first step of histopathological examination technique. In order to obtain the closest appearance to the living tissue in the tissues to be examined histopathologically, it is necessary to stop autolysis and deterioration in the tissue. This process is called fixation, and the agents that provide this are called fixatives [4,5]. Various agents are used for fixation, the most preferred of which is formalin. Formalin contains 40% (or 37%) formaldehyde gas dissolved in water. This colorless, suffocating-smelling, liquid-form agent, which is mostly buffered with phosphate salts and diluted 10 times with water, provides detection in a wide range of tissues [6]. Bouin solution consisting of formaldehyde, picric acid and acetic acid provides detection by cross-linking proteins. It is preferred for the fixation of sensitive, soft and especially small tissues such as biopsy materials [7]. Carnoy solution consisting of acetic acid, alcohol and chloroform provides fixation in many tissues and is mostly preferred in emergency applications with its fast penetrating feature [8]. Glutaraldehyde provides detection by the reaction of aldehyde in its structure and amine groups of tissue proteins. This colourless, pungent, oily and liquid-form agent is successful in the detection of carbohydrates, proteins and enzymes, and is mostly preferred in electron microscopic examinations [9].

Since paraffinized tissue sections are colourless, they need to be stained for histological examination. The most commonly used histochemical staining technique in routine examination of tissues is hematoxylin-eosin (HE) staining. HE staining is based on blue-purple (nucleus)

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and red-pink (cytoplasm) staining of tissue components at different Ph [10]. Another staining method used in routine is Masson trichrome (MTC) staining. This stain offers a more selective observation especially by staining collagen connective tissue fibres [11]. In nerve tissue histological examination, more detailed observation of myelinated/unmyelinated axon structures and nissil bodies may be needed. The most preferred nerve tissue-specific histochemical stain is luxol fast blue (LFB) staining. This staining shows details of neural nuclei, axoplasm, and myelination [12]. Another method that can be used for shows the components of the nervous system is aniline blue (AB) staining. This stain gives details about the connective tissue between neurofibrils and axon extensions [13].

In this study, it was aimed to determine the most ideal fixative in different histochemical stains applied in histological examination of peripheral nerve tissue. For this purpose, histochemical staining of tissues fixed with four different fixatives was performed. Stained preparations were evaluated for morphological details of nuclei, axons, and intercellular structures in the nervous tissue and staining quality, and comparisons were made between fixatives.

2. Materials and Methods

Seven healthy, adult, male, male, Wistar Albino rats weighing 240±20 g were used in the study. Animals were euthanized under general anesthesia (Xylazine 10mg/kg and Ketamine 5mg/kg). The thighs of the animals were shaved and after a deep incision, the right and left sciatic nerves were separated from the truncus, and removed by dissection of the peripheral tissues in the middle part of the thigh. The sciatic nerves from each animal were divided into four equal parts, and placed in four different fixatives.

2.1. Processing of Tissues

Sciatic nerve tissue samples taken from rats were fixed in the fixatives specified in Table 1, and then taken to routine tissue follow-up. In routine tissue follow-up, tissues were dehydrated (in alcohol), cleared (in xylene) and infiltrated (in paraffin), respectively. Then, the tissues were converted into made paraffin blocks using a paraffin dispersion device (Leica, TP 1020, Germany). From each paraffin block, tissue sections of 4µ thickness for different staining were taken on adhesive slides using a rotary microtome (Leıca, RM 2125, Germany). The sections, which were kept in the oven for a while, were prepared for different stainings after deparaffinization (xylene) and rehydration (in alcohol, and distilled water).

2.2. Hematoxylin-Eosin (HE) staining

After deparaffinization and rehydration, the tissues prepared for staining were kept in hematoxylin (Weigert's iron hematoxylin) for 5 min. Then the sections were washed in water for 10 min and stained with eosin (polyvinyl alcohol eosin yellow) for 1 min. The hematoxylin and eosin-stained tissues were passed through increasing concentrations of alcohols (70%, 80%, 90%, 96%) and kept in absolute alcohol for 2 min. Finally, the sections cleaned in two different xylol for 5 min each were covered with a coverslip by dropping Entellan™ [14].

2.3. Masson's Trichrome (MTC) Staining

MTC (Cat. no. 04-010802; Bio-Optica, Italy) staining was performed according to the protocol specified in the commercial staining kit. A solution consisting of equal mixtures of Weigert iron hematoxylin (solution A) and Weigert iron hematoxylin (solution B) was added to the tissue sections prepared for staining and incubated for 10 min. Sections were kept in alcohol-based picric acid solution for 4 minutes without washing. It was then quickly washed in distilled water. It was incubated in ponceau acid fuchsin solution for 4 min and then washed again.The tissue sections were incubated with phosphomolybdic acid solution for 10 min, and aniline blue was added without washing and incubated for 5 min. The sections were dehydrated (in alcohol) and cleaned (in xylol) and then sealed [15].

2.4. Luxol Fast Blue (LFB) Staining

LFB (Cat. no. 04-200812; Bio-Optica, Italy) staining was performed according to the protocol specified in the commercial staining kit. After deparaffinization, reagent A (alcohol-based luxol fast blue) solution was added to the tissues kept in 95% ethanol and incubated overnight in an oven at 56°C. The tissue sections removed from the oven were washed in 95% alcohol. Reagent B (basic buffer solution) solution was added to the tissues washed in distilled water and kept for 30 s. The tissues were washed in 70% alcohol and washed again in distilled water. Then, the solution prepared from reagents C (Cresyl violet solution) and D (acid activation buffer) in 2;1 ratios was dripped onto the tissues and incubated in an oven at 56oC for 20 min. Finally, the tissues differentiated in 95% ethanol were

Table 1. Fixatives used in the study and their contents.

Contents	Fixatives			
	NBF	**BS**	**CS**	**G**
Formaldehyde	100 ml	250 ml		
Glutaraldehit %25				100 ml
Acetic acid		750 ml	100 ml	
Picric acid		50 ml		
Chloroform			30 ml	
Ethanol (95%)			600 ml	
Distle su	900 ml			900 ml
$NaH_2PO_4*H_2O$	4 g			
Na2HPO4	6.5 g			

NBF; Neutral Buffer Formaldehyde BS; Bouin solution CS; Carnoy solution G; Glutaraldehyde.

dehydrated (in alcohol), cleaned (in xylol) and sealed [16].

2.5. Aniline Blue (AB) staining

Aniline staining of tissue sections prepared for staining after deparaffinization and rehydration was performed by modification of the method used by Santiago-Moreno et al. For this, tissue sections were incubated in PBS containing 3% buffered glutaraldehyde for 30 min at room temperature. Then the tissues were incubated in a solution containing 5% aniline blue (distilled water-aniline blue, Ph=3.5) mixed with 2% acetic acid for 5 min. Finally, the tissue sections were washed in distilled water, dehydrated (in alcohol), cleaned (in xylol) and sealed [17].

2.6. Histochemical parameters and scoring

The histological appearance quality of the tissues fixed in different fixatives was examined under a light microscope (Olympus BX 53, Japan) in terms of nucleus (schwan cell), axoplasm and intercellular morphological details and staining contrasts. In order to obtain a more rigorous result in the evaluation of these histological parameters, a wide scoring scale was adopted. For this purpose, a scoring scale of 1-10 was used according to the quality of histological appearance and the findings of each animal were averaged [18].

2.7. Statistical analysis

Statistical analyses were performed using SPSS 24.0 for Windows software (SPSS Inc., NY, USA). Variance analysis (One-way Anova) was used to determine the difference between the groups at a 95% confidence interval. The statistical significance level in the values of the research results was evaluated as $P \leq 0.05$.

3. Results

In HE, MTC, LFB, and AB staining of peripheral nerve tissues fixed with different fixatives, the cell (nucleus, axon plasma) and intercellular structures (epineural, perineural and endoneurial connective tissue sheaths) forming the tissue were evaluated in terms of morphological detail and staining quality in order to obtain the most ideal histological appearance.

3.1. Histological findings in formaldehyde-fixed tissues

It was observed that HE staining of tissues fixed with formaldehyde, it was observed that nuclear, axoplasmal and intercellular connective tissue morphologies were in the best manner preserved. Morever, It was observed that the selective staining contrast also between these structures was better than other fixatives. Accordingly, it was determined that formaldehyde offers the most ideal histological observation opportunity in HE staining, compared to other fixatives, in terms of both morphological detail and staining quality.Similar findings were obtained in MTC and LBF staining of these tissues as in HE staining. Formaldehyde fixation has been found to be more effective than other fixatives, particularly in visualizing myelin structures and staining Schwann cell nuclei. Similar results to LFB staining were obtained in AB staining of formaldehyde-fixed tissues. However, AB staining did not give a good nuclear detail as in LFB staining. Formaldehyde was found to be the most ideal fixation agent for all stains made in the study (Fig. 1).

3.2. Histological findings of tissues fixed with Bouin solution

It was determined that the morphological detail preservation of cell and intercellular structures and staining quality were not as good as formaldehyde and Carnoy in HE staining of tissues fixed with Bouin. In MTC staining, it was similar and it was found that overstaining, especially in connective tissue, caused breaks in cell details. However, unlike LFB staining, Bouin solution provided a better histological visualisation than Carnoy fixative. In AB staining, Bouin fixative was not as good as formaldehyde and Carnoy. It was determined that intense staining of tissues fixed with Bouin in an irregular distribution with AB caused loss of morphological detail. The staining of tissues fixed with Bouin did not provide a histological appearance as good as formaldehyde and Carnoy, but it was successful in LFB staining (Fig. 2).

3.3. Histological findings of tissues fixed with Carnoy solution

Although the morphological preservation and staining quality of diffrent compartment in cell, and intercellular structures in HE staining of tissues fixed with Carnoy were not as good as formaldehyde, better histological visuals were obtained compared to other fixatives. The same situa-

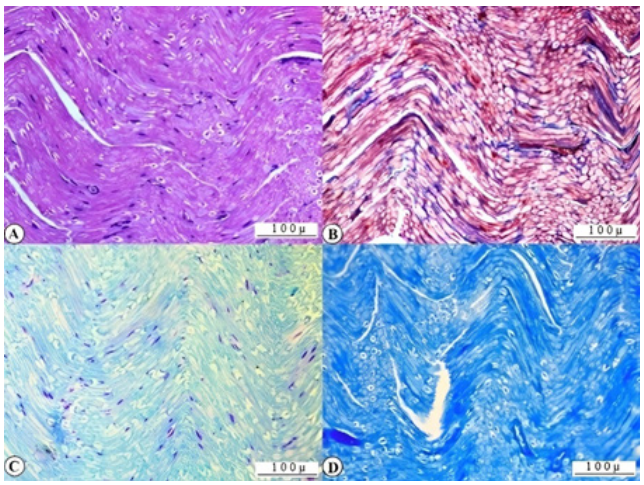

Fig. 1. Different histochemical stains of peripheral tissues fixed with 10% buffer formaldehyde solution, X200, Bar; 100µ A. HE B. MTC C. LFB D. AB.

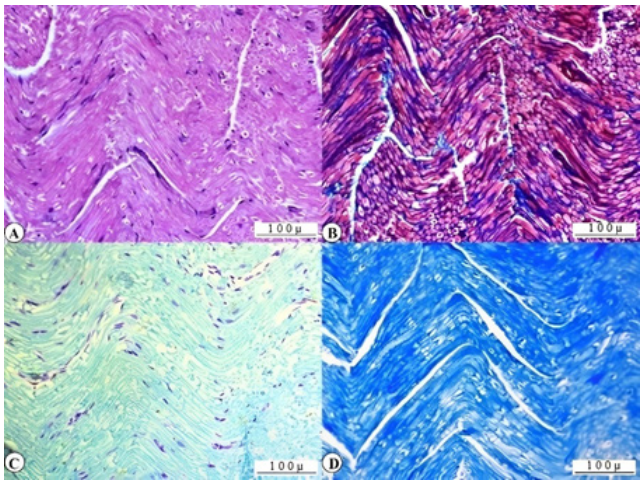

Fig. 2. Different histochemical stains of peripheral tissues fixed with Bouin solution, X200, Bar; 100µ A. HE B. MTC C. LFB D. AB.

tion was also valid for MTC staining, but it was observed that Carnoy did not provide sufficient morphological detail in LFB staining, especially when the nuclear detail was weak. In AB staining, Carnoy fixative provided a better visualization compared to other fixatives, although not as good as formaldehyde. Although AB staining was successful in showing myelinated structures, nuclear detail was not sufficient. In the histochemical staining of Carnoy-fixed tissues, although an optimal histological appearance was not obtained as much as formaldehyde, it provided a good histological observation opportunity compared to other fixatives. However, it was not as successful as Bouin in LFB staining (Fig. 3).

3.4. Histological findings of the tissues fixed with glutaraldehyde

It was observed that cell and intercellular morphology was not well preserved with due to excessive shrinkage in HE staining of the tissues fixed with glutaraldehyde. In addition, it was determined that the staining density and distribution in tissue were not suitable for a healthy histological examination. It was determined that glutaraladehyde did not provide as good histological visualisation as other fixatives, both in terms of inefficient in morphological detail and poor selective staining quality. This was not only valid for HE staining but also for MTC, LBF, and AB stainings (Fig. 4). The findings related to nuclear, axoplasmal and intercellular morphology and staining quality in staining with different fixatives were presented in tables and graphs (Fig. 5, Table 2).

In the statistical analysis, it was determined that the difference between the fixatives in terms of the presentation of the nuclei and cytoplasm of the cells and intercellular morphological details was statistically significant ($P \leq 0.05$). Likewise, it was determined that the difference between the fixatives in terms of the staining quality of the nuclei and cytoplasm of the cells and intercellular structures was statistically significant ($P \leq 0.05$).

4. Discussion

The peripheral nervous system is an important structure that transmits external stimuli to the central nervous system and helps to shape the possible response. Due to this function, in to damage made this tissue has important consequences, and has become the center of interest in the scientific World [19]. The sciatic nerve is the largest of the peripheral nerve appendages that innervate the organism, and it regulates the sensory and motor functions of the hind limbs. The sciatic nerve is often preferred in

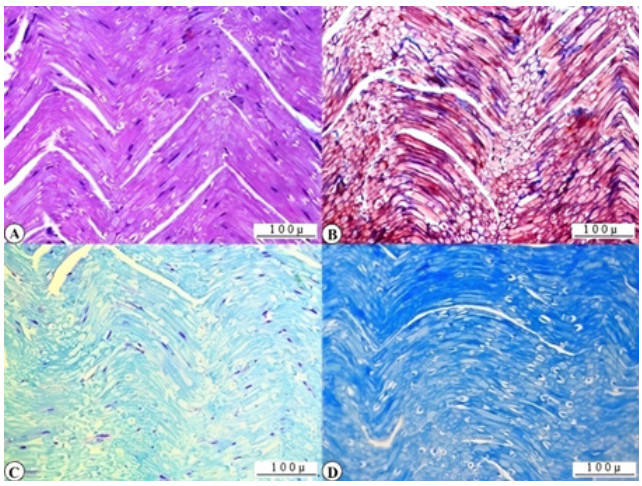

Fig. 3. Different histochemical stains of peripheral tissues fixed with Carnoy solution, X200, Bar; 100µ A. HE B. MTC C. LFB D. AB.

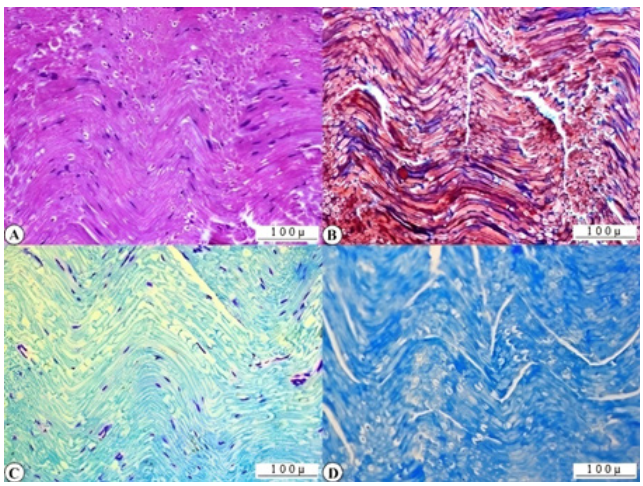

Fig. 4. Different histochemical stains of peripheral tissues fixed with glutaraldehyde, X200, Bar; 100µ A. HE B. MTC C. LFB D. AB.

Table 2. Scoring the quality of histological appearance in histochemical staining with different fixatives.

		HE	MTC	LFB	AB
NMD/NSC	**F**	9.2/9.5	7.8/8.0	9.1/9.2	8.0/8.1
	B	8.5/8.7	7.2/7.5	8.8/9.0	7.4/7.5
	C	9.0/9.1	7.5/7.8	8.1/8.4	7.7/7.8
	G	7.8/7.8	6.0/6.1	6.4/6.4	6.1/6.2
AMD/ASC	**F**	9.5/9.5	8.0/8.2	9.2/9.4	8.1/8.2
	B	8.7/8.8	7.4/7.5	9.0/9.1	7.5/7.7
	C	9.1/9.2	7.8/8.0	8.2/8.4	7.8/8.0
	G	7.8/7.8	6.2/6.4	6.5/6.7	6.4/6.5
İMD/İSC	**F**	9.7/9.8	8.1/8.2	9.4/9.5	8.4/8.7
	B	8.8/9.0	7.5/7.7	9.0/9.1	7.5/7.7
	C	9.2/9.5	7.8/8.1	8.4/8.8	8.2/8.4
	G	7.8/8.0	6.2/6.4	6.7/6.7	6.4/6.7

NMD; Nuclear morphological detail, NSC; Nuclear staining contrast, AMD; Axoplasmal morphological detail, ASC; Axoplasmal staining contrast, IMD; Intercellular morphological detail, ISC; Intercellular staining contrast.

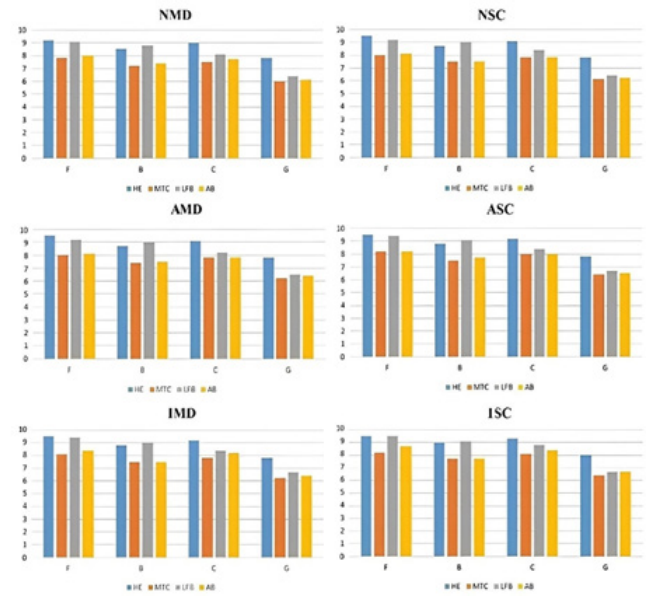

Fig. 5. Comparison of different fixatives in terms of some histological parameters.

peripheral nerve tissue studies due to its easy accessibility, traceability and its susceptibility to various modeling. Most of the peripheral nerve tissue studies are on experimental neural damage. Histopathological examination is an indispensable technique in these studies. Although the studies in this field have focused on peripheral nerve tissue damage, histotechnological developments have been insufficient [20-22].

Histopathological examination, which is frequently used in clinical routines and observation of experimental processes, is performed with a technical application including a series of stages. The first stage in histopathological technique is fixation, and the main purpose of this step, which affects the result completely, is to prevent autolysis and decay in the tissue as a manifestation of a biological process and to obtain the closest image to the living tissue. Today, there are many fixatives used for fixation. The most commonly used of these is formaldehyde. Although this fixative, which is mostly used in buffered form, is very successful, it is now beyond discussion that it has a number of disadvantages in terms of biosafety. For this reason, the development of fixatives that can be an alternative to formaldehyde has been a particular focus of interest. In addition to safe use, a fixative is also expected to present the ideal histological appearance [4,7,23]. In this study, it was aimed to determine the fixative that provides the most ideal appearance in histological examination of peripheral nerve tissue.Some researchers who state that formaldehyde is the best fixation agent as well as those who state the opposite. Foot, reported that even the buffered form of formaldehyde produced misleading details due to deterioration of neurons, excessive coagulation of myelin and chemical changes [24]. Zanni et al. stated that the view that formaldehyde is the gold standard has no scientific basis and that there are many fixatives available as alternatives to formaldehyde [25]. Some researchers have reported that modified forms of formaldehyde are more successful. Bamisi et al. stated that alcoholic formalin gave more successful results than buffered formaldehyde and paraformaldehyde in liver and brain tissue fixation [26]. Ulucan et al. reported that the most successful fixative in HE staining was buffered formaldehyde in their study comparing the effects of different fixatives. They also emphasized that buffered formaldehyde does not lyse erythrocytes, unlike other fixatives [27]. In this study, it was determined that buffered formaldehyde provides good morphological detail and staining quality and provides an optimal histological appearance in accordance with many previous studies.

Bouin solution developed with the combination of formaldehyde is preferred for the fixation of sensitive and soft tissues, especially small biopsy materials. Mirzaei et al. evaluated the fixation of different tissues with different fixatives and stated that Bouin solution provided better fixation in testis, liver and brain tissues compared to Carnoy and formaldehyde and was more successful, especially in showing nuclear detail [28]. Peterson et al. stated that Bouin solution provides strong cytoplasmic staining with eosin, but acetic acid-containing fixatives such as Bouin may lead to protein and nucleic acid denaturation due to hydrogen bonding [29]. Singhal et al. reported that Bouin is the most ideal fixative for MTC staining [30]. Ulucan et al. reported that Bouin solution lyses erythrocytes and does not give as successful results in fixation as formaldehyde [27]. In this study, bouin solution was not as successful as formaldehyde and Carnoy in HE, MTC and AB staining. It was observed that overstaining of connective tissue overshadowed the cellular detail. However, in LFB staining, it was observed that the Bouin solution provided a better histological appearance than the Carnoy solution, although it was not as effective as formaldehyde.

The acetic acid contained in Carnoy solution provides rapid fixation when infusion into the tissue is increased. It has been reported that this fixative, which can be used in many tissue fixation, causes dehydration, and binding hydrogen in the tissue due to the alcohol and chloroform contained in it, which causes denaturation and coagulation of proteins and nucleic acids [29]. Mirzaei et al. reported that Carnoy solution was better than other fixatives in spleen and kidney tissue fixation in their study investigating the effect of different fixatives on different tissues [28]. Rowley et al. reported that Carnoy is a suitable fixative for the preservation of hyaluronan in ovarian and liver tissue [31]. In their study comparing the fixation of Carnoy and formalin in various tissues, Ahmed et al. stated that although Carnoy is safe and penetrates the tissues faster, it does not provide as successful fixation as formalin [18]. Ulucan et al. stated that Carnoy solution provides intense nuclear staining due to agglutination of nucleic acid, but the most ideal staining is performed with buffered formalin and other fixatives produce different staining intensities [27]. In this study, it was found that Carnoy was better than other fixatives in terms of morphological detail and staining quality in HE, MTC and AB staining, although not as good as formalin, but not as good as Bouin solution except formalin in LFB staining.

Glutaraldehyde, an aldehyde group fixative, is not as widely used as formaldehyde. However, it is preferred in applications in the mostly electron microscopic examinations. This is thought to be due to the fact that glutaraldehyde preserves molecular detail better than morphology [9]. No studies comparing glutaraldehyde with other fixatives other than formaldehyde were found in previous studies. Hopwood stated that glutaraldehyde inhibited enzyme activity more than formaldehyde. He

also reported that it preserved glycogen in rat liver similar to formaldehyde [32]. Srinivasan et al. stated that since glutaraldehyde has a larger molecular structure than formaldehyde, it penetrates into the tissue twice slower than formaldehyde [33]. Fix and Garman stated that the ability of glutaraldehyde to cross-link proteins is higher than formaldehyde and therefore glutaraldehyde may be more effective in the fixation of nerve tissues that are resistant to cross-linking due to their high lipid content [34]. In this study, it was observed that glutaraldehyde was not better than other fixatives in terms of tissue detail and staining quality.

5. Conclusion

As a result, it was observed that different fixatives produced different results in histochemical staining of peripheral nerve tissue. In this study, the most successful fixative in terms of morphological detail and staining quality in all histochemical stains was buffered formaldehyde. This was followed by Carnoy solution, but Bouin solution presented a better histological appearance than Carnoy in LFB staining. Glutaraldehyde did not present a better histological appearance than the other fixatives used in the study. It is predicted that this comprehensive study will provide basic information and reference to histotechnology to be used in peripheral nerve tissue studies.

Conflict of interests

The authors have no conflicts with any step of the article preparation.

Consent for publications

The authors read and approved the final manuscript for publication.

Ethics approval

This study was performed with the ethical approval of the Harran University Animal Experiments Local Ethics Committee (Approval No: 2023/008/11).

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

Author Contributions

Conceptualization (MBD and US); Formal analysis (MBD and MD); Investigation (MD-İD); Methodology (MBD and MD); Supervision (İD); Writing – review & editing (MBD, US, MD and İD).

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