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Application of Several Special Staining Methods for Paraffin Sections on Epon-Embedded Semithin Sections

Yarı-İnce Epon Kesitlere Parafin Kesitler İçin Önerilen Çeşitli Özel Boyama Yöntemlerinin Uygulanması

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ABSTRACT

Aim: This study aimed to compare several specific staining protocols recommended for paraffin sections and toluidine blue and light green double staining combination to be tried for the first time with routine toluidine blue staining on semithin epon sections.
Material and Methods: Samples of 1x1x1 mm were taken from the liver, skin, and aorta tissues of Wistar albino adult rats. Tissue samples were fixed with 5% glutaraldehyde at +4º C overnight, postfixed with 1% osmium tetroxide for one hour, and then, blocked with Epon 812 after processing. Semithin sections of 1 µm thickness were obtained from the epon blocks. Sections were stained with Altmann's method (for mitochondria), Verhoeff's method (for elastic fibers), Gordon&Sweets' silver impregnation method (for type III collagen), toluidine blue and light green double staining combination (for type I collagen) and routine toluidine blue method.
Results: In liver sections, mitochondria in hepatocytes were differentiated by the Altmann method, and stromal type III collagen fibers were distinguished with Gordon&Sweets' method. Elastic lamellar structures were easily observed in black in the aortic sections stained with the Verhoeff method. Successful results were obtained in the staining of dermal type I collagen with toluidine blue and light green double staining in skin sections.
Conclusion: Since the specific staining tried for the first time gave positive results in epon sections, it was concluded that these methods can be used to determine the localization of cellular and intercellular components that are aimed to be examined at the ultrastructural level.
Keywords: Epon section; special stainings; light green.

ÖZ

Amaç: Bu çalışmada, parafin kesitler için önerilen çeşitli spesifik boyama protokollerinin ve ilk kez denenecek toluidin mavisi ve açık yeşil ikili boyama kombinasyonunun yarı-ince epon kesitlerde rutin toluidin mavi boyamasıyla karşılaştırılması amaçlandı.
Gereç ve Yöntemler: Wistar albino türü erişkin sıçanlara ait karaciğer, deri ve aort dokularından 1x1x1 mm boyutlarında örnekler alındı. Alınan doku örnekleri %5 gluteraldehit ile bir gece süreyle +4º C'de fikse edildi, ardından bir saat %1 osmiyum tetroksit ile postfiksasyon uygulandı ve takip işlemi sonrası Epon 812 ile bloklandı. Epon bloklardan 1 µm kalınlıkta yarı-ince kesitler elde edildi. Kesitler Altmann yöntemi (mitokondri için), Verhoeff yöntemi (elastik lifler için), Gordon&Sweet gümüşleme yöntemi (tip III kollajen için), toluidin mavisi ve açık yeşil ikili boyama kombinasyonu (tip I kollajen için) ve rutin toluidin mavisi yöntemi ile boyandı.
Bulgular: Karaciğer kesitlerinde, Altmann yöntemi ile hepatositlerdeki mitokondriler, Gordon&Sweets yöntemi ile stromal tip III kollajen lifler belirgin bir şekilde ayırt edildi. Verhoeff yöntemi ile boyanan aort kesitlerinde elastik lamellar yapılar siyah renkte kolaylıkla izlendi. Deri kesitlerinde toluidin mavisi ve açık yeşil ikili boyaması ile dermal tip I kollajenin boyanmasında başarılı sonuçlar elde edildi.
Sonuç: İlk kez denenen spesifik boyanmalar epon kesitlerde olumlu sonuçlar verdiği için, bu metodlardan ultrastrüktürel düzeyde incelenmesi hedeflenen hücresel ve hücrelerarası bileşenlerin lokalizasyonunun belirlenmesinde yararlanılabileceği sonucuna varılmıştır.
Anahtar kelimeler: Epon kesit; özel boyamalar; light green.

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INTRODUCTION

Semithin sections occupy an important place in histological and clinical diagnosis studies. They act as links in between light microscopic images and transmission electron microscopy (TEM) images. Protecting the cellular compounds of tissues better than those embedded in paraffin and enabling further identification of them on the light microscopic level are the biggest advantages of semithin sections. TEM studies employ a double fixation procedure that applies a glutaraldehyde fixation followed by a postfixation, with which osmium tetroxide is generally used. Obtaining staining in different colors is very difficult in semithin sections of tissues fixed with osmium tetroxide. The presence of reducted osmium complicates the penetration of the dyes into the sections or blocks certain reactive groups in the tissues. For these reasons, glutaraldehyde fixation alone is often preferred when targeting lipids is not an objective (1-3).

The advantages of epoxy resins make them the most preferred embedding medium in TEM studies. Resin sections have plastic and hydrophobic structures, and therefore, do not show affinity for or are stained by the dyes used in many histological methods. Epon also limits the penetration of the staining agents used in light microscopic examination into tissues. In routine electron microscopy techniques, semithin sections are usually subjected to monochromatic staining methods. Basic dyes such as toluidine blue, methylene blue, basic fuchsin, azure II, crystal violet, safranin O, and thionin are used for this purpose. Increased alkalinity enhances the penetration of dyes into the tissues in plastic sections (2-6). Toluidine blue, which is the most widely used stain in this context, is a metachromatic, cationic thiazine dye (7,8). Alkaline solutions of toluidine blue are used to stain semithin epon sections. These solutions yield elaborate structural details, are easily prepared, and do not deteriorate for a long time. Polychromatic staining methods cannot be routinely used on epon sections because of disadvantages such as the need for numerous complicated reactive agents, long incubation period, difficulty in stabilizing the color tone, short duration of use of the stain solutions, dominance of the last stain, shedding of the sections due to multistep processes, and wrinkles or precipitates forming in the sections. This leads to the inability to identify specific tissue components in semithin sections. Fixative solutions, buffer solutions, pH, temperature, concentrations of the stain solutions, staining duration, section thickness, and properties of the embedding medium are the factors that affect differential staining in polychromatic staining methods (2-6).

This study aimed to i) demonstrate the general tissue morphology of the control sections of various tissues by routine monochromatic toluidine blue staining, ii) determine at the cellular/intercellular level the localization, quantification, and density of specific tissue components that cannot be completely identified with toluidine blue by utilizing the polychromatic staining methods proposed for paraffin sections, iii) identify type I collagen fibers with the combination of toluidine blue and light green, which this information was not found in the literature, and, iv) localize cellular/intercellular specific tissue components in semithin epon sections, thus allowing ultrastructural correlation.

MATERIAL AND METHODS

In this study, archived epon blocks of liver, aorta, and skin tissues of Wistar albino male and female adult rats that were prepared in the Bursa Uludağ University, Faculty of Medicine, Department of Histology and Embryology, Transmission Electron Microscopy Unit were used.

Tissue samples were fixed overnight in 5% glutaraldehyde buffered with 0.13 M Sörensen's phosphate buffer at 4° C and postfixed with 1% osmium tetroxide in the same buffer at 4° C for one hour. After postfixation, the tissues were washed with buffer solution and dehydrated in an alcohol series of increasing concentrations. The clearing was performed in a propylene oxide solution. Afterwards, they were kept in a mixture of propylene oxide:epon at a ratio of 1:1. After overnight impregnation in pure epon, tissues were embedded in Epon 812. The epon blocks were trimmed using an ultra-trim device (Reichert). Afterward, semithin sections of 1 µm thickness were obtained using glass knives and an ultramicrotome (Reichert Supernova). All sections were mounted on gelatin-coated slides to minimize shedding risks and applied etching by acetone before staining (9).

Staining Methods

Classical 1% toluidine blue with borax staining (3) was applied to all the tissue samples of the control sections. Liver sections were stained with Altmann's method (10) and Gordon&Sweets' silver impregnation method, aorta sections were stained with Verhoeff's method, and skin sections were stained with toluidine blue (3) and light green (10,11) double staining combination. The optimum durations and temperatures of the dyes were determined after preliminary studies. Photographs were taken using an Olympus BX50 photomicroscope.

RESULTS

Identification of the Mitochondria with Altmann's Method

The mitochondria could not be specifically observed in the semithin epon-embedded liver sections stained with the conventional toluidine blue stain (Figure 1A). After Altmann's protocol was applied to the sections, the mitochondrial profile could clearly be distinguished as granular structures in brilliant cyclamen color, contrasting on a pale pinkish-yellow cytoplasmic background (Figure 1B).

Identification of the Reticular (Type III Collagen) Fibers with Gordon&Sweets' Silver Impregnation Method

The reticular (type III collagen) fibers in the liver stroma could not be differentiated with toluidine blue (Figure 1A), while they could clearly be observed in black color with Gordon&Sweets' silver impregnation method (Figure 1C).

Identification of the Elastic Fibers with Verhoeff's Method

Elastic fibers were observed as bundles in their classic dark blue-violet in semithin epon-embedded aorta sections stained with the conventional toluidine blue (Figure 2A). However, they were differentiated as black bundles on a clear background in sections stained with Verhoeff's hematoxylin (Figure 2B).

Identification of the Type I Collagen Fibers with the Toluidine Blue and Light Green Combination

Type I collagen fibers present in the dense irregular connective tissue of the dermis (stratum reticulare) layer of skin were distinguished as light violet irregular bundles

in semithin epon-embedded sections stained with toluidine blue. Specific differentiation could not be achieved with this staining (Figure 3A). In the epon sections stained with the toluidine blue and light green combination, however, type I collagen fibers were observed to be stained a brilliant green color on a clear background and could be easily identified from other contrasting structures stained violet-dark blue with toluidine blue (Figure 3B).

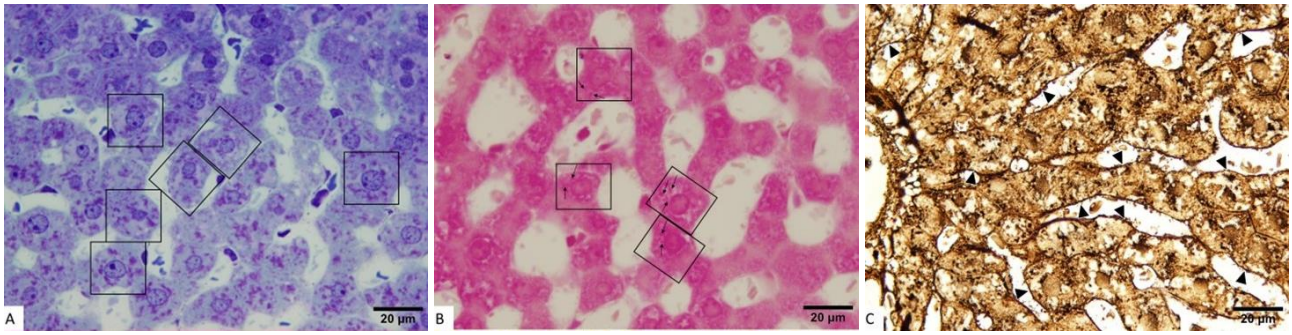

Figure 1. A) Toluidine blue staining, **B)** Altmann's method, and **C)** Gordon&Sweets' silver impregnation method in semithin epon sections of liver
black boxes: hepatocyte cells (A), arrows in black boxes: mitochondria in hepatocytes (B), black arrowheads: reticular fibers (C)

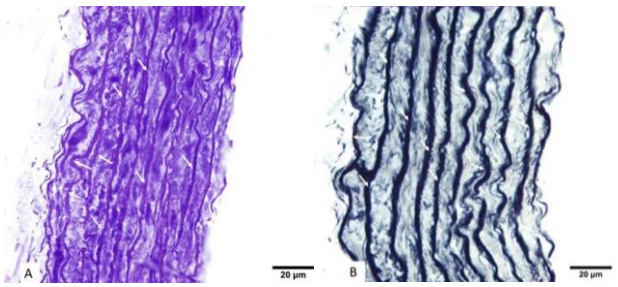

Figure 2. A) Toluidine blue staining and **B)** Verhoeff's hematoxylin staining in semithin epon sections of the aorta
white arrows: elastic bundles

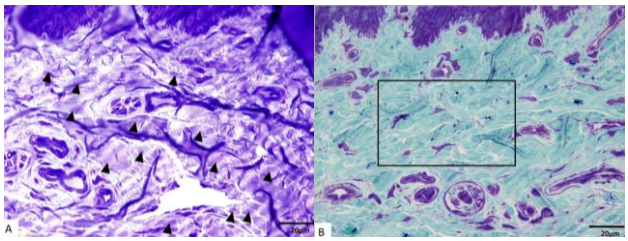

Figure 3. A) Toluidine blue staining and **B)** toluidine blue and light green combination in semithin epon sections of skin
black arrowheads: type I collagen bundles (A), black box: type I collagen bundles (B)

DISCUSSION

In the field of monochromatic stainings of plastic sections, toluidine blue was initially tested by a group of researchers for staining semithin epon-embedded sections obtained after osmium tetroxide postfixation (12). Their staining method utilizing toluidine blue's high alkaline solution and the positive results therefore initialized the current extensive usage of the protocol and stain in all the laboratories worldwide.

Toluidine blue is still used today for staining semithin sections. It is especially preferred in the morphological evaluation of the peripheral nerves (13-16). For this purpose, histopathological analysis of age-related changes, different damages (e.g., axonal degeneration, autolysis, swelling of myelinated fibers, and loss of the myelin sheath), and the effect of different treatment agents (e.g., axonal regeneration or nerve regeneration) in the sciatic nerve, sural nerve or phrenic nerve were investigated. In these studies, quantitative histomorphometric analyses were performed by evaluating the myelinated nerve fiber density (17-21). These sections are also used in the evaluation of demyelination and remyelination levels according to the results of G-ratio (the ratio of axon diameter and myelin sheath thickness) after damage, lesion, and treatments in the central nervous system (22).

Combined histological methods such as conventional light microscopy, non-conventional light microscopy (semithin sections stained with toluidine blue), TEM, and immunohistochemistry are very important for understanding and interpreting the examined tissue components. Correlations of these methods are used both in research and clinical practice (23). For example, the histological identification of M-cells in Peyer's plaques in the small intestine of albino rats (24), the identification of telocytes in the oviduct of mares (25), the correlative analysis of hard tissue and large pieces by light microscopy and electron microscopy (26), in improving visualization and interpretability of tooth cementum annulation (27), and the optimal diagnostic interpretation of epoxy-resin-embedded bone marrow biopsies (28) have been reported between routine light microscopy and TEM.

Many studies on factors like pH, temperature, and microwave radiation have been conducted in order to further increase the practical use of the classic basic dyes utilized in the field of staining plastic sections, which started in the 1960s. The studies in this field, which aim towards improving the current methods or the new

methods to be developed to be faster, easier, more practical, and more reliable and enabling the acquisition of the highest histological details, are still observed to be continuing. In one of the studies of semithin plastic sections, differential staining and distinction of spermium acrosome, mitochondrial sheaths in the midpiece, and tail were achieved with toluidine blue and basic fuchsin. Proceeding the protocol with phosphotungstic acid and light green further allowed the collagen to be stained in a separate color (29). In a dichromatic staining study, semithin sections were stained with borax methylene blue and basic fuchsin. On account of the observed staining of the nucleus, cytoplasm, collagen, elastin, myelin, and axoplasm, the method was especially recommended for peripheral nerve and blood vessel research (30). In another study, semithin epon-embedded sections of various tissues were stained with a one-step Mallory-Heidenhein stain. Intracytoplasmic components such as the nucleus, cytoplasm, collagen and elastic fibers, glycogen, and mucus were reported to be stained in different colors after nuclear staining with celestine blue (31). In a study that investigates a version of Mallory's phloxin B-methylene blue-azure II technique, the triple staining combination was carried out both unaltered and after phloxin B was removed from it, and the results of these two experiments were compared. Phloxin B was reported to have stained collagen and elastic fibers only (32). Fritsch (33), stained epon-embedded sections with a methylene blue-azure II solution and reported that following a counter-staining with basic fuchsin, cartilage, collagen fibers, elastic fibers, and muscle fibers could be stained in separate colors and easily distinguished. In another study, after a phosphotungstic acid-methyl green combination staining was applied to semithin epon sections, some mucin granules and the glycocalyx were observed to have stained in the same color, while other mucin granules, luminal mucin, and collagen fibers were stained in a different and contrasting color (34). Another study utilized microwave radiation on Bodian's silver staining, resorcin fuchsin, and later picrosirius red F3BA staining methods of nervous and connective tissue sections obtained after embedding into glycol methacrylate. The study reported that the staining durations were notably shortened and the nerve, elastic, and collagen fibers were stained in different colors, making their differentiation easier (35). In an experiment on semithin sections (36), Harris hematoxylin, silver methenamine, light green, eosin, and safranine stains were applied to the same section consecutively. The application was evaluated as a histological, histochemical, and immunocytochemical method that allows a thoroughly specific identification of the whole cellular tissue architecture. It was reported that following staining with carbol methylene blue-carbol gentian violet solution and a counter-staining with pararosaniline, vascular connective tissue, and elastic laminae were stained in separate colors and thus could easily be distinguished (37). In a dichromatic staining study aiming the demonstration of the collagen composition of peripheral nerves in semithin epon sections, the quantity and orientation of the collagenous connective tissue in horizontal and vertical nervous tissue sections could easily be distinguished with a toluidine blue staining followed by a basic fuchsin application (38). D'Amico (39) in his study, applied to semithin epon-embedded sections a methylene blue-azure B mixture and a succeeding basic fuchsin. He obtained separate colors differentiating cytoplasm, nucleus, collagen, elastin, mucus, and lipid components and evaluated the results positively. Another study reported that a mixture of azure B and basic fuchsin allowed the differentiation of many intra- and extracellular (collagen and elastic fibers) components in diverse colors and tones and that the method could also be useful for pathological tissues (40). Twort's staining method (neutral red and fast green FCF mixture) was adapted to epon-embedded sections in a study, which highlighted that in addition to many intracellular structures, extracellular collagen and elastic fibers could also be easily distinguished in many colors (41). A solution consisting of a toluidine blue and malachite green mixture and basic fuchsin for counter-staining was utilized in another study, in which discernible staining of nuclei, erythrocytes, mitochondria, collagen and elastic fibers, and cartilaginous structures was recorded (42). Light microscopic results were compared with electron microscopic results in a study that researches, following a methylene blue and sodium tetraborate application to semithin sections of heart muscle, and mitochondria morphometry in cardiomyocytes. The study was stated as quite successful, without further need for advancement towards the electron microscopic level (43).

In the literature, similar studies related to the specific staining of connective tissue fibers (elastic fibers, type I and type III collagen fibers) and mitochondria (Verhoeff hematoxylin, toluidine blue-light green combination, Gordon&Sweets' silver impregnation, Altmann's protocol; respectively) applied to epon sections were not found within the scope of the present study. However, within the scope of staining methods applied for general or different purposes, there are studies that mention the staining results of tissue components in the present study. Therefore, the results of the present study are in agreement with the studies on light green and fast green FCF dyes that only stain type I collagen, especially trichrome techniques, and the identification of collagen and elastic fibers (29,31,36,41).

We think that our study will be a guide in this field. However, as a limitation of our study, the stainings we applied were not studied on different tissues. We believe that applying the staining in our study on more tissue types would be more beneficial.

CONCLUSION

Some staining techniques recommended for paraffin sections (Altmann's method for mitochondria, Gordon&Sweets' silver impregnation method for type III collagen/reticular fibers, Verhoeff's method for elastic fibers) can also be applied to semithin epon sections. Toluidine blue-light green double staining, which we tried as a new combination, can be used to identify type I collagen fibers in semithin epon sections. Quantitative histomorphometric analyses of cell and tissue components can be easily performed at the light microscopic level in semithin epon sections. Epon-embedded tissues with osmium postfixation can be advanced to ultrastructural examination. Thus, light and electron microscopic correlation becomes possible.

Ethics Committee Approval: In this study, archived epon blocks of rats were used. Therefore, ethical approval was not required with the approval of the animal experiments local ethics committee of Uludağ University (01.08.2023).

Conflict of Interest: None declared by the authors.

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