SHORT COMMUNICATION

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A MODIFIED METHOD FOR THE SELECTIVE STAINING OF ELASTIC SYSTEM FIBERS IN METHACRYLATE TISSUE SECTIONS

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ABSTRACT

The elastic system fibers are abundant elements of the extracellular matrix found in organs such as skin, blood vessels, lung and in elastic cartilage and elastic tendons. These fibers have been studied by several selective staining methods, such as resorcin-fuchsin for light microscopy and hematoxylin-eosin plus fluorescence and confocal scanning laser microscopy. However, most of these techniques are only efficient for tissues embedded in paraffin or paraplast, since most dyes show low penetration into glycol methacrylate resins. In this report, we describe a variation of Weigert's resorcin-fuchsin method that involves the pretreatment of resin sections with potassium permanganate. This procedure increased the affinity between the dye and elastic fibers, and stained the elastic fibers in black or dark violet, the nuclei in purple and other structures in light blue. Thus, this modification of the original method provided excellent artifact-free demarcation of elastic fibers in well-preserved tissues.

Key words: Elastic system fiber, extracellular matrix, methacrylate resin, resorcin-fuchsin, selective stain

The elastic system fibers are complex structures that contain elastin, microfibrillar proteins, lysyl oxidase, and, occasionally, proteoglycans. Elastin is the predominant protein of mature elastic fibers and provides the fibers with their characteristic property of elastic recoil. Elastic fibers can be identified in light microscopy by selective staining methods. This selective staining has allowed the visualization of elastic structures in many tissues and organs. In elastic tendons, lung and skin, the fibers are slim, rope-like, and variable in length. In major arteries, such as the aorta, elastic fibers form concentric sheets or lamellae, while in elastic cartilage these fibers are arranged in a three-dimensional network [9].

The elastic fibers in all of these organs and tissues have been studied by several selective staining methods such as resorcin-fuchsin [15] and orcein [14] for ordinary light microscopy, and hematoxylin-eosin staining in conjunction with fluorescence and confocal laser microscopy [3,4,5]. However, most of these techniques are only efficient for tissues embedded in paraffin or paraplast since many dyes show poor penetration into glycol methacrylate resins and their derivates [8].

Various methods have been proposed to facilitate the penetration of stains into resin sections, including microwave irradiation [2] and plasticizing clearing agents [6]. However, these methods are complex and can compromise the quality of the histological sections.

In this report, we describe a modification of resorcin-fuchsin staining method proposed by Weigert in which the resin sections are pretreated with potassium permanganate and oxalic acid. This procedure enhances the dye penetration and improves the detection of elastic fibers.

Male and female adult gerbils (*Meriones unguiculatus*, Gerbilinae, Muridae) were sacrificed by CO_2 inhalation followed by decapitation and the aorta, epiglottis, esophagus, skin and prostate were removed and fixed in Karnovsky fixative (5% paraformaldehyde and 2.5% glutaraldehyde in Sörensën phosphate buffer, pH 7.2), embedded in glycol methacrylate resin (Leica historesin embedding kit) or paraplast (Histosec, Merck) and sectioned (3 μm) on a Leica automatic rotative microtome. The choice of these organs was based on the abundance of elastic system components in their histological architecture.

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The sections were subsequently treated with 1% potassium permanganate for 10 min, washed in distilled water for 1 min, immersed in 3% oxalic acid for 1 min and washed again in distilled water for 1 min. After one wash with 95% ethanol for 1 min, the sections were stained with Weigert's resorcin-fuchsin [15] for 5 h, immersed in 95% ethanol for 1 min, counter-stained with Harris' hematoxylin for 3 min, washed in tap water for 10 min, dehydrated in ethanol, clarified in xylol and mounted in Canada balsam. This procedure is summarized in Table I.

Table 1. Steps for the modified Weigert resorcin-fuchsin method for the detection of elastic fibers in hydrophilic resins.

Step	Treatment	Time
1	Potassium permanganate (1%)	10 min
2	Distilled water	1 min
3	Oxalic acid (3%)	1 min
4	Distilled water	1 min
5	Ethanol (95%)	1 min
6	Weigert´s Resorcin-fuchsin	5 h
7	Ethanol (95%)	1 min
8	Harris's hematoxylin	3 min
9	Tap water	10 min
10	Dehydration in ethanol (70%, 95% and 100%)	5 min each
11	Clearing in xylol (3 washes)	10 min each
12	Mounting in Canada balsam	-

Since the elastic fibers of glycol methacrylate resin sections are not stained by hematoxylin-eosin, this stain was used for comparison with Weigert´s original method [1]. Gömöri's reticulin method [1], which employs the same pretreatment as that described here, was used to demonstrate the selectivity of the resorcin-fuchsin stain for elastic fibers. In addition, some sections were stained with resorcin-fuchsin without pretreatment with potassium permanganate in order to demonstrate that prior oxidation was essential for detecting the elastic fibers.

All of the stained sections were observed and documented with either a Zeiss Jenaval or an Olympus light microscope.

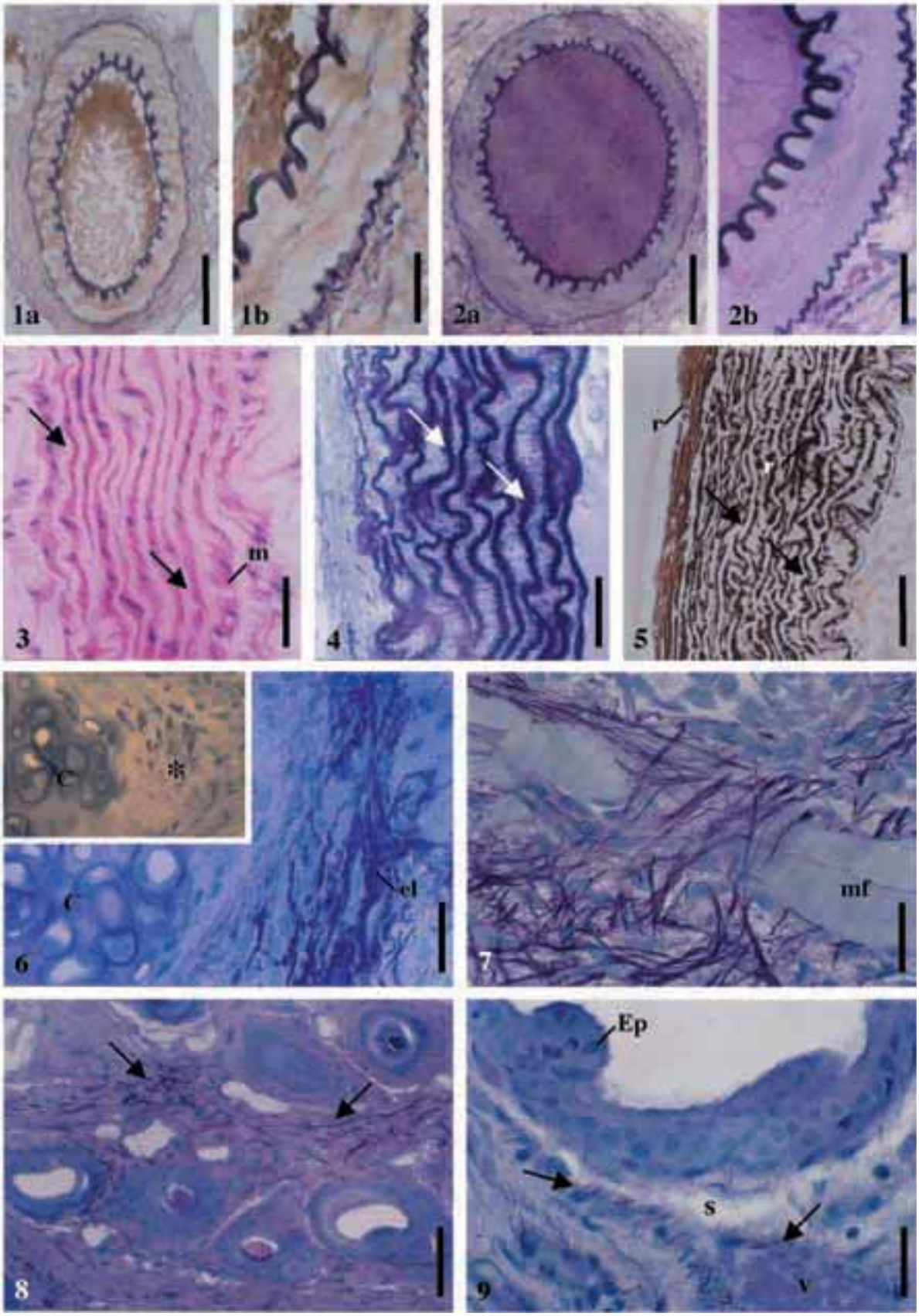
Paraplast sections of the prostate muscle artery stained by Weigert's original method are shown in Figure 1a and b. Figure 2a and b shows the same tissue embedded in resin and stained by the Weigert's modified method described here. Both techniques stained the conspicuous internal and external elastic lamina of the *intima* and the thin fibers of the *adventitia*. However, the demarcation and preservation of the elastic fibers was better with the modified method.

Figures 3, 4 and 5 show the aorta wall stained with hematoxylin-eosin, Weigert's modified resorcin-fuchsin and Gömöri's silver impregnation, respectively. The elastic fibers were not stained in the hematoxylin-eosin method, which stains only smooth muscle cells and collagen fibers of the tunica media (Fig. 3). After Gömöri's staining method (Fig. 5), the reticular fibers were stained in black whereas elastic fibers were not stained. On the other hand, Weigert's modified resorcin-fuchsin staining selectively revealed only elastic fibers (Fig. 4). Thus, the differential selectivity of Gömöri's and Weigert's modified resorcin-fuchsin methods is determined by steps subsequent to the pretreatment with potassium permanganate which oxidizes sugar residues.

Figures 6, 7, 8 and 9 show resin sections from epiglottis, esophagus, skin and prostate, respectively, that were stained by the modified Weigert resorcin-fuchsin method. A large amount of strongly stained elastic elements was seen in all cases.

The elastic system fibers cannot be observed by conventional hematoxylin-eosin staining. Rather, these extracellular matrix elements have been observed by selective staining methods such as Weigert's resorcin-fuchsin, Verhöeff´s ferric hematoxylin and Unna´s orcein stains. These methods

Figures 1a,b. A muscle artery from gerbil (*M. unguiculatus*) prostate embedded in paraplast and stained by Weigert's original resorcin-fuchsin method. Bars = a, 52 µm; b, 13 µm. **Figures 2a,b.** A muscle artery from gerbil prostate embedded in historesin and stained by the modified resorcin-fuchsin method. Bars = a, 40 µm; b, 13 µm. **Figure 3.** Elastic elements (**arrows**) in gerbil aorta embedded in historesin and stained with hematoxylin-eosin, which in negatively stains elastic fibers. (**m**) muscle fibers. Bar = 13 µm. **Figure 4.** The modified resorcin-fuchsin method shows intensely stained aortic elastic fibers (**arrows**). Bar = 13 µm. **Figure 5.** Gömöri's reticulin method does not stain aortic elastic fibers (**arrows**), but demarcates reticular fibers in brown (**r**). Bar = 13 µm. **Figures 6-9.** Some organs and tissues from *M. unguiculatus* embedded in historesin and stained by the modified Weigert resorcin-fuchsin method. **6**. Epiglottis with elastic fibers (**el**) around the cartilage (**C**). Bar = 52 µm. The inset shows that staining without pretreatment does not detect the elastic fibers (*****). Bar = 52 µm. **7**. Irregular distribution of elastic fibers in the esophageal submucosa. The association between elastic and skeletal muscle fibers (**mf**) can be seen. Bar = 20 µm. **8**. Histological section of skin showing the relatively thick elastic fibers (**arrows**). Bar = 52 µm. **9**. General distribution of elastic fibers in gerbil prostate. Note the very thin elastic fibers (**arrows**) of the stroma (**s**) and at the base of the epithelium (**ep**). (**v**) blood vessel. Bar = 16 µm.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generally serve only for material embedded in paraffin or paraplast. The use of hydrophilic resins for embedding tissues provides excellent microscopic resolution, but the most histological stains cannot be used with such samples because the resin polymer network limits access of dye molecules to the tissue.

The modification of the staining method described here involves pretreatment with potassium permanganate. This treatment results in the formation of free radical aldehydes. Feulgen and Voit [7] reported the presence of aldehyde groups in elastic fibers. These groups are responsible for cross-linking during elastic fiber maturation [11]. Active aldehyde groups frequently appear during desmosin and isodesmosin formation but are later reduced and may even disappear [13].

Aldehyde groups react positively with Schiff's reagent and resorcin-fuchsin, and are responsible for staining of elastic fibers. Lillie *et al*. [10] and Mc Callum [12] demonstrated that the staining of elastic fibers by resorcin-fuchsin and orcein was mediated by the presence of free aldehyde groups in the elastic extracellular matrix.

The use of potassium permanganate as an oxidizing agent is well established and involves a mechanism known as Casella's reaction [11]. This reaction oxides structures containing carbohydrate to produce aldehydes that are detected by Schiff's reagent.

Since elastic fibers naturally contain aldehydes, the use of Casella's reaction increases the number of free aldehyde groups [11]. This increase in reactive groups facilitates binding of the dye molecule and provides intense staining of elastic and pre-elastic fibers.

The results obtained here were very satisfactory since it was not necessary to remove the resin prior to staining, and excellent artifact-free staining of elastic fibers was observed in well-fixed tissues. This enhanced staining should facilitate morphometric stereological analyses.

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