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NEW METHOD FOR DYEING OF ELASTIC TISSUES: MODIFICATION OF VERHOEFF'S METHOD

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Summary. *The classical methods for dyeing of elastica in the light microscopy understand the differentiation of the stain from tissue without accomplishing the selective removing of the stain from the elastic tissue surrounding. By our modification or Verhoeff's method in which the concentration of iron (III) - chloride combined with 1% water solution of iodine in potassium-iodide (1:1) is lessened, the successful dyeing of elastica is accomplished. The differentiation of the sedimentous excess of Fe-hematein by iron (III) - chloride usually used is successfully replaced by 0,5 % water solution of Bismark Brown Y diazo dye in acetate buffer (2:1). The suggested diazo dye in acid solution shows a great affinity to environment and amli to elastic tissue differentiating indirectly the exessive Fe-hematein from the surrounding of elastic tissues. The time of contrasting is not strictly determined because there is no danger of rinsing the stain out of the elastic tissues and lamellas. The elastica dyed by our modified method shows a greater number of tissues and lamellas, it is more noticeable and its appearance reflects their real width, length and net.*

Key words: *Elastic tissue, hystochemical identification*

Introduction

Elastic tissues make a special kind of binding fibrilar proteins with a specific structure which is determined by the presence of the dominant amorphine component, elastin and fibrilin composed of several types of glycoproteins (1,2). Specific composition of elastica as well as the total changes caused by aging or various diseases (3) prevented the reliable identification of these structures in the light microscopy regardless of their position in tissues.

Displaying of the elastic tissues is mostly based on the usage of numerous empirical methods which were evolved and modified along with the introduction of chemical structure of the elastic tissues components.

Metal-hematein dyeings have been widely used since the beginning of XX century (Harris, 1902: Verhoeff, 1908) as well as the orcein method according to Tanzer (1891) or the recent aldehid-fuksin method according to Gomori (4). Other attempts to establish a representative method for dyeing of elastic tissues to date are mostly the modifications of the basic established techniques and still remain only more or less applicable attempts (5,6,7,8,9,10). The crucial point with most mentioned dyeings is the process of removing of the excessive stain from the preparation where the decisive factor is the experience of a researcher as well as his subjective evaluation of the obtained results.

For these reasons there is still a need for investigations which would eliminate subjectivity as much as possible, namely, establish a technique that would always and in the same way, regardless of the type and multitude of elastic tissues and lamellas with a certainty, repeatedly accurately, show their structure, number and net.

Material and Methods

Experiments were carried out on human autopsy material. Samples of the middle parties/parts of the aorta were fixed in 10% buffered neutral formalin for 24 h, dexydrated and embedded in paraffin. Sections were cut at 5 μm.

According to our modified method, the samples were stained by Verhoeff's 6 % hematein with reduced concentration of iron (III) chloride if compared to original method (4%) combined with Lugol's iodine (1% water solution with 2 g calcium iodide) for 5 minutes . The removing of colour was not done but contrasting by 0,5 % water solution of Bismark Brown Y (C.I.21000) colour in acetate buffer of pH-4 (0.1 mol/L).

Procedure:

Blocks were fixed in 10% buffered neutral formaline (approximatively 4% formaldehyde). Sections were cut at 5 μm after routine dehydration and embedding in paraffin.

Solutions:

Picric acid solution

Picric acid	1.2 g
Glac.acetic acid	30 ml
Dist.water	970 ml

Verhoeff's hematoxylin (modified)

A: Hematoxylin (C.I.75290)	6 g
Ethanol abs.	100 ml
B: $FeCl_3$	4 g
Dist.water	100 ml
C: Lugol's iodine	
KI	2 g
I_2	1 g
Dist.water	100 ml

Solutions A, B and C are mixed in proportion 1:1:1

Bismark Brown Y solution

A: Bismark Brown Y (C.I.21000)	0.5 g
Dist.water	100 ml
B: Acetic buffer (pH-4)	0.1 mol/L

Solutions A and B to be mixed in proportion 2:1 before usage.

Method:

1. Bring paraffin sections to water.
2. Treat sections with 3%picric acid for 5 minutes.
3. Wash gently in distilled water for 5 minutes.
4. Stain with modified Verhoeff's solution for 5 minutes.
5. Wash in three changes of distilled water for 5 minutes in each.
6. Immerse sections in Bismark Brown Y solution for 3 minutes.
7. Wash in running tap water.
8. Dehydrate in alcohols, clear in xylene and mount in Permount.

Result: Elastica is dyed dark brown and environment yellow

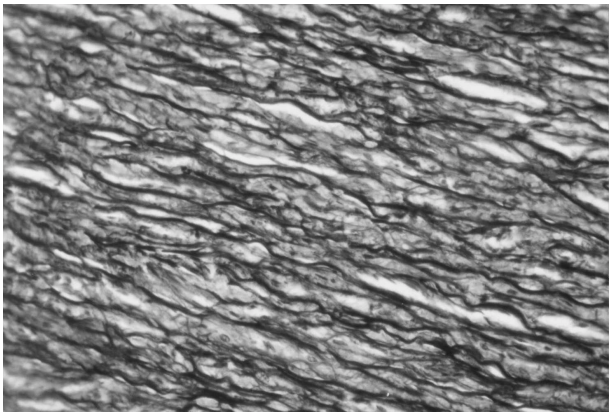

Fig. 1. Significant affinity of all elastic fibers and lamellas to stain with solid contrasting (modified Verhoeff's method , x120)

Results

Modifying existing Verhoeff's method for dyeing of elastica combined with Lugol's iodine, where the background was dyed with 0,5% Bismark Brown Y water solution in acetic buffer, we tried in both preliminary procedure (the application of picric acid solution in 3% glacial acetic acid solution) and in the final dyeing to achieve better identification of elastic tissues and lamellas.

Obtained results (Fig. 1, 2) speak in favour of a very good applicability of modified Verhoeff's stain to tissues and lamellas, which is dark brown having good contrast with yellow background.

Discussion

Paraffin sections of thoracic aorta samples, rich in elastica fixed in 10% buffered neutral formalin were deparaffined with xylene and taken through decreasing level of ethanol. Before dyeing the additional denaturation of all used preparations saturated with picric acid solution in 3% glacial acetic acid solution was done giving the tissues more natural appearance. Picric acid also increases tissue basophilia with positive influence on binding of diazo dyes to elastic components surrounding.

In order to determine the effect of Fe hematein dyeing on material prepared in this way, we applied Verhoeff's stain in the form of 6%hematoxylin. The compounds metal-hematein are marked as catione dyes in histochemistry. According to Puchtler (1961) if a water solution of hematoxylin in mixture with iron (III)-chloride is used chelates which are formed are changed by aging in relation and composition. The four phenol OH groups of hematoxylin are strong spots which can be bound by hydrogen bonds. This bond is first made to the oxygen of water molecule, and the bond formed is 10 times stronger than the bond to the ethanol oxygen. The replacement of water as a dissolvent results in favouring of the Fe-hematein complex formation.

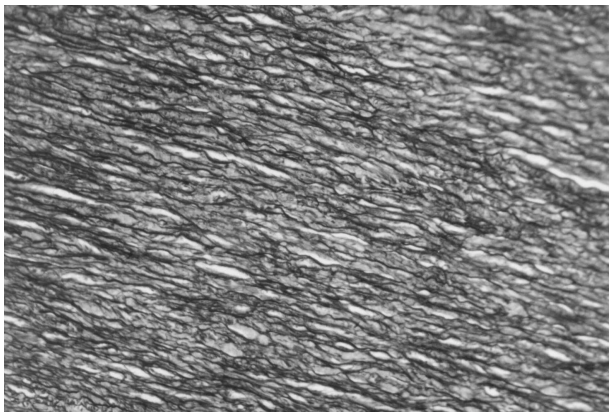

Fig. 2. Elastic tissue stained dark brown with very good contrasting (modified Verhoeff's method, x100)

Most authors (11) consider that the dyeing of elastica with Verfoeff's solution primary depends on the presence of Fe3+ ion. The ion of iron has little affinity for amino group but it tends to react to ligands that coordinate with the oxygen of phosphate, polyol or saccharide. If the carbonyl and hydroxyl groups are blocked by appropriate methods, dyeing won't occur. On the contrary, according to Puchtler et al., such effect cannot be obtained by blocking reactions such as benzoilation, acetylation, metylation (12).

The experiments done by Puchtler et al. in 1979 establish that the dyeing of elastin by Fe hematoxylin is caused by hematein (12). These dyes as well as other dyes for elastica contain the phenol OH group which should be responsible for making the bond dye - substrate. The first investigations of the bonding of resorcin-fuxin to elastica speak of forming of hydrogen bonds between phenol OH groups of dyes and suitable bonding spots such as carbonyl groups in tissues. Similar claim applies to the bond orcein-elastin too. Since the elastin is almost nonpolar having very few free carboxyl groups the possible hydrogen bonding depends on the accessible peptide bonds. Also, since the structures of the complex formed during the aging of Fe-hematoxylin are not known at any moment of the reaction, it is dubious which of them would be responsible for making the bond to elastin.

The final procedure in dyeing understands the differentiation of iron(III)-chloride with 2% water solution. According to this, Verhoeff's method doesn't have the defined end of reaction; i.e. it is left to the conductor of reaction to determine the final limit of decoloration. This rise a question if the coloured structures can be regarded as elastic tissues and lamellas.

In order to find out whether the affinity of elastica is greater to the aromatic dye or Fe-hematein, we combined Verhoeff's solution with Bismark Brown Y aromatic dye. Diazo dye shows lower affinity to elastica than the iron complex so, the elastica is separated in the form of thin and short tissues. Contrasting obtained in this way was not completely histochemically acceptable. The Bismark Brown Y dye competed with Fe-hematein prevented the bonding of the complex and that's why elastin remained uncoloured. In contrast to the amorphine elastin, microfibrils showed insignificant affinity to applied dye.

If the procedure is performed gradually, initially by application of Verhoeff's solution and afterwards is treated by the water solution of aromatic dye then the differentiation of iron from the environmental tissue occurs but partly from elastica too, so the intensity of the dye bonded to elastica is somewhat weaker.

If Lugol's iodine, which is not needed according to some authors, is added to Verhoeff's solution by application of Verhoeff's formula the complex of Fe-hematein binds to a greater extent to the tissue and the applied solution of diazo dye is not able to wash away the excessive bonded hematoxylin to surrounding tissue (13).

Puchtler et al., consider that Lugol's solution is not needed for the realization of dyeing with complex of Fe-hematein (13). In their opinion iodine changes the selective binding of the complex i.e. decreases the binding of dye to elastica and increases the binding to "pseudo elastica". At the same time, in this process iodine would rather be a means of reduction then oxidation for iron.

$$Fe^{+3} + I^{-} \rightarrow Fe^{+2} + 1/2\ I_2$$

If the process of iron reduction would be prior, it is most likely that the bonding of iron complex would be prevented. All attempts to dye elastica with compounds which substitute iron (III)-chloride in Verhoeff's formula with iron (II)-sulfate haven't given positive results. As the halogen ligands could donate the electronic pair in bonding to metals in several different ways, it is most probable that for the firm bonding of the complex to elastica almost selective responsibility has one of the possible reactions of this type.

The reinforced settlement of iron by the application of Lugol's solution is lowered by reduction of iron(III) - chloride concentration from 10% to 4% solution. Attempts to remove Fe-hematoin complex from the surrounding tissue but not from elastica by application of Bismark Brown Y dye in the solution with adjusted pH (so that it shows the least affinity to elastica and bigger to surrounding tissue) gave sufficiently acceptable results which empirically in the indirect way eliminated the differentiation as the process accompanying Verhoeff's dyeing. The stain used for the additional dyeing of tissues was in the form of 0,5% solution mixed with acetate buffer in proportion 2:1 (14,15).

By application of this procedure, on elastic tissue of the aortic wall, the greatest number of elastic fibers and lamellas in the dyed samples is shown including the greatest part of lamella width. Elastica is dyed dark brown and the surrounding yellow. Elastic components in other tissues are of different chemical composition and in our opinion each method for elastica dying needs adequate modification.

References

1. Jacob MP, Seances CR. Elastin: preparation, characterisation, structure, biosynthesis and catabolism. Soc Biol Fil 1990; 187: 166-80.
2. Sakai LY, Keene DR, Engvall E. Fibrilin, a new 350-kD glycoprotein, is a component of extracellular microfibrils. J Cell Biol 1986; 103: 2499-509.
3. Rustin MHA, Papadaki L, Rode J, Dowd PM. Elastic fibers in patients with systemic sclerosis. Arch. Pathol. Anat. 1989; 416: 115-20.
4. Gomori G. Aldehide-fuchsin: a new stain for elastic tissue. Amer J Clin Path 1950; 20: 665-6.
5. Sulthouse TN. Selective staining of collagen and elastin by

Luxol Fast Blue G in methanol. J Histochem Cytochem 1965; 13: 133-40.
6. Lillie RD. Exploration of dye chemistry in tanzer unna orcein type elastin staining. Histochemie 1969; 19: 1-12.
7. Disbrey BD, Rack JH. Hystological laboratory methods. Ex S Livingstone. Edinbourgh and London 1970.
8. Musto L. Improved iron hematoxylin stain for elastic tissue. Stain Tecnol 1981; 56: 185-7.
9. Garvwey W. Modified elastic tissue-Masson trichrome stain. Stain Techn 1984; 59: 213-6.
10. Žarkov VP, Lunjkov VD. Metodika isledovanija: Bistroe gistohimičeskoe okrašivanie gistologičeskih preparatov. Arh Anat Gistol Embriol 1990; 99: 54-5.
11. Brissie RM, Spicer SS, Hall BJ, Thampson NT. Ultrastructural staining of thin sections with iron hematoxylin. J Histochem Cytochem 1974; 22: 895-907.
12. Puchtler H, Sweat F, Bates R and Brown JH. On the mechanism of resorcin-fuchsin staining. J Histochem Cytochem 1961; 9: 553-9.
13. Puchtler H, Waldrop FS. On the mechanism of Verchoeffs elastica stain a convenient stain for myelin sheats. Histochemistry 1979; 62: 233-47.
14. Horobin RW, James NT. The staining of elastic fibers with direct blue 152. A general hypothesis for the staining of elastic fibers. Histochemie 1970; 22: 324-36.
15. Horobin RW, Fleming L. Structure-staining relationships in histochemistry and biologic stains. II Mechanistic and practical aspects of the staining of elastic fibers. J Microsc 1980; 119: 357-72.

NOVA METODA ZA BOJENJE ELASTIČNIH VLAKANA MODIFIKOVANOM VERHOEFF-OVOM METODOM

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Kratak sadržaj: *Klasične metode za bojenje elastike, u svetlosnoj mikroskopiji, podrazumevaju diferencijaciju boje sa tkiva pri čemu se ne može postići selektivno odstranjivanje boje isključivo sa okoline elastičnih vlakana. Našom modifikacijom Verhoeff-ovog postupka u kome je smanjena koncentracija gvožđe (III)-hlorida u kombinaciji sa 1% vodenim rastvorom joda u kalijum-jodidu (1:1) postignuto je uspešno bojenje elastike. Diferencijacija viška istaloženog Fe-hemateina pomo}u gvožđa (III)-hlorida koja se rutinski koristi uspešno je zamenjena 0.5% vodenim rastvorom diazo boje Bismark Brown Y u acetatnom puferu (2:1). Predložena diazo boja u kiselom rastvoru pokazuje veliki afinitet za okolinu a amli za elastična vlakna pri čemu se na posredan način vrši diferencijacija viška Fe hemateina sa okoline elastičnih vlakana. Vreme kontrastiranja nije strogo određeno, jer ne postoji opasnost od ispiranja boje sa elastičnih vlakana i lamela. Obojena elastika prema našem modifikovanom postupku ispoljava veći broj vlakana i lamela, uočljivija je a njen izgled odslikava njihovu stvarnu širinu, dužinu i umreženost.*

Ključne reči: *Elastična vlakna, histohemijska identifikacija*

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