

A Comparative Assessment of Methods of Demonstrating Amyloidosis in Human Tissue

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ABSTRACT

This study compared methods of demonstrating amyloidosis in human tissues to recommend suitable staining methods in resource-poor settings. Human Liver and kidney tissues were collected and fixed in 10% formal saline for 24 hours. Liver and kidney sections were obtained from post-mortem samples. Samples were cut with a thickness of 3mm in the cutting-up room. The selected tissues were placed in tissue baskets carefully labeled and processed histologically. The tissues were processed using an automatic tissue processor. The staining methods employed in staining the sections were modified Highman's Congo Red, Metachromatic Crystal Violet method, and Toluidine blue methods. The results showed the different staining reactions of the liver and kidney tissues to special these stains. The demonstration of amyloidosis using modified Highman's Congo Red method appeared as red in both the liver and kidney tissue micrographs. The demonstration of amyloidosis using Metachromatic Crystal Violet stain appeared as blue in both the liver and kidney tissue micrograph. Furthermore, the demonstration of amyloidosis using Toluidine blue stain appeared as blue and red pigments in both the liver and kidney tissue micrograph. However, this staining was properly differentiated in all the tissues.

Keywords: amyloidosis; human; tissues; staining liver; kidney

INTRODUCTION

Amyloidosis is not a single disease entity but rather a diverse group of disease processes characterized by the abnormal extracellular deposition of insoluble fibrillar protein, generically termed amyloid, in one or many organs [1]. Amyloid deposits contain the fibrillar protein that defines the type of amyloidosis and several common components such as the serum amyloid P and glycosaminoglycans. Glycosaminoglycans stain blue with iodine, and in 1854 Virchow was the first to use iodine stain to study cerebral amyloidea under the microscope. He described its appearance as that of starch or cellulose, thus giving the disease its name; amyloid is Greek for starch. All of the components have been claimed to be involved in amyloid fibril stability as well as the acceleration of amyloid formation. Amyloid fibrils are arranged in an antiparallel conformation with a β -sheet structure [1 - 3]. The classification of amyloidosis is based upon the tissue distribution of amyloid deposits (local or systemic amyloidosis), the absence or presence of preexisting disease (primary or secondary amyloidosis), and the chemical type of amyloid protein fibril [1].

Localized amyloidosis affects only one organ or tissue in the body in contrast to systemic amyloidosis. Age-related localized amyloid is common in vascular tissue, and the human aorta may be the most common site for this type of amyloid deposition. Two biochemically different forms of localized amyloid have been identified. The most common form of localized amyloid is aortic medial amyloidosis, which occurs in most people older than 60. The amyloid fibril protein is called medin (AMed). Medin is a 50 amino acid fragment of its precursor lactadherin, a glycoprotein expressed by the mammary epithelium. Lactadherin is a 378 amino acid protein, and its normal function is unknown [4, 5]. Intimal amyloid is less common and is seen in association with severe arteriosclerosis. Based on a study of one patient with aortic intimal amyloid, the fibrillar protein was claimed to consist of a 69 amino acid residues long N-terminal fragment of Apo lipoprotein A1. However, trials to confirm this result have not been successful. Whether or not amyloid is involved in the pathogenesis of arteriosclerosis is not known [6, 7].

Considering the importance of studying amyloidosis in human tissues, this study is set to compare the methods of demonstrating amyloidosis in human tissues and evaluate various staining methods that will be suitable to stain it. Furthermore, there is a paucity of literature as regards the suitable staining methods of amyloidosis in human tissues, especially in resource-poor settings where sophisticated methods are not available advanced hence this study is justified. This study aims to carry out a comparative assessment of methods of demonstrating amyloidosis in human tissues to recommend the best and most suitable staining method in such settings. Furthermore, this study will provide insight into the suitable staining techniques for demonstrating amyloid in humans with the view of helping histopathologists in prompt diagnosis and faster demonstration of amyloid as it relates to disease conditions that might affect the area.

MATERIALS & METHODS

Research Design and Procedure

30 Liver and kidney tissues used for this research were obtained from post-mortem samples of suspected amyloidosis cases and selected randomly. The tissues were collected and fixed in 10% formal saline for 24 hours. Samples were cut with a thickness of 3mm in the cutting-up room. The selected tissues were placed in tissue baskets carefully labeled and processed histologically.

Histological Processing

The tissues were processed using an automatic tissue processor. The fixed plastic cassette tissues in 10% formalin were automatically processed by passing them through different grades of alcohol as follows:

- 70% alcohol 2hrs
- 80% alcohol 2hrs
- 90% alcohol 2hrs
- 90% alcohol 2hrs
- 95% alcohol 2hrs
- Absolute 2hrs
- Xylene I 2hrs
- Xylene II 2hrs
- Molten paraffin wax 1 2hrs
- Molten paraffin Wax II 2hrs [8, 9, 10, 11, 12]

After the last timing, the tissues were removed from their plastic cassettes, placed at the center of the metallic tissue mold, and filled with molten paraffin wax. They were also left to solidify, after which they were now placed in the refrigerator at 5oC for 15 minutes. After the blocks were cool in the refrigerator for the time stated above (15 minutes), the blocks were removed from the metallic case using a knife, after which the paraffin wax at the side of the blocks was removed.

The blocks were then trimmed and cut serially at 3mm on a rotary microtome. The sections were floated in a water bath at 55oC and picked up by the use of clean frosted end slides. The frosted end slides were now placed on the hot plate for 40 minutes for adequate attachment of the sections on the slides, after which the sections were de-waxed, hydrated, air dried and stored in a slide box ready for the staining process.

Staining Procedures

The staining methods employed in staining the sections were Highman's Congo Red, Crystal violet, and Toluidine blue staining methods.

Modified Highman's Congo Red Method

- 1) Section was taken to water via xylene and ethanol.
- 2) It was placed into Congo red solution for 5 minutes or longer.
- 3) It was differentiated in alkaline ethanol (about 5-30 seconds).
- 4) It was washed well with tap water.
- 5) Nuclei were counter-stained with Alum hematoxylin.
- 6) It was washed in water
- 7) It was dehydrated with ethanol.
- 8) It was clear with xylene and mount with a resinous medium [9].

Metachromatic Staining Method with Crystal Violet

- 1) The section was taken to water
- 2) It was stained in 1% aqueous methyl or crystal violet for 2 - 5 minutes
- 3) It was rinsed in water
- 4) It was differentiated in 0.5 - 1% acetic acid until the amyloid is purplish - red or red in good contrast with blue-violet staining of nuclei and normal tissue.
- 5) It was rinsed in running tap water for at least 5 minutes.
- 6) The slide was drained, and while it was still moist, it was mounted with modified Apathys gum syrup.
- 7) It was coverslipped with nail varnish [10].

Standard Toluidine Blue Method

- 1) The section was taken to the water
- 2) It was placed in toluidine blue solution at 37°C for 30 minutes.
- 3) It was rinsed in water.
- 4) It was placed into absolute iso-propanol for one minute.
- 5) It was blotted carefully and allowed to air dry.
- 6) It was cleared with xylene and covered coverslip using Canada balsam.
- 7) It was examined microscopically using crossed polarizing filters [11].

RESULTS

The results showed the different staining reactions of the liver and kidney to modified Highman's Congo Red, Crystal Violet, and Toluidine Blue stains. Table 1 below shows that the nuclear demonstration has good demonstration while that of amyloid shows an excellent demonstration using modified Highman's Congo Red method on the Kidney section that the nuclear demonstration while that of amyloid shows no demonstration using the standard toluidine blue method in the kidney and also explain the nuclear demonstration while that of amyloid also shows a fair demonstration by the metachromatic method using crystal violet on the kidney.

TABLE 1

STAINS	Nuclear demonstration	Demonstration of amyloid and contrast
Highman's Congo Red	Positive (+++)	Positive (+++)
Toluidine blue	Positive (+)	Negative (-)
Crystal violet	Positive (+)	Positive (+)

KEY:

+ = Fair demonstration

- = No demonstration

DISCUSSION

Demonstration of amyloid in tissues had been possible in the past using a wide range of staining methods of Highman's modified Congo Red method, standard Toluidine blue method, and the metachromatic method using crystal violet. Each of these methods has its limitations, for example, their non-specificity to amyloid proteins, false positivity due to microscope type, room darkness, and interpreter bias. There have been advances in laboratory diagnosis like the recently introduced free light chain assay, which is >10 times more sensitive than immunofixation electrophoresis. However, in resource-poor settings, there is the challenge of availability or affordability and level of competency, causing local laboratories to fall back on the affordable traditional staining techniques, often running out of stains and needing alternative staining techniques. The result in table 1 above found that the average nuclear demonstration of all the tissues was best in the Highman's Congo Red method, followed by the metachromatic method using the crystal violet method, and lastly, the Toluidine blue method. On the other hand, the Amyloid demonstration was best in the Congo Red method while fairly demonstrated by the metachromatic staining using crystal violet and standard Toluidine blue methods. This demonstration agrees with other works of many writers who have classified amyloid as an acidophil, a hyaline substance mostly composed of protein but which may also include other components, making it usually identifiable by different staining procedures that attach to its molecular pattern like Birchall et al., [13], Howie, [14] and Linke, [15]. However, in using the methods above, the amyloid demonstration was best by the modified Highman's Congo Red method, especially in the kidney and Liver. This is in agreement with the works of Missmahl [16], Puchtler, Howie & Owen-Casey, [17], Sweat, & Levine [18], Yakupova, Bobyleva, Vikhlyantsev, & Bobylev [19] and Birchall et al., [13]. In the same vein, the metachromatic method gave a fairly good demonstration, while that of the standard Toluidine blue was fair. The deposition in terms of occurrence, as shown in table 2 below, was found to be highest in the kidney out of the fifteen (15) post-mortem samples, five (6) were positive, and only nine (9) were negative for amyloidosis and for the liver were out of the fifteen (15) post mortem samples, six (5) were positive, and only eight (10) were negative for amyloidosis. With the controversy surrounding false positives and the variability of in-vivo vs. in-vitro uptake of these dyes, due to a number of factors, including the choice of biopsy site, staining, and tissue analysis, several reports of visceral amyloid deposits have favored more deposits in the kidney than in the liver [18] [19]. This work is also in agreement with the works of Sen & Başdemir [20], Kaminsky, Jin, & Powell, [21], El-Meanawy, Mueller, & Iczkowski, [22], Jacobson et al., [2], Birchall et al., [13] and Bely & Makovitzky, [23]. These researchers reported a preponderance of amyloid deposits in tissue samples contained from the kidney compared to the liver. Researchers like Shin & Robinson-Papp, [24] disagree with each other while placing the renal samples second to the renal sample and followed by the kidney and liver, respectively. Regarding the association in causality between chronic inflammation and amyloidosis, out of about thirty (30) post-mortem samples (15 for each organ) collected from 15 different individuals.

TABLE 2

Sample	Total number of samples	Positive for amyloidosis	Positive for amyloidosis
Liver	15	5	10
Kidney	15	6	9

It was observed that those that died due to chronic inflammatory diseases had more positives for amyloidosis. It was also shown that these works agree with other workers like El-Meanawy, Mueller, & Iczkowski [22], Birchall, et al. [13], and Bely & Makovitzky [23] that chronic inflammatory disease is a major cause of amyloidosis in certain organs. Our result shows that the nuclear demonstration has a good demonstration while that of amyloid and contrasts shows an excellent demonstration by using the modified Highman's Congo Red method on the kidney section. The nuclear demonstration has a fair demonstration, while that of amyloid and contrasts shows no demonstration by using the standard Toluidine blue method on the kidney section, and the nuclear demonstration and that of amyloid and contrast also shows a fair demonstration by a metachromatic method using crystal violet on the kidney section.

LIMITATION

Effect of sample size; Given the sample size, it may be difficult to tell whether this particular outcome is a true finding, and it's possible that a type II error occurred. Also, despite the study's samples being chosen randomly, selection bias may have influenced the study's findings. Observation bias also may have played a role in the findings as well.

CONCLUSIONS

Based on the findings of this study, we discovered more amyloid deposits in kidney samples, as best demonstrated more by Highman's Congo red, then by metachromatic staining using the crystal violet method, and lastly by the standard Toluidine blue method. Furthermore, the kidney tissues stained more for amyloid deposits than the liver tissues, and most of these positives were found in necroptic samples from patients who died of chronic inflammatory diseases. In resource-poor settings, where sophisticated diagnostic methods are unavailable, these staining techniques may play a useful role in helping pathologists make timely diagnostic decisions. The use of in these resource-poor settings, Highman's Congo Red method, which is used in histological processing, is still widely used to demonstrate amyloidosis. More research should be conducted to determine the use of Highman's Congo Red method in other tissue demonstrations.

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