Standardization of grocott’s methenamine (hexamine) silver method for glycogen demonstration in liver tissue

Atheelah Mohammed Idris
University of Khartoum

Hibatalla Elshazli Elgamri
University of Khartoum

Samah Abdelrahim Batran
University of Khartoum

Yosef Mohamed-Azzam Zakout (yosifzakot@yahoo.com)
University of Hail

Research Article

Keywords: Fixation, fixative, glycogen, Grocott methenamine (hexamine) silver, liver

Posted Date: March 9th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2657019/v1

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Abstract

Purpose

Demonstration of glycogen can be done in different lesions and is considered diagnostically significant, mainly in some tumors. Glycogen staining is affected by the type of fixative, the temperature of fixation, and the staining technique.

Methods

Grocott

Grocott's liver and thirty-two paraffin sections were prepared and stained with Grocott's methenamine (hexamine) silver technique.

Results

Eighty percent ethanol gave better results at both RT and 4°C in comparison to the other fixatives.

Conclusion

80% alcohol at 4°C seems to provide the best staining results for glycogen with the Grocott methenamine (hexamine) silver technique at the level of this study.

Introduction

Glycogen is demonstrated in different lesions including glycogen storage disease [1], Ewing sarcoma [2] and Juvenile Rhabdomyosarcoma [3]. Furthermore, glycogen can be demonstrated in some carcinomas including bladder cancer [4].

Gomori's methenamine-silver nitrate was first described for glycogen and mucin demonstration [5], then was used by Grocott (1955) as a fungal stain in both smears and tissue sections [6]. However, Grocott's methenamine (hexamine) silver technique is a valuable technique for glycogen demonstration, which can provide staining results similar to those obtained by PAS reaction, when they used in combination with diastase or amylase control [7].

There are two theories regarding glycogen fixation; one by the importance of removing water molecules from the tissue by alcoholic fixatives to avoid the dissolving of glycogen. The other by fixing the protein that is associated with glycogen, and assumes that aqueous fixatives are sufficient for glycogen fixation [7]. Furthermore, some workers recommend picric acid-containing fixatives for glycogen fixation [7]. In the current study, we examined the effect of three types of fixatives (picric acid-containing fixatives, alcoholic and aqueous fixatives) at 4°C and room temperature (RT) to determine the ideal combination for demonstrating of glycogen using Grocott's methenamine (hexamine) silver method.

Methods

Study design

In this study, we examined the quality of the Grocott methenamine (hexamine) silver method in tissues fixed in different fixatives and temperatures of fixation.

Four fixatives were used: 10% NBF, Bouin's solution, 80% alcohol, and Rossman's solution at RT and 4°C.

In this study, we used archived liver paraffin blocks of known preparation methods. The blocks remained from another group in our department. These blocks were prepared from one rabbit as follows:

Sampling

The liver was washed in normal saline, cut into eight parts 5 X 3 X 3 mm approximately, and transferred to the required fixative.

Fixation

Each piece was fixed for 24 h in one of four fixatives: NBF, Bouin's solution, 80% alcohol, and Rossman's solution at one of two temperatures, 4°C in a refrigerator and RT.

Post-fixation treatment

Tissues that have been fixed in Rossman's solution were treated with 95% alcohol 2 h, while those fixed in Bouin's solution were treated with 75% alcohol 2 h [8]. After that, each specimen was transported to a labeled cassette according to the temperature of fixation and type of fixative.

Tissue processing
The specimens were transferred to the automated tissue processor. For dehydration, specimens fixed in 80% alcohol were transferred to one change of 80% alcohol followed by four changes of absolute alcohol 1 h each. The specimens fixed in Bouin’s solution were dehydrated in one change of both 75% and 90%, followed by four changes of absolute alcohol 1 h each. Specimens fixed in Rossman’s solution were dehydrated in one change of 95% alcohol followed by four changes of absolute alcohol for 1 h each. Specimens fixed in 10% NBF were dehydrated in one change of both 70% and 90% alcohol, followed by four changes of absolute alcohol for 1 h each. All specimens were cleared in two changes of xylene for 2 h each, impregnated in two changes of melted paraffin wax at 60°C for 2 h each, embedded in paraffin wax using metal molds and left to harden at room temperature, then in the refrigerator.

Cutting

Thirty-two albuminized and labeled slides were prepared and 3µ sections were cut using a rotary microtome, four sections from each block (3 tests and 1 negative control for each variable).

Staining

All sections were de-waxed with xylene for 6 min, hydrated in descending concentrations of alcohols from absolute through 90% and 70% to distilled water (DW) for 2 min each. Four sections from each block were stained by Grocott’s methenamine (Hexamine) silver technique (3 tests and 1 control).

For Grocott’s methenamine (Hexamine) silver technique, sections were treated with 5% aqueous chromic acid for 1 h, washed in tap water, rinsed in 1% sodium metabisulfite, washed for 5 min in tap water, rinsed in DW, placed in preheated (56°C) incubating solution in the dark for up to 1h (composed of 25 ml DW, 5 ml borax solution and 25 ml methenamine silver solution). Sections were then rinsed in DW, treated with 3% sodium thiosulfate for 5 min, counterstained with Arzac’s stain for 15 sec, blotted, dehydrated in alcohol, cleared in xylene, and mounted in DPX [9].

Control sections

The negative control sections were brought to water, treated with saliva (a source of amylase) for 1 h at 56°C in a water bath, then washed in running tap water and stained with the required technique (Photo. 1).

Scoring system

A subjective assessment of staining results was performed by two examiners, in which scores from zero to 10 were given. Scores were assigned as follows: from 0-4 (poor staining results), 5-6 (satisfactory staining results), 7-8 (good staining results), and 9-10 (excellent staining results). The average score of two examiners was calculated for each test section and the staining quality results were written as follows:

Poor: 0 - < 5: glycogen is weakly stained, the granules appear small and indistinct, and identifying glycogen is hard.

Satisfactory: 5 - < 7: the glycogen is faintly stained however it is visible.

Good: 7 - < 9: Glycogen is visible and distributed fairly evenly throughout the section.

Excellent: 9 – 10: Glycogen is visible and the granules are large and well stained which are distributed fairly evenly all through the section.

Ethics approval

 Archived liver paraffin blocks of known preparation methods were used in this study. The Department of Histopathology and Cytology, Faculty of Medical Laboratory Sciences, University of Khartoum, has accepted the current study and no ethical approval was required.

Results

Eighty percent alcohol at 4°C showed the highest staining quality (average score: 8.7) in comparison to other fixatives at the same temperature (Table 1, Fig. 1, photos. 2-5). However, Bouin’s solution gave good results at this temperature (average score: 7.5), but with less average score comparing to 80% alcohol. Furthermore, when fixation was conducted at RT, 80% alcohol was also found to be the best fixative for glycogen demonstration (average score: 8.3), where both Bouin’s solution and NBF fixatives gave satisfactory results and Rossman’s solution provided poor results at the same temperature.

<table>
<thead>
<tr>
<th>Fixatives &amp; Temperatures</th>
<th>Bouin’s solution at RT</th>
<th>Bouin’s solution at 4°C</th>
<th>10% NBF at RT</th>
<th>10% NBF at 4°C</th>
<th>80% Alcohol at RT</th>
<th>80% Alcohol at 4°C</th>
<th>Rossman’s solution at RT</th>
<th>Rossman’s solution at 4°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexamine (methamine)</td>
<td>6.8</td>
<td>7.5</td>
<td>5</td>
<td>5.6</td>
<td>8.3</td>
<td>8.7</td>
<td>2.8</td>
<td>6.2</td>
</tr>
<tr>
<td>Silver</td>
<td>Satisfactory</td>
<td>good</td>
<td>Satisfactory</td>
<td>Satisfactory</td>
<td>good</td>
<td>good</td>
<td>poor</td>
<td>Satisfactory</td>
</tr>
</tbody>
</table>

The averages of staining quality from three sections for each variable examined by two observers for Grocott’s methenamine (Hexamine) silver technique following 4 fixatives at 2 degrees of temperature.
Discussion

For the accurate preservation and demonstration of glycogen, it is fundamental to choose an appropriate fixative, the temperature of fixation, and the staining method. This study is one of the leading studies which optimize Grocott's methenamine (Hexamine) silver technique, using 80% alcohol, NBF, Bouin's fluid and Rossman's fluid at both RT and 4°C.

In this study, 80% alcohol at 4°C gave the highest average score of staining quality for glycogen demonstration using the hexamine silver method. By using this technique, glycogen appears black and is distributed in hepatocytes. These findings support the results obtained by Kugler and Wilkinson (1964) [10]. In their study, they reported that ice-cold 80% alcohol was a good preserver for glycogen present in the tissue when using different histochemical reactions. However, Bouin's solution at 4°C provided good staining results too, but its average score was less than 80% alcohol. Nevertheless, Kinsley et al. 2013 studied different fixatives to identify the best one for the preservation of glycogen in mouse liver. They evaluated 4 different types of fixatives: 100% ethyl alcohol, 1% periodic acid in 10% NBF, alcoholic formalin, and 10% NBF. They concluded that 1% periodic acid in 10% NBF for 48 hr at 4°C was the best fixative to preserve glycogen [11]. Trott 1961 evaluated several types of fixatives for fixing glycogen in rats’ liver. He used Rossman's solution, alcohol formalin, Carnoy's fluid, 10% neutral formalin, Bouin's, acetic alcohol formalin, and 10% formalin, formal-saline for different durations. He concluded that good glycogen staining with the PAS method can be obtained after three months of fixation with acetic alcohol formalin. Furthermore, he reported that the aqueous-based fixatives tend to be unreliable for demonstrating the maximum amount of glycogen after 24 to 48 h of fixation [12].

Moreover, Trott 1961 observed that the appearance of glycogen can be affected by the type of fixation. He found that tissues fixed in acetic alcohol formalin provide the most obvious polarization. However, he mentioned that less polarization is noticed following aqueous-based fixatives. Additionally, larger and coarser glycogen granules were observed following alcohol-based fixatives [12].

Also, we found that Grocott’s methenamine (Hexamine) silver technique is a useful method for glycogen demonstration, particularly when tissue was fixed in 80% alcohol, in which, it demonstrated glycogen as black clumps or granules in hepatocytes. This conclusion is supported by the finding of Murgatroyd (1971), who recommended this method as a good technique for glycogen demonstration [13]. In conclusion, 80% alcohol seems to be the best fixative for glycogen demonstration by Grocott’s methenamine (Hexamine) silver technique, which provides good staining results at both RT and 4°C.

Declarations

Acknowledgments:

We would like to thank all who assessed this study.

Funding

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Atheelah Mohammed Idris and Hibatalla Elshazli Elgamri performed the technical work and histological techniques including cutting, and staining. Samah Abdelrahim Batran was responsible of the assessment of the results and the revising of the manuscript. Yosef Mohamed-Azzam Zakout was responsible of planning, designing, and directing the study, writing and preparing the manuscript.

Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent to publish

Not applicable.

References


**Photos**

Photo 1 to 5 are available in the Supplementary Files section

**Figures**

![Hexamine Silver technique graph](https://example.com/figure1.png)

**Figure 1**

Glycogen average scores for Grocott methenamine (Hexamine) silver technique using 4 fixatives at two fixation temperatures.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- P1.png
- P2.png
- P3.png
- P4.png
- P5.png