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SHORT COMMUNICATION

Optimization of PAS stain and similar Schiff's based methods for glycogen demonstration in liver tissue

Yosef Mohamed-Azzam Zakout[1,2] · Marwah Abdelrahman Abdellah[2] · Masia Aldai Abdallah[2] · Samah Abdelrahim Batran[2]

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Abstract

Demonstration of glycogen in tissue holds considerable diagnostic relevance across various pathological conditions, particularly in certain tumors. The histochemical staining of glycogen using methods utilizing Schiff's reagents is subject to influences arising from the type of fixative, fixation temperature, and oxidizing agents employed. This study aimed to assess diverse fixatives, fixation temperatures, and oxidizing agents, each with variable treatment durations, in conjunction with Schiff's reagent for optimal glycogen demonstration. Paraffin blocks derived from a rabbit's liver served as the experimental substrate, encompassing 340 paraffin sections subjected to different procedures. For tissues fixed at 4 °C, good staining outcomes, as determined by the periodic acid–Schiff (PAS) stain, were observed with 10% neutral buffered formalin (NBF), 80% alcohol, and Bouin's solution. Tissues fixed at room temperature (RT) demonstrated good PAS staining results with both 10% NBF and 80% alcohol. Notably, other oxidizing agents exhibited poor outcomes across all fixatives and fixation temperature, with two exceptions, as satisfactory staining results were obtained when using 5% chromic acid. Consequently, Both 10% NBF and 80% emerge as preferred fixatives of choice for glycogen demonstration when coupled with PAS stain. It is noteworthy that Bouin's solution could also provide good outcomes when fixation occurred at 4 °C.

Keywords Fixation · Glycogen · Oxidizing agent · PAS

Introduction

Glycogen, a branched glucose polymer, functions as a crucial energy reservoir during periods of nutritional abundance, providing a reserve for subsequent utilization during times of metabolic demand (Roach et al. 2012). The PAS stain, originally reported for mucin demonstration (McManus 1946), was subsequently adapted for the demonstration of various substances, including glycogen. Periodic acid induces the formation of up to three aldehyde substituents on each 1,2-linked carbohydrate unit within the tissue, with the resulting aldehydes amenable to visualization through the application of Schiff's reagent (McManus 1948). While alternative oxidizers such as potassium permanganate and chromic acid can also yield these aldehydes from polysaccharides, their robust oxidizing nature surpasses that of periodic acid, potentially leading to degradation of the produced aldehydes over time (Lillie 1951, Della Speranza and Fail 2005). The fixation of glycogen remains a subject of contention, with varying recommendations ranging from alcohol or picric acid-containing fixatives to aqueous fixatives (Totty 2002). Additionally, the selection of the fixative type is known to impact the visual manifestation of glycogen (Totty 2002). Consequently, this study systematically incorporated a spectrum of fixatives, including aqueous, alcoholic, and picric acid-containing solutions, to comprehensively evaluate and compare staining outcomes. The main objective is to discern the optimal fixative for glycogen demonstration utilizing the PAS stain and selected Schiff's reagent-based methods.

✉ Yosef Mohamed-Azzam Zakout
yosifzakot@yahoo.com; y.zakout@uoh.edu.sa

[1] Department of Pathology, College of Medicine, University of Hail, Hail, Kingdom of Saudi Arabia

[2] Department of Histopathology and Cytology, Faculty of Medical Laboratory Sciences, University of Khartoum, Khartoum, Sudan

Materials and methods

Study design

This study employed four distinct fixatives: 10% NBF, Bouin's solution, Rossman's solution, and 80% alcohol, each applied at both RT and 4 °C. Four oxidizing agents were incorporated into the experimental design: 5% chromic acid (w/v aqueous) for duration of 10, 20, 30, and 60 min at RT, as well as for 10 min at 60 °C; 10% chromic acid (w/v aqueous) for durations of 20 and 30 min at RT; 2% $FeCl_3$ (w/v aqueous) for 5, 10, and 15 min; and 1% periodic acid (w/v aqueous) for 5 min.

Sample acquisition

Archived paraffin blocks, prepared using established methods, served as the primary material for this study. These blocks were procured from another research group at the Department of Histopathology and Cytology at the Faculty of Medical Laboratory Sciences, University of Khartoum, Sudan. Originating from a rabbit's liver, the archived blocks were meticulously prepared by the aforementioned research group as follows:

Sampling

The rabbit's liver was rinsed with normal saline before being sliced into eight pieces, each measuring approximately 5 × 3 × 3 mm. These tissue specimens were then promptly transmitted to specific fixatives.

Fixation

Each specimen was meticulously assigned to one of four distinct fixatives: 80% alcohol, 10% NBF, Bouin's solution, and Rossman's solution. Fixation procedures were carried out at both RT and 4 °C, the latter in a refrigerator, with each specimen left in the respective fixative for a standardized period of 24 h. To ensure traceability, each tissue specimen was placed in a labeled cassette, appropriately denoting the fixative type and the temperature of fixation. Following fixation, the specimens were further transferred to the processing machine.

Postfixation treatment

Subsequent to fixation in Rossman's solution, the samples underwent a transitional step involving immersion in 95% alcohol for a duration of 2 h. In the case of Bouin's fixation, a comparable postfixation treatment was administered, with the specimens undergoing treatment in 75% alcohol for 2 h (Hopwood 2002). Following these postfixation procedures, the specimens were meticulously transferred to labeled cassettes clearly indicating the specific type of fixative employed and the corresponding temperature of fixation. This systematic labeling ensured precise documentation for subsequent analytical stages.

Tissue processing

During the tissue processing phase, the following procedures were applied:

1. **Dehydration:**
   - ***80% alcohol fixed samples:*** Subjected to one change of 80% alcohol, followed by four changes of absolute alcohol, each for a duration of 1 h.
   - ***Bouin's solution fixed samples:*** Dehydrated through one change of 75% and 90% alcohol, succeeded by four changes of absolute alcohol, each lasting 1 h.
   - ***Rossman's solution fixed samples:*** Treated with one change of 95% alcohol, followed by four changes of absolute alcohol each for 1 h.
   - ***10% NBF fixed samples:*** Treated with one change of 70% and 90% alcohol, succeeded by four changes of absolute alcohol, each lasting 1 h.
2. **Clearing:** All samples underwent clearing with two changes of xylene, each spanning 2 h.
3. **Impregnation:** The samples were impregnated through two changes of melted paraffin wax, with each change lasting 2 h.
4. **Embedding:** Subsequently, the samples were embedded in paraffin wax utilizing metal molds. The embedded samples were allowed to harden at RT and were subsequently placed in a refrigerator.

Sectioning

A total of 340 labeled slides were prepared. Using a rotary microtome from Leica Biosystems (Wetzlar, Germany), 4-µm sections were cut from the paraffin-embedded tissue blocks. To ensure comprehensive representation, each block underwent sectioning for three distinct tests and one control for each variable, resulting in the extraction of 11 sections from each block. This meticulous sectioning process laid the foundation for subsequent histological examination and evaluation of the various fixatives, oxidizing agents, and temperature conditions.

Staining

All sections underwent successive treatments with two changes of xylene, each for 3 min, followed by absolute alcohol, 90% alcohol, 70% alcohol, and distilled water, each for 2 min. Subsequently, they were stained using the following techniques:

PAS stain (Survana et al. 2013): Sections were immersed in 1% periodic acid for duration of 5 min, followed by rinsing in distilled water (DW). Subsequently, the sections were treated with Schiff's reagent for 15 min, followed by washing in running tap water for 10 min. The sections were then stained with Harris's hematoxylin for 3 min, differentiated in 1% acid alcohol, followed by bluing in running tap water for 10 min. Sections were dehydrated in absolute ethanol, cleared in xylene. Finally, the treated sections were mounted in D.P.X.

Chromic acid–Schiff method (Carson and Hladik 2009): Sections were treated with 5% chromic acid (w/v aqueous) for varying durations, including 10, 20, 30, and 60 min at RT. Additionally, a treatment at 60 °C for 10 min was administered. The other treatment involved the use of 10% chromic acid (w/v aqueous) for 20 and 30 min at RT. Following the chromic acid treatments, sections were thoroughly washed in three changes of DW. Subsequently, Schiff's reagent was applied to the sections for 15 min. The sections were then washed in running tap water for 10 min and stained with Harris's hematoxylin for 3 min. Differentiation was achieved through immersion in 1% acid alcohol, followed by bluing in running tap water for 10 min. Sections were dehydrated in absolute ethanol, cleared in xylene, and mounted in D.P.X.

Ferric chloride–Schiff reaction: Sections were treated with 2% ferric chloride (w/v aqueous) for specified durations, including 5, 10, and 15 min. Following the ferric chloride treatment, thorough rinsing in DW was carried out. Subsequently, sections were treated with Schiff's reagent for 15 min, washed in running tap water for 10 min, and stained with Harris's hematoxylin for 3 min. Differentiation was accomplished by immersion in 1% acid alcohol, followed by bluing in running tap water for 10 min. Sections were dehydrated in absolute ethanol, cleared in xylene, and mounted in D.P.X.

For negative controls (Fig. 1), the glycogen was extracted from the negative control sections by treating them with saliva for 1 h at 37 °C and washed in tap water, distilled water, then stained according to the required technique (Coimbra 1966).

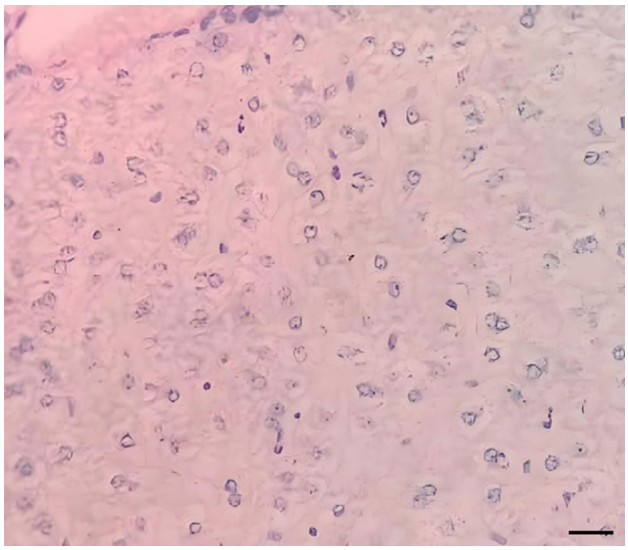

Fig. 1 PAS negative control, 80% alcohol at RT. Scale bar 100 µm

Scoring system

Two examiners conducted a subjective evaluation of the staining outcomes. The staining results were assigned scores on a scale ranging from 0 to 10 (Idris et al. 2023). Slides with technical errors that rendered them difficult to evaluate were excluded. The average score for each test slide was calculated by summing the scores assigned by both examiners and subsequently dividing this sum by the total number of evaluations. This scoring system, based on a combination of expert judgment and numerical scores, enhances the objectivity and robustness of the staining evaluation, ensuring the credibility of the findings reported in this study. The significance of these scores is as follows:

Poor staining quality (score 0 to < 5): Scores falling within the range of 0 to less than 5 indicate poor staining quality, characterized by weakly stained glycogen. In this category, glycogen granules appear small and indistinct, posing challenges in their recognition.

Satisfactory staining quality (score 5 to < 7): Staining results falling within the range of 5 to less than 7 are considered satisfactory, with visible glycogen but the staining is faint.

Good staining quality (score 7 to < 9): Staining results falling within the range of 7 to less than 9 are classified as good. In this category, glycogen is distributed fairly evenly throughout the stained section and is visible. The staining intensity is notably improved compared with the satisfactory range, allowing for clearer and more distinct identification of glycogen deposits.

Excellent staining quality (score 9–10): Staining results falling within the range of 9 to 10 are classified as excellent. In this category, glycogen is visible and has well-stained large granules which are fairly evenly distributed throughout the stained section.

Results and discussion

The evaluation of different fixatives, temperatures, and oxidizing agents for glycogen demonstration revealed distinct staining outcomes. Notably, 10% NBF and 80% alcohol emerged as superior fixatives, consistently providing good staining results for the PAS stain (Table 1; Fig. 2).

For Bouin's solution at 4 °C, the highest average score of staining quality was detected with 1% periodic acid for 5 min, which scored 7.7 (good) (Table 1). For Rossman's solution at both 4 °C and RT, the highest average score of staining quality was detected with 1% periodic acid for 5 min, which provided satisfactory staining results (Table 1). For 10% NBF at both RT and 4 °C, the highest average score of staining quality was detected with 1% periodic acid for 5 min, which scored 8.7 (good) for each (Table 1). Similarly, periodic acid provided the highest score for 80% alcohol at both 4 °C and RT, which scored 8 and 7.7 (good), respectively (Table 1; Fig. 3). Interestingly, 5% chromic

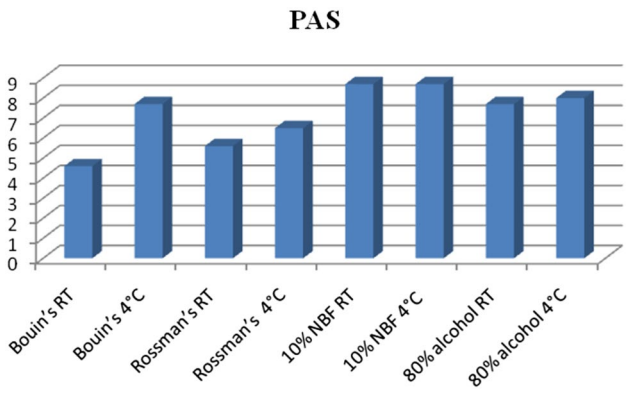

Fig. 2 Average scores of staining quality for PAS stain with different fixatives and temperatures of fixation

Table 1 Average scores of staining quality from three stained sections for each studied variable examined by two examiners for different oxidizing agents—Schiff reactions, following four fixatives at two level of temperatures of fixation

Oxidizing agent, concentration, and duration	Fixatives and temperatures of fixation							
	Bouin's solution		Rossman's solution		10% NBF		80% alcohol	
	RT	4 °C	RT	4 °C	RT	4 °C	RT	4 °C
1% periodic acid, 5 min	4.6* Poor	**7.7** ***Good***	**5.6**** **Satisfactory**	**6.5*** **Satisfactory**	**8.7** ***Good***	**8.7** ***Good***	**7.7** ***Good***	**8** ***Good***
5% chromic acid at RT, 10 min	4.2 Poor	4.5 Poor	1.33 Poor	3.1 Poor	3.3 Poor	3.7 Poor	1.8 Poor	**5** ***Satisfactory***
5% chromic acid at RT, 20 min	3 Poor	2.2 Poor	0.5 Poor	1.2 Poor	2.8 Poor	4.2 Poor	1 Poor	3.7 Poor
5% chromic acid at RT, 30 min	2.83 Poor	1.3 Poor	0,7 Poor	0,83 Poor	3.2 Poor	3.3 Poor	0.5 Poor	3.3 Poor
5% chromic acid at RT, 1 h	2.6 Poor	0.83 Poor	0.5 Poor	0.8 Poor	1 Poor	3 Poor	0.5 Poor	0.2 Poor
5% chromic acid at 60 °C, 10 min	3.8 Poor	3 Poor	0.7 Poor	0.5 Poor	3.7 Poor	3.2 Poor	2.7 Poor	**5.7** ***Satisfactory***
10% chromic acid at RT, 20 min	2.3 Poor	0.7 Poor	1.2 Poor	0.83 Poor	0.7 Poor	2.3 Poor	0.3 Poor	0.2 Poor
10% chromic acid at RT, 30 min	1.2 Poor	0.7 Poor	0.83 Poor	0.5 Poor	0.7 Poor	1.2 Poor	0.2 Poor	1 Poor
2% $FeCl_3$, 5 min	0.7 Poor	0.5 Poor	0.5 Poor	***	0.3 Poor	0 Poor	0.3 Poor	0.3 Poor
2% $FeCl_3$, 10 min	1.7 Poor	0.7 Poor	0.2 Poor	***	0.2 Poor	0.2 Poor	0 Poor	0 Poor
2% $FeCl_3$, 15 min	0.7 Poor	0.3 Poor	0.33 Poor	***	0 Poor	0.5 Poor	0.2 Poor	0.5 Poor

*The mean was calculated from three slides (three slides were excluded)

**The mean was calculated from five slides (one slide excluded)

***Excluded

Bold italic indicates fixatives and temperature of fixation that gave (Satisfactory) or (Good) staining results

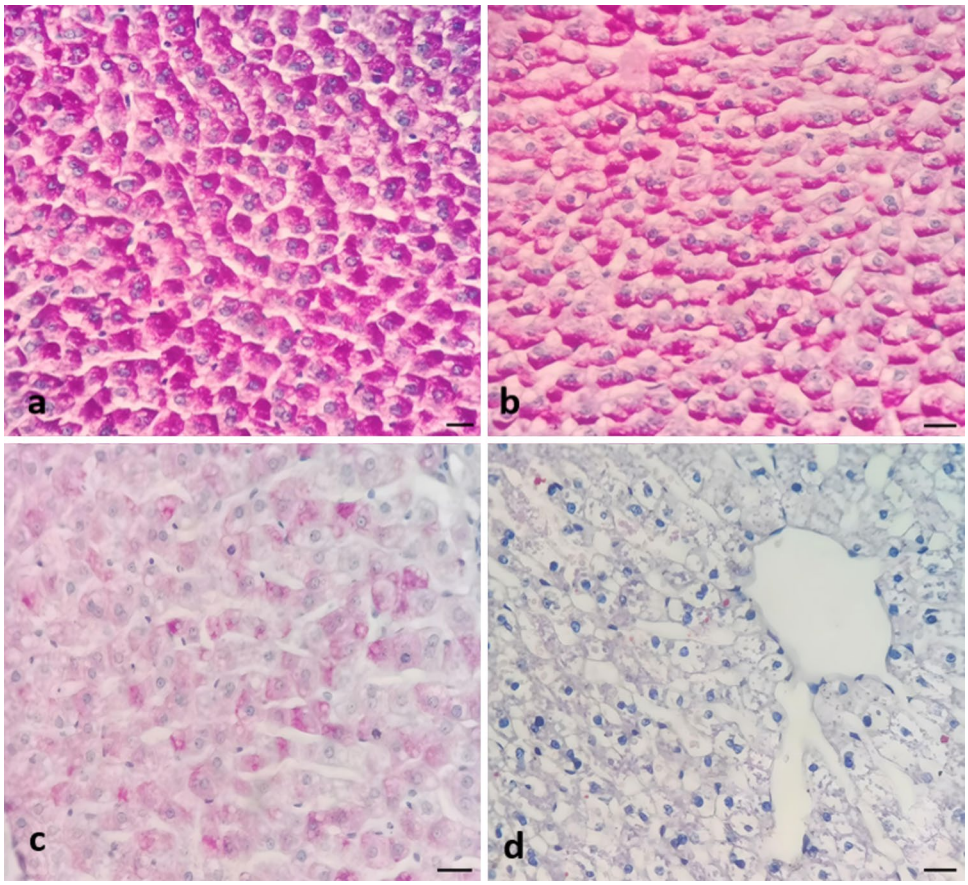

Fig. 3 **a** PAS stain, 80% alcohol at 4 °C, good, **b** PAS stain, 80% alcohol at RT, good **c** 5% chromic acid at 60 °C for 10 min-Schiff reaction, fixed in 80% alcohol at 4 °C, satisfactory, **d** 2% $FeCl_3$–Schiff reaction for 5 min, 10% NBF at 4 °C, poor. 40×. Scale bar 100 µm

acid–Schiff reaction at both RT and 60 °C for 10 min provided satisfactory staining results with 80% alcohol at 4 °C (Table 1; Fig. 3). However, 10% chromic acid at RT for 20 and 30 min provided poor staining results with all fixatives (Table 1). The 2% $FeCl_3$ for 5, 10, and 15 min provided poor staining results with all fixatives and temperatures of fixation (Table 1; Fig. 3).

The efficacy of glycogen staining is influenced by the choice of fixative, fixation temperature, and the selection of an appropriate oxidizing agent in conjunction with Schiff's solution. Our study highlights 10% NBF and 80% alcohol as optimal fixatives for PAS stain, consistently providing good staining results at both RT and 4 °C. Notably, Bouin's solution also demonstrated effectiveness, particularly when fixation occurred at 4 °C. These findings align with work of Kugler and Wilkinson (1964), who noted that ice-cold 80% alcohol preserves 81% of glycogen found in the tissue in various histochemical reactions. The success of 10% NBF fixation can be attributed to formalin's action, effectively fixing proteins associated with glycogen (Totty 2002), resulting in robust staining results. Understanding the nuanced interplay between fixatives and staining outcomes is crucial for refining histopathological techniques. Our results contribute to this understanding by emphasizing the reliability of 10% NBF and 80% alcohol as top fixatives for glycogen visualization through PAS stain, with Bouin's solution demonstrating efficacy under specific conditions. This insight enhances the precision of glycogen detection in tissue analysis, underscoring the importance of methodological considerations in achieving optimal staining quality.

Our investigation into oxidizing agents revealed that periodic acid stands out as the superior choice when compared with other agents assessed in this study. This aligns with existing literature where periodic acid consistently emerges as the predominant oxidizing agent paired with Schiff's reagent for glycogen demonstration (Stankler and Walker 1976, Fairchild and Fournier 2004, Tabatabaei Shafiei et al. 2014). Our endorsement of periodic acid as the optimal oxidizing agent in our study reinforces its standing in the scientific community. Researchers and histopathologists can confidently adopt periodic acid in their protocols, leveraging its demonstrated efficacy for precise and reproducible glycogen staining outcomes.

Chromic acid is a strong oxidizing agent that can oxidize numerous groups beyond the reactive aldehyde stage (Carson and Hladik 2009). Graf and Klessen's (1981) observation that glycogen is not easily detected through chromic acid–Schiff's technique resonates with our findings. Chromic acid's capacity to destroy the produced aldehyde, as

noted by Lillie (1951), underscores the intricacies associated with its use in glycogen staining. Our study concurs with the acknowledged challenges of the chromic acid–Schiff method. However, our nuanced findings shed light on the method's potential viability under specific conditions. Notably, a 5% chromic acid–Schiff reaction at both RT and 60 °C for 10 min demonstrated satisfactory staining results when coupled with 80% alcohol at 4 °C. This underscores the importance of meticulously considering fixative choice, fixation temperature, oxidizing agent concentration, and duration of treatment when employing the chromic acid–Schiff method for glycogen demonstration.

In conclusion, our study positions 10% NBF and 80% alcohol as the top fixatives for glycogen demonstration via PAS stain, followed by Bouin's solution at 4 °C. Periodic acid emerges as the superior oxidizing agent in comparison with the other oxidizing agents explored in this study. These insights contribute to refining histopathological techniques, emphasizing the nuanced interplay of fixative and oxidizing agent selection in optimizing glycogen staining quality.

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Author contributions Marwah Abdelrahman Abdellah and Masia Aldai Abdallah conducted the technical work and histological techniques, which involved cutting and staining. Samah Abdelrahim Batran was responsible for assessing the results and revising the manuscript. Yosef Mohamed-Azzam Zakout took charge of planning, designing, and directing the study, as well as assessing the results and writing and preparing the manuscript.

Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Conflict of interest The authors have no relevant financial or nonfinancial interests to disclose.

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