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Effects of various fixatives and temperature on the quality of glycogen demonstration in the brain and liver tissues

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ABSTRACT

The visualization of glycogen deposits in cells and tissues is important for studying glycogen metabolism as well as diagnosis of glycogen storage diseases. Evidence suggests that the demonstration of glycogen can better be enhanced by factors such the choice of fixative and temperature during fixation. Here, we assessed efficacy of neutral buffered formalin (NBF), alcoholic formalin (AF) and paraformaldehyde (PFA) at 4 °C, 37 °C and 40 °C using Periodic Acid Schiff's staining method. Each liver specimen was fixed in NBF and AF while the brain tissues were fixed in NBF, AF and PFA. We found that there was a better PAS staining intensity with the liver tissues fixed in AF compared with NBF. Also, there was no difference in the quality of the staining for tissues fixed in AF at 37 °C, 4 °C and 40 °C, but fixation with NBF at 4 °C gave the best staining quality when compared with 40 °C and 37 °C. Furthermore, hippocampal tissues fixed in AF showed better quality of PAS staining compared with NBF and PFA. A significant increase in staining intensity was observed for PFA when compared with NBF. Superior staining intensity for PAS was observed at 4 °C for hippocampal tissues fixed with NBF, AF and PFA. Taken together our results show that AF at a temperature of 4 °C gave the best result. Hence, glycogen demonstration can better be enhanced by the choice of fixative and temperature during fixation.

1. Introduction

Glycogen is a large, branched polymer of glucose which provides a readily mobilized form of energy in many living organisms. Glycogen concentration varies considerably among different types of cells with its storage markedly occurring in the liver, muscle and brain [1]. The visualization of glycogen deposits in cells and tissues under the microscope is important for the study of normal glycogen metabolism as well as diagnosis of glycogen storage diseases. Its demonstration in the body is very important for a number of lesions and is significant, especially in the diagnosis of certain tumours [2-4]. It also serves as a useful tool in the detection of intracellular accumulation of excessive glycogen in the liver as a manifestation of metabolic dysfunction [5].

The aqueous fixatives and processing solvents used in paraffin embedding for routine hematoxylin and eosin stains remove both lipid and glycogen, leaving clear vacuoles of which the precise content is unknown in routine hematoxylin and eosin-stained sections. Intracellular glycogen in liver sections is readily diminished by aqueous fixatives and processing solvents commonly used in routine tissue processing. As a result, glycogen masses appear as clear vacuoles within the cytoplasm of hepatocytes in histology sections [5]. The precise content of these vacuoles is therein undetermined and, in most instances, the identification of glycogen can be challenging [5]. Demonstration of brain glycogen poses some unique challenges to experimental observation. Its relatively low concentration in the brain makes it difficult for histochemical stains to pick up [6]. The process of glycogenolysis which begins almost instantly with cessation of blood flow and continues until the brain is frozen, acidified, or otherwise treated to inactivate glycogen phosphorylase pose challenges to glycogen brain demonstration [6]. The demonstration of glycogen using histological sections depends on a number of factors including choosing a suitable fixative at a suitable temperature.

Fixation, a physiochemical process in which cells or tissues are fixed chemically, results in the tissue or cells being able to withstand the successive treatment by different reagents with negligible change in morphology [7]. This process differs for the different chemical substances found in tissues. An ideal fixative involves a complicated process of chemical episodes [8]. It stabilizes the cell component by making them insoluble, thereby reducing the alteration by subsequent treatment and also preventing osmotic damage of tissues [8].

The temperature at which tissues are fixed also play a pivotal role in either increasing the rate of fixation or decreasing the diffusion rate of the fixative both of which affect tissue architecture. By tradition, fixation of tissues occurs at room temperature, while for some processing

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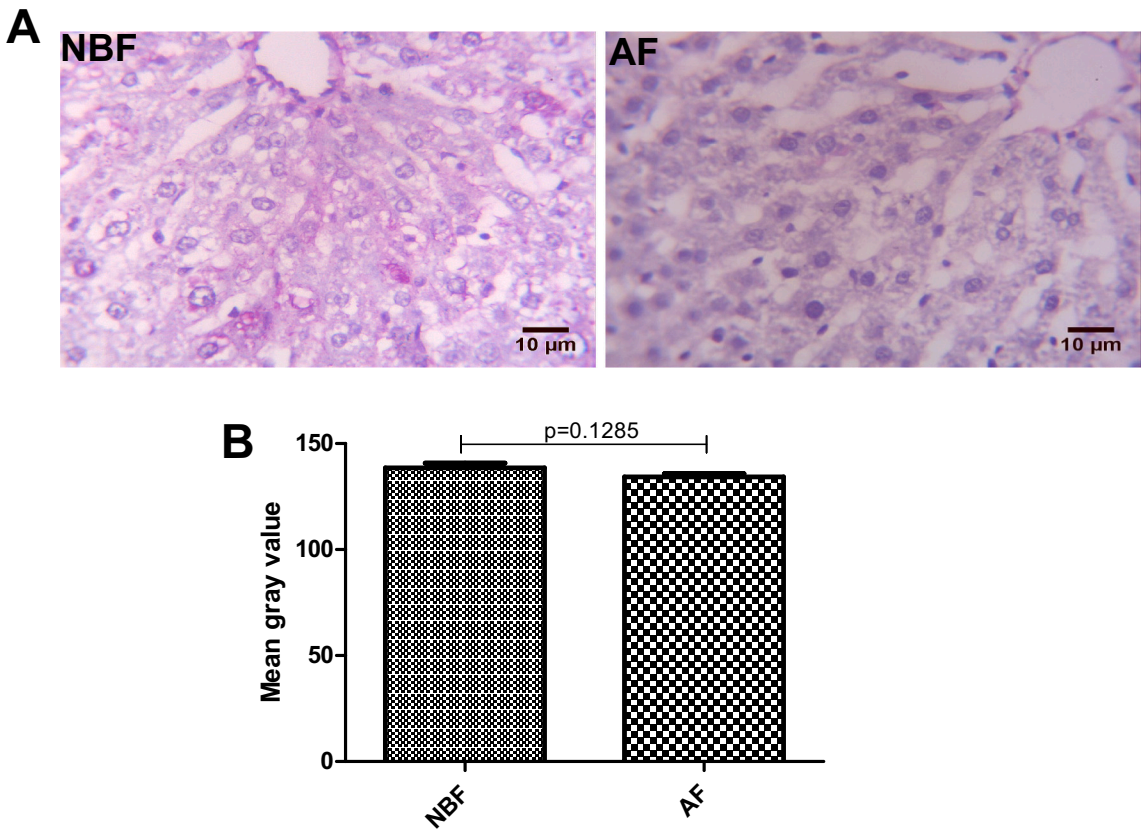

Fig. 1. A: Photomicrographs of PAS stained liver tissues fixed with NBF and AF. B: Image J count of PAS staining intensity of the liver of rats fixed with NBF and AF. Staining intensity is quantified using mean gray values, in a scale of 0 to 255 from dark (more staining intensity) to bright (less staining intensity). $p < 0.05$. Here and in further figures, the staining intensity is quantified as mean gray value in a scale from 0 to 255 from dark (that is more intense stain) to bright (that is less intense stain).

for histochemistry and electron microscopy it occurs between 0 and 4 degrees. The lower temperature of fixatives results in slowing down autolysis and diffusion of various cellular components, whereas chemical reactions including those involved in fixation occur faster at a higher temperature. Increasing the temperature of the fixative increases the rate of diffusion of the fixative into the tissue thereby speeding up the rate of chemical reaction between the tissue and the fixative [9]. This study sought to investigate the effect of three different fixatives: neutral buffered formalin (NBF), alcoholic formalin and paraformaldehyde (PFA) applying different temperatures (4 °C, 37 °C, 40 °C) on histochemical staining of Periodic Acid Schiff staining for glycogen.

2. Materials and method

2.1. Experimental animals

Following ethical approval from the institutional ethics committee (ORD/AD/EAC/19/050), six adult male Wistar rats (180–200 g) were used in this study. The animals were obtained from the Animal holding facility of the College of Medicine, Ekiti State University, Ado-Ekiti. The animals were kept in a cage under normal light and dark cycle for a week with free access to feed and water ad libitum. Thereafter, the rats were euthanized via intraperitoneal injection of ketamine (30 mg/kg); the liver and brain were quickly harvested and fixed in the different fixatives at the required temperatures.

2.2. Fixation

Each liver specimen was fixed in neutral buffered formalin (NBF) and 10% alcoholic formalin (AF) while the brain tissues were fixed in NBF, AF and paraformaldehyde (PFA) at 4 °C, 37 °C and 40 °C. The tissues preserved in fixatives at 40 °C were placed in an incubator; those fixed at 4 °C were in a refrigerator while those at 37 °C were left to stand on a laboratory bench for 24 h.

The labelled specimens were dehydrated using ascending grades of alcohol for 1 h each, and then placed in four changes of absolute alcohol for 1 h each. The specimens were cleared in two changes of xylene for 2 h each and impregnated in paraffin wax. Embedding will be carried out using paraffin wax and left to solidify at room temperature, then cooled in the refrigerator. Sections of 4 µm were gotten by cutting blocks with a rotary microtome.

2.3. Staining

For PAS (Periodic Acid Schiff) reaction, sections were treated with 1% periodic acid for 5 min, rinsed in distilled water, stained with Schiff's reagent for 20 min, then washed in running tap water for 10 min, counterstained with hematoxylin for 8 min, dehydrated in alcohol, cleared in xylene and mounted in DPX [10]. The presence of glycogen was confirmed with negative control sections using diastase digestion.

2.4. Photomicrography and image analysis

Photomicrographs of the tissue sections were studied and taken at various magnifications under an OMAX 40×–2000× digital light microscope. Image J software (National Institute of Health, USA) was used to analyse and quantify photomicrographs. Intensity of PAS staining was performed by measuring the mean gray value of pixels. The settings were such that a higher staining intensity corresponded to a lower mean

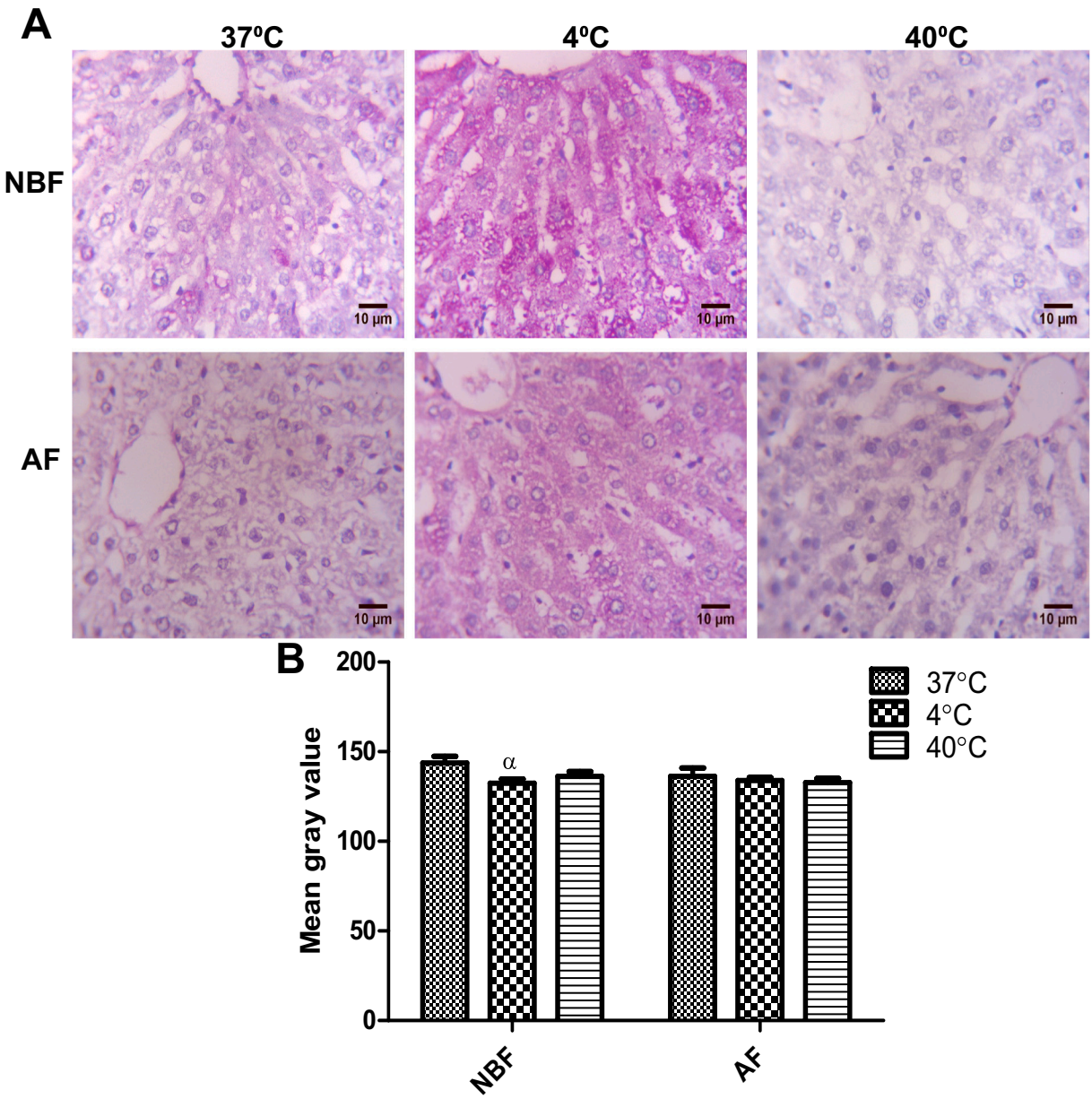

Fig. 2. A: Photomicrographs of PAS stained liver tissues fixed in NBF and AF at different temperatures. B: Image J count of PAS staining intensity of the liver of rats fixed with NBF and AF at 37 ˚C, 4 ˚C and 40 ˚C. $p < 0.05$, α — significant difference compared to 37 ˚C.

gray value.

2.5. Statistical analysis

Data was expressed as mean ± standard error (SEM) and were analysed using GraphPad Prism version 5.00 for Windows (GraphPad Software, USA). Group comparisons were performed using Analysis of variance (ANOVA) as appropriate, followed by Bonferroni tests for multiple comparison. Comparisons of two groups were performed using Student's *t*-test for unpaired data. Statistical significance was assumed at p values < 0.05.

3. Results

3.1. Effect of fixative on the staining intensity of PAS in the liver

As seen in Fig. 1A, there was a better morphologic detail of PAS staining with the liver tissues fixed in AF compared with NBF. Results from image analysis show an increase in the mean gray value of NBF (139 ± 2.3) when compared to AF (134 ± 1.5) (Fig. 1B) but it was not statistically significant ($p = 0.1285$).

3.2. Effect of temperature on the staining intensity of PAS in the liver

Fig. 2A shows photomicrographs of the liver fixed with NBF, AF and PFA stained with PAS at different temperatures. Fixation with NBF at 4 ˚C gave the best staining quality when compared with 40 ˚C and 37 ˚C. Also, there was no difference in the quality of the staining for tissues fixed in AF at 37 ˚C, 4 ˚C and 40 ˚C. As seen in Fig. 2B, Student *t*-test showed a significant difference ($p = 0.0366$) in the mean gray value between NBF at 37 ˚C (144 ± 3.6) and 4 ˚C (132 ± 1.7). This indicates an increase in the staining intensity of PAS for NBF at 4 ˚C compared to 37 ˚C. However, there was no significant difference between the mean gray value of NBF at 37 ˚C and 40 ˚C ($p = 0.4762$). Also, for AF, there was no significant difference between the mean gray value of at 37 ˚C and 4 ˚C ($p = 0.7302$); similar results was obtained for

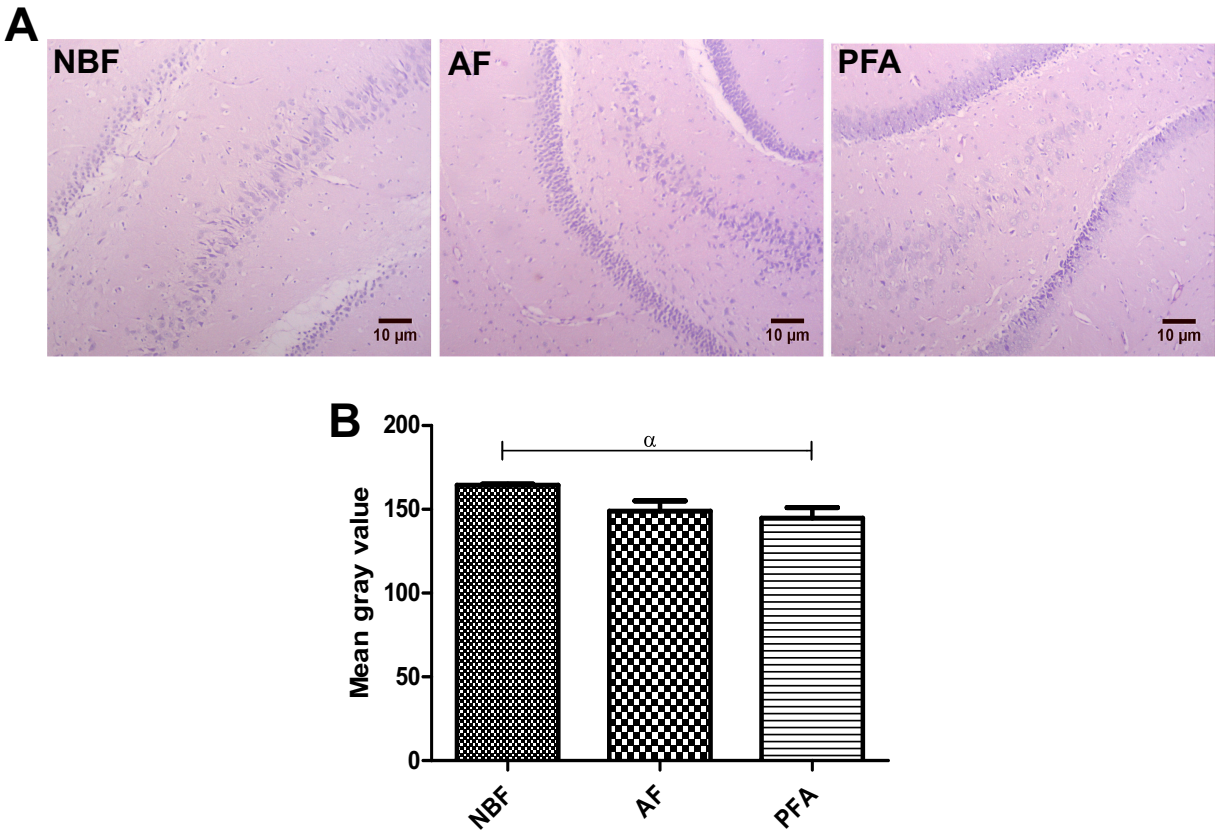

Fig. 3. A. Photomicrographs of PAS stained hippocampal tissues fixed in NBF, AF and PFA. B: Image J count of PAS staining intensity of the hippocampus of rats fixed with NBF, AF and PFA. α — significant difference compared to AF and PFA.

the mean gray value between 37 °C and 40 °C ($p = 0.4762$).

3.3. Effect of fixative on the staining intensity of PAS in the hippocampus

As seen in Fig. 3A, photomicrographs show better quality of PAS staining with hippocampal tissues fixed in AF compared with NBF and PFA. Image analysis results show that there was a significant difference in the mean gray value of PAS across the groups ($p = 0.0298$). As seen in Fig. 3B, Students' *t*-test showed that there was a significant difference in the mean gray value of PAS stained with NBF (164 ± 0.62) and AF (149 ± 6.2) ($p = 0.0236$). This indicates a higher staining intensity with AF. Also, a significant difference ($p = 0.0073$) was observed between NBF and PFA (145 ± 6.4) showing a higher staining intensity with PFA. However, there was no significant difference between the mean gray value between AF and PFA ($p = 0.6458$).

3.4. Effect of temperature on the staining intensity of PAS in the hippocampus

Fig. 4A shows a superior staining intensity with PAS for tissues fixed with NBF, AF and PFA at 4 °C. This was followed by better staining quality with PAS at 40 °C for tissues fixed with NBF and AF when compared with fixation at 37 °C while staining intensity with tissues fixed with PFA was better at 37 °C when compared with 40 °C. As seen in Fig. 4B, there was a significant difference in the staining intensity between for NBF ($p < 0.05$), AF ($p < 0.001$) and PFA ($p < 0.05$) at 37 °C and 4 °C. However, there was no significant difference in the staining intensity of PAS for NBF ($p > 0.05$), AF ($p > 0.05$) and PFA ($p > 0.01$) at 37 °C and 40 °C.

4. Discussion & conclusion

The natural structural integrity of a tissue is preserved by fixation, which employs the use of chemicals that maintain the cell structure from degradation. The chemical composition of the tissue is retained during this phase in which hardening of the tissues for sectioning and prevention of degradation also occur [11]. Appropriate fixation is paramount for the demonstration of morphological features and localization of cellular components. Asides the mechanism of action of different fixatives, temperature is also an important factor in achieving good results. Hence, the histological preparation of sections for the demonstration of glycogen depends on the quality of staining taking into account the fixative and the temperature at which fixation is carried out.

Although formaldehydes are the most commonly used fixatives for histochemical studies, alcohol based fixatives have been found to produce excellent results and also devoid of attendant hazards associated with the former [12]. From this study, we found out that AF stained glucose better in the liver and brain compared to NBF in the liver and NBF and PFA in the brain. Mostly, NBF is used almost universally for fixation because of its ability to preserve consistently the morphological details of cells and tissues [13]. The better staining results with AF could be as a result of the two-phased fixation it employs viz. an initial alcohol fixation phase which precedes a cross-linking phase mediated by formalin. Formaldehyde-based fixatives penetrate the tissue well, but are slow. The use of alcohol to dilute the concentration of formaldehyde increases the speed of tissue penetration. This could further help prevent the loss of glycogen from the tissue, leading to a better demonstration. Result from our study is in agreement with that of Holda et al. [14] who studied the penetration depth of different fixatives and found out that AF solution was the fastest fixative among the studied ones. Alcoholic formalin can be considered as a good fixative,

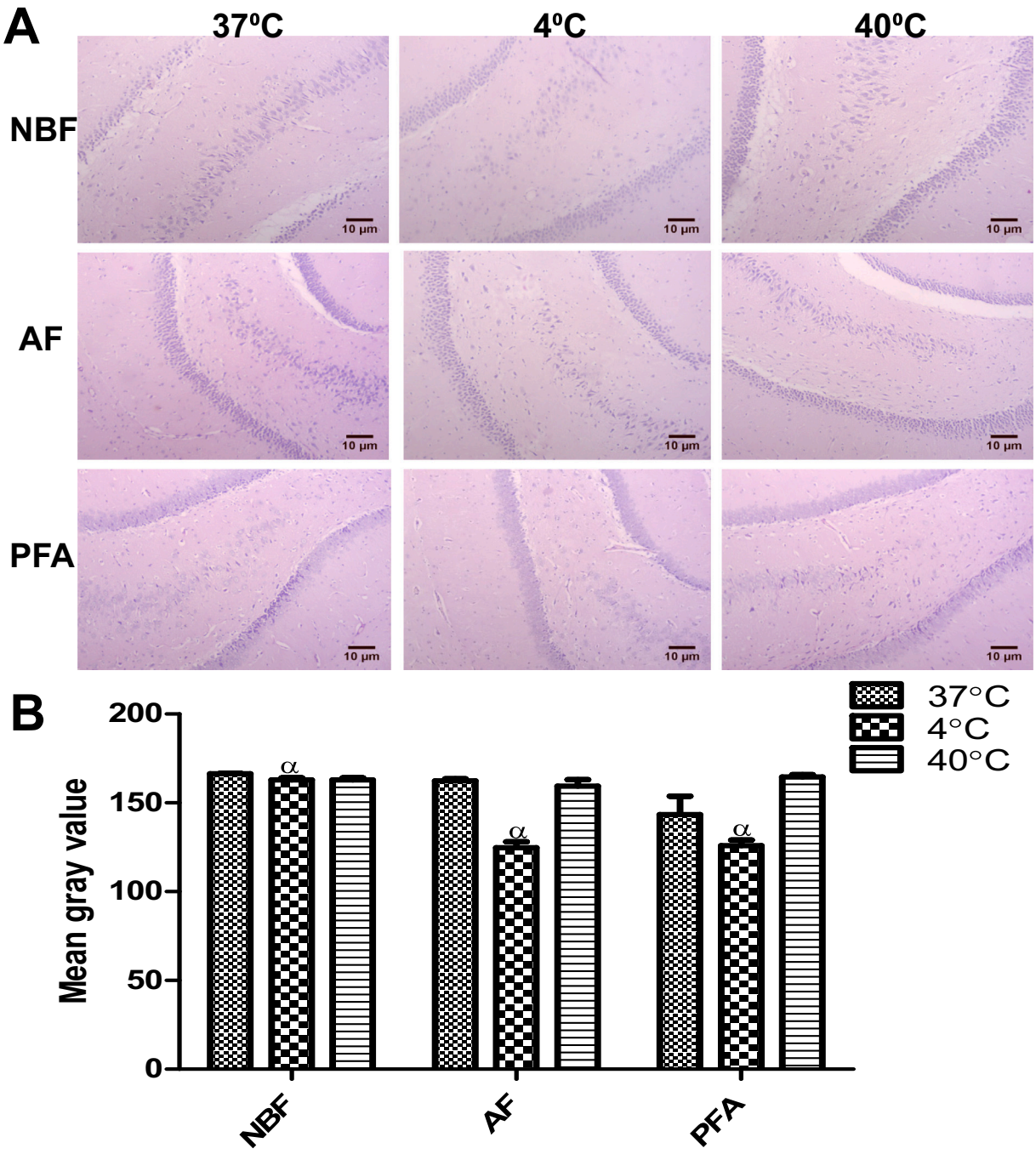

Fig. 4. A: Photomicrographs of PAS stained hippocampal tissues fixed in NBF, AF and PFA at different temperatures. B: Image J count of PAS staining intensity of the hippocampus of rats fixed with NBF, AF and PFA at 37 °C, 4 °C and 40 °C. $p < 0.05$, α — significant difference compared to 37 °C.

because in addition to fixing the tissue, the process of dehydration already began. One of the suggested methods of fixing glycogen which is through dehydration seems tenable, because it results in decreased solubility. The process of dehydration of glycogen initiated by the alcohol portion of AF probably rendered the glycogen denatured resulting in a better staining quality. A contrary result was gotten by [15] who found out that AF was not the best fixative for preserving glycogen because it left the tissues brittle upon sectioning and showed cell lysis at the tissue periphery. Although the authors acknowledged that AF was better than other fixatives been compared together because little glycogen was lost, but it left the tissue in a brittle state for further processing.

Paraformaldehyde acts as a cross-linking agent that reacts with proteins and nucleic acids in the cell. It has a rapid rate of penetration and prevents extraction of glycogen provided fixation time is not prolonged [16]. In this study, PFA was able to retain tissue glycogen better than NBF, probably because formaldehyde generated from PFA is the most effective monoaldehyde. It is preferred over formalin because the later contains undetermined amounts of impurities such as methanol and formic acid in other to prevent formaldehyde polymerization over time [16]. These findings are in line with the work of Guth and Watson [17] whose quantitative studies showed that during initial ischemia, glycogen decreased to approximately 60%, but after the introduction of paraformaldehyde, glycogen levels remained.

Temperature is an important factor for fixation. The temperature of fixatives during tissue processing may affect the architecture. An

increase in the temperature of fixation will result in an increase in the rate of diffusion of the fixative into the tissue. This results in speeding up the rate of chemical reaction between the tissue and fixative. The rate of fixation is increased with increase in temperature, but increased temperature also increases autolysis rate for unfixed parts of the tissue. If the temperature of the fixative is low or decreased, the diffusion rate also decreases, which results in extended penetration time. The results obtained from this study show that NBF fixative gave the best staining quality in the liver at 4 °C when compared to 37 °C and 40 °C. It has been reported that chilling the fixative and tissue has been shown to prevent enzymatic loss of glycogen, which could be the reason why NBF at 4 °C gave a better staining intensity [18]. In our study, there was no difference in the staining intensity of glycogen in the liver at 4 °C, 37 °C and 40 °C which could be indicative of the fact that AF fixes well under both high and low temperatures.

Similarly, all 3 fixatives (NBF, AF and PFA) showed a better staining intensity at 4 °C in the brain. This was closely followed by 40 °C for NBF and AF. Also, PFA stained best at 4 °C followed by 37 °C. Results from our study are in agreement with that of Zakout et al. [19] who demonstrated an increase in the staining intensity of glycogen in the liver at 4 °C. They found that there was polarization of glycogen granules in hepatic cells at 37 °C. This is suggestive of the fact that the reduced rate of penetration of the tissue which occurs at a low temperature reduces glycogen dilution which enhances the containment of glycogen within the cells.

In conclusion, alcoholic formalin at low temperature gave the best staining results in our study. Hence, the choice of fixative and appropriate temperature are essential determinants for the proper localization of glycogen in paraffin sections.

Declaration of competing interest

The authors stated that there are no conflicts of interest that can affect the decision to publish this manuscript.

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References

[1] Brown AM, Ransom BR. Astrocyte glycogen and brain energy metabolism. Glia 2007;55(12):1263–71.
[2] Shen GM, Zhang FL, Liu XL, Zhang JW. Hypoxia-inducible factor 1-mediated regulation of PPP1R3C promotes glycogen accumulation in human MCF-7 cells under hypoxia. FEBS Lett 584 (20): 4366–4372.
[3] Thwe PM, Pelgrom LR, Cooper R, Beauchamp S, Reisz JA, D'Alessandro A, et al. Cell-intrinsic glycogen metabolism supports early glycolytic reprogramming required for dendritic cell immune responses. Cell Metab 2017;26:558–67.
[4] Dauer P, Lengyel E. New roles for glycogen in tumour progression. Trends Cancer 2019;5(7):396–9.
[5] Gates LL, Adler RR, Elangbam CS. Osmium tetroxide post-fixation and periodic acid-Schiff dual-staining technique to demonstrate intracellular lipid and glycogen in the mouse liver section – a novel method for co-visualization of intracellular contents in paraffin-embedded tissue. J Histotech 2016;39(1):2–7. https://doi.org/10.1080/01478885.2015.1106072.
[6] Wu L, Wong CP, Swanson RA. Methodological considerations for studies of brain glycogen. Neuro Res 2019;97:914–22.
[7] Bancroft JD, Gamble M. Theory and practice of histological techniques. 5th ed. Philadelphia, PA: Churchill Livingstone Elsevier; 2002. p. 63–108.
[8] Singh H, Bishen KA, Garg D, Sukhija H, Sharma D, Tomar U. Fixation and fixatives: roles and functions — a short review. Dent J Adv Stud 2019;7(2):51–5.
[9] Bancroft JD, Gamble M. Theory and practice of histological techniques. 5th ed. Philadelphia, PA: Churchill Livingstone Elsevier; 2001. p. 63–240.
[10] Bancroft JD, Stevens A. Theory and practice of hitological techniques. New York: Churchill Livingstone; 1996. p. 4.
[11] Tatford M. Progress in the development of Microscopical techniques for diagnostic pathology. J Histol 2009;32(1):9–19.
[12] van Essen HF, Verdaasdonk MA, Elshof SM, de Weger RA, van Diest PJ. Alcohol based tissue fixation as an alternative for formaldehyde: influence on immunohistochemistry. J Clin Pathol 2010;63(12):1090–4. https://doi.org/10.1136/jcp.2010.079905.
[13] Grizzle WE. Special symposium: fixation and tissue processing models. Biotechnic Histochem 2009;84(5):185–93.
[14] Holda MK, Klimek-Piotrowska W, Koziej M, Tyrak K, Holda J. Penetration of formaldehyde based fixatives into heart musculature. Folia Med Cracov 2017;57(4):63–70.
[15] Kinsley D, Everds N, Arp L, Becker J, Geraci M. Optimization of techniques for the preservation of glycogen in paraffin embedded mouse liver. J Histotech 2000;23(1):51–5.
[16] Hayat MA, editor. Fixation for electron microscopy. Philadelphia, PA: Churchill Livingstone Elsevier; 2012.
[17] Guth L, Watson PK. A correlated histochemical and quantitative study on cerebral glycogen after brain injury in the rat. Exp Neurol 1968;22(4):590–602.
[18] Presnell JK, Schreibman MP, Humanson GL. Humason's animal tissue technique. Baltimore and London: The John Hopkins Univesity Press; 1997.
[19] Zakout YM, Salih MM, Ahmed HG. The effect of fixatives and temperature on the quality of glycogen demonstration. Biotecnic Histochem 2010;85(2):93–8. https://doi.org/10.3109/10520290903126883.