Limitation of cane sugar solution as a tissue fixative for glycogen preservation in mouse liver

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ABSTRACT

Introduction: Sugar-based natural fixatives have been proposed as substitutes for formalin in histopathological practice because of their promising results in preserving tissue structures. However, they have not yet been applied. This study aimed to determine the optimal concentration of cane sugar solution (CSS) for tissue fixation, histochemical staining, and glycogen storage in mouse liver tissues.

Materials and Methods: A total of 24 mouse liver tissues from six mice were divided into four groups and fixed with 10% neutral buffered formalin (NBF), 30%, 50% and 70% CSS, respectively, for 24 h at room temperature. After tissue processing and sectioning, the samples were stained with hematoxylin and eosin (H&E), methyl green pyronin Y (MGPY) and peroxidase acid-Schiff (PAS). The tissue sections were evaluated under a light microscope by two blinded pathologists. Data were analyzed using one-way analysis of variance (ANOVA) and a post-hoc test to assess the differences among the experimental groups.

Results: The gross morphology of all the samples showed minimal shrinkage without color changes. Microscopic examination revealed that the 50% and 70% CSS groups showed comparable efficacy in cytoplasmic and nuclear staining, H&E staining intensity, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) staining, and collagen staining with the 10% NBF group. The 30 percent CSS group showed significantly poorer nuclear and DNA staining than the 10% NBF group. All CSS groups failed to preserve glycogen in mouse liver tissues ($p < 0.001$) compared with the 10% NBF.

Conclusions: Our study concluded that 50% and 70% CSS are potentially replaceable, eco-friendly fixatives for 10% NBF in small tissue samples for tissue fixation and histochemical staining. However, 10% NBF was still superior to CSS for glycogen preservation in mouse liver tissue.

KEYWORDS:
Eco-friendly fixatives; Glycogen preservation; Cane Sugar Solution; Histochemical staining

INTRODUCTION

Microscopic evaluation of tissue biopsies has been considered the gold standard for diagnosis for years.[1] Before the tissues can be viewed on a slide, they undergo several processes, of which fixation is the first and arguably the most important one, as it sets the foundation for the subsequent steps. Fixation is a process in which a fixative penetrates the respective tissue, causing physiochemical reactions leading to three main outcomes: prevention of enzyme autolysis, inhibition of bacterial putrefaction, and preservation of cellular components as close to their living state as possible. Since its discovery, formalin has become the most established fixative in the field owing to its several advantages, such as being easily accessible, inexpensive, and having a high degree of accuracy.[2] The most commonly available fixative is 10% neutral buffered formalin (NBF), which crosslinks amino acids and forms methylene bridges.[3,4] Despite its benefits, the major drawback of formalin is its toxicity to human health. Upon contact, it can trigger allergic dermatitis in the skin or irritate the mucous membrane, leading to conjunctivitis.5 In addition, it has been classified as a group 1 carcinogen due to its ability to cause sinonasal and nasopharyngeal cancers.[6]

Thus, attention needs to be shifted towards effective alternative fixatives that do not threaten human health. Recent studies have shown that natural fixatives such as honey, jaggery, cane, and sugar are comparable to formalin in preserving the tissue and allowing for satisfactory staining and visualization of cellular outline and nuclear morphology.[7–10] These fixatives are non-toxic, eco-friendly, and economical, and do not require special equipment.

Sugarcane is a well-known source of sugar that is rich in sucrose. Various sugarcane products contain 13%–15% sucrose. According to the theory based on the study by Patil et al., sucrose breaking down at low pH produces aldehydes and crosslinks with tissue amino acids, which is similar to the action of formaldehyde in tissue fixation.[8] Moreover, sugarcane has antiseptic/antibacterial properties and cytoprotective activities.[11,12]

In recent years, honey- or sugar-based natural fixatives have become promising alternatives to formalin for tissue fixation because of their sucrose content. Recent studies have shown

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that natural fixatives can fix small tissues without interfering with diagnosis.[13,14] Chittemsetti et al. showed that sugar-based fixatives reveal better nuclear and cytoplasmic staining than formalin.[7] Other studies revealed that sugar-based fixatives were comparable with formalin for special collagen stains, such as periodic acid-Schiff (PAS) and Masson's trichrome, etc..[15] Moreover, sugar-based fixatives revealed promising results in immunohistochemical staining.[16,17] However, formaldehyde is still used in daily clinical practice due to conflicting results among the studies. In addition, no study has been conducted on the detection of nucleic acids using histochemical staining and intracellular glycogen storage.

Therefore, this study aimed to focus on the fixative efficacy of cane sugar solution (CSS) in tissue fixation, morphological preservation, and histochemical staining. Mouse liver tissue morphology was determined using hematoxylin and eosin (H&E), nucleic acid methyl green-pyonin Y (MPGY), collagen staining, and glycogen preservation by peroxidase acid-Schiff (PAS) staining.

MATERIALS AND METHODS

Preparation of Cane Sugar Fixatives

Commercially available unrefined raw cane sugar (Country Farms Sdn Bhd, Malaysia) was dissolved in distilled water to prepare 30, 50, and 70% CSS. Commercially available 10% NBF (Thermo Scientific, USA) was used as the control.

Sample Collection and Fixation

A total of 24 fresh liver tissues from six mouse carcasses were obtained from the IMU University (IMU) research laboratory with the approval of the International Medical University-Joint Committee on Research & Ethics (IMU-JC).[18,19] Fresh liver tissues from each carcass were dissected into 1-cm-thick pieces[20] and immersed into four different fixatives: 30%, 50%, and 70% CSS and 10% NBF with a minimum of 1:10 tissue to fixative volume. The samples were then fixed for 24 hours at room temperature on a shaker. Tissue samples were processed using an automated Tissue-Tek processor with 90-minute per station protocol. Tissues were dehydrated in an ethanol series, cleared with xylene, and infiltrated with molten paraffin. Paraffin-embedded tissues were then sectioned for microscopic examination.[21]

Histological and Histochemical Staining Evaluation

Commercially available H&E and PAS were purchased from Sigma-Aldrich (M) Sdn. Bh. For the MGPY solution, 0.5% methyl green solution was prepared by adding 0.025 g of methyl green to 5 ml of 0.2 M acetate buffer at pH 4.2, followed by the preparation of an MGPY solution through the addition of 0.0025 g of pyronin Y to 5 ml of the previously prepared 0.5% methyl green solution.[22] Five-µm-thick mouse liver tissues were stained with H&E, MGPY, and PAS according to the manufacturer's protocol. The slides were examined under a light microscope by two pathologists who were blinded to avoid subjective bias. With H&E staining, the tissue sections were assessed for cellular outline, cytoplasmic and nuclear staining, and staining intensity. DNA and RNA staining were assessed using the MGPY stain, whereas glycogen preservation, collagen fiber staining, and nuclear staining were analyzed using PAS staining. Each criterion was rated on a scale of 1–4 (1 - poor, 2 - satisfactory, 3 - good and 4 - excellent). At least five different fields of view were observed at x400 magnification. Photomicrographs were taken using a Nikon Eclipse 80i light microscope (Nikon Elements version 4.13 software).

Statistical Analysis

All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS, version 29; Chicago, IL, USA). One-way analysis of variance (ANOVA) and post-hoc tests were used to assess the differences between the experimental groups. Statistical significance was set at $P < 0.05$.

RESULTS

Gross Morphology Findings

After 24-hour fixation, all specimens showed minimal shrinkage with no obvious color changes (Figure 1). However, the 30% CSS-fixed tissues produced gas and a fermentation smell after 24 hours of fixation, suggesting sugar fermentation by bacteria.

Evaluation of Microscopic Morphology and Preservation of RNA and DNA in CSS-Fixed Mouse Liver Tissues

Under microscopic examination, mouse liver tissues fixed in 10% NBF (control) revealed well-preserved nuclei and cytoplasm with distinct cellular outlines (Figure 2a). Thirty percent CSS group showed identifiable cellular and nuclear morphology, with areas of cellular degeneration, cytoplasmic vacuolation, and cellular hyalinization (Figure 2b). Moreover, the 50% and 70% CSS groups showed well-preserved nuclear and cytoplasmic morphology, with a few areas of tissue degeneration. In addition, 70% CSS-fixed tissues showed darkened nuclei, shrunken cytoplasm, and widened sinusoids, suggesting tissue shrinkage compared with the control group (Figure 2c and d).

MGPY was used to evaluate DNA and RNA preservation in mouse liver tissues. It is a cationic dye that stains highly polymerized nucleic acid (DNA) with methyl green and slightly polymerized nucleic acids (RNA) with pyronin Y.23 The representative picture of the 30% and 50% CSS groups showed low staining intensity and uneven staining patterns compared to the control. The representative image of the 70% CSS group showed similar staining density to that of the control group (Figure 3e–h).

Evaluation of Glycogen Preservation in CSS-Fixed Mouse Liver Tissues

Next, the preservation of glycogen and collagen was evaluated using PAS staining in the control and CSS groups. None of the CSS concentrations tested was able to stain glycogen, suggesting that CSS failed to preserve glycogen integrity in mouse liver tissues. However, the control and all concentrations of CSS groups exhibited compatible collagen staining. (Figure 3i–l).

Table I: Analysis of cellular morphology, DNA and RNA staining, collagen staining and glycogen preservation in the control and CSS groups

Staining methods	Parameters	Control (10% NBF)	30% CSS	50% CSS	70% CSS	p-value	
						One-way ANOVA	Post Hoc Test
H&E	Cellular outline	2.58	1.67	1.83	2.5	0.043*	(Control,70%) vs
	Mean ± SD	(±0.86)	(± 0.26)	(±0.61)	(±0.63)		(30%,50%) p<0.05
	Cytoplasmic staining	2.67	2.00	2.16	2.5	0.396	
	Mean ± SD	(± 0.88)	(± 0.45)	(± 0.98)	(± 0.18)		
	Nuclear staining	3.33	2.00	2.33	2.75	0.019*	Control vs 30% CSS
	Mean ± SD	(± 0.52)	(± 0.63)	(± 0.98)	(± 0.52)		p<0.05
	Staining intensity	3.08	2.50	2.33	2.75	0.306	
	Mean ± SD	(± 0.74)	(± 0.45)	(± 1.08)	(± 0.27)		
MGPY	DNA staining	2.67	1.33	1.58	2.42	0.017*	Control vs 30% CSS
	Mean ± SD	(± 0.88)	(± 0.26)	(± 0.58)	(± 1.07)		p<0.05
	RNA staining	3.08	2.00	2.17	2.83	0.71	
	Mean ± SD	(± 0.92)	(± 0.45)	(± 0.68)	(± 0.93)		
PAS	Collagen staining	3.25	2.42	2.75	2.67	0.206	
	Mean ± SD	(± 0.76)	(± 0.80)	(± 0.69)	(± 0.26)		
	Glycogen preservation	3.25	0.25	0.25	0.17	<0.001*	Control vs (30% CSS,
	Mean ± SD	(± 1.36)	(± 0.61)	(± 0.42)	(± 0.41)		50%CSS, 70%CSS) p<0.05

Footnote: NBF = Neutral Buffer Formalin, CSS = Cane Sugar Solution, ANOVA = Analysis of variance, H&E = Haematoxylin and Eosin, MGPY = Methyl Green Pyronin Y, PAS = Periodic Acid-Schiff, *p < 0.05

Analysis of Microscopic Morphology, Preservation of DNA, RNA, Collagen and Glycogen in CSS-Fixed Mouse Liver Tissues

The slides underwent a blindfolded assessment independently by two pathologists. The morphological scoring of H&E-stained CSS-fixed tissues was investigated for cellular architecture preservation, and 10% NBF-fixed mouse liver tissues were used as controls. The control group exhibited a mean value of 2.58 for the cellular outline with a standard deviation of 0.86. This value significantly decreased to 1.66 (±0.26) at 30% CSS, slightly increased to 1.83 (±0.61) at 50% CSS and approached the control value again at 2.5 (±0.63) with 70% CSS. Cytoplasmic staining revealed a similar trend. The control group had a mean of 2.67 (±0.88), which dropped to 2.0 (±0.45) at 30% CSS, slightly increased to 2.17 (±0.98) at 50% CSS and was close to the control at 2.5 (±0.45) at 70% CSS. The nuclear staining notably reduced from 3.33 (±0.52) in the control group to 2.0 (±0.63) at 30% CSS. This value modestly increased to 2.33 (±0.98) at 50% CSS and 2.75 (±0.52) at 70% CSS, even though it did not return to control levels. For staining intensity, the control mean was 3.08 (±0.74), which decreased to 2.5 (±0.45) at 30% CSS, further dropped to 2.33 (±1.08) at 50% CSS and then slightly increased to 2.75 (±0.27) at 70% CSS (Table I).

Analysis of the DNA content by MGPY staining revealed that the control group had a mean of 2.67 (±0.88). This significantly decreased to 1.33 (±0.26) at 30% CSS, then slightly increased to 1.58 (±0.58) at 50% CSS and further approached the control value at 2.42 (±1.07) at 70% CSS. The RNA content in the control group had a mean of 3.08 (±0.92). This decreased to 2.0 (±0.45) at 30% CSS, slightly increased to 2.17 (±0.68) at 50% CSS, and further increased to 2.83 (±0.93) at 70% CSS, even though it remained lower than the control (Table I).

The glycogen content stained by PAS showed a dramatic reduction from the control mean of 3.25 (± 1.36) to 0.25 (± 0.61) at 30% CSS. This low level was maintained at 50% CSS with a mean of 0.25 (±0.42) and decreased further to 0.17 (± 0.41) at 70% CSS. The mean collagen level in the control group had a mean of 3.25 (±0.76). This decreased to 2.41 (±0.80) at 30% CSS, then slightly increased to 2.75 (±0.69) at 50% CSS and was 2.67 (±0.26) at 70% CSS, which was still lower than the control value (Table I).

Furthermore, we analyzed the preservation efficacy of CSS using a one-way ANOVA. In H&E staining, the mean cytoplasmic staining and staining intensity of the 30% CSS and 50% CSS groups were lower than those of the control group; however, there was no significant difference among the groups. The cellular outline and staining intensity were significantly different between the groups. Further analysis with the post hoc test revealed that the mean cellular outline was higher in the control and 70% CSS groups than in the 30% and 50% CSS groups. In addition, the 30% CSS group showed significantly poorer nuclear staining than the control group (Table I). Similarly, DNA staining with MGPY in the 30% CSS group showed a significant difference. RNA staining by MGPY and collagen staining by PAS showed no significant differences between the groups. However, glycogen preservation at all concentrations of CSS groups was significantly different from that of the control group (Table I).

DISCUSSION

In this study, we investigated the fixative properties of cane sugar solutions in mouse liver tissues using histochemical staining, H&E, MGPY, and PAS staining, and determined the optimal concentration of CSS. Among the three different concentrations (30%, 50%, and 70% CSS), 50% and 70% CSS showed promising fixative efficiency, which can be compared

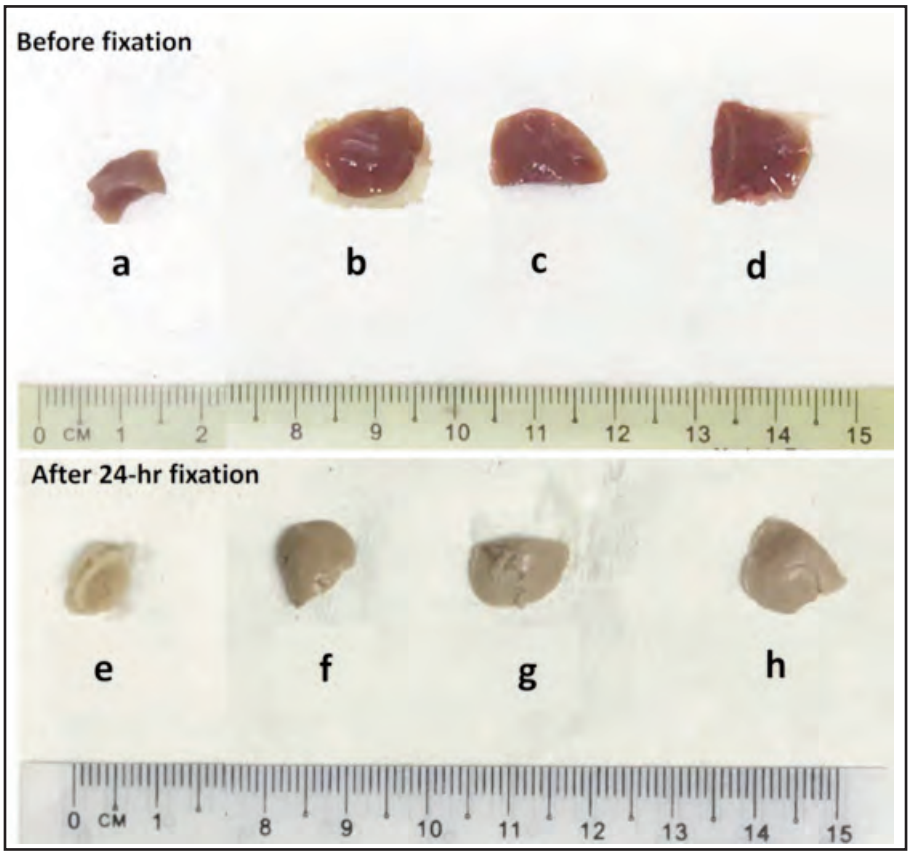

Fig. 1: Gross morphology of mouse liver tissues before fixation (a, b, c, d) and after fixation with 10% NBF (e), 30% CSS (f), 50% CSS (g) and 70% CSS (h)

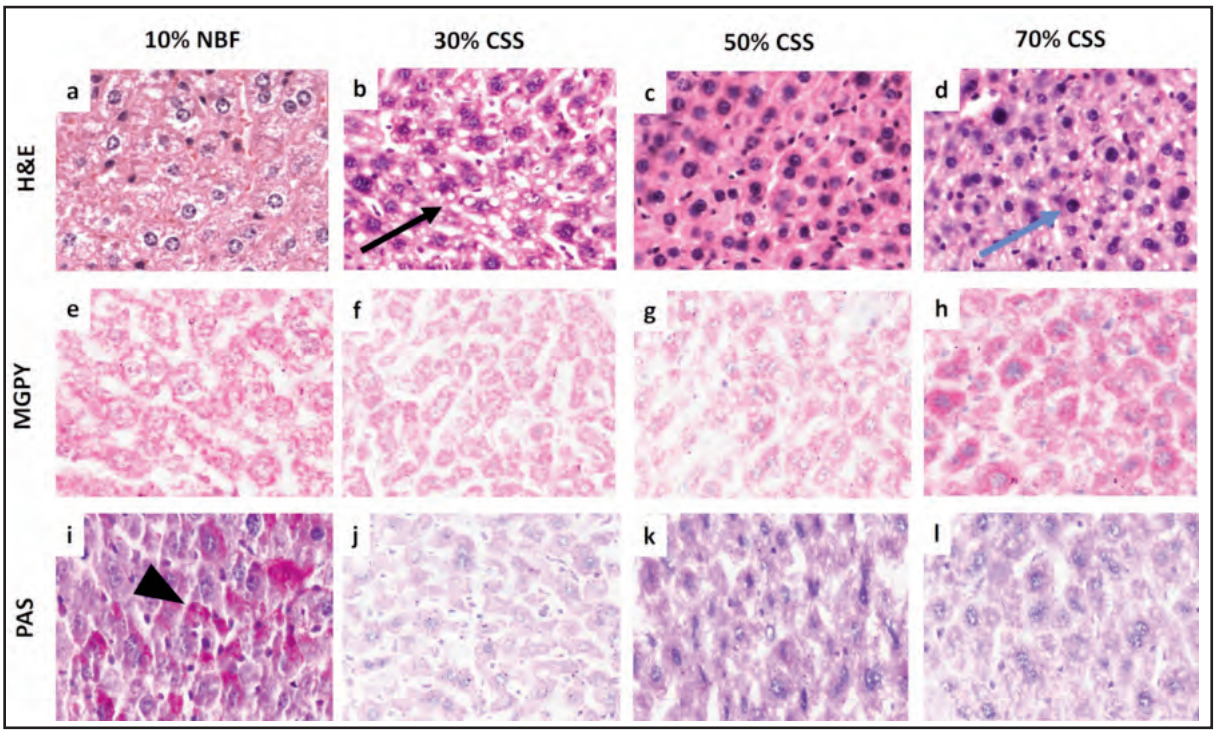

Fig. 2: Mouse liver tissues were fixed with 10% NBF (a, e, i), 30% CSS (b, f, j), 50% CSS (c, g, k) and 70% CSS (d, h, l). H&E stain (a-d): cytoplasmic vacuolation was seen in the 30% CSS group (b, black arrow), 50% and 70% CSS groups (c, d) showed well-preserved cellular morphology, and 70% CSS group (d) showed darkened nuclei (blue arrow) compared to 10% NBF group (a). MGPY stain (e-h): 30% and 50% CSS groups (f, g) showed low staining- intensity in DNA and RNA staining. PAS stain (i-l): 10% NFB group showed well-preserved glycogen in the cytoplasm of mouse liver (i, black arrowhead), while 30%, 50%, and 70% CSS groups failed to preserve glycogen (j-l)

with formalin in terms of cellular morphology and DNA, RNA, and collagen preservation. However, all CSS concentrations failed to preserve glycogen in the mouse liver tissues.

Despite being the most widely used ideal fixative for more than a century, 10% NBF has been associated with several significant drawbacks: hardening and discoloration of tissues, unpleasant and irritating odor, and formation of formic acid, which is corrosive and poses serious health risks to laboratory personnel upon exposure.[24,25] Moreover, 10% NBF remains popular in routine laboratory practice due to its low cost, ability to quickly fix large amounts of tissue, and germicidal properties that prevent autolysis and putrefaction.[24] However, health and environmental concerns have driven the search for safer alternatives.

Natural products such as sugar and honey have been used in the food industry as natural preservatives to extend the shelf life of various foods owing to their properties, such as inhibition of microbial growth, reduction of water activity, and preservation of color, texture, and flavor. Higher fructose content in sugar, jaggery, and honey suggests a potential fixation mechanism by converting fructose to aldehyde and creating a cross-link with tissue amino acids.[8]

Our study revealed that 50% and 70% CSS had potential fixative effects on mouse liver tissues and their histochemical staining, whereas 30% CSS failed to preserve these tissues. Our findings are consistent with those of Udonkang et al. (2018), who observed that tissue fixed in a higher concentration of a honey solution (70% and above) showed similar nuclear and cytoplasmic staining qualities to formalin-fixed tissues in 48-h to 6-month of fixation.16 Jaggery, a sugarcane derivative, is compromised with a high content of fructose, glucose, and 20% water. The high osmolarity of sucrose solution causes water to be drawn out of the tissue, leading to dehydration. This dehydration helps to fix and preserve the tissue morphology by preventing swelling and distortion.[26] Aligned with these findings, we observed that CSS-fixed tissues were more dehydrated than 10% NBF-fixed tissues, which led to the challenge in microtome sectioning. However, it did not affect the morphology of mouse liver tissues or the further staining process. The concentration of CSS differs from previous studies in which lower concentrations (20–30%) showed a promising fixative effect on tissue preservation.[7,8,10,26,27] Previous studies on the fixative activity of sugar indicated that a concentration of approximately 195 g of sugar per 100 g of water is needed to achieve a water activity level that is sufficient to inhibit the growth of bacteria.[28,29] Although fixation duration was 24 h in all studies, sucrose and glucose composition and additional components might be different among these studies and might affect fixative efficiency.

Majumdar et al. (2016) reported that the reaction of reducing sugars and amino acids in an acid medium or under thermal stress led to a Maillard reaction, which resulted in an intermediate product, a furanic compound. The furanic compound was further accelerated into a hydroxymethylfurfural (HMF) compound with an aldehyde group. The fixation process involves the cross-linking of the proteins present in the tissues by HMF through a di-Schiff base reaction. Nucleic acid fixation may also occur by the reaction of MHF with the free amino groups of nucleotides.[30] MGPY is a classic histological stain that uses basic dyes to differentiate between DNA and RNA. Methyl green binds to phosphate radical in the DNA double helix to stain it green-blue, whereas pyronin Y binds to RNA to stain it red.[31] As first evidence, we showed that 30% CSS failed to preserve, but 50% and 70% CSS preserved the nucleic acid, similar to formalin by MGPY staining. High sugar concentrations in CSS create a hypertonic environment that reduces water activity and inhibits the activity of nucleases that can degrade nucleic acids.[8,32]

Interestingly, our findings revealed that all concentrations of CSS failed to preserve glycogen in the mouse liver tissues. It has been reported that glycogen is not directly fixed, but is trapped with fixed proteins.[33,34] The penetration rate of formaldehyde in 10% NBF is approximately 1 mm per hour for the first hour, followed by a slower rate of about 1 mm every 3 hours. While specific quantitative data on the penetration rate of CSS fixatives are less documented, it is possible that CSS may have a slower penetration rate into the tissue, which leads to protein digestion and results in an inability to preserve glycogen.

This study demonstrated that CSS at concentrations of 50% and 70% offers comparable efficacy to 10% NBF in preserving tissue morphology, nucleic acids, and collagen in small tissue samples. These findings suggest that CSS is a viable and safe alternative to formalin for short-term fixation, particularly for small tissue specimens. However, it is important to acknowledge the limitation that CSS is ineffective in preserving glycogen. Further research is needed to determine whether this limitation can be addressed through modified fixation protocols or if alternative sugar solutions might offer better glycogen preservation. In addition, the long-term stability of tissues fixed with CSS compared to those fixed with 10% NBF remains unclear. Moreover, it is important to address the technical challenges encountered in CSS-fixed tissue. Common artifacts such as scratch lines appearing in sessions, opaque sections, and tissue-block separation during microtomy are frequently observed. The dehydrating properties of CSS render the tissue brittle, likely causing over-dehydration and resulting in artifacts during sectioning.[35]

Overall, CSS holds promise as a safe and effective fixative for specific applications, particularly for short-term fixation of small tissue samples where glycogen preservation is not a critical factor. However, further studies are required to fully define its suitability for broader use in tissue pathology.

While providing insights into the use of CSS as a tissue fixative, this study had several limitations. This study focused solely on mouse liver tissue, and the results may not be applicable to other tissue types of species. A recent study revealed that tissue fixation at 4°C with 80% alcohol showed better glycogen preservation compared to fixation at RT.36 The fixation time was fixed at 24 h at RT, and exploring different fixation durations and temperatures could reveal optimal conditions for tissue fixation and glycogen preservation. This study did not directly address the clinical

relevance of CSS as a fixative. Further research is required to determine whether CSS can be effectively used for diagnostic pathology.

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CONFLICT OF INTEREST

There is no actual or potential conflict of interest in relation to this article.

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