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Original Article

Comparative Evaluation of Common Fixatives on Histomorphological Preservation in Mouse Tissues Using Hematoxylin and Eosin Staining

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Abstract

Background: This study aimed to evaluate and compare the histological effects of three commonly used fixatives (10% neutral buffered formalin, Bouin's solution, and Carnoy's solution) on mouse liver, heart, and testis tissues. **Methods**: A total of twelve healthy adult male mice were used, with four mice allocated to each fixative group. From each animal, the liver, heart, and testis were surgically excised and fixed in the designated solutions for standard durations. Tissues were processed using an automated tissue processor, embedded in paraffin, sectioned at 3 µm thickness, and stained with hematoxylin and eosin (H&E). Histological sections were then evaluated microscopically for nuclear clarity, cytoplasmic preservation, staining quality, and structural integrity. **Results**: The results demonstrated notable differences among the fixatives. Bouin's solution provided superior nuclear detail in testicular tissue but was associated with mild tissue hardening. Formalin exhibited optimal preservation of overall morphology, particularly in liver and heart sections, with minimal artifacts. Carnoy's fixative offered sharp nuclear contrast but was associated with noticeable tissue shrinkage and distortion. **Conclusion**: These findings highlight that the choice of fixative significantly affects histological outcomes, and selection should be guided by tissue type and diagnostic objectives. The study underscores the importance of appropriate fixation in enhancing tissue preservation and microscopic interpretation.

Keywords: Histological preservation; Formalin fixation; Bouin's fixation; Carnoy's fixation; H&E staining.

Introduction

Histology and cytology are fundamental disciplines in diagnostic pathology, each providing complementary insights into tissue and cellular structure. Histology focuses on the preservation and examination of tissue architecture, where fixation represents a critical initial step in tissue processing. Proper fixation prevents autolysis and microbial degradation [1] while maintaining structural integrity through chemical stabilization of cellular components [2]. The quality of subsequent procedures including embedding, sectioning, staining, and immunohistochemical analysis largely depends on the adequacy of fixation , as inadequate fixation may lead to artifacts and compromised diagnostic accuracy [3].

In contrast, cytology involves the microscopic evaluation of isolated cells and is widely used as a minimally invasive, rapid, and cost effective diagnostic approach [4]. Techniques such as the Papanicolaou (Pap) test and fine needle aspiration (FNA) have significantly improved early disease detection, particularly in cancer screening [5]. Advances in immunocytochemistry and molecular diagnostics have further expanded the role of cytology in modern precision medicine [6].

Fixation remains a cornerstone in histopathology, directly influencing tissue morphology and staining characteristics [7]. Fixatives are broadly classified into cross linking and coagulating agents, each interacting differently with tissue components [8]. Factors such as fixation time, tissue type, and fixative composition play a crucial role in determining the quality of histological outcomes . Inadequate or delayed fixation can result in structural distortion and suboptimal staining [9].

Among the most commonly used fixatives in histopathology are 10% neutral buffered formalin [10], Bouin's solution [11], and Carnoy's solution [16]. Formalin is widely regarded as the gold standard due to its ability to preserve overall tissue morphology and allow long term storage [10]. Bouin's solution is known for its superior nuclear detail, particularly in delicate tissues [11], while Carnoy's solution is valued for its rapid fixation and

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excellent preservation of nucleic acids [16]. However, each fixative has inherent limitations, and their effects may vary depending on the type of tissue being examined. The liver, heart, and testis are commonly studied tissues in histological research due to their distinct structural and functional characteristics. The selection of an appropriate fixative for each tissue type is essential to ensure optimal preservation of cellular and structural details, which directly impacts the accuracy of microscopic evaluation. Therefore, this study aims to evaluate and compare the histological effects of three commonly used fixatives 10% neutral buffered formalin, Bouin's solution, and Carnoy's solution on mouse liver, heart, and testis tissues using hematoxylin and eosin (H&E) staining.

Materials and Methods

Animal Selection and Housing Conditions

Twelve healthy adult male mice (Mus musculus), aged 2–3 months and weighing between 25–30 g, were used in this study. The animals were housed in standard laboratory cages under ambient room temperature with a natural light–dark cycle. Bedding was composed of albino type wood shavings. The mice were obtained from the Animal House Facility, University of Benghazi.

Preparation of Solutions

Preparation of 10% Formalin

In a clean measuring container, 900 mL of distilled water was added first. Then, 100 mL of 40% formaldehyde solution was gradually added to the water while stirring gently. The mixture was thoroughly mixed to ensure complete and uniform dilution. The solution was then transferred into a sealed and clearly labeled container. It was stored at room temperature, preferably in a well ventilated area or under a fume hood.

Preparation of Bouin's Fixative

Inside a fume hood, 3000 mL of saturated picric acid solution was poured into a clean glass container. While stirring gently, 1000 mL of formaldehyde (37–40%) was slowly added with continuous mixing. Then, 200 mL of glacial acetic acid was carefully added drop by drop while maintaining constant stirring. Mixing was continued until the solution became uniform and appeared consistently yellow.

Preparation of Carnoy's Fixative

In a clean, dry glass container, 120 mL of absolute ethanol was added first. Then, 60 mL of chloroform was slowly added with gentle stirring. After that, 2 mL of glacial acetic acid was added drop by drop while continuously stirring. The solution was mixed thoroughly until a clear and homogeneous mixture was obtained. Finally, the fixative was stored in a tightly sealed amber glass bottle at room temperature, protected from light.

Table 1: Composition of Common Histological Fixatives (NBF, Bouin's, and Carnoy's)

Fixative	*Component*	*Volume (mL)*
10% Formalin	**Formaldehyde solution (40%)**	**100**
	Distilled water	**900**
	Total volume	**1000**
Bouin's Fixative	**Saturated picric acid solution**	**3000**
	Formaldehyde (37–40%)	**1000**
	Glacial acetic acid	**200**
Carnoy's Fixative	**Absolute ethanol**	**120**
	Chloroform	**60**
	Glacial acetic acid	**2**

Experimental Design

The study included three experimental groups based on the type of fixative used: 10% neutral buffered formalin (NBF), Bouin's solution, and Carnoy's solution. Each group consisted of four mice (n = 4 per group). From each animal, the liver, heart, and testis were surgically excised for histological analysis.

Anaesthesia and Tissue Collection

Mice were anesthetized using diethyl ether via inhalation in a well ventilated chamber. Following anesthesia induction, a midline incision was performed to expose internal organs. The liver, heart, and testis were carefully dissected, rinsed in physiological saline, and immediately transferred into the respective fixatives.

Fixation Protocols

Tissues from each group were fixed according to standard histological protocols. In the formalin group, specimens

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were immersed in 10% neutral buffered formalin for 24–48 hours. In Bouin's group, tissues were fixed for 24 hours, followed by thorough rinsing in 70% ethanol to remove residual picric acid. In Carnoy's group, tissues were placed in Carnoy's solution (ethanol, chloroform, and acetic acid in a 6:3:1 ratio) for 2–4 hours.

Tissue Processing and Embedding

After fixation, tissues were dehydrated, cleared, and infiltrated using an automatic tissue processor (SLEE Tissue Processing System). Samples were embedded in paraffin wax using a SLEE embedding centre. Serial sections of 3 µm thickness were cut using a rotary microtome (SLEE Microtome). The sections were then floated on a water bath (Bio Optica) to allow spreading and flattening before being mounted onto slides for microscopic analysis.

Staining Procedure

Sections were stained with hematoxylin and eosin (H&E) according to a standardised histological protocol adapted from a previously published study (Muhammet Bahaeddin *et al*., 2024). The procedure began with deparaffinization in xylene, followed by rehydration through descending grades of alcohol. Sections were then stained with Weigert's iron hematoxylin for 5 minutes, washed, and appropriately differentiated. This was followed by eosin (Bio Optica) counterstaining for approximately 2 minutes. Finally, the sections were dehydrated, cleared, and mounted with coverslips. The Hematoxylin and Eosin (H&E) stainer (Bio Optica) was used to enhance visualisation of cellular and tissue structures.

Microscopic Evaluation

Prepared slides were examined using a digital light microscope. Images were captured for comparison of nuclear detail, cytoplasmic integrity, tissue architecture, and staining quality. Evaluation was qualitative and focused on fixation artefacts, cellular preservation, and clarity of histological structures.

Statistical analysis

Histological scores were expressed as median (interquartile range, IQR). Differences between fixatives were analysed using the Kruskal–Wallis test, followed by Dunn's post hoc test with Bonferroni correction for multiple comparisons. Interobserver agreement was assessed using Cohen's kappa coefficient. Exact p-values were reported, and $p < 0.05$ was considered statistically significant.

Ethical approval

This study was reviewed and approved by the Scientific Committee of the Faculty of Biomedical Sciences, University of Benghazi, Benghazi, Libya, and was conducted in accordance with institutional ethical standards.

Results

Microscopic Description

Liver tissue fixed in 10% formalin

Histological examination of liver tissues from four mice, fixed in 10% neutral-buffered formalin, revealed well-preserved architecture. Hepatocytes showed uniform morphology with distinct nuclei and intact cytoplasmic boundaries. Sinusoidal spaces were clearly defined, and hepatic cords appeared orderly and continuous. H&E staining demonstrated balanced nuclear-cytoplasmic contrast, indicating effective preservation of liver tissue architecture. These histological observations are illustrated in Figure 1.

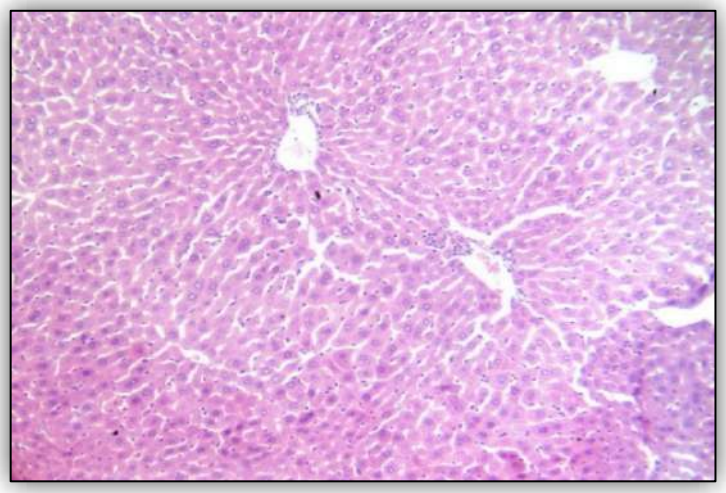

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Figure 1: Histological section of a liver fixed from four mice with Formalin (control) section stained with H & E X40.

Liver tissue fixed in Bouin's solution

Liver tissue fixed in Bouin's solution showed generally preserved architecture with some variations compared to formalin. Hepatocytes retained their structure, with mild cytoplasmic granularity and slight nuclear distortion in some areas. Staining intensity was increased with enhanced nuclear visibility, accompanied by slight background staining. Hepatic cords remained mostly organized, with occasional irregularities in sinusoidal spaces. These histological observations are illustrated in Figure 2.

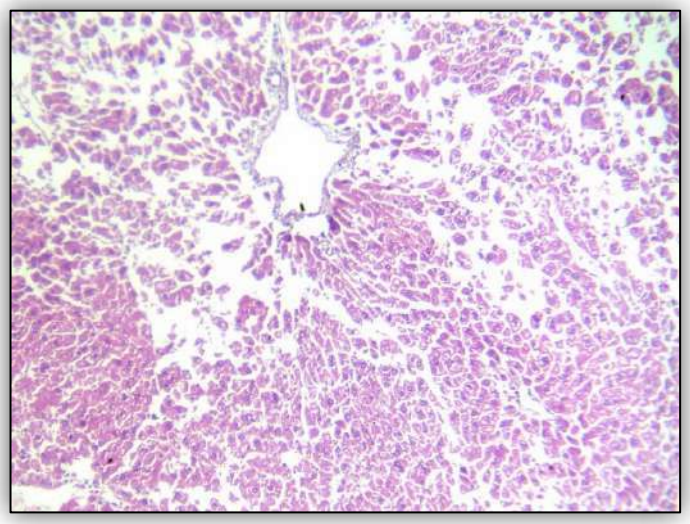

Figure 2 :Liver section fixed from four mice in with Bouin's solution and stained with H & E at X40 magnification

Liver tissue fixed in Carnoy's solution

Carnoy's fixed liver tissues showed well preserved morphology with distinct hepatocytes and clearly defined nuclei. Hepatic cords were well organized, and sinusoidal spaces were uniformly maintained. Minimal artifacts such as mild cytoplasmic shrinkage were observed. Nuclear detail was more pronounced with reduced background staining compared to Bouin's.

These histological observations are illustrated in Figure 3.

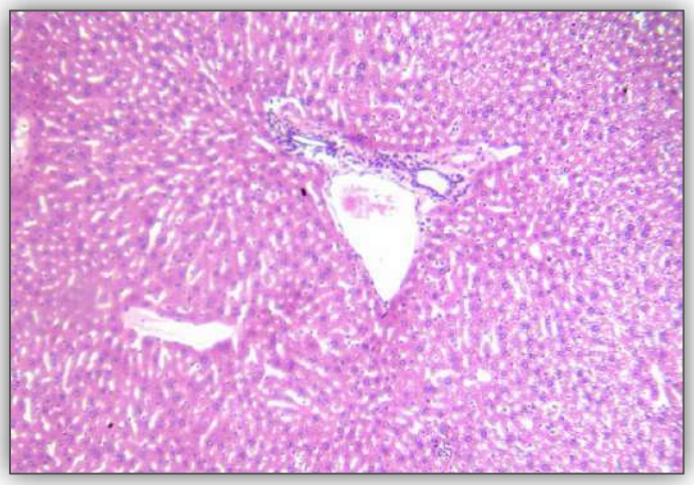

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Figure 3: Liver section fixed with Carnoy's solution and stained with H & E at X40 magnification.

Heart tissue

Heart tissue fixed in 10% formalin

Formalin-fixed cardiac tissue showed excellent preservation of myocardial structure. Cardiac muscle fibres were clearly arranged with distinct striations. Cardiomyocyte nuclei were centrally located with uniform chromatin distribution. Intercalated discs and connective tissue components were well preserved without distortion. These histological observations are illustrated in Figure 4.

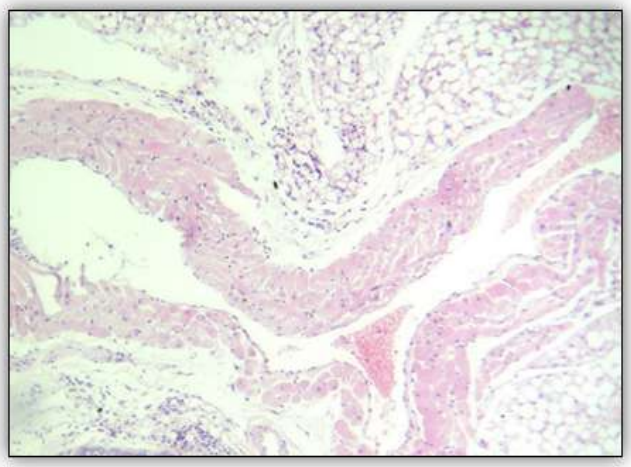

Figure 4: Histological section of a Heart from mice fixed with Formalin

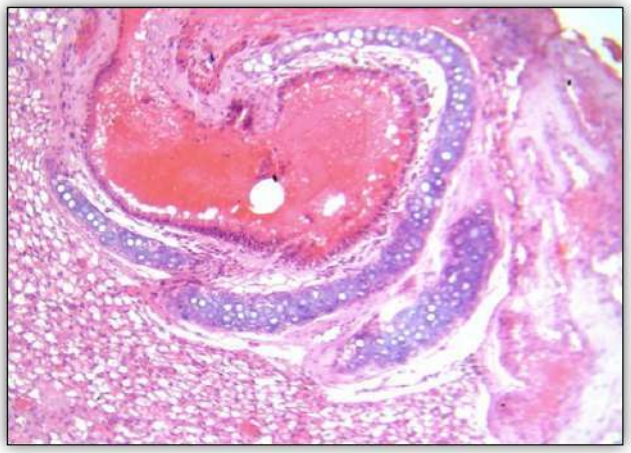

Figure 5: Cardiac Tissue Morphology fixed with Bouin's solution and stained with H & E at X40 magnification

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Heart tissue fixed in Carnoy's solution

Carnoy's fixed cardiac tissue showed moderate preservation of myocardial architecture. Muscle fibres were identifiable with moderate striation visibility. Nuclear detail was enhanced compared to Bouin's, with improved contrast. Mild shrinkage and occasional fibre separation were observed. Cytoplasmic staining was balanced without overstaining.

These histological observations are illustrated in Figure 6.

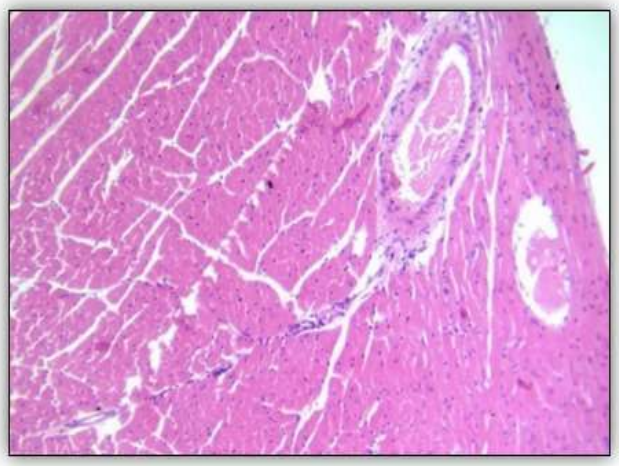

Figure 6: Cardiac Tissue Morphology fixed with Carnoy's solution and stained with H & E at X40 magnification

Testicular tissue

Testis fixed in 10% formalin

Formalin-fixed testicular tissue showed well-preserved seminiferous tubules with intact architecture. Spermatogenic layers were clearly organised at different developmental stages. Sertoli and Leydig cells were identifiable with normal nuclear morphology. Basement membranes were distinct and continuous, allowing clear visualisation of the testicular structure.

These histological observations are illustrated in Figure 7.

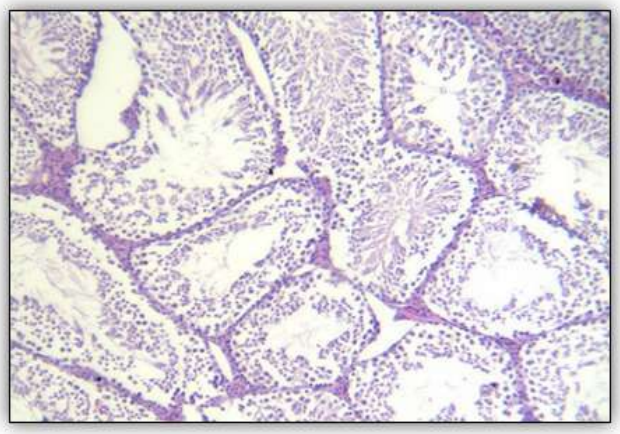

Figure 7: Histological section of a Testis from mice Fixed with Formalin (control) section stained with H & E X40.

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Testis fixed in Bouin's solution

Bouin's fixed testicular tissue showed excellent preservation of seminiferous tubules with highly distinct nuclear detail. Spermatogenic layers were clearly defined, and chromatin patterns were sharp. Increased eosin staining enhanced contrast between cell layers. Mild background staining and slight tissue hardening were occasionally observed.

These histological observations are illustrated in Figure 8.

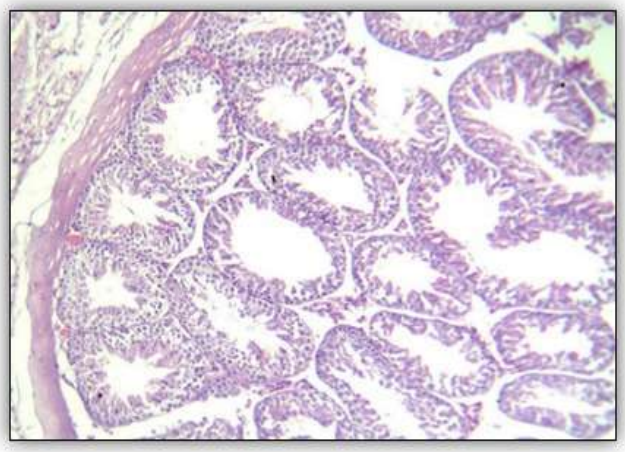

Figure 8: Testis Microstructure fixed with Bouin's solution and stained with H & E at X40 magnification

Testis fixed in Carnoy's solution

Carnoy's fixed testicular tissue showed enhanced nuclear staining with clear chromatin patterns and identifiable spermatogenic stages. Some intercellular and intertubular spaces were observed, consistent with mild shrinkage artefacts. Overall tubular architecture was moderately preserved but less compact than formalin and Bouin's.

These histological observations are illustrated in Figure 9.

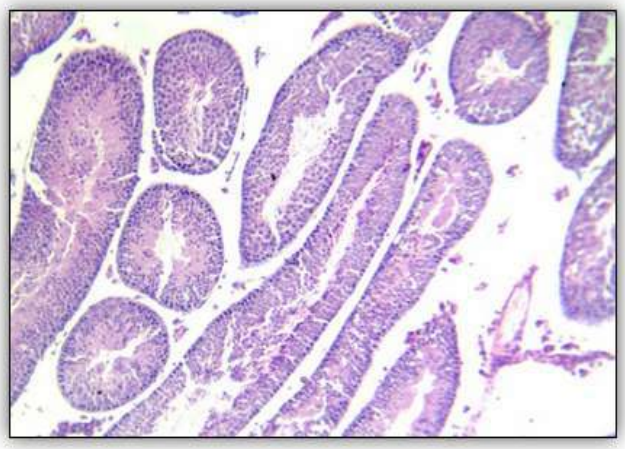

Figure 9: Testis Microstructure fixed with Carnoy's solution and stained with H & E at X40 magnification

Comparative summary of fixatives and tissues

Formalin provided the most consistent preservation across liver, heart, and testis, maintaining overall tissue architecture with minimal artefacts. Bouin's solution enhanced nuclear and cytoplasmic staining, particularly in testicular tissue, but showed occasional background staining and mild structural distortion. Carnoy's solution provided superior nuclear contrast in all tissues but was

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associated with mild shrinkage and separation artefacts. Semi-quantitative scoring revealed significant differences between fixatives. In liver tissue, nuclear detail scores differed significantly (Kruskal–Wallis, p = 0.021), with Bouin's showing lower median values compared to formalin. Post hoc analysis demonstrated a significant difference between Bouin's and Carnoy's (p = 0.01). Interobserver agreement was excellent for nuclear detail (κ = 0.82) and good for cytoplasmic preservation (κ = 0.75).

Table 2: Histological Effects of Formalin Fixative on Mouse Tissues (Liver, Heart, and Testis)

Fixatives	Tissue	Autolysis	Variations of nucleus	Cytoplasm
Formalin	Liver	• **Mild** ✓ • Moderate • Marked	• **Fine** ✓ •Moderate • Strong	•**Eosinophilic** ✓ •Basophilic
	Heart	• Mild • Moderate • Marked • **No Autolysis** ✓	• **Fine** ✓ •Moderate • Strong	•**Eosinophilic** ✓ •Basophilic
	Testies	• **Mild** ✓ •Moderate • Marked	• **Fine 20%** ✓ •Moderate • Strong	•**Eosinophilic** ✓ •Basophilic

Table 3: Histological Effects of Bouin's Solution on Mouse Tissues (Liver, Heart, and Testis)

Fixatives	Tissue	Autolysis	Variations of nucleus	Cytoplasm
Bouin's solution	Liver	•Mild •Moderate •**Marked** ✓	• Fine • **Moderate** ✓ • Strong	**Eosinophilic** ✓ Basophilic
	Heart	•**Mild 20%** ✓ •Moderate •Marked	• **Fine** ✓ • moderate • strong	**Eosinophilic** ✓ Basophilic
	Testies	•**Mild** ✓ •Moderate •Marked	• **Fine** ✓ • moderate • strong	**Eosinophilic** ✓ Basophilic

Table 4: Histological Effects of Carnoy's Solution on Mouse Tissues (Liver, Heart, and Testis)

Fixatives	Tissue	Autolysis	Variations of nucleus	Cytoplasm
Carnoy's solution	Liver	• **mild 20%** ✓ • moderate • marked	• Fine • moderate • Strong •**mild** ✓	**Eosinophilic** ✓ Basophilic
	Heart	• **mild** ✓ • moderate • Marked	• Fine • moderate • strong • **mild** ✓	**Eosinophilic** ✓ Basophilic

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	Testies	• mild • **moderate** ✓ • marked	• Fine • **moderate** ✓ • strong	**Eosinophilic** ✓ Basophilic

Table 5: Semi-quantitative scoring of histological parameters

Fixatives	Tissue	Nuclear Detail Median (IQR)	Cytoplasm Median (IQR)	Architecture Median (IQR)
10% Formalin	Liver	3(3-3)	3(2-3)	3(3-3)
Bouin's solution	Liver	2(2-3)	2(2-2)	2(2-3)
Carnoy's solution	Liver	3(2-3)	2(2-2)	2(2-2)

Table 6: Interobserver agreement

Interpretation	**Cohen's Kappa**	**Parameter**
Excellent	0.82	Nuclear detail
Good	0.75	Cytoplasm
Good	0.79	Architecture

Table 7: Comparison between fixatives (Kruskal–Wallis + Dunn)

Tissue	Liver
Parameter	Nuclear
Kruskal-Wallis p-value	0.021
Formalin vs Bouin	0.04
Formalin vs Carnoy	0.32
Bouin vs Carnoy	0.01

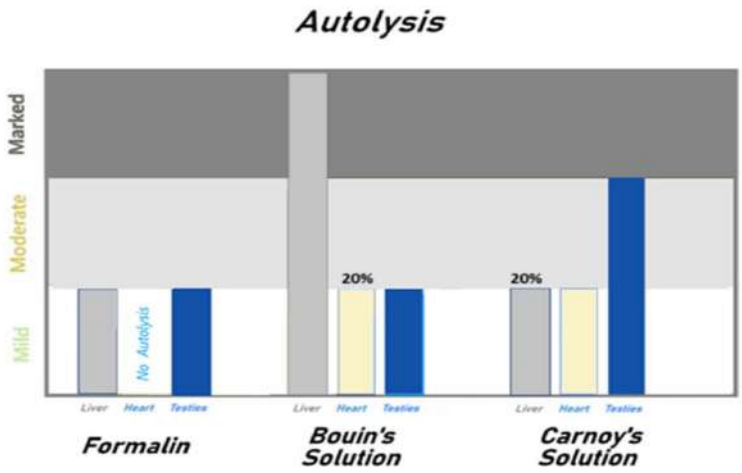

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Figure 10: Distribution of the highest fixative effects on tissues (Autolysis)

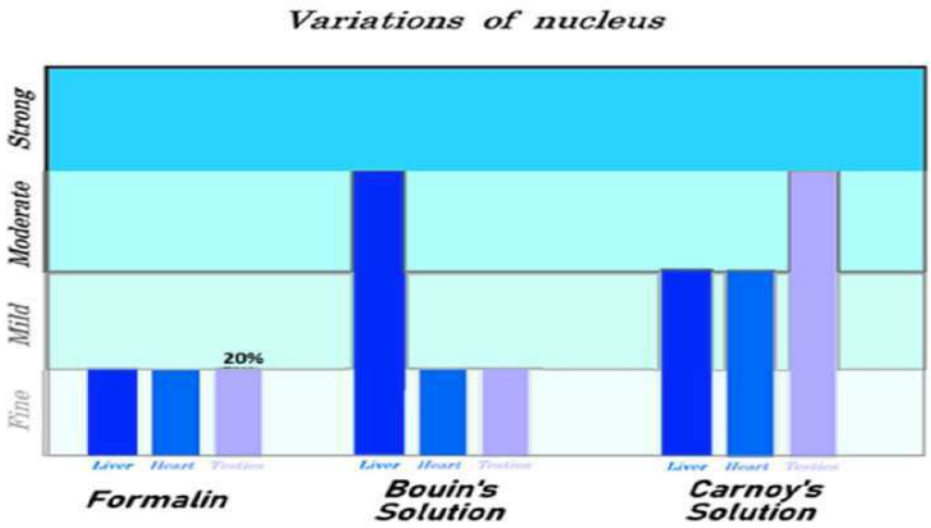

Figure 11 Distribution of the highest nuclear variation by fixatives

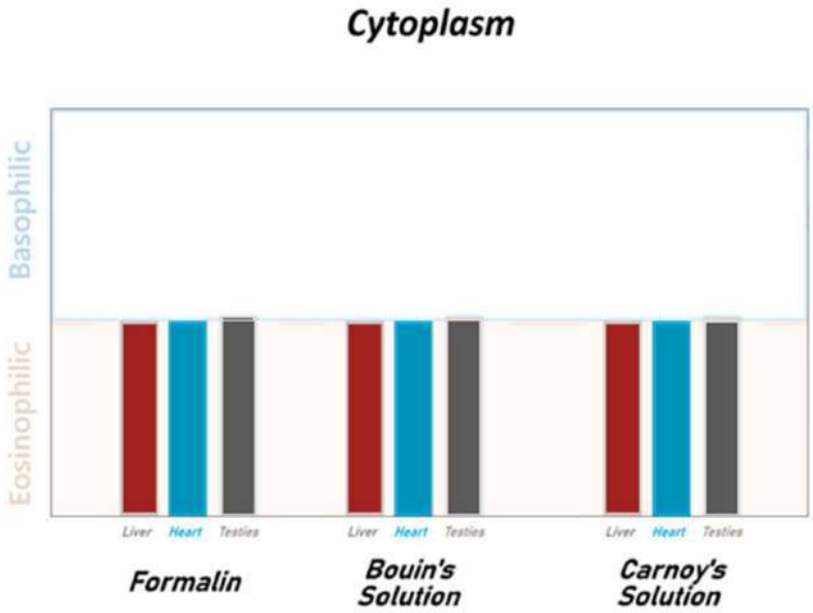

Figure 12: Distribution of cytoplasm by fixatives

Discussion

The present study aimed to evaluate and compare the histological preservation qualities of three commonly used fixatives 10% neutral buffered formalin, Bouin's solution, and Carnoy's solution on liver, heart, and testis tissues from adult mice. Each fixative demonstrated distinct effects on tissue architecture, nuclear clarity, and staining characteristics, depending on the organ type. In liver tissue, formalin provided the most balanced preservation. It maintained cellular morphology, preserved sinusoidal structure, and produced uniform staining with minimal artefacts. Bouin's fixative, while enhancing nuclear detail, introduced mild background staining and occasional nuclear distortion. Carnoy's offered improved nuclear

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contrast and acceptable cytoplasmic preservation but was associated with minor shrinkage artefacts. These findings support the continued use of formalin as a reliable standard for hepatic histology, with Carnoy's as an alternative where nuclear detail is prioritised [1,2].

In heart tissue, formalin again preserved architecture effectively, maintaining fibre alignment and nuclear structure with minimal distortion. Bouin's increased eosin staining intensity, which improved cytoplasmic visibility but occasionally obscured finer details. Carnoy's offered a balanced approach with stronger nuclear contrast than formalin and moderate eosin uptake, although some fiber separation was observed. This suggests that while formalin is ideal for overall cardiac morphology, Carnoy's may be better suited for studies requiring nuclear resolution [1,2,3].

In testis tissue, Bouin's solution proved superior in highlighting spermatogenic layers and nuclear details, making it particularly advantageous for evaluating stages of spermatogenesis. Formalin preserved both cellular and extracellular structures well, serving as a general purpose fixative. Carnoy's enhanced hematoxylin uptake and nuclear contrast but induced intercellular spaces and slight shrinkage. Therefore, while Bouin's remains the fixative of choice for testicular histology, formalin provides sufficient detail for routine analysis, and Carnoy's may be employed where sharp nuclear visualization is essential despite minor artifacts [4,5,6].

Collectively, these findings underscore that no single fixative is universally optimal across all tissues. The choice of fixative should be tailored to the histological features under investigation nuclear vs. cytoplasmic detail, structural integrity vs. contrast making an organ specific approach to fixation both necessary and scientifically justified.

The findings of our study align with several previous investigations concerning fixative performance on different tissues. For instance, Thavarajah *et al*. (2012) and Ajileye & Esan (2022) confirmed the superior preservation of liver and heart architecture by 10% formalin, consistent with our observation of minimal artifacts and well maintained morphology in those tissues [20,7].

Regarding testicular tissue, Aziz *et al*. (2023) and Singhal *et al*. (2016) emphasized the enhanced nuclear clarity and spermatogenic layer definition offered by Bouin's solution findings that were echoed in our results. However, similar to Ahmed *et al*. (2011), we also noted slight tissue hardening and background staining caused by picric acid, which limits Bouin's suitability for long term preservation [10,18,6].

As for Carnoy's solution, our findings support Pereira *et al*. (2015) and Rowley *et al*. (2020) who reported excellent nuclear detail but also observed shrinkage and intercellular gaps. This confirms the dual nature of Carnoy's: ideal for nuclear evaluation but problematic for overall tissue compactness, particularly in heart and testis [16,17].

Overall, these comparisons reinforce the conclusion that fixative selection must be tissue specific and guided by the desired histological outcome whether it's nuclear detail, general morphology, or staining contrast.

Additionally, the study by Ellenburg *et al*. (2020) reported that Bouin's fixative provided excellent nuclear clarity in testicular biopsies, making it superior for evaluating spermatogenesis exactly as observed in our testis samples [10].

Likewise, Dortbudak *et al*. (2024) observed minimal artifacts and optimal cytoplasmic clarity in liver and heart tissues fixed with 10% formalin, affirming its reliability for routine histopathology closely matching our findings [13].

Furthermore, Tian *et al*. (2024) emphasized the usefulness of Carnoy's solution for enhancing chromatin visibility, especially in testicular tissue, while also reporting tissue separation artifacts, which were similarly detected in our Carnoy fixed slides [19].

However, not all findings from the literature aligned with the outcomes of our study. Ellenburg *et al*. (2020) found that formalin fixation preserved nuclear and cytoplasmic structures in testicular tissue better than Bouin's, which contrasts with our finding that Bouin's provided clearer nuclear detail for spermatogenic stages [10].

In a study by Aziz *et al*. (2023), Carnoy's solution was described as providing compact and undistorted testicular tissue sections, while our results showed intercellular spaces and mild tissue shrinkage, suggesting variability possibly due to fixation time or tissue handling [10].

Ahmed *et al*. (2011) concluded that Bouin's fixative is unsuitable for high quality liver preservation due to excessive yellow staining and structural disruption. While we observed some artifacts, we still found acceptable preservation of hepatic architecture in Bouin fixed samples [6].

Some literature such as Singhal *et al*. (2016) reported formalin's limitations in nuclear detail compared to Carnoy's and Bouin's, but in our cardiac tissue analysis, formalin outperformed the others in structural clarity and artifact reduction [18].

5. Conclusion

This study demonstrated that the choice of fixative significantly affects histological preservation depending on tissue type. 10% neutral buffered formalin provided the most consistent preservation of liver and heart tissues with good structural integrity. Bouin's solution was superior for testicular tissue, offering enhanced nuclear detail and better visualization of spermatogenic layers. Carnoy's fixative

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improved nuclear staining but was associated with tissue shrinkage and increased intercellular spaces. Overall, the findings emphasize that no single fixative is optimal for all tissues, and fixation protocols should be selected according to the specific histological requirements of each organ to ensure accurate morphological evaluation.

Conflict of interest: The authors declare that there is no conflict of interest regarding the publication of this paper.

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