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# Effect of Fixative Type and Duration on Staining Quality in Wistar Rat Testicular Tissue

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## ABSTRACT

**Background and Objective:** Fixatives and fixation protocols are critical determinants of histological staining quality, especially for sensitive tissues like the testis. However, comprehensive comparative evaluations of commonly used fixatives and fixation durations remain limited. This study aimed to assess the effects of four fixatives-Bouin's fluid, Davidson's fluid, formalin, and neutral buffered formalin (NBF)-at three fixation durations (48 hours, 7 days, and 4 weeks) on the staining fidelity of Wistar rat testicular tissue using different histological techniques. **Materials and Methods:** Testicular tissues from Wistar rats were fixed using the specified fixatives for each duration. Sections were stained with Hematoxylin and Eosin (H&E), Gordon and Sweet (G&S), and Periodic Acid-Schiff (PAS) techniques. Staining quality was evaluated based on fixation effectiveness, tissue integrity, and artefact presence. Data were analyzed using descriptive statistics and appropriate comparative tests at $p<0.05$. **Results:** Bouin's fluid yielded superior results for H&E and G&S at 48 hours but performed poorly with PAS staining. Davidson's fluid maintained consistent G&S staining across all durations and demonstrated high initial PAS quality. Formalin showed optimal H&E staining at 48 hours but declined with prolonged fixation. The NBF was moderately effective for short-term H&E but lacked versatility across staining methods. **Conclusion:** This study emphasizes the importance of selecting appropriate fixatives and fixation durations tailored to specific staining techniques. Bouin's and Davidson's fluids are preferable for short- and long-term use respectively, depending on the staining method. These findings can guide histologists and researchers in optimizing testicular tissue processing for enhanced diagnostic and research accuracy.

## KEYWORDS

Fixative type, fixation duration, testicular histology, Wistar rats, staining fidelity, histological techniques



## INTRODUCTION

Fixatives and fixation protocols are critical in histological and histopathological studies, as they ensure the structural and molecular integrity of tissues for subsequent staining and microscopic evaluation[1]. The choice of fixative and the duration of fixation significantly influence the quality and diagnostic value of stained tissue sections[2]. This is particularly relevant for specialized staining techniques

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such as Hematoxylin and Eosin (H&E), Gordon and Sweet (G&S), and Periodic Acid-Schiff (PAS), which are widely used for studying morphological, connective tissue, and carbohydrate-related structures, respectively.

Testicular tissue presents unique challenges for histological assessment due to its complex architecture and sensitivity to handling and fixation conditions[3]. Fixatives such as Bouin's fluid, Davidson's fluid, formalin, and neutral buffered formalin (NBF) are commonly employed for preserving testicular tissues[4]. However, the efficacy of these fixatives varies based on their chemical composition and their ability to stabilize specific cellular and extracellular components[4]. For instance, Bouin's fluid, known for its picric acid and acetic acid components, provides excellent nuclear and cytoplasmic contrast but may alter glycogen-rich structures[5]. In contrast, formalin-based fixatives are widely used due to their availability and cost-effectiveness but are less effective for preserving delicate structures over extended durations[6].

The fixation time further complicates the choice of fixative, as prolonged fixation can result in over-hardening, shrinkage, and loss of antigenicity, while insufficient fixation leads to poor staining outcomes and artifacts[7]. These factors are especially critical for techniques like G&S, which rely on the preservation of argentophilic structures, and PAS, which depends on intact carbohydrate moieties[8]. Despite the critical role of fixation in tissue preparation, limited studies comprehensively compare the combined effects of fixative types and fixation durations on multiple staining techniques for testicular tissues[9].

This study aims to evaluate the effects of four commonly used fixatives (Bouin's fluid, Davidson's fluid, formalin, and NBF) and varying fixation durations (48 hours, 7 days, and 4 weeks) on the quality of H&E, G&S, and PAS staining in testicular tissue of Wistar rats. The findings will provide insights into optimizing fixation protocols for testicular histology, with implications for both research and diagnostic practices.

## MATERIALS AND METHODS

**Study area:** This study was conducted between February and March 2024 at the Animal House of the Department of Anatomy, Federal University of Technology, Akure, Nigeria.

**Experimental design:** This study was designed to assess the effects of four fixatives (Bouin's fluid, Davidson's fluid, formalin, and neutral buffered formalin and three fixation durations (48 hours, 7 days, and 4 weeks) on the staining quality of testicular tissues in adult male Wistar rats using Hematoxylin and Eosin (H&E), Gordon and Sweet (G&S), and Periodic Acid-Schiff (PAS) staining techniques.

**Animal model:** A total of 15 adult male Wistar rats, weighing between 200 to 300 g were used for this study. The animals were housed in standard laboratory conditions with a 12-hour light/dark cycle, adequate ventilation, and access to food and water ad libitum for 2 weeks.

**Tissue collection:** The Wistar rats were anaesthetised with 50 mg/kg of ketamine, and the testes were excised by making a midline incision in the abdomen, deepened to the peritoneum in the pelvic region, to expose the testes, which were then immediately rinsed in cold phosphate-buffered saline (PBS) to remove blood and debris[10].

**Fixation protocol:** The testicular tissues were randomly divided into four groups based on the fixative used:

- Bouin's fluid
- Davidson's fluid
- Formalin (10%)
- Neutral Buffered Formalin (10%)

Table 1: Semi-quantitative evaluation of staining quality of testicular tissue using self-designed scale

| Criteria/score | 0-2 | 3-4 | 5-6 | 7-8 | 9-10 |
|---|---|---|---|---|---|
| Cellular details | Poor visibility of cellular details | Vague cellular outlines | Fair resolution of structural details | Good visibility of key structures | Crisp cellular morphology; robust |
| Score | 0 | 1 | 2 | 3 | 4 | 5 |
| Fixation quality | No fixation; tissue/cells are degraded or autolyzed | widespread shrinkage, poor preservation of morphology | Suboptimal fixation; partial preservation, some cytoplasmic and nuclear distortion | Moderate fixation; structures generally visible but with some artifacts | Good fixation; well-preserved morphology with minor flaws | Excellent fixation; uniform, artifact-free preservation of cells/tissues |
| Staining quality | No staining or extremely poor contrast | Incomplete or uneven staining; difficult to interpret | Weak staining; some structures are difficult to differentiate | Adequate staining; most structures are visible with effort | Good staining; clear contrast, minor inconsistencies | Optimal staining; high contrast, all features well-demarcated |
| Score | 0 | 1 | 2 | 3 |
| Artifact presence | Numerous or large artifacts impairing interpretation | Moderate artifacts present, but interpretation still possible | Few minor artifacts; interpretation largely unaffected | No observable artifacts; slide is clean and interpretable |

Each group was further subdivided into three subgroups corresponding to fixation durations of 48 hours, 7 days, and 4 weeks. Tissues were immersed in their respective fixatives in labeled containers and stored at room temperature under appropriate conditions.

**Tissue processing:** After fixation, tissues were dehydrated through graded ethanol series, cleared in xylene, and embedded in paraffin wax. Paraffin blocks were sectioned at 5 µm thickness using a rotary microtome[11].

**Staining techniques:** The following staining techniques were performed on tissue sections:

- **Hematoxylin and Eosin (H&E):** For general tissue morphology and nucleocytoplasmic contrast[12]
- **Gordon and Sweet (G&S):** For the demonstration of reticular and connective tissue fibers
- **Periodic Acid-Schiff (PAS):** For carbohydrate-rich structures and basement membranes[13]

All staining procedures were conducted according to standard protocols, with strict adherence to reagent preparation and timing.

**Semi-quantitative evaluation of staining quality:** The stained slides were evaluated under a light microscope by three independent observers who are experienced anatomists, blinded to the experimental groups. Staining quality was scored on a self-designed scale with a minimum score of 0 and a maximum of 23. The scoring criteria and values are in Table 1.

**Statistical analysis:** Data were analyzed using GraphPad Prism version 8.0 and expressed as Mean±Standard Deviation. Comparisons were made using Two-way ANOVA. A $p<0.05$ was considered statistically significant.

**Ethical considerations:** All experimental procedures adhered to the guidelines of the International Animal Care and Use Committee (IACUC), ensuring humane treatment of animals and minimizing distress during handling and euthanasia. Ethical approval was obtained from the Ethical Committee of the Federal University of Technology, Akure, under the approval number FUTA/ETH/24/23.

## RESULTS

**Bouin's fluid:** Bouin's fluid preserved testicular morphology effectively at 48 hours, with high staining scores in H&E and G&S. However, PAS staining was consistently poor across all time points. Staining quality declined progressively at 7 days and 4 weeks, indicating Bouin's fluid is best suited for short-term fixation, particularly for H&E and G&S (Fig. 1(a-i), 5a). This was supported by a significant time-dependent effect ($F = 7.98$, $p = 0.0402$).

**Davidson's fluid:** Davidson's fluid produced optimal H&E and PAS staining at 48 hours, with reduced quality at 7 days and 4 weeks. In contrast, G&S staining improved progressively, peaking at 4 weeks, indicating enhanced preservation of connective tissue elements over time. These trends, illustrated in (Fig. 2(a-i), 5b), showed no statistically significant differences across durations ($F = 0.02$, $p = 0.9851$), suggesting time-stable but stain-specific effects.

**Formalin:** Formalin fixation resulted in high H&E staining quality at 48 hours, which declined sharply at 7 days and 4 weeks, indicating reduced morphological preservation over time. G&S staining was consistently poor, while PAS staining showed slight improvement with prolonged fixation. As shown in (Fig. 3(a-i), 5c), these patterns were not statistically significant ($F = 7.98$, $p = 0.6308$), suggesting limited long-term efficacy across staining techniques.

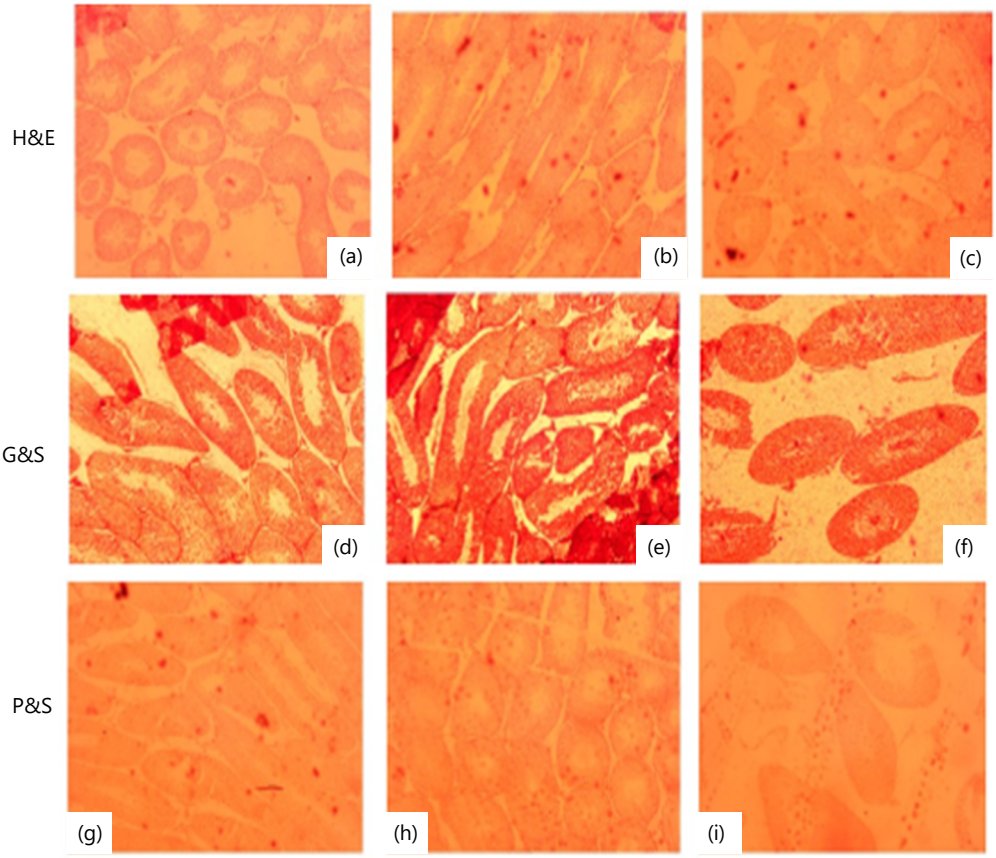


Fig. 1(a-i): Representative histological slides of testicular tissue fixed with Bouin's fluid for 48 hours, 7 days, and 4 weeks. Magnification is ×40, (a) 48 hours, Seminiferous tubules are well preserved, nuclear detail is still sharp, and cytoplasmic staining is fairly uniform, (b) At 7 days, Tubules begin to look less distinct; nuclear details are slightly blurred. There is some diffusion of eosin staining, giving the tissue a more homogenous "washed out" look, (c) At 7 days, Tissue shows over-fixation artefacts, nuclear details are faded (hematoxylin penetration reduced), (d) At 48 hours, seminiferous tubules are well preserved with distinct collagen distribution, (e) At 7 days, staining intensity increases with irregular collagen deposition and reduced tubular clarity, (f) At 4 weeks, over-fixation leads to exaggerated collagen staining, clumping, and poor tissue contrast, (g) At 48 hrs, showed normal myocardial fibers with intact architecture and moderate PAS-positive glycogen deposits, (h) At 7 days, displayed mild disorganization of myofibers and patchy glycogen accumulation and (i) At 4 weeks, revealed distorted myocardial fibers with irregular, diminished PAS staining and loss of glycogen uniformity

(a-c) Representative histological slides of testicular tissue fixed with Bouin's fluid for 48 hours, 7 days, and 4 weeks, respectively. Magnification is ×40, (d-f) Gordon and Sweet's (G&S) staining of Wistar rat testicular tissue at different fixation durations and (g-i) PAS staining of Wistar rat testicular tissue at different fixation duration ×40 magnification

**Neutral buffered formalin:** The NBF showed strong initial H&E staining at 48 hours, with gradual decline at 7 days and 4 weeks. A G&S staining was moderate at 48 hours, dropped at 7 days, and slightly improved at 4 weeks. PAS staining remained poor across all durations. As illustrated in (Fig. 4(a-i), 5d), these changes were not statistically significant ($F = 1.70$, $p = 0.2918$), indicating limited consistency over time.

## DISCUSSION

Bouin's fluid showed distinct patterns in its effects on different staining techniques (H&E, G&S, and PAS) based on fixation time. The staining quality with Bouin's fluid was initially high (16 score at 48 hours), indicating its excellent ability to preserve tissue morphology and maintain nucleocytoplasmic contrast. Over time, the staining quality decreased slightly (15.5 score at 7 days and 14.5 score at 4 weeks),

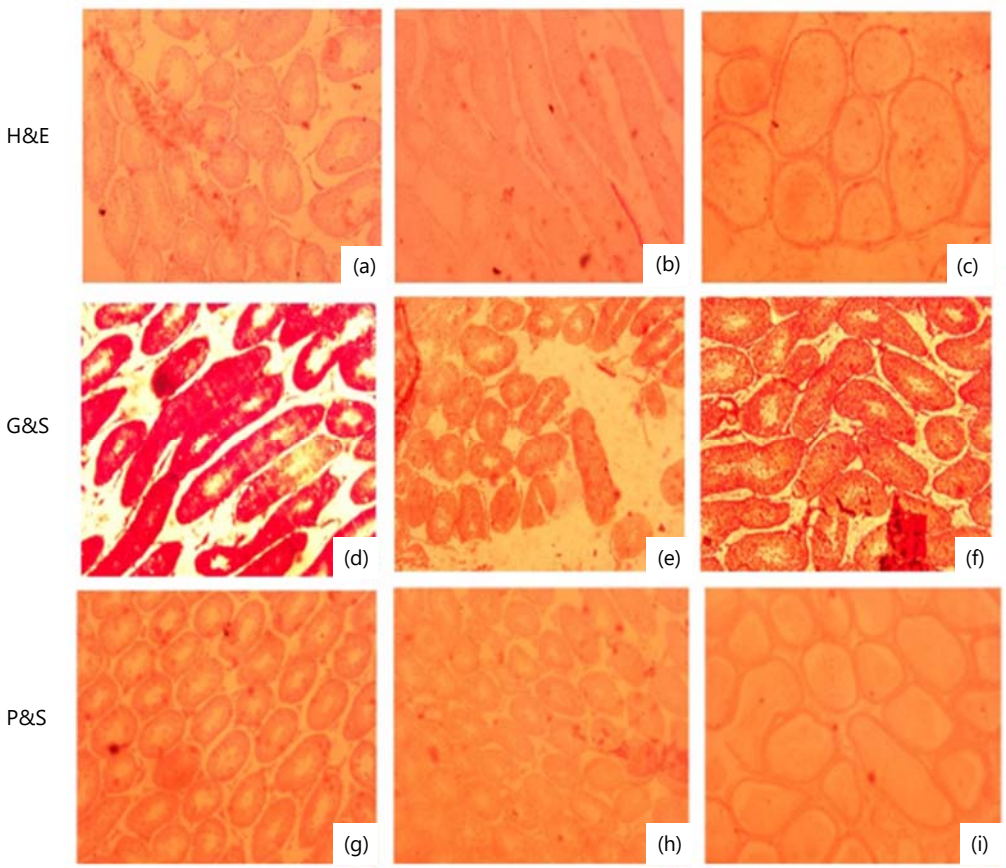


Fig. 2(a-i): Histological slides of testicular tissue fixed with Davidson's fluid for 48 hours, 7 days, and 4 weeks. Magnification is ×40, (a) At 48 hours, seminiferous tubules appear well-preserved with clear cellular outlines and intact lumen, (b) At 7 days, tubular architecture is retained but with slight reduction in cellular clarity and increased interstitial spaces, (c) At 4 weeks, prolonged fixation results in shrunken seminiferous tubules with exaggerated outlines and loss of distinct cellular details, (d) At 48 hours, seminiferous tubules show preserved structure with distinct collagen distribution and clear interstitial spaces, (e) At 7 days, collagen staining appears irregular with reduced tubular clarity and mild background staining, (f) At 4 weeks, prolonged fixation results in exaggerated collagen deposition, clumping of fibers, and poor tissue contrast, (g) At 48 hours, seminiferous tubules show clear cellular structure with strong PAS-positive staining highlighting the basement membranes and glycogen-rich cytoplasm, (h) At 7 days, staining intensity diminishes slightly with some loss of definition in the tubular basement membranes and a more diffuse cytoplasmic PAS signal and (i) At 4 weeks, prolonged fixation results in reduced PAS reactivity, with faint basement membrane staining and less distinct cellular morphology, indicating tissue over-fixation effects

(a-b) Histological slides of testicular tissue fixed with Davidson's fluid at different durations (H&E, ×40), (d-f) Histological slides of testicular tissue fixed with Davidson's fluid at different durations (Gordon and Sweet's stain ×40) and (g-i) PAS staining of Wistar rat testicular tissue at different fixation durations ×40 magnification

suggesting a gradual decline in fixation effectiveness for prolonged durations. This trend aligns with studies highlighting Bouin's as ideal for short-term preservation due to its picric acid content, which provides sharp nuclear staining[14] (Fig. 1(a-i), 5a). The performance of Bouin's fluid for G&S staining showed a similar pattern. At 48 hours, the staining quality was moderate (16 score), but a decline was observed at 7 days (15.5 score) and 4 weeks (14.5 score). This indicates that while Bouin's provides good initial preservation, the fixative may not support the prolonged structural integrity required for silver-based staining. Kiernan *et al.*[15] corroborated this, noting that Bouin's fixation could result in loss of fine structural details over time due to protein denaturation (Fig. 1(a-i), 5a).

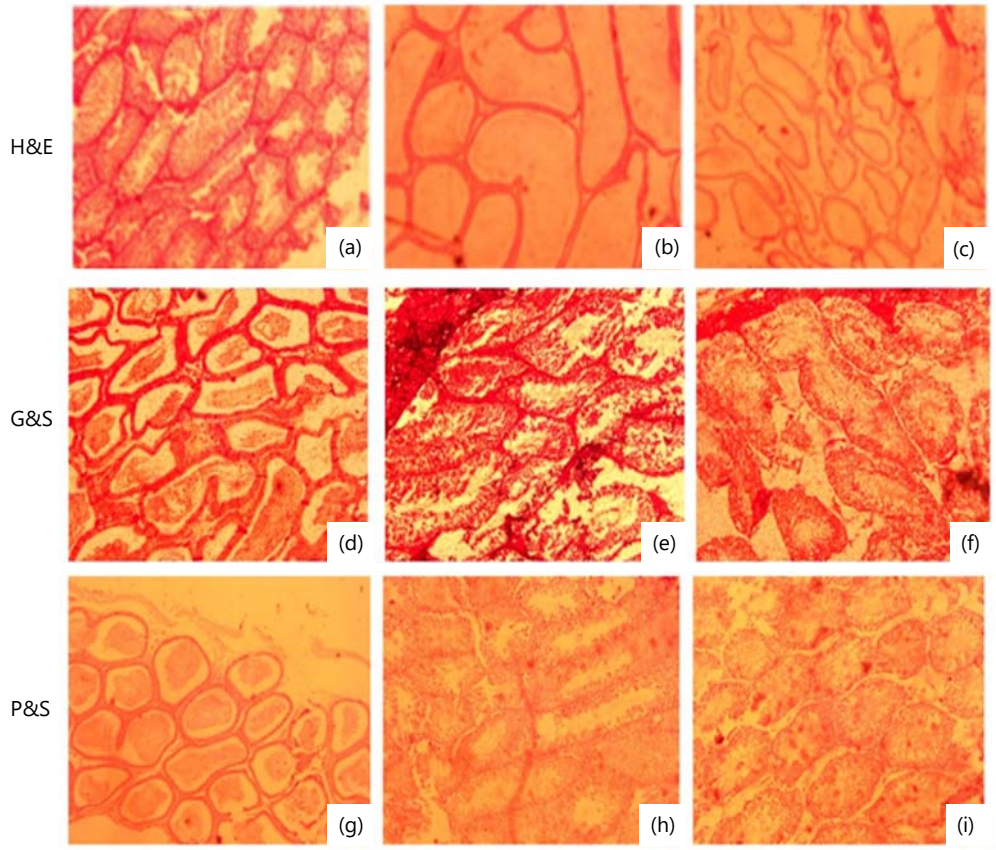


Fig. 3(a-i): Slides of testicular tissue fixed with formalin for 48 hours, 7 days, and 4 weeks using Magnification is ×40, (a) At 48 hours, seminiferous tubules exhibit well-preserved cellular details with distinct nuclei and clear interstitial spaces, (b) At 7 days, tissue shows moderate cellular shrinkage, with some loss of nuclear clarity and mild interstitial widening, (c) At 4 weeks, prolonged fixation causes noticeable tissue degradation, with faded cellular details, disrupted tubular architecture, and increased interstitial space, indicating over-fixation effects, (d) At 48 hours, seminiferous tubules are well-defined with clear collagen distribution demarcating tubule boundaries, (e) At 7 days, staining intensity becomes uneven, showing irregular collagen accumulation and partial loss of tubular structure clarity, (f) At 4 weeks, over-fixation leads to diffuse collagen staining with clumping and significant disruption of the overall tissue architecture and tubular delineation, (g) At 48 hours, the tissue shows clear and distinct cellular structures with well-preserved morphology and sharp boundaries, (h) At 7 days, staining intensity becomes uneven with partial loss of cellular detail and some blurring of structural definition and (i) At 4 weeks, prolonged fixation results in diffuse staining, loss of cellular detail, and significant disruption of tissue architecture

(a-c) H&E staining of Wistar rat testicular tissue fixed with formalin for different durations (Magnification: ×40), (d-f) Gordon and Sweet's (G&S) staining of Wistar rat testicular tissue at different fixation durations, ×40 magnification and (g-i) PAS staining of tissue at different fixation durations ×40 magnification

Bouin's showed the poorest performance for PAS staining (7.5 at 48 hours, 4.5 at 7 days, and 4 at 4 weeks). This highlights its inadequacy in preserving polysaccharides and other PAS-reactive substances. The picric acid component may interfere with carbohydrate-rich structures, reducing staining intensity. This finding aligns with Buesa and Peshkov[16], who found Bouin's less effective for glycogen-rich tissues, especially after extended fixation durations (Fig. 1(a-i), 5a). Davidson's fluid demonstrated robust preservation and staining outcomes across techniques and durations. The H&E staining was initially high at 48 hours (16 score), but it decreased significantly at 7 days (8) and 4 weeks (8). Davidson's fluid contains acetic acid, which enhances nuclear detail, but prolonged fixation may lead to over-hardening, reducing staining

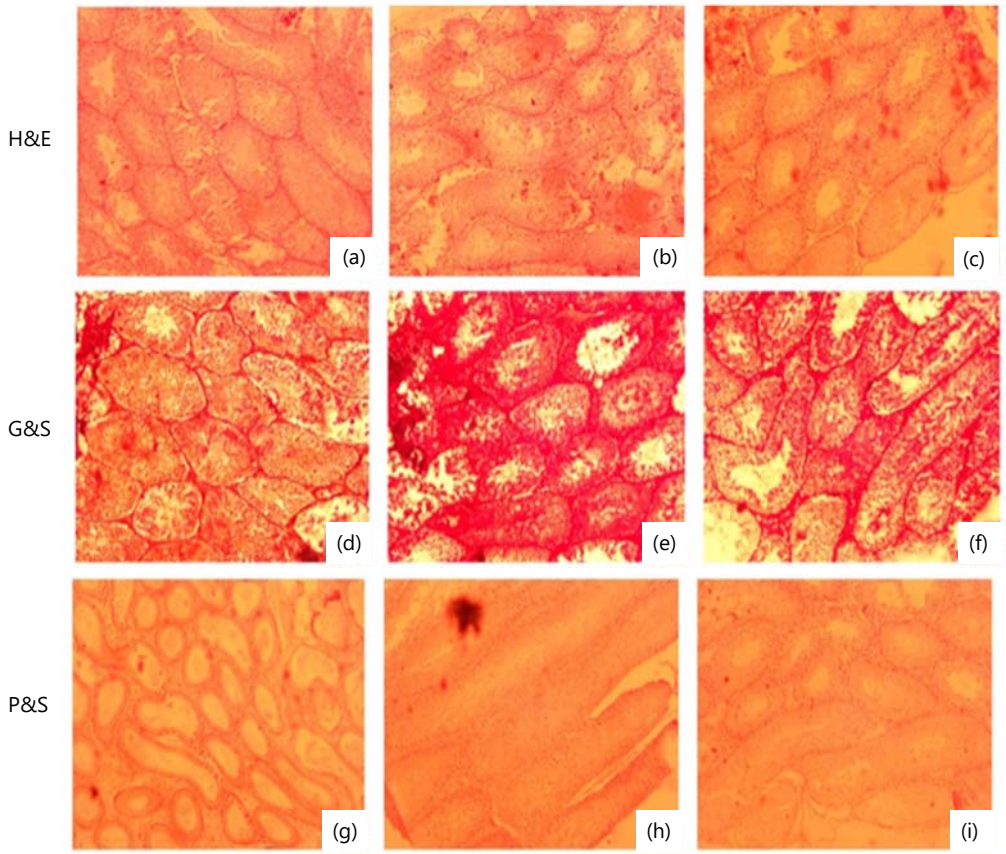


Fig. 4(a-i): Testicular tissue slides were fixed with NBF for 48 hours, 7 days, and 4 weeks. Magnification is ×40, (a)At 48 hours, seminiferous tubules exhibit well-defined cellular morphology with clear nucleus and cytoplasm contrast, (b) At 7 days, there is a noticeable reduction in staining clarity, with some loss of nuclear detail and slight blurring of tubular boundaries, (c) At 4 weeks, prolonged fixation leads to diffuse staining, diminished cellular resolution, and significant disruption of normal tissue architecture, (d) At 48 hours, seminiferous tubules are well-defined with clear collagen distribution demarcating tubule boundaries, (e) At 7 days, staining intensity becomes uneven, showing irregular collagen accumulation and partial loss of tubular structure clarity, (f) At 4 weeks, over-fixation leads to diffuse collagen staining with clumping and significant disruption of the overall tissue architecture and tubular delineation, (g) At 48 hours, renal tubules exhibit well-defined basement membranes with clear PAS-positive staining highlighting the brush borders and glomerular structures, (h) At 7 days, there is a noticeable reduction in staining intensity, with some loss of definition in tubular brush border clarity and less distinct glomerular outlines and (i) At 4 weeks, prolonged fixation results in diffuse and weaker PAS staining, diminished visibility of renal tubule structures, and overall loss of tissue architectural detail

(a-c) H&E staining of Wistar rat testicular tissue fixed with NBF for different durations (Magnification: ×40), (d-f) Gordon and Sweet's (G&S) staining of testicular tissue at different fixation durations ×40 magnification and (g-i) PAS staining of kidney tissue slides fixed with NBF for different durations at ×40 magnification

intensity. This is consistent with Mills[17], who noted Davidson's excellent performance for short-term H&E staining but suggested time-sensitive optimization (Fig. 2(a-i), 5b). G&S staining showed a unique improvement over time (7 at 48 hours, 16.5 at 7 days, and 21 at 4 weeks). This suggests that Davidson's fluid stabilizes components relevant to silver staining during prolonged fixation. Carson *et al.*[18] observed a similar trend, attributing the improvement to reduced tissue artifacts and enhanced impregnation efficiency over time (Fig. 2(a-i), 5b). Davidson's provided the highest PAS staining intensity at 48 hours (18.5), but a decline was observed at 7 days (17) and 4 weeks (10). While its initial performance is attributed to effective carbohydrate preservation, the gradual decline over time might result from

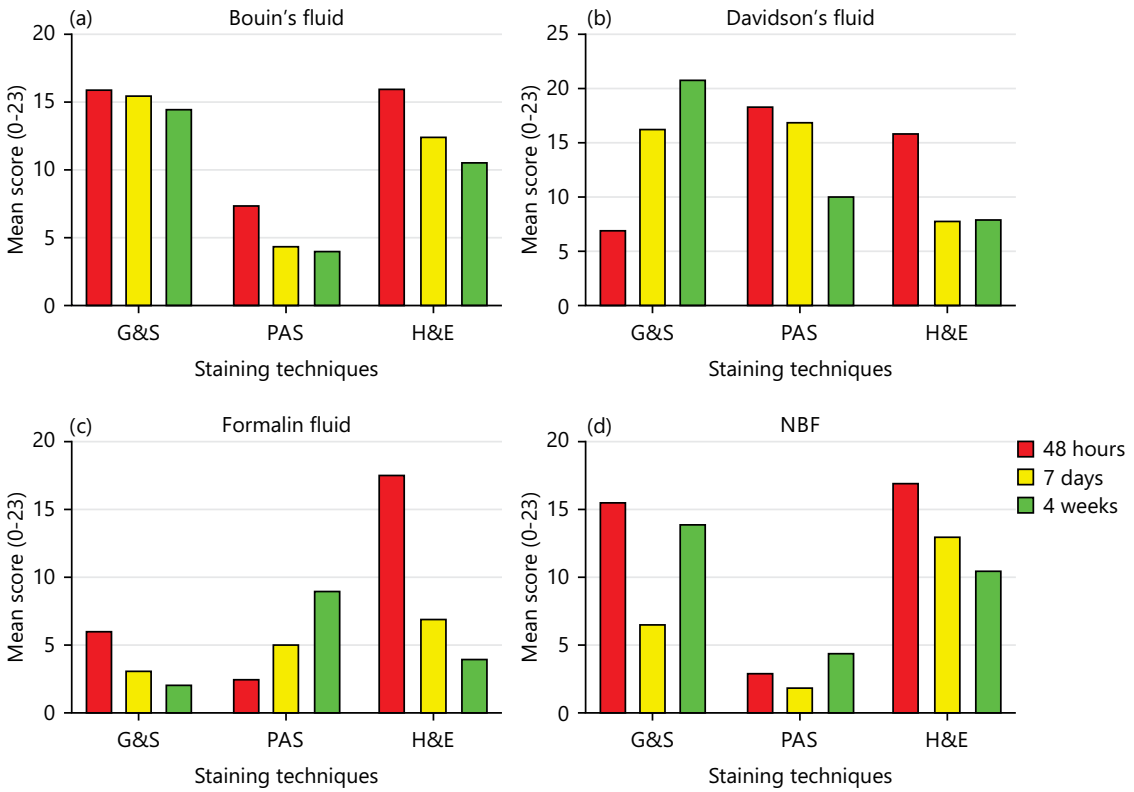


Fig. 5(a-d): Effect of fixation duration (48 hours, 7 days, and 4 weeks) on staining quality of testicular tissue using different fixatives (a) Bouin's fluid, (b) Davidson's fluid, (c) Formalin and (d) Neutral buffered formalin (NBF) across Hematoxylin and Eosin (H&E)

Gordon and Sweet (G&S), and Periodic Acid-Schiff (PAS) staining techniques. Statistical analysis was performed using Ordinary Two-Way ANOVA with the following results: (a) F = 7.98, DFn = 2, DFd = 4, p = 0.0402, (b) F = 0.02, DFn = 2, DFd = 4, p = 0.9851, (c) F = 7.98, DFn = 2, DFd = 4, p = 0.6308 and (d) F = 1.70, DFn = 2, DFd = 4, p = 0.2918

fixative-tissue interactions that degrade PAS-reactive components (Fig. 2(a-i), 5b). Formalin showed variable effects depending on the staining technique and duration. A H&E staining was most effective at 48 hours (17.5), but it decreased significantly at 7 days (7) and 4 weeks (4). This aligns with its known limitations, where prolonged fixation causes over-hardening and reduced staining quality due to extensive crosslinking. A Fox *et al.*[19] reported similar findings, emphasizing formalin's suitability for short-term fixation in routine H&E staining (Fig. 3(a-i), 5c). The G&S staining performance was poor across all durations (6 at 48 hours, 3 at 7 days, and 2 at 4 weeks). Formalin's inability to preserve structures necessary for silver staining likely accounts for this decline. This trend is supported by Kiernan[15], who noted that formalin's reactive aldehyde groups interfere with argentophilic components (Fig. 3(a-i), 5c). PAS staining performance was initially poor (2.5 at 48 hours) but improved slightly over time (5 at 7 days and 9 at 4 weeks). This unusual trend may be due to secondary crosslinking effects stabilizing carbohydrate-rich structures over prolonged fixation. Buesa and Peshkov[16] noted that while formalin is not the best choice for PAS, extended fixation could improve staining outcomes with optimization (Fig. 3(a-i), 5c). The NBF provided moderate results across staining techniques but varied significantly with duration. The NBF showed strong initial staining (17 at 48 hours), with a decline over time (13 at 7 days, 10.5 at 4 weeks). This suggests good nucleocytoplasmic preservation initially but reduced quality with prolonged fixation, similar to plain formalin. Mills[17] observed comparable trends, recommending NBF for short-term fixation in H&E protocols (Fig. 4(a-i), 5d). A G&S staining was moderate at 48 hours (15.5) but declined sharply at 7 days (6.5) before slightly improving at 4 weeks (14). The decline at 7 days suggests tissue artifacts, while the improvement at 4 weeks may be due to secondary stabilization of silver-reactive structures (Fig. 4(a-i), 5d). The PAS staining was poor across all durations (3 at 48 hours, 2 at 7 days, and 4.5 at 4 weeks). This indicates that NBF, like formalin, is unsuitable for preserving PAS-reactive substances, particularly for long-term fixation (Fig. 4(a-i), 5d).

## CONCLUSION

Each fixative displayed unique interactions with staining techniques and fixation times. Bouin's and Davidson's fluids excelled for short-term preservation, particularly for H&E and PAS, while Davidson's proved superior for G&S over extended periods. Formalin and NBF, although widely used, demonstrated limited versatility and suboptimal results for specialized staining protocols like PAS and G&S. The findings emphasize the importance of tailoring fixative and fixation duration to specific histological objectives.

## SIGNIFICANCE STATEMENT

This study provides comparative evidence on the influence of fixative type and fixation duration on the staining quality of Wistar rat testicular tissue using H&E, Gordon and Sweet, and PAS techniques. The findings highlight that appropriate selection of fixatives and fixation periods significantly affects tissue preservation, staining fidelity, and histological interpretation. The study offers practical guidance for histologists, anatomists, and researchers in optimizing fixation protocols for improved diagnostic accuracy and experimental reliability in testicular tissue studies.

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