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Research Paper

Formalin Versus Bouin Solution for Rat Testicular Tissue Fixation: A Histochemical and Immunohistochemical Evaluation

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ABSTRACT

Background: An accurate histopathological assessment and reporting of testicular biopsies require an appropriate tissue fixative. We assessed the histological, histochemical, and immunohistochemical quality of testicular biopsies, comparing 10% formalin versus Bouin solution as tissue fixatives.

Methods: This experimental study utilized 20 adult male albino rats equally divided into five cages for 30 days. By the end of the experiment, all animals were anesthetized, and both testes were removed and weighted; one testicle was fixed in 10% formalin and the other testicle in Bouin solution, offering 40 specimens and then subjected to histological, morphometric, histochemical, and immunohistochemical assessments.

Results: Formalin revealed high-quality cytological details and better nuclear chromatin detail (P=0.03). At the architectural level, the Bouin solution showed better quality details with less cytoplasmic shrinkage of seminiferous tubule germ cells (P=0.001). Bouin's fixed tissues were more suitable for staining by trichrome methods but unsuitable when subsequent immunohistochemistry was requested. The diagnostic concordance between the Bouin solution versus formalin-fixed biopsies was 91.7%.

Conclusion: This study supports that the morphology of testicular tissue fixed with Bouin solution was nearly comparable to those fixed with 10% neutral buffered formalin. However, the Bouin solution cannot substitute formalin when subsequent immunohistochemistry is considered.

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1. Introduction

Testicular biopsy is an invasive procedure performed for diagnostic and therapeutic purposes [1]. It is the cornerstone in assessing infertile males with azoospermia to distinguish obstructive from non-obstructive cases, with an accuracy rate of over 90% [2]. Nowadays, testicular biopsies are also performed for sperm extraction for in vitro fertilization, where part of this biopsy is also sent for histopathological examination to confirm the visualization of mature spermatids. It can further evaluate infertile men with risk factors for testicular malignancy [3].

An accurate histopathological assessment and reporting of testicular biopsy require the appropriate tissue fixative [4]. Fixation is a crucial step in the histological evaluation of tissue biopsies [5]. It is a physical or chemical method involving the gradual diffusion of reagents into specimens [6]. It helps in the maintenance of cellular and architectural details of cells and prevents autolysis during tissue processing [5]. Furthermore, fixative components may easily affect the localization sensitivity and quantification of tissue proteins when an immunohistochemical study is planned [7].

Aldehydes, particularly formalin (4% formaldehyde), have long been regarded as the "gold standard" fixative to preserve testicular tissue morphology, antigenicity, high-quality immunostaining, and molecular characteristics of many tissues [7]. It is a cross-linking chemical fixative act by forming covalent bonds among the amino acid chains of proteins [8, 9]. Due to some drawback issues, formalin may cause shrinkage and distortion of the architecture of testicular tissues. Recent articles have discouraged the use of formalin as the fixative of preference in testicular biopsies and reported that the Bouin solution should be used as a testicular fixative [10-12]. Bouin solution is a compound fixative composed of picric acid, formaldehyde, and acetic acid in an aqueous solution [10]. However, Bouin fixative is laboratory time-consuming and requires alcohol multiple washes to eradicate picric acid for better tissue staining. Furthermore, it needs handling precautions due to its explosive potential and mutagenic effect [13].

The use of formalin in tissue fixation has a long history of more than 150 years and has long been used as a routine fixative in many labs including our institution [9]. To the best of our knowledge, this is the first research in our locality aimed to assess the histological, histochemical, and immunohistochemical quality of testicular biopsies, comparing 10% formalin versus the Bouin fixative solution.

2. Materials and Methods

Animals

A total of 20 adult male albino rats weighing 190-230 g (13-15 weeks old) were used in this experiment. All rats were obtained from the Animal Breeding House of the laboratory animal, College of Veterinary Medicine, Mosul University. Rats were kept in plastic cages (47×34×18 cm) lined with wood chips, with five rats per cage under standard environmental conditions (12-hour light/dark cycles, humidity: 50%-56%, and temperature: 22±2˚C), and supplied with rodent laboratory food (pellets) and had free access to water until the end of the experiment. On day 30, animals were sacrificed by cervical dislocation. A laparotomy was made to expose the abdominal cavity, and contents and testes were carefully excised and weighted.

Tissue fixation

We prepared a 10% buffered formalin by the dilution of one part of the stock solution (40% formaldehyde) with nine parts of distilled water. To adjust the formalin solution to a neutral pH, we added 4 gm of sodium phosphate. Bouin solution was prepared by a mixture of 750 mL picric acid with 250 mL of 40% formaldehyde and 50 mL of glacial acetic acid [14].

Testicular tissue was harvested post-mortem and fixed within seconds to prevent histological changes secondary to ischemia. Both testes were removed; one testicle was fixed in 10% formalin and another in Bouin solution, offering 40 specimens. The rat testicular tissues were allowed to be fixed for 24 hours and then weighed before processing. Bouin solution tissues were washed with tap water and 50% alcohol to remove the yellow color and then transferred to 70% ethanol before processing.

Tissue processing

The testes were processed through graded alcohols (70% ethanol, 90% ethanol, and 100% ethanol) in two steps of 30 min each, cleared with xylene in two steps of 45 min each, and embedded in paraffin to obtain tissue blocks [9]. Eight sections with a thickness of 5 µm (two sections per slide) were taken from each block and stained with hematoxylin and eosin (HE), Masson trichrome (MT), and periodic acid Schiff (PAS) stains before monitoring for morphological changes. Masson trichrome highlights the normal blue distribution of collagen fibers in the capsule, basal lamina of seminiferous tubules, and the wall of blood vessels [15]. The PAS reveals the magenta-red

seminiferous tubule basement membrane and elongated spermatids [16]. Other sections were saved for immunohistochemical (IHC) staining. Briefly, sections were cut onto charged slides, dewaxed, and rehydrated. To expose the target protein, heat-induced epitope retrieval was carried out in a citrate bath (pH 6) using a steamer for 8-30 minutes, according to the selected antibody instructions. Primary antibodies practiced at the dilutions given below:

• Vimentin monoclonal antibody; clone-J144;1/20 dilution; Catalog: MA3-745, Thermo Fisher Scientific, USA.

• Calretinin monoclonal antibody; clone-SP13; 1/100 dilution; Catalog: PA1-31163, Thermo Fisher Scientific, USA.

• Ki-67 polyclonal antibody; 1/100 dilution; Catalog: PA5-16785, Thermo Fisher Scientific, USA.

• Alpha-smooth muscle actin monoclonal antibody; clone-1A4 (asm-1); 1/200 dilution; Catalog: MA5-11547, Thermo Fisher Scientific, USA.

Positive and negative controls were included for each immune marker analysis. Detection was performed using biotin-conjugated secondary antibodies and HRP, followed by a DAB colorimetric detection kit. Sections were counterstained with hematoxylin and prepped for mounting.

The positive immune reactivity appears as a brown stain in the cytoplasm of Sertoli cells and peritubular myoid cells, surrounding the seminiferous tubules, using vimentin and alpha-smooth muscle actin, respectively [17, 18]. Ki-67 reveals positive nuclear staining of spermatogonia, primary spermatocytes, Leydig cells, and endothelium while calretinin highlights the nucleus and/ or cytoplasm of Leydig cells [19, 20].

Histological analysis

The following cytological features were evaluated under X10 and X40 magnifications and then graded as poor quality (0) or good quality (1) based on the morphological appearance of the nuclear cell membrane, nuclear chromatin, cytoplasmic membrane, cytoplasmic granularity, cell basement membrane and, clarity of Sertoli and Leydig cells. Architectural features include the sloughing of the germinal epithelium into the lumens of the tubules, the shrinkage of seminiferous germinal cells, the shrinkage of seminiferous tubules, and the width of the interstitium (shrinkage of seminiferous tubules from one another) further graded on 0 to 3 scores (0=null, 1=mild (<33%), 2=moderate (34%-66%), and 3=severe (>67%).

The morphometric analysis was calculated with the max ToupView Image software using a digital camera linked to an Olympus light microscope. The total number of images per animal was ten images (five fields/slide). Accordingly, the round or almost round seminiferous tubules were selected. The mean diameters of the tubules were obtained by estimating across the minor and major axes. The height of germ cells epithelium was estimated as the space from the tunica propria to the edge of the seminiferous lumen and two opposed readings were analyzed using their mean value [21].

Histochemical and immunohistochemical analysis

The outcomes of PAS, MT, and immune stains were analyzed concerning the intensity and localization of histochemical and immunohistochemical reactions. The intensity was recorded as mild=1, moderate=2, and strong=3 [22]. The distribution of staining (localization) was graded as bad=0 and good=1. The information from the protein data sheets was utilized to evaluate the expected immune protein localization and staining pattern.

The microscopical examination was performed by two anatomical pathologists, without knowing the type of fixative used per testes. The final reports were averaged, taking into account inter-observer variability, to help give an idea of tissue quality.

Statistical analysis

All data were analyzed using SPSS software, version 18. Data are presented as Mean±SE. The concordance rate between the Bouin solution and 10% formalin was estimated. The student's t-test was practiced. Differences were statistically significant when $P<0.05$.

3. Results

After tissue fixation with formalin, the testes' weight declined by 66.6% independence of testicular length and width, Figure 1. The testis weight loss before or after fixation was 33.4% and 11.2% by formalin and Bouin solution, respectively. However, the testicular volume (length and width) didn't change significantly by both fixatives.

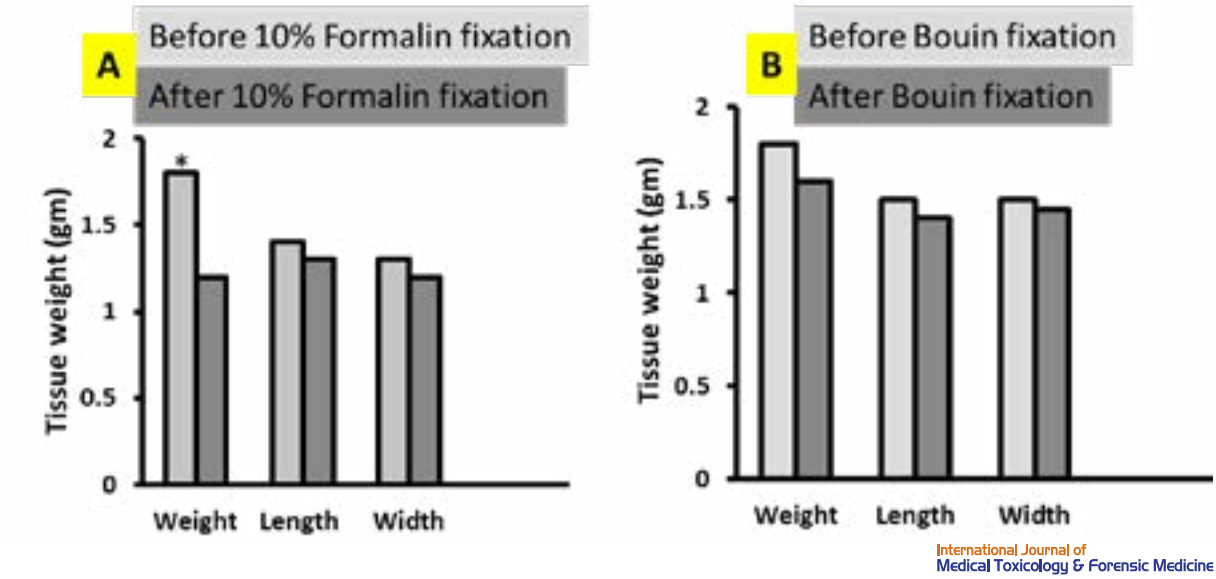

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Figure 1. Effects of testicular biopsy processing using 10% formalin and Bouin solution as fixatives

A) Testicular weight, length, and width changes using 10% formalin fixation (before 1 min and after 24 hr), B) Testicular weight, length, and width changes using Bouin solution (before 1 min and after 24 hr). The testis weight decreased significantly in response to formalin fixation compared to that in response to Bouin fixation (*P<0.05).

Histological analysis

A summary of cytological features observed in testicular morphology, comparing 10% formalin and Bouin solution, is illustrated in Table 1. Briefly, there was no significant difference in the quality of the cytology of tissue specimens. Regardless of the type of fixative, all sections were appropriate for histologic assessment of spermatogenesis. The samples fixed with the Bouin solution revealed high-quality cell details. However, some degree of nuclear chromatin clumping and clearing was identified (P=0.03), Figure 2.

At the level of architectural details, there was shrinkage of the cytoplasm of seminiferous tubule germ cells, significantly pronounced in tissues fixed with formalin (P=0.001), (Table 2, and Figure 3). Furthermore, sloughing germinal cells into the lumens of seminiferous tubules was at the edge of significance in formalin-fixed tissue, P=0.06.

The cytological, and architectural variables were assessed by two pathologists with a high consensus between them, P=0.0001.

Table 1. The cytological features of testicular tissue comparing 10% neutral buffered formalin and Bouin solution fixatives

Cytological Features	The Mean Score		P
	Formalin 10%	Bouin Solution	
Nuclear membrane	0.8	0.9	0.429
Nuclear chromatin	0.9	0.45	0.03
Cytoplasmic membrane	0.9	0.8	0.710
Cell basement membrane	1	0.9	0.826
Sertoli cells clarity	0.75	0.95	0.537
Leydig cells clarity	1	0.9	0.826

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The cytological features graded as poor quality (0) or good quality (1). Differences are statistically significant at P<0.05.

Table 2. The architectural features of testicular tissue comparing 10% neutral buffered formalin and Bouin solution fixatives

Architectural Features	The Mean Score		P
	Formalin 10%	Bouin Solution	
Sloughing of the germinal epithelium into the lumens of seminiferous tubules	0.6	0.15	0.06
Shrinkage of seminiferous germinal cells	0.9	0.15	0.001
Shrinkage of seminiferous tubules	0.5	0.15	0.073
Width of interstitium	0.75	0.3	0.461

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The architectural features were graded as 0 to 3: 0=null, 1=mild, 2=moderate, and 3=severe. Differences are statistically significant at P<0.05.

Morphometric analysis

The morphometric of testes fixed with 10% formalin and Bouin were assessed by HE staining. The results revealed that the parameters of seminiferous tubules fixed with 10% formalin were smaller than that fixed with the Bouin solution. The cross-sectional area of seminiferous tubules fixed with formalin was smaller than that fixed with Bouin solution, a decrease was highly significant (P<0.001) (Table 3, Figure 4).

Histochemical and immunohistochemical analysis

The histochemical staining of testicular tissue using Masson trichrome revealed better staining intensity and localization for tissues fixed with Bouin solution than formalin-fixed specimens, with (P=0.002) and (P=0.001), respectively. Formalin-fixed tissues revealed a clear separation of collagen fibres in the tunica albuginea with clear delineation of the fibres surrounding the seminiferous tubules (Figure 5 A, B). The PAS stain revealed no significant disparity in staining quality(intensity and localization of the stain) in both tissue fixatives with (P=0.24), and (P=0.731), respectively (Figure 5 C, D).

To assess the antigens preservation quality in testicular tissues fixed in formalin and Bouin, we did immunostaining for the proteins located in the cytoplasm (Vimentin and alpha-smooth muscle actin), in nuclei (Ki76), and both nucleus and or cytoplasm (Calretinin). Table 4 and Figure 5 summarize the difference in the staining intensity and localization labelling vimentin, Calretinin, Ki67, and SMA proteins expression in testicular biopsies fixed in Bouin versus formalin. In general, no statistical discrepancies of immunostaining quality were observed in both fixatives, apart from ki-67 which revealed the least immune reactivity in Bouin fixed tissues with both poor localization and weak intensity (P=0.001), and (P=0.03), respectively (Figure 5 F). For calretinin, formalin-fixed samples exhibited slightly intense expression in the cytoplasm and or the nucleus of Leydig cells compared with those fixed in the Bouin solution

(Figure 5 G, H). Vimentin expression quality was slightly superior in testes fixed in 10% formalin. It revealed positive immunostaining in the midportion of Sertoli cells and radiates toward the apices (Figure 5 I). Immunostaining for SMA showed near similar expressions in the cytoplasm of cells in 10% formalin and Bouin solution samples (Figure 5 K, L). The overall diagnostic concordance between the Bouin solution and formalin-fixed biopsies was 91.7%.

Table 3. The morphometric parameters of testicular tissue comparing 10% neutral buffered formalin and Bouin solution fixatives

Parameters	Type of Fixative		P
	Formalin 10%	Bouin Solution	
Diameter of the seminiferous epithelium (Mm)	103.00±11.30	111.00±5.52	<0.001
Height of the seminiferous epithelium (Mm)	70.061±8.316	76.520±5.594	<0.001
The cross-sectional area of the seminiferous tubules (Mm^2)	240.265±18.826	253.423±17.534	<0.001

Values expressed as Mean±SE. Differences are statistically significant at P<0.05.

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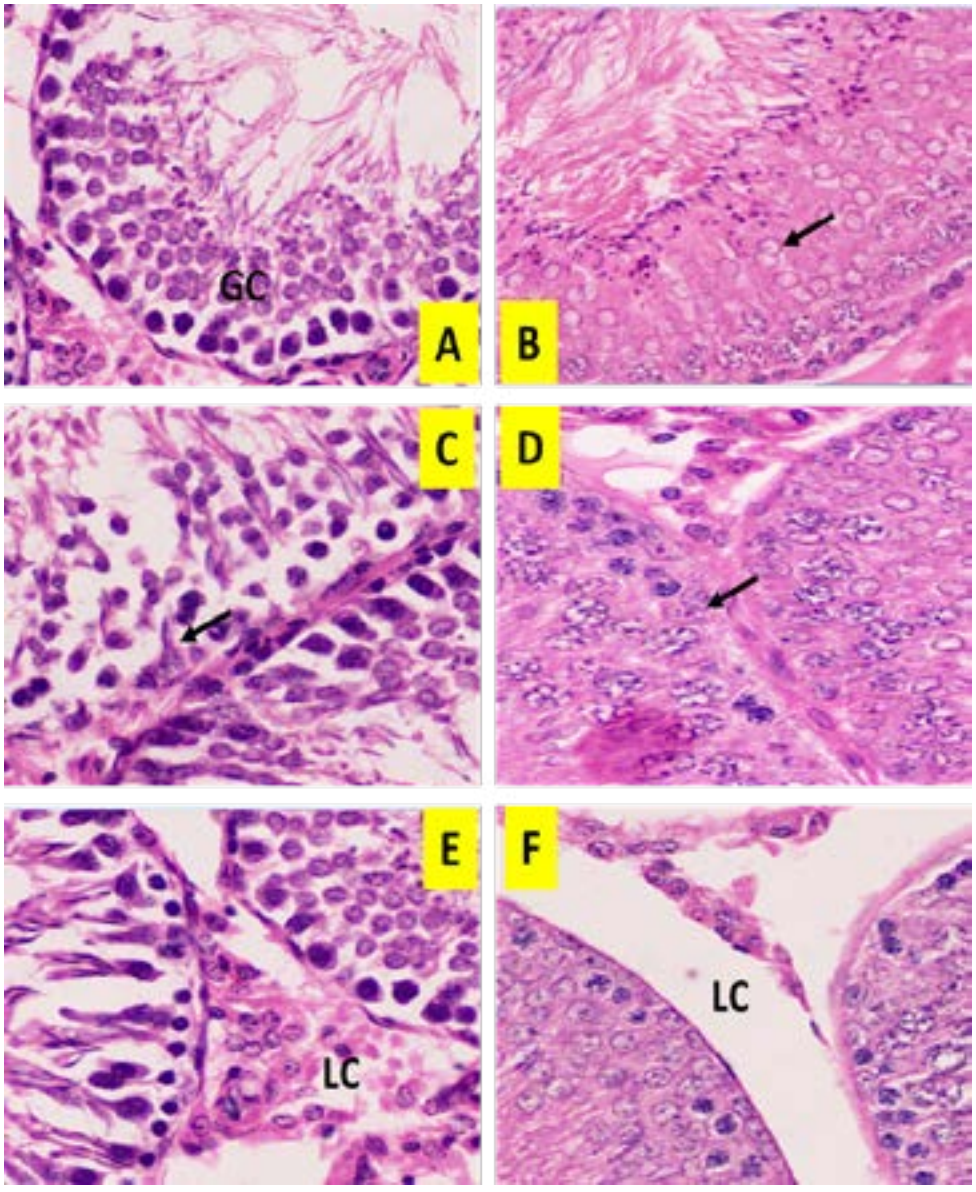

Figure 2. The cytological quality of H&E stained testicular tissue sections

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A, C, E) Formalin fixed rat testis showing high quality cytological features of germ cells, especially nuclear detail (A), C) Clarity of sertoli cells (arrow), E) Interstitium leydig cells.

B, D, F) Bouin solution fixed rat testis showing nuclear chromatin clearing and clumping of germ cells (arrow) (B),

D) Sertoli cell clarity (arrow), F) Leydig cells.

Bars A-F=200 µm. Germ cells: GC; Leydig cells: LC;

4. Discussion

The histological interpretation of testicular biopsies requires an adequate tissue fixative to maintain the nuclear and cytoplasmic details, conserve the architectural organization of germ cells within the seminiferous tubule, and preserve proteins and nucleic acid for immunohistochemical analysis. Formalin has been the "gold standard" fixative for decades, and the most widely used in routine histopathology practice [23]. However, some resources preferred the Bouin solution for the fixation of testes [8].

In the present experiment, we estimated a reduction in the testis's weight for formalin-fixed specimens compared to those fixed in the Bouin solution. This may be attributed to the testicle being a water-rich organ;

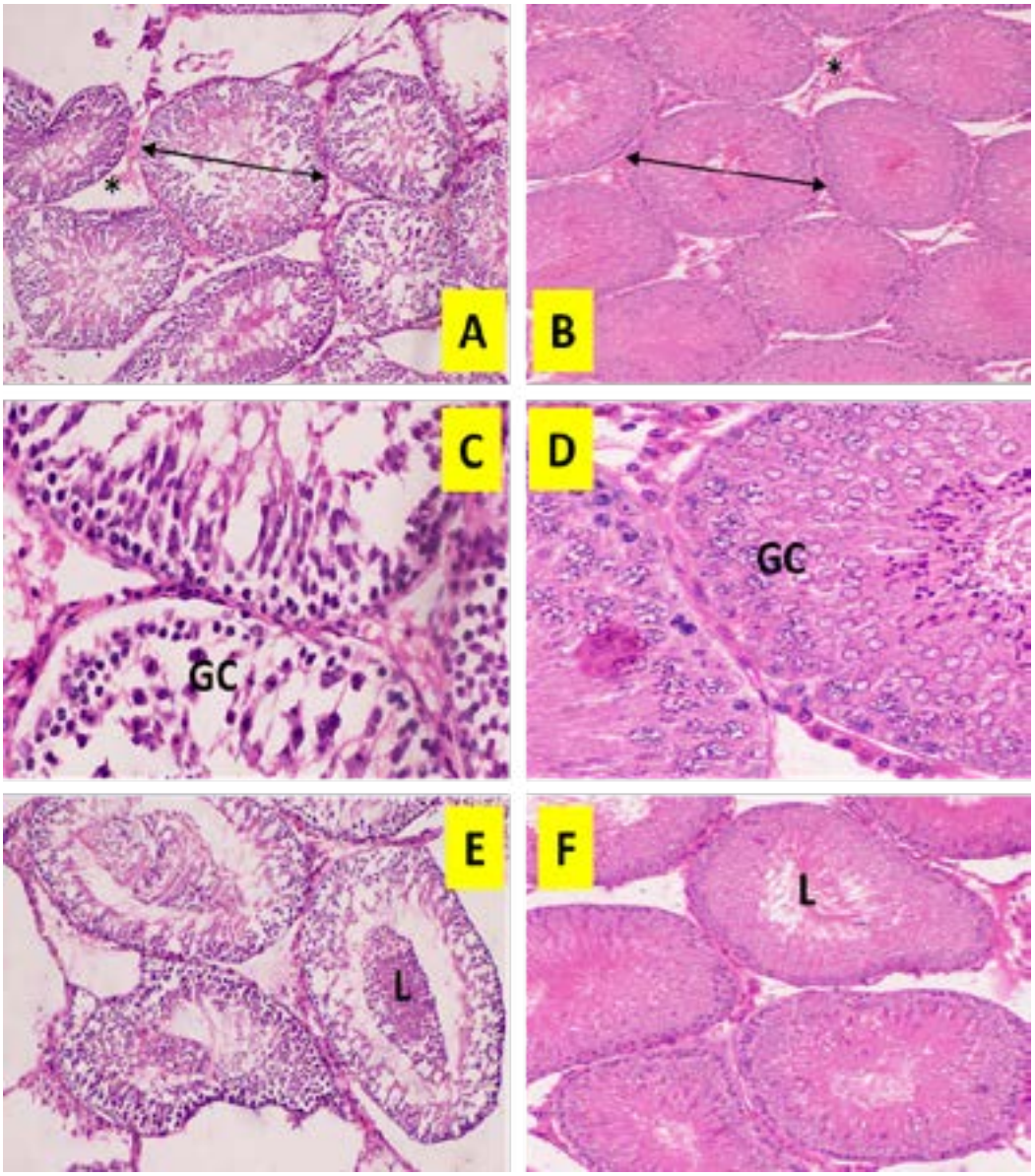

Figure 3. The architectural quality of H&E stained testicular tissue sections

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A, C, E) Formalin fixed rat's testis showing seminiferous tubules (double headed arrow) and interstitial tissue (*), expansion of interstitsial space (A). Shrinkage of seminiferous GC (C). Sloughing of germinal epithelium into the lumens (L) of seminiferous tubules (E).

Bouin solution fixed rat's testis showing seminiferous tubules (double headed arrow) and interstitial tissue (*) (B). Absence of shrinkage of seminiferous GC (D). Absence of sloughing of germinal epithelium into the lumens of seminiferous tubules (F).

Bars A-F=150 µm.

therefore, it can be expected that the high water-content Bouin solution would exhibit less tissue shrinkage [13]. Further, this finding suggested the differential diffusion capacity of fixatives in the testicular specimens over the given period [13]. These results were agreed upon by Wang et al. [22].

The microscopical examination of the testis, regardless of the fixative, revealed that all sections were appropriate for histological assessment of the stages of spermatogenesis. This was consistent with Cabrera et al., who showed a good morphology of tissues fixed with formalin, close to those detected in the Bouin solution [6].

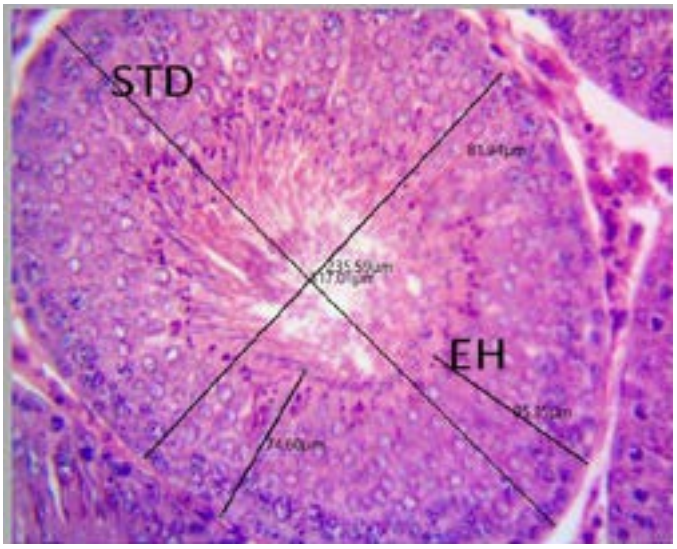

Figure 4. A photomicrograph of rat testis' seminiferous tubule shows the morphometric parameter measurements of seminiferous tubule diameter (STD), and epithelium height (EH), using the Toupview software. H&E stain, 400X.

In the current study, the samples fixed with Bouin solution revealed high-quality nuclear and cytoplasmic cytological details. This was consistent with Bultitude et al., who found better preservation of nuclear details in sections fixed with the Bouin solution than formalin [24]. This good preservation of nuclear detail made Bouin solution a favorable fixative for testicular tissue [25]. However, some degree of nuclear chromatin clumping and clearing was noticed in Bouin fixed specimens. This was in agreement with the reports by Latendresse et al., who detected chromatin aggregation and clumping in Bouin solution-fixed testis [7]. It is well-known that Bouin solution is a mixture of different chemical ingredients: Picric acid, acetic acid, and formaldehyde, each of which has a specific action. Picric acid is a slowly penetrating fixative that affects proteins causing excessive tissue shrinkage [26]. Acetic acid coagulates the nucleic acids. It is included in mixtures to preserve chromatin and is particularly useful for better visualization of meiotic chromosomes in the spermatocytes. It is also a rapidly penetrating ingredient that can counteract the picric acid shrinkage effect. Formaldehyde cross-links proteins but a relatively slow tissue penetrating compared with most fixatives [26].

Formalin is a notable fixative to cause shrinkage of cells due to its hyperosmolality and probable effects on the protein filaments in the cell membrane [12, 27]. In our work, the testicle preserved in 10% formalin had a significant cytoplasmic shrinkage in germ cells, as well as interstitial cells, especially those lying below the tunica albuginea. These results were in agreement with those described by Howroyd et al. [27]. In contrast to formalin, only minimal shrinkage was present in germ

Table 4. The overall immunohistochemical staining quality of testicular tissue comparing 10% neutral buffered formalin and Bouin solution fixatives

Effects	Type of Fixative							
	Formalin 10%				Bouin Solution			
	Vim	Cal	Ki67	SMA	Vim	Cal	Ki67	SMA
Intensity of staining	3	3	2.8	2.6	2.6	2.1	1.3*	2.7
Localization of staining	1	0.9	1	1	1	0.8	0.3*	0.9

Abbreviations: Vim: Vimentin; Cal: Calretinin; SMA: Smooth muscle actin. The intensity of staining was graded on 1 to 3 scores: 1=mild, 2=moderate, and 3=strong. The localization of staining was graded as 0 to 1: 0=Bad, and 1=Good.

*Differences are statistically significant at P<0.05.

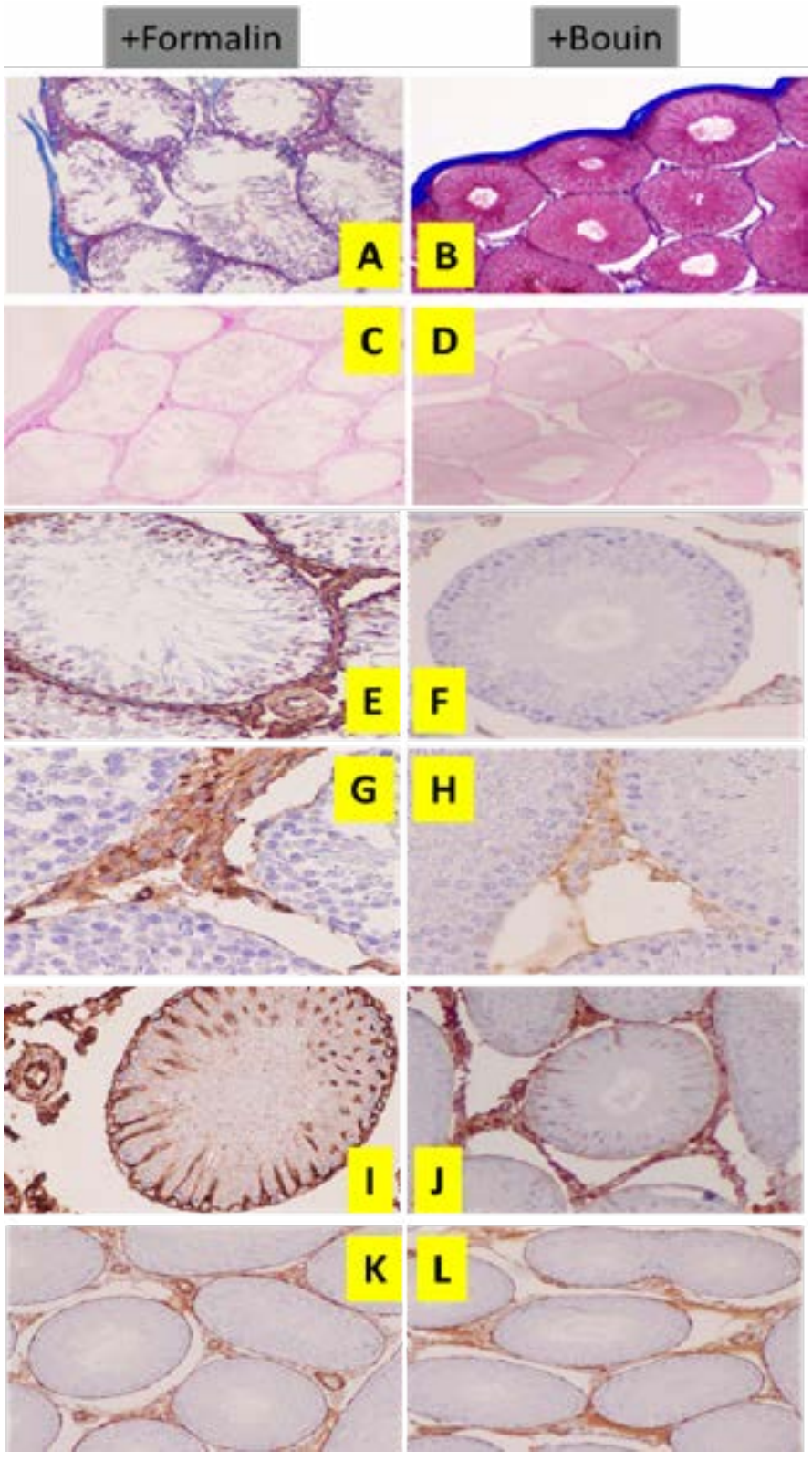

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Figure 5. The histochemical and immunohistochemical analyses of testicular tissue sections fixed in 10% formalin versus bouin solution, and stained with Masson trichrome stain (A and B), and periodic acid Shiff stain (C and D). The tissue sections were immunostained with Ki-67 (E and 5 F), calretinin (G and H), vimentin (I and J), and smooth muscle actin (K and L) antibodies. Bars A-L=100 µm.

cells and interstitial cells fixed in the Bouin solution. The sloughing of the germinal epithelium into the lumen of tubules was more evident in formalin-fixed sections, although it was focal and did not affect the assessment of sperm maturation. This finding was also mentioned in the study by Dhakal et al. [28]. The weak infiltration of formaldehyde in 10% formalin likely caused germ cell sloughing to the lumen of seminiferous tubules, which may be an acceptable explanation [22]. For Bouin fixative, although the microscopical evaluation was not affected by the shrinkage of the seminiferous tubules and interstitium compartments of rat testes, it was not recommended in our labs due to the presence of picric acid [29]. Problems associated with picric acid include the explosive properties of this substance and time-spending protocols [7]. Another problem is technicians and medical students in their practice, during the processing and dissection of specimens, all were rigorously exposed to this chemical, which in certain circumstances, reaches the threshold for toxicity [7].

The altered morphometric parameters of seminiferous tubules in formalin-preserved specimens were probably related to the previously mentioned explanations [12, 22, 25]. Such findings were supported by wang et al. [22]. However, another study argued that Bouin-fixed specimens were unsuitable when morphometry was considered [25].

Regarding histochemistry, it is widely reported that formalin fixative had a bad effect on trichrome stains [30]. In parallel to our results, Bouin solution-fixed testes gave better Masson's Trichrome staining results in terms of color, contrast, and positivity, while samples fixed with 10% formalin offered less definition. This result was in contrast to that of Gatta et al., who observed that formalin was superior to the Bouin solution in terms of color brightness and localization [31].

So far, histopathological evaluation might involve not only the use of traditional methods, like histochemical stains but also special ancillary tests, such as immunohistochemistry and molecular methods. We compared the quality of immune staining of testicular tissues prepared separately in both fixatives. Ki-67, a nuclear protein involved in cell proliferation, showed poorly localized and weak expression with Bouin specimens, while the 10% formalin-fixed biopsies showed a strong ki-67 protein immune expression in the spermatocytes, Leydig cells, and endothelium, as reported previously [19]. We could not achieve satisfactory staining for Bouin fixed specimens even when overnight incubation, higher concentrations of the primary antibody, and prolonged and different antigen retrieval techniques, were used. These observations were in parallel to those of Gutta et al., who reported weak and focal immune staining for Bouin-fixed tissues [31]. We speculate that the loss of nuclear antigenicity might be attributed to the presence of picric acid, which is known to cause the denaturation and precipitation of DNA [31]. Hayat mentioned that the Bouin solution is not a suitable substitute for formalin for tissue fixation because it causes an artifactual shift in immune expression [32]. However, in disagreement with us, Ananthanarayanan et al. noticed that the ki-67 antibody worked in Bouin's fixed specimens [25]. This discrepancy might be attributed to the fixation time. In the present work, we used a consistent fixation time of 24 hr for both fixatives, a fixation time with the Bouin solution might have a deleterious effect on nuclear protein preservation. This aspect has been investigated by Chung et al., who found that under or over-fixation of tissue leads to low-quality DNA preservation [33].

Although other immune markers exhibited a satisfactory immune expression for both fixatives and provided evidence that the corresponding epitopes retain their antigenicity, the 10% formalin-fixed testis revealed stronger vimentin and calretinin staining quality than those fixed in the Bouin solution, an observation raised by Howroyd et al. [27]. The clarification is that the acetic acid component of Bouin might disrupt cytoskeletal proteins during fixation, thus reducing the immune detection of their antigens. Overall, it seems that the better nuclear cytology attained with Bouin solution in testicular tissue implicates serious drawbacks in terms of diminished quality of immune staining [25]. The present study indicated that although 10% mformalin revealed morphological shrinkage, IHC expression of testicular proteins was superior to that of Bouin-fixed tissues. Thus, we assumed that these differences in immune expression might depend on the type of fixative itself rather than on protein localizations [18]. Therefore, we suggest the need for precaution in using Bouin as a fixative for testicular specimens when the immunohistochemical study is planned.

The main limitation of the present work is the small sample size. Further studies are required with variable and larger sample sizes and with human specimens to authenticate the observations of the present work for clinical practice. Also, additional studies are advised to assess the ultrastructural changes in testis caused by different fixations.

5. Conclusion

This study supports that the morphology of testicular tissue fixed with Bouin solution was nearly comparable to those fixed with 10% neutral buffered formalin. However, the Bouin solution cannot substitute formalin when subsequent IHC is considered.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of the Institutional Animal Care and Use, College of Veterinary Medicine, University of Mosul (Code: UM.VET.2022.05).

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Authors' contributions

All authors equally contributed to managing this research article.

Conflict of interest

The authors declared no conflict of interest.

References

[1] Bakhtiyar K, Beiranvand R, Ardalan A, Changaee F, Almasian M, Badrizadeh A, et al. An investigation of the effects of infertility on Women's quality of life: A case-control study. BMC Womens Health. 2019; 19(1):114. [DOI:10.1186/s12905-019-0805-3] [PMID] [PMCID]

[2] Andrade DL, Viana MC, Esteves SC. Differential diagnosis of azoospermia in men with infertility. Journal of Clinical Medicine. 2021; 10(14):3144. [DOI:10.3390/jcm10143144] [PMID] [PMCID]

[3] Karbel Hadeel A, Al-Bdairi Adnan A, Khairullah Ahmed R, Al-Humairi Ameer K. Histopathological evaluation of non-obstructive azoospermic males using testicular aspirate (TESA) biopsy. Indian Journal of Forensic Medicine & Toxicology. 2020; 14(4):2993. [DOI:10.37506/ijfmt.v14i4.12046]

[4] Panzacchi S, Gnudi F, Mandrioli D, Montella R, Strollo V, Merrick BA, et al. Effects of short and long-term alcohol-based fixation on sprague-dawley rat tissue morphology, protein and nucleic acid preservation. Acta Histochemica. 2019; 121(6):750-60. [DOI:10.1016/j.acthis.2019.05.011] [PMID] [PMCID]

[5] Suvarna KS, Layton C, Bancroft JD. Bancroft's theory and practice of histological techniques. Edinburgh: Elsevier health sciences; 2018. [Link]

[6] Cabrera NC, Espinoza JR, Vargas-Jentzsch P, Sandoval P, Ramos LA, Aponte PM. Alcohol-based solutions for bovine testicular tissue fixation. Journal of Veterinary Diagnostic Investigation. 2017; 29(1):91-9. [DOI:10.1177/1040638716672252] [PMID]

[7] Latendresse JR, Warbrittion AR, Jonassen H, Creasy DM. Fixation of testes and eyes using a modified davidson's fluid: Comparison with bouin's fluid and conventional davidson's fluid. Toxicologic Pathology. 2002; 30(4):524-33. [DOI:10.1080/01926230290105721] [PMID]

[8] Cerilli LA, Kuang W, Rogers D. A practical approach to testicular biopsy interpretation for male infertility. Archives of Pathology & Laboratory Medicine. 2010; 134(8):1197-204. [DOI:10.5858/2009-0379-RA.1] [PMID]

[9] Carson FL, Cappellano C. Histotechnology a self instructional text. Chicago: ASCP Press; 2020. [Link]

[10] Dohle GR, Elzanaty S, van Casteren NJ. Testicular biopsy: Clinical practice and interpretation. Asian Journal of Andrology. 2012; 14(1):88-93. [DOI:10.1038/aja.2011.57] [PMID] [PMCID]

[11] McLachlan RI, Rajpert-De Meyts E, Hoei-Hansen CE, de Kretser DM, Skakkebaek NE. Histological evaluation of the human testis--approaches to optimizing the clinical value of the assessment: Mini review. Human Reproduction. 2007; 22(1):2-16. [DOI:10.1093/humrep/del279] [PMID]

[12] Tran T, Sundaram CP, Bahler CD, Eble JN, Grignon DJ, Monn MF, et al. Correcting the shrinkage effects of formalin fixation and tissue processing for renal tumors: Toward standardization of pathological reporting of tumor size. Journal of Cancer. 2015; 6(8):759-66. [DOI:10.7150/jca.12094] [PMID] [PMCID]

[13] Rahman MA, Sultana N, Ayman U, Bhakta S, Afrose M, Afrin M, et al. Alcoholic fixation over formalin fixation: A new, safer option for morphologic and molecular analysis of tissues. Saudi Journal of Biological Sciences. 2022; 29(1):175-82. [DOI:10.1016/j.sjbs.2021.08.075] [PMID] [PMCID]

[14] Paavilainen L, Edvinsson A, Asplund A, Hober S, Kampf C, Pontén F, et al. The impact of tissue fixatives on morphology and antibody-based protein profiling in tissues and cells. The Journal of Histochemistry and Cytochemistry. 2010; 58(3):237-46. [DOI:10.1369/jhc.2009.954321] [PMID] [PMCID]

[15] El-Salam A, Faruk E, Manawy SM, Nafie NY. Microscopic structural alterations in response to mesenchymal stem cells injection in experimentally induced oligospermia in adult rat testis (histological and immunohistochemical study). Egyptian Journal of Histology. 2019; 42(4):826-37. [DOI:10.21608/ejh.2019.7960.1080]

[16] Hasanin NA, Sayed NM, Ghoneim FM, Al-Sherief SA. Histological and ultrastructure study of the testes of acrylamide exposed adult male albino rat and evaluation of the possible protective effect of vitamin E intake. Journal of Microscopy and Ultrastructure. 2018; 6(1):23-34. [DOI:10.4103/JMAU.JMAU_7_18] [PMID] [PMCID]

[17] ElGhamrawy TA, Helmy D, Elall HF. Cadherin and vimentin immunoexpression in the testis of normal and induced infertility models of albino rats. Folia Morphologica. 2014; 73(3):339-46. [DOI:10.5603/FM.2014.0050] [PMID]

[18] Moustafa AM. Age-related changes in the immunohistochemical localization pattern of α-smooth muscle actin and vimentin in rat testis. Egyptian Journal of Histology. 2012; 35(3): 412-23. [DOI:10.1097/01.EHX.0000418134.14989.85]

[19] He M, Wang L, Chen Y, Zhang T, Guo J. Effect of wuziyanzong pill on levels of sex hormones, and expressions of nuclear- associated antigen Ki-67 and androgen receptor in testes of young rats. Journal of Traditional Chinese Medicine. 2016; 36(6):743-8. [DOI:10.1016/s0254-6272(17)30009-2] [PMID]

[20] Shan LI, Wen-dan XU, Xiao-nan DA, Jing WA, Jian LU, Chao GA, et al. Expression of calretinin in testes of rats at different development stages. Journal of International Reproductive Health/Family Planning. 2014; 33(6):410-14. [Link]

[21] Hussein AM, Mustafa HN, Badawoud MH. Ameliorative potentials of a combination of fenugreek and alpha-tocopherol on cadmium induced testicular toxicity: An ultrastructural study. Folia Morphologica. 2015; 74(3):325-34. [DOI:10.5603/FM.2015.0049] [PMID]

[22] Wang WY, Meng LJ, Xu YJ, Gong T, Yang Y. Effects of 4% paraformaldehyde and modified davidson's fluid on the morphology and immunohistochemistry of Xiang pig testes. Journal of Toxicologic Pathology. 2020; 33(2):97-104. [DOI:10.1293/tox.2019-0072] [PMID] [PMCID]

[23] Shetty JK, Babu HF, Hosapatna Laxminarayana KP. Histomorphological assessment of formalin versus nonformalin fixatives in diagnostic surgical pathology. Journal of Laboratory Physicians. 2020; 12(4):271-5. [DOI:10.1055/s-0040-1722546] [PMID] [PMCID]

[24] Bultitude MF, Ghani KR, Horsfield C, Glass J, Chandra A, Thomas K. Improving the interpretation of ureteroscopic biopsies: Use of bouin's fixative. BJU International. 2011; 108(9):1373-5. [DOI:10.1111/j.1464-410X.2011.10641.x] [PMID]

[25] Ananthanarayanan V, Pins MR, Meyer RE, Gann PH. Immunohistochemical assays in prostatic biopsies processed in Bouin's fixative. Journal of Clinical Pathology. 2005; 58(3):322-4. [DOI:10.1136/jcp.2004.019299] [PMID] [PMCID]

[26] Kiernan J. Histological and histochemical methods. Banbury: Scion Publishing Ltd; 2015. [Link]

[27] Howroyd P, Hoyle-Thacker R, Lyght O, Williams D, Kleymenova E. Morphology of the fetal rat testis preserved in different fixatives. Toxicologic Pathology. 2005; 33(2):300-4. [DOI:10.1080/01926230590896145] [PMID]

[28] Dhakal HP, Coleman J, Przybycin CG. A novel dual immunostain to characterize sloughed cells in testicular biopsies for infertility. The American Journal of Surgical Pathology. 2019; 43(8):1123-8. [DOI:10.1097/PAS.0000000000000128] [PMID]

[29] Tu L, Yu L, Zhang H. Morphology of rat testis preserved in three different fixatives. Journal of Huazhong University of Science and Technology. 2011; 31(2):178-80. [DOI:10.1007/s11596-011-0247-0] [PMID]

[30] Howat WJ, Wilson BA. Tissue fixation and the effect of molecular fixatives on downstream staining procedures. Methods. 2014; 70(1):12-9. [DOI:10.1016/j.ymeth.2014.01.022] [PMID] [PMCID]

[31] Benerini Gatta L, Cadei M, Balzarini P, Castriciano S, Paroni R, Verzeletti A, et al. Application of alternative fixatives to formalin in diagnostic pathology. European Journal of Histochemistry. 2012; 56(2):e12. [DOI:10.4081/ejh.2012.12] [PMID] [PMCID]

[32] Hayat MA. Microscopy, immunohistochemistry, and antigen retrieval methods: For light and electron microscopy. Berlin: Springer Science & Business Media; 2002. [Link]

[33] Chung JY, Song JS, Ylaya K, Sears JD, Choi L, Cho H, et al. Histomorphological and molecular assessments of the fixation times comparing formalin and ethanol-based fixatives. The Journal of Histochemistry and Cytochemistry. 2018; 66(2):121-135. [DOI:10.1369/0022155417741467] [PMID] [PMCID]