Int. J. Morphol.,
44(1):350-357, 2026.

Effect of Different Fixatives and Duration of Fixation on the Histological Integrity of Mouse Ovarian Tissue

Efecto de Diferentes Fijadores y Duración de la Fijación Sobre la Integridad Histológica del Tejido Ovárico de Ratón

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SEIDLI, H.; SHIRI, E.; BAKHTIARI, A.; MOHAMMADI, Y. & MIRZAEI, F. Effect of different fixatives and duration of fixation on the histological integrity of mouse ovarian tissue. *Int. J. Morphol., 44(1)*:350-357, 2026.

SUMMARY: Histopathological analysis is critical for diagnosing disease and needs high-quality tissue preservation. Effective fixation is particularly crucial for ovarian tissue to maintain the detailed structures of the ovaries. This study systematically evaluated the effectiveness of three common fixatives, Neutral Buffered Formalin (NBF), Bouin's solution, and Carnoy's solution, on the morphological preservation of mouse ovarian tissue across fixation periods of 12, 24, and 48 hours. Ovarian samples from 27 adult NMRI female mice were fixed, processed, and stained with hematoxylin and eosin (H&E). Morphological preservation and stromal and follicular artifacts were estimated using a scoring system. Carnoy's solution showed superior preservation at 12 hours, showing few artifacts and clearly distinct cellular components, although its quality notably deteriorated with prolonged fixation. Bouin's solution delivered the best results at 24 hours, reaching a balance in cytoplasmic preservation and tissue staining, but it did not perform as well as Carnoy's at 12 hours. NBF consistently produced poor results at all-time intervals, with a gradual decrease in artifacts over time, but inadequate preservation of follicles. Statistical assessment confirmed significant differences in the percentage of artifacts among the various fixatives and durations ($p < 0.05$). These results underscore the necessity of choosing fixatives according to the fixation duration: Carnoy's solution for short-term (≤12 hours), Bouin's for medium-term (24 hours), and NBF for prolonged preservation. This research offers essential insights for improving ovarian tissue fixation protocols, thereby increasing the accuracy of diagnostics and research in reproductive pathology.

KEY WORDS: Ovarian histology; Fixation artifacts; Carnoy's solution; Bouin's solution; Neutral Buffered Formalin; Follicular preservation.

INTRODUCTION

Microscopic examinations and primary observations evaluate cells and tissue structure, may represent changes in tissue cell shape, and reveal functional abnormalities. This method is a standard method for diagnosing various diseases (Musumeci, 2014; Sikandar, 2018).

The ovary, as the main reproductive organ in women, is sensitive to fixation in histopathology due to its mixture of different cells in the follicles and stromal cells, as well as its cyclical function. Inadequate fixation can deform the structure of the follicles, cause shrinkage of the nuclei, and reduce the quality of staining (Garg *et al*., 2017; Pandey *et al*., 2023). According to the National Cancer Institute (NCI), more than 19,000 cases of ovarian cancer occur annually, resulting in more than 13,000 deaths (Levine, 2020). It is not possible to diagnose the stage of neoplasms exclusively through clinical evaluations and imaging tests. Consequently, an accurate histopathological diagnosis is essential for initiating routine treatment. Ovarian tumors typically exhibit a diverse range of clinical, morphological, and histological features (Batool *et al*., 2022).

Tissue fixation is a critical step in histological evaluations and significantly impacts the quality and accuracy of the results. Different types of fixatives present advantages and disadvantages (Van der Loos, 2007). Finding the best fixation of any tissue is essential for achieving optimal specimen evaluation. The optimal

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Received: 2025-10-08 Accepted: 2025-11-29

fixative should exactly preserve morphological features and support histopathological changes in the unbiased diagnosis of diseases (Qidwai *et al*., 2014). Natural buffered formalin (NBF) is a routine fixative which frequently used for fixation because of its accessibility and its ability to maintain tissue morphology (Howat & Wilson, 2014). Bouin's solution, made up of formaldehyde, picric acid, and acetic acid in water, may be more effective than NBF in preserving the structure of different tissues (Howat & Wilson, 2014). Carnoy's fixative, made up of alcohol, acetic acid, formalin, and chloroform, is used to preserve cellular structures, especially in fragile samples (Pereira *et al*., 2015).

A comprehensive and systematic assessment of these effects can refine fixation methods and consequently enhance the quality of histological results for disease diagnosis and scientific research. This study aims to investigate and compare the impacts of NBF, Bouin, and Carnoy's fixatives and fixation duration on the morphology and quality of ovarian histological slides.

MATERIAL AND METHODS

Animal Adaptation and Ethics. This experimental study utilized 27 adult female N/MRI mice, aged 8 weeks, with an average body weight of 30–35 g. Before experimentation, the mice were adapted to laboratory conditions for one week. During this period, they were housed under controlled environmental conditions, including ad libitum access to food and water, adequate ventilation, and a standardized 12-hour light/dark cycle.

The experimental protocol was approved by the Animal Ethics Committee of Hamadan University of Medical Sciences (approval number: IR.UMSHA.AEC.1404.008). All stages of experimental trials were supported in full compliance with international regulations regarding the care and use of laboratory animals (for instance, Directive 2010/63/EU of the European Parliament or the NIH Guide for the Care and Use of Laboratory Animals).

Fixation Groups & Subgroups. After the adaptation phase, the animals were anesthetized via intraperitoneal injection of a ketamine-xylazine (100 mg/kg and 10 mg/kg, respectively). Ovarian samples were dissected from both sides, resulting in 54 samples in total. In summary, the samples were categorized into three groups and preserved in three different fixatives (n:18): NBF, Bouin's solution, and Carnoy's solution. Each group was further divided into three subgroups based on fixation duration of 12, 24, and 48 hours.

Tissue processing. The process of tissue preparation, which includes dehydration, paraffin infiltration, paraffin embedding, and blocking, was performed using a tissue processor, and after processing, sectioning was done. In summary, ovarian samples were exposed to progressively ascending concentrations of ethanol (70 %, 90 %, and 100 %) for one hour each, with two rounds conducted for each concentration. Following this, the sections were placed in xylene twice, each lasting 15 minutes, and then subjected to two rounds of liquid paraffin embedding, each lasting one hour, before proceeding to blocking. The resultant blocks were sectioned into 5 μm in thickness, and these sections were positioned on glass slides. Finally, the slides will be stained using hematoxylin and eosin (Azmoonfar *et al*., 2024).

Hematoxylin and eosin staining (H&E). After deparaffinizing the sections in xylene, the rehydration was done through a series of descending alcohols for 15 minutes each. After rinsing in distilled water, the nuclei were stained with Harris' hematoxylin for 8 minutes, followed by differentiation using acid alcohol. Eosin Y was used for cytoplasmic staining for 3 minutes. Ultimately, the sections were dehydrated through ascending alcohol, cleared in xylene, and mounted with coverslip using Entellan. H&E-stained slides were then analyzed under a light microscope for histopathological assessment (Mirzaei *et al*., 2025).

Morphological Integrity Assessment for Ovarian Follicles. To evaluate the integrity of ovarian follicles, a standardized scoring system was used. Two independent, blinded observers directed the scoring. For each follicle, all stage-specific criteria were assessed, with artifacts. Primordial follicles were evaluated for intact flattened granulosa layers; primary follicles for the stratification of cuboidal granulosa (>1 layer), theca definition, and zona pellucida; secondary follicles for granulosa layering, early antrum formation, separation of theca interna/externa, cumulus oophorus, and central positioning of the oocyte; tertiary follicles for uniform membrana granulosa, thecal vascularization, and clear definition of the cumulus stalk; and Graafian follicles for free-floating cumulus complexes, organization of mural granulosa, and vascularized theca interna. The integrity of the corpus luteum was scored if it was present.

Each criterion received a score from 0 to 3 based on the quality of preservation: 0 (Poor) signified severe distortion (unidentifiable structures, nuclear pyknosis, or tearing); 1 (Fair) represented moderate distortion (partial shrinkage/blurred boundaries); 2 (Good) indicated minimal artifacts (identifiable structures); and 3 (Excellent) reflected

optimal preservation (sharp cellular/nuclear boundaries, with no shrinkage/vacuolization).

Histological artifact assessment. The evaluations consisted of assessing the artifact of follicles and the stroma. Areas categorized as artifacts were measured using ImageJ and calculated after converting images to 8 bits and Image thresholding. All assessments were conducted blindly.

In the evaluation of follicle integrity, which refers to the degree of transparent regions caused by cellular shrinkage due to fixation observed within the follicle, artifact areas were pinpointed, and their overall area within the follicle was calculated as a percentage of the total follicular area. The assessment of stromal integrity involved calculating the area of artifacts within the stroma as a percentage of the total stromal area (Adeniran *et al*., 2021).

Statistical analyses. All statistical analyses were conducted using SPSS statistical software version 22. The data are expressed as mean ± SEM, and statistical significance was set at $p< 0.05$. One-way ANOVA and Tukey post hoc test will be used to compare between groups.

RESULTS

Ovary morphological integrity and staining quality.

As shown Table I, qualitative assessment of the slides revealed a marked variation in morphological integrity and staining quality among the different fixatives and time duration of fixation. As shown in Figures 1 to 3, Carnoy's fixative, with a 12-hour fixation period, yielded the most superior staining results, characterized by distinct cellular boundaries, absence of shrinkage, vacuolization and nuclear pyknosis and tissue tearing, well-defined circular nuclei, and prominent nucleoli. However, extending the fixation time to 24 and 48 hours with Carnoy's resulted in a significant decline in staining quality, evidenced by cellular shrinkage to the point where nuclei appeared as condensed, dark masses. Thus, Carnoy's fixative appears optimal for short-term (≤12 hours) ovary fixation.

Bouin’s solution showed the best fixation results after 24 hours; both shorter and longer fixation times resulted in a significant decline in staining quality. While 24 hours was the ideal time, the quality of staining obtained with Bowen's fixative did not reach the standard achieved with Carnoy's fixative after 12 hours.

Natural buffered formalin was found to be inadequate for mouse ovary fixation at 12- and 24-hour durations. Longer fixation durations decreased artifacts, but the alterations in staining quality over time were minimal. Although a reduction in artifacts and a slight enhancement in staining were observed over time, natural buffered formalin was deemed unsuitable for use in mouse ovarian tissue preparation.

Table I. Comprehensive ovarian tissue morphology scoring.

Fixative/duration		Nuclear Detail	Cytoplasmic Integrity	Stromal Preservation	Artifact Severity	total Score
NBF	12 h	1 (Pyknotic/clump)	2 (Diffuse boundaries)	1 (Moderate	1 (Severe)	**5**
	24 h	1 (Partially obscured)	3 (Mild vacuolization)	3 (Mild tearing)	3 (Moderate)	**10**
	48 h	2 (Minimal enhancement)	4 (Residual vacuolization)	3 (Inconsistent)	3 (Moderate)	**12**
Carnoy's solution	12 h	5 (Circular nuclei, prominent nucleoli)	5 (Distinct boundaries, no vacuolization)	5 (Intact architecture)	5 (None)	**20**
	24 h	2 (Condensed, dark nuclei)	2 (Moderate shrinkage)	3 (Partial	2 (Minimal)	**9**
	48 h	1 (Pyknotic nuclei)	1 (Severe shrinkage)	1 (Fragmented)	2 (Minimal)	**5**
Bouin's solution	12 h	2 (Partially defined)	3 (Mild vacuolization)	3 (Mild edema)	3 (Moderate)	**3**
	24 h	4 (Well-defined nuclei)	4 (Minor irregularities)	4 (Mild	4 (Minimal)	**16**
	48 h	3 (Faded chromatin)	2 (Shrinkage/vacuolization)	2 (Disorganize)	2 (Significant)	**2**

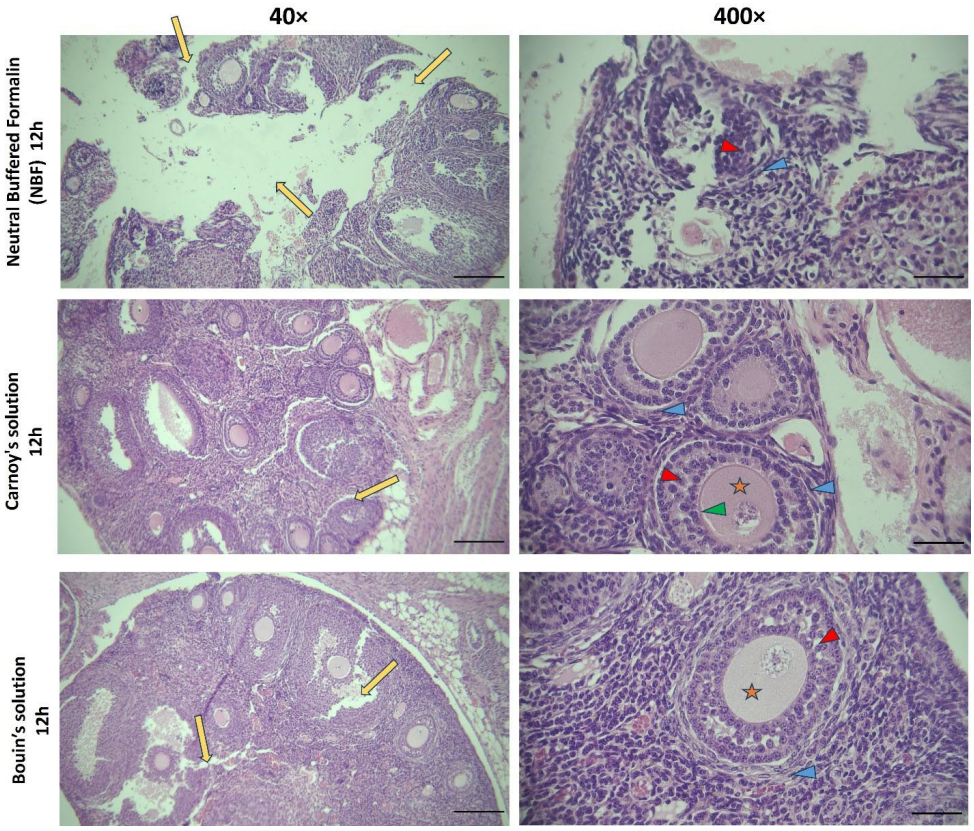

Fig. 1. Representative images of haemotoxylin & eosin stained mouse ovarian sections prepared with different fixatives (10 % neutral buffered formalin (NBF), Carnoy's and Bouin's solution for 12 h, ×40 and 400× magnification). Yellow color: Artifact/ Blue color: Outer and inner theca/ Red color: Zona granulosa / Green color: Zona pellucida / Orange color: Oocyte.

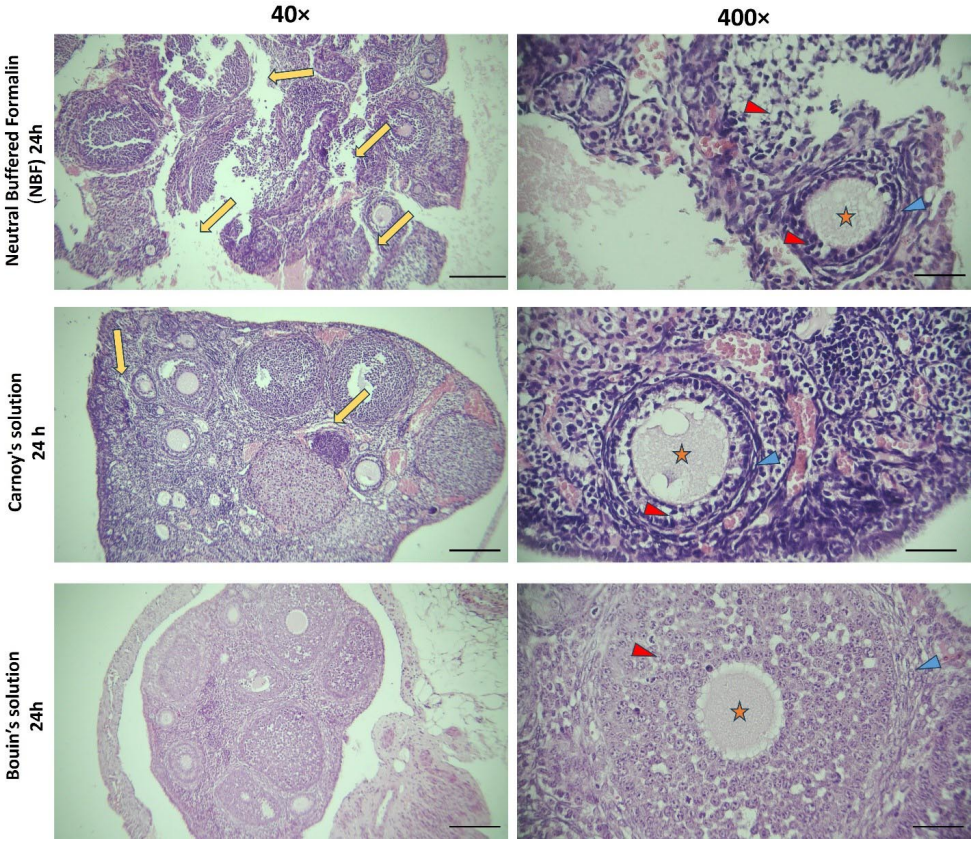

Fig. 2. Representative images of haemotoxylin & eosin stained mouse ovarian sections prepared with different fixatives (10 % neutral buffered formalin (NBF), Carnoy's and Bouin's solution for 24 h, ×40 and 400× magnification). Yellow color: Artifact/ Blue color: Outer and inner theca/ Red color: Zona granulosa / Green color: Zona pellucida / Orange color: Oocyte.

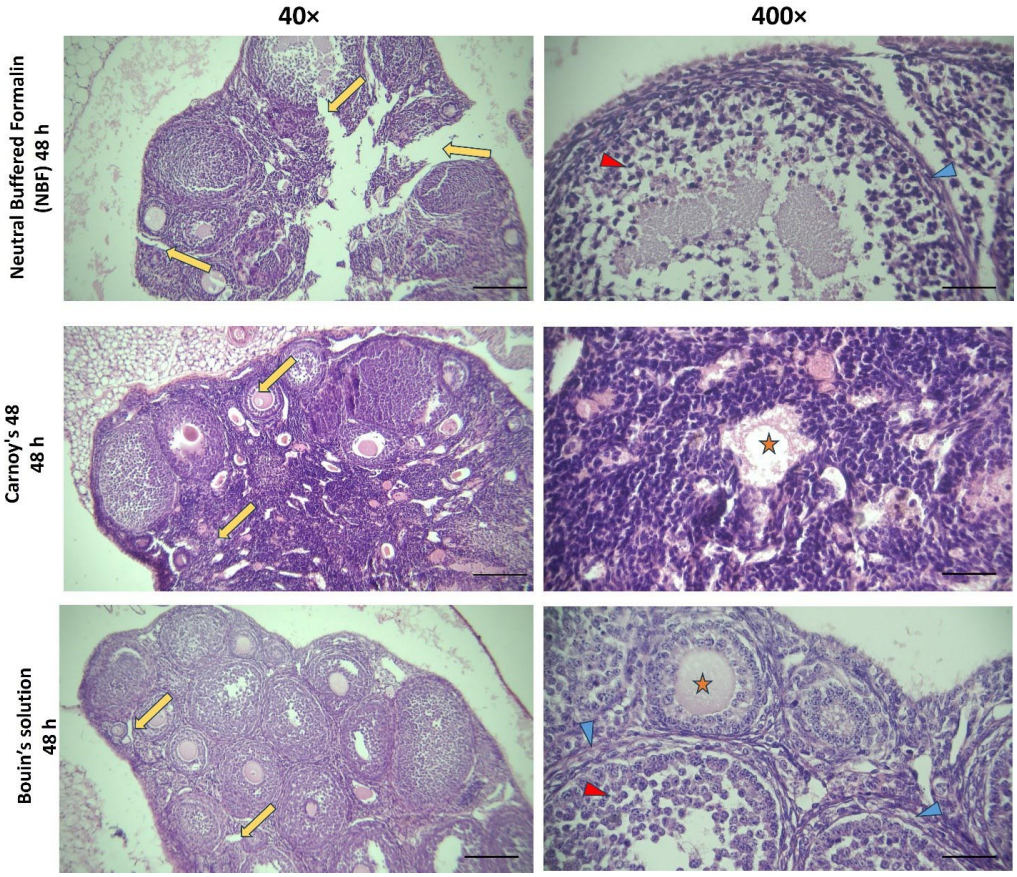

Fig. 3. Representative images of haemotoxylin & eosin stained mouse ovarian sections prepared with different fixatives (10 % neutral buffered formalin (NBF), Carnoy's and Bouin's solution for 48 h, ×40 and 400× magnification). Yellow color: Artifact/ Blue color: Outer and inner theca/ Red color: Zona granulosa / Green color: Zona pellucida / Orange color: Oocyte.

Morphological integrity of follicles.

The structural integrity of ovarian follicles showed considerable variations depending on the choice of fixative and the duration of fixation (Table II). Evaluation of the samples utilized according to a scoring system (0 = Poor, 3 = Excellent) to examine how well the stage-specific architectural features were preserved for primordial, primary, secondary, tertiary/antral, and Graafian follicles, as well as the corpus luteum. Important assessment criteria included the integrity of the layers (e.g., granulosa cell layer in primordial/primary follicles, distinct theca layers in secondary and beyond, and the formation of antrum in tertiary and Graafian follicles), contact with the oocyte, and the overall structure of the follicle. Fixation using NBF consistently produced poor outcomes (scores 0-1.0) across all follicular stages and time points (12h, 24h, 48h), exhibiting substantial architectural distortion that obstructed clear identification of the follicle stages.

Carnoy's solution demonstrated the best preservation when used for a fixation period of 12 hours (average score: 3.0), sustaining excellent structural integrity and providing a clear distinction of stage-specific characteristics for all types of follicles. However, its efficacy sharply decreased at 24 and 48 hours (score: 1.0), resulting in structural collapse and the

Table II. Morphological integrity scoring of follicles.

Follicle Stage	NBF (12h)	NBF (24h)	NBF (24h)	Carnoy's (12h)	Carnoy's (24h)	Carnoy's (48h)	Bouin's (12h)	Bouin's (24h)	Bouin's (48h)
Primordial	1	0	1	3	3	1	1	3	3
Primary	1	2	2	3	2	1	1	3	2
Secondary	0	2	2	3	2	1	1	2	2
Tertiary	0	1	1	3	2	1	1	2	2
Graafian	0	0	0	3	1	2	1	2	1
Corpus Luteum	1	0	0	3	2	2	1	2	2
Total score	**3**	**5**	**6**	**18**	**12**	**7**	**6**	**14**	**12**

loss of defining features. Bouin's solution yielded the best results at 24 hours (average score: 2.0), enabling the identification of follicular structures across different stages, although with less distinct separation of layers in comparison to Carnoy's solution at 12 hours. The performance of Bouin's was diminished at both the 12 and 48-hour marks (score: 1.0), leading to architectural blurring and loss of features. These results underscore the vital significance of selecting the appropriate fixative and ensuring precise fixation duration for the reliable histological evaluation of ovarian follicle staging based on their architecture.

Ovarian tissue artifact considerations.

Analysis of the slides and the calculation of artifact percentages reveal a notable impact of various fixatives on the levels of stromal and follicular artifacts.

The percentage of stromal artifacts after 12 h of fixation using NBF was approximately 30 %. This percentage reduced over time, with a significant reduction in stromal artifacts observed at 24 h when compared to the 12 h fixation ($p \leq 0.01$). Furthermore, the decrease in the percentage of stromal artifacts at 48 h was also significant when analyzed against the 24 h fixation ($p \leq 0.05$) (Fig. 4A).

The percentage of stromal artifacts in Carnoy's solution and Bouin's solution fixatives was below 5 %. A shorter fixation duration (12 h) was the optimum time for Carnoy's solution, while increasing the fixation duration significantly increased the artifact compared to 12 h fixation (Fig. 4B).

About Bouin's solution, the 24 h fixation significantly reduced the percentage of stromal artifacts compared to 12 h fixation, and 48 h (Fig. 4C).

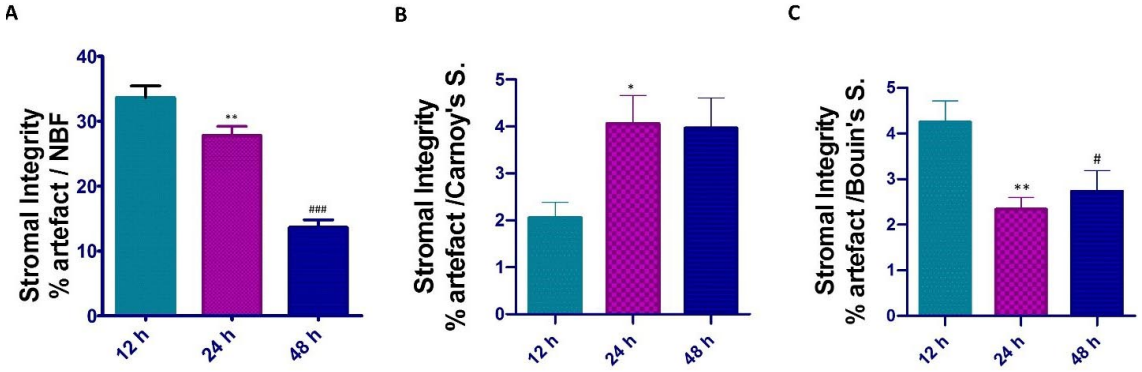

Fig. 4. Histological assessment of stromal integrity in fixed mouse ovarian sections using H&E staining. Ovarian sections were fixed in NBF(A), Carnoy's (B), and Bouin's solution (C) for 12 h, 24 h, and 48h. The percentage of clear space in stroma was measured to determine stromal integrity. The data is shown as mean ± SD, with ** $p<0.01$ and * $p<0.05$ compared with NBF group. ###$p<0.001$ and # $p<0.05$ compared with Carnoy's solution group.

The percentage of follicular artifacts exhibited a similar pattern to that of stromal artifact; however, in the case of Carnoy's and Bouin's fixatives, the variation in fixation duration did not lead to notable differences in follicular artifact, and only extending the fixation period to 24 h and 48 h of NBF resulted in a significant reduction in follicular artifact (Fig. 5A,B,C).

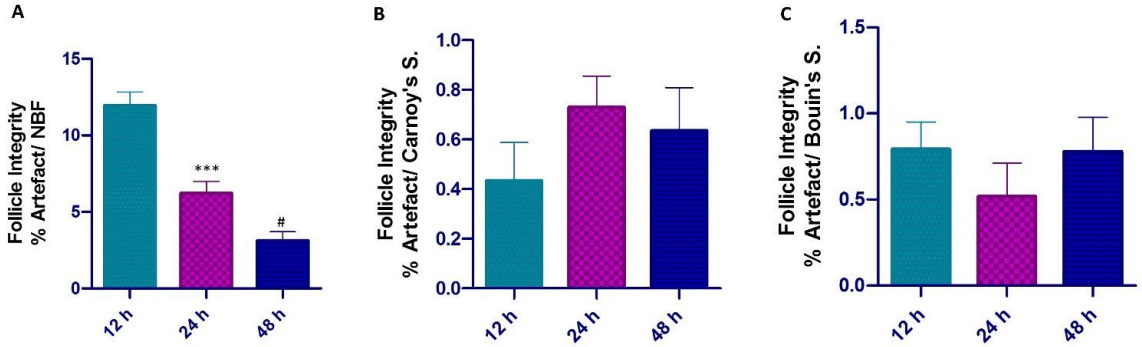

Fig. 5. Histological assessment of follicular integrity in fixed mouse ovarian sections using H&E staining. Ovarian sections were fixed in NBF(A), Carnoy's(B) and Bouin's solution(C) for 12 h, 24 h, and 48h. The percentage of clear space in follicles was measured to determine stromal integrity. The data is shown as mean ± SD, with *** $p<0.001$ compared with NBF group. # <0.05 compared with carnoy's solution group.

DISCUSSION

The present study evaluated the effects of three commonly used fixatives in ovarian fixation, neutral buffered formalin (NBF), Carnoy's solution, and Bowen's solution, on the histological preservation of mouse ovarian tissue at different fixation periods (12, 24, and 48 h).

Our findings demonstrated the critical role of Carnoy's solution as a fast-acting fixative. The excellent efficiency of Carnoy's solution for short-term fixation can be attributed to its rapid penetration, which avoids autolysis, and its dehydration properties reduce contractile artifacts. This fixative is composed of ethanol, chloroform, and acetic acid. Ethanol, one of its main components, acts as a coagulant fixative that rapidly denatures proteins and precipitates nucleic acids, thereby preserving cellular structure (Kiernan, 2015). Chloroform, another main component, improves lipid solubility, enhancing the stabilization of cell membranes and cytoplasmic components (Carson & Cappellano, 2009). Additionally, acetic acid preserves chromatin by preventing the excessive hardening of nuclear elements, thus keeping the crucial DNA-protein interactions (Impraim *et al*., 1987). Previous studies on reproductive organs have similarly reported that Carnoy's solution is ideal for small, delicate tissues requiring quick fixation (Pereira *et al*., 2015). One research has indicated that Carnoy's solution is more effective than formalin-based fixatives in maintaining the integrity of small biopsy samples and fine-needle aspirates, particularly under circumstances where prompt processing is essential (Dapson, 1993). However, a limitation of Carnoy's solution, as observed in our 24 and 48-hour samples, is that prolonged fixation (>12 hours) can lead to excessive tissue hardening and brittleness. This aligns with findings from Johnson *et al*. (2020), who showed that ethanol-based fixatives can cause over-dehydration and structural distortion over time (Rowley *et al*., 2020).

Bowen's solution showed the highest cytoplasmic preservation, the least shrinkage, and the best quality of stromal staining at 24 hours. The gradual and effective penetration into the tissue due to the special formulation of Bowen's solution, which is a combination of picric acid, formaldehyde, and acetic acid, allows for optimal fixation, especially at medium fixation times. Unlike fast-acting fixatives such as Carnoy's solution, Bowen's solution offers a balance between speed of fixation and preservation of structure during medium fixation times, reducing tissue distortion while providing adequate protein crosslinking (Bancroft & Gamble, 2008). One significant benefit of Bouin's solution is the function of picric acid, which serves both as a fixative and a mordant. Picric acid improves the retention of glycogen and cytoplasmic proteins by creating soluble complexes with basic amino acids, thus preventing their loss during processing. This characteristic is particularly important in fixing ovarian tissue, where it is vital to preserve the integrity of follicular cells and oocytes for reliable histological and immunohistochemical evaluations (Impraim *et al*., 1987). In addition, the picric acid in Bowen's solution enhances the visibility of collagen fibers by increasing contrast in Masson's and Mallory's trichrome stains, making this solution suitable for examining ovarian fibrosis or extracellular matrix (ECM) remodeling in conditions such as polycystic ovary syndrome (PCOS) or post-ovulatory scarring. This fixative also increases compatibility with elastin dyes, facilitating the exploration of vascular and stromal adaptations in ovarian tissues (Briley *et al*., 2016; Carson & Cappellano, 2020). Despite its benefits, Bouin's solution, due to its acidic pH, does have the risk of nucleic acid degradation, which may limit its usage in molecular studies that require intact DNA and RNA (Sarma *et al*., 2020).

NBF, the gold standard in histopathology, provided moderate preservation across all time points but was consistently outperformed by Carnoy's and Bouin's solutions at their respective optimal durations. While NBF's buffering capacity prevents excessive acidity and maintains general tissue architecture, its slow penetration rate may explain why it was less effective in preserving fine ovarian structures compared to the other fixatives.

Previous research has noted that NBF can cause mild nuclear shrinkage and reduced antigenicity in immunohistochemical studies, which may be a limitation if further molecular analyses are planned (Taha *et al*., 2024). Nevertheless, NBF remains a reliable choice for long-term storage, as it does not induce the extreme brittleness seen with Carnoy's or Bouin's solutions after extended fixation.

CONCLUSION

Our results indicate that there is not a single fixative that is ideal for ovarian tissue preservation at every time point. Researchers should choose fixatives according to the desired fixation time and subsequent applications. Carnoy's solution is most effective for quick fixation (24 hours), Bouin's solution is superior for moderate time frames (48 hours), while NBF is a reliable option for long-term storage.

ACKNOWLEDGMENTS. Special thanks to the Hamadan University of Medical Sciences, Hamadan, Iran.

SEIDLI, H.; SHIRI, E.; BAKHTIARI, A.; MOHAMMADI, Y. & MIRZAEI, F. Efecto de diferentes fijadores y duración de la fijación sobre la integridad histológica del tejido ovárico de ratón. *Int. J. Morphol., 44(1)*:350-357, 2026.

RESUMEN: El análisis histopatológico es fundamental para el diagnóstico de enfermedades y requiere una conservación tisular de alta calidad. Una fijación efectiva es particularmente crucial en el caso del tejido ovárico para mantener sus estructuras detalladas. Este estudio evaluó sistemáticamente la eficacia de tres fijadores comunes —Formol Amortiguado Neutro (FAN), la solución de Bouin y la solución de Carnoy— en la preservación morfológica del tejido ovárico de ratón, considerando periodos de fijación de 12, 24 y 48 horas. Muestras ováricas de 27 ratones hembras adultas de la cepa NMRI fueron fijadas, procesadas y teñidas con hematoxilina y eosina (H&E). La preservación morfológica y los artefactos estromales y foliculares se evaluaron mediante un sistema de puntuación. La solución de Carnoy demostró una preservación superior a las 12 horas, presentando escasos artefactos y componentes celulares claramente distinguibles, aunque su calidad se deterioró notablemente con la fijación prolongada. La solución de Bouin produjo los mejores resultados a las 24 horas, alcanzando un equilibrio entre la preservación citoplasmática y la tinción tisular, pero con un resultado inferior a la solución de Carnoy a las 12 horas. El FAN produjo resultados consistentemente deficientes en todos los intervalos temporales, con una disminución gradual de artefactos con el tiempo, pero con una preservación inadecuada de los folículos. La evaluación estadística confirmó diferencias significativas en el porcentaje de artefactos entre los distintos fijadores y duraciones ($p < 0,05$). Estos resultados subrayan la necesidad de seleccionar el fijador de acuerdo con la duración de la fijación: la solución de Carnoy para fijaciones a corto plazo (≤12 horas), la de Bouin para mediano plazo (24 horas) y FAN para una preservación prolongada. Esta investigación ofrece perspectivas esenciales para optimizar los protocolos de fijación de tejido ovárico, incrementando así la precisión del diagnóstico y la investigación en patología reproductiva.

PALABRAS CLAVE: Histología ovárica; Artefactos de fijación; Solución de Carnoy; Solución de Bouin; Formalina tamponada neutra; Preservación folicular.

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