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Research Article

# Assessment if Time-Alteration in the Fixative Procedure of Five Types of Fixatives on the Output Precision and Digital Analysis of Tissue Architecture Images

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**Abstract** | The necessity of obtaining high-quality samples supplying correct information in histological and histopathological examinations is evident, also anyone with an interest in these fixatives needs to know their characteristics and how they work, which will greatly help them in choosing the appropriate fixatives for particular uses. This study aimed to assess the influences of five common fixatives, which include 10% NBF, Bouin's, Carnoy's, Zenker's, and Clarke's fixatives, on liver tissue at five distinct fixation durations, inclusive of 6, 12, 24, 48, and 72 hours. Routine histological preparations included H and E stains to evaluate the quality and structure of the tissues. Competitive results exhibited that amongst all the fixatives, Formalin and Zenker,s had higher effectiveness for short-term fixation (6,12 and 24 hrs). For Bouin's fluid, the tissues were far more preferable at 12- and 24-hours fixation, while Carnoy's and Clarke's solutions were preferred at 6- and 12-hours fixation. The efficiency of these fixatives was also poor after forty-eight hours, as the tissue became hard and crumbly. This study has made practical suggestions about the best use of each of the fixation's types and fixative time, generally all results were needed to emphasize the choice of suitable fixatives and fixation periods depending on the histological analysis relevant to various samples.

**Keywords** | Buffered formalin (10%), Bouin's fluid, Zenker,s solution, Carony,s solution, Clarke'ssolution, fixation times, Image J

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## INTRODUCTION

Chemical fixation involves the use of organic or inorganic solutions to achieve proper histological preservation. There are two main types of chemical fixatives: coagulant and non-coagulant (cross-linking) fixatives. Both organic and inorganic solutions may coagulate proteins and render them insoluble (Isam, *et al*., 2001; Titford, 2001). Incorrect or inadequate tissue fixation causes issues with tissue processing, microtome, staining, and conducting ancillary research (Fox *et al*., 1985). Chemical fixation is critical for histological and histopathological examinations because it maintains tissue integrity by stabilizing cellular and extracellular components. This method involves utilizing organic or inorganic solutions to coagulate proteins, rendering them insoluble and preserving tissue structure as similar to the in vivo state as possible (Isam, *et al*., 2001; Titford, 2001). Despite the crucial role of fixation, no universal fixative has been established that is suitable for all histological applications, necessitating a better understanding of the

properties and mechanisms of diverse fixatives (Fox *et al.*, 1985; Bancroft and Gamble, 2019). The fixation aims to maintain cellular and extracellular components as close to the in vivo tissue structure as possible. Fixatives are normally classified as coagulants or non-coagulants (cross-linking agents). Both types seek to protect tissue against microbial invasion, inhibit enzymatic and metabolic activities, and prevent autolysis and putrefaction (Park *et al.*, 2016). However, all fixation procedures produce abnormalities such as color changes, stiffness, swelling, and shrinking, which might impede future tissue processing and staining (Rai *et al.*, 2016). The tissue's macromolecular structure is balanced by forming cross-links between its constituent parts during the fixation process, which also kills the bacteria present in the tissues and stops autolysis. Fixing a specimen without creating deformation is difficult because all fixation methods produce artifacts such as color change, hardening, swelling, and shrinking. Furthermore, fixation and fixative selection may be crucial throughout the tissue processing stages, especially for cross-sectional sampling and staining procedures (Bancroft and Gamble 2019). Fixatives can often be divided into four kinds according to how they function. Aldehydes, oxidizing agents, alcohol-based fixatives, and among them is the metallic group of fixatives (Matsuda *et al.*, 2011). In the fixation process, the tissue is shielded from microbes, all enzymatic and other metabolic processes are rapidly reduced, decomposition, putrefaction, and autolysis are prevented, and the tissue is hardened and strengthened to the greatest extent feasible to reduce damage (Park, *et al.*, 2016). The tissue or cell's semi-fluid condition is converted throughout this process into rigid specimens where their original states are maintained (Rai, *et al.*, 2016). This study may be a helpful resource for researchers interested in human and model organism histology, quality, consistency, sectioning, staining, and histological visualization. (Bancroft and Gamble, 2019). However, each fixative and tissue processing methodology preserve some molecular and macromolecular characteristics of the tissue better than others. Currently, no universal or ideal fixative has been established; fixatives are chosen based on the final product required to demonstrate a specific aspect of a tissue. Our goal is to perfect fixation methods and staining procedures to create optimal histology slides and photos that clearly illustrate typical challenges by identifying and comparing the impact of different fixative chemicals and fixative times on histological structure in the liver and muscle and noticing the specific changes in cell shapes and stain density, utilizing macroscopic measurements and computer analysis with the Image J program.

### Aims and hypotheses

The primary goal of this study is to examine the efficacy of various fixatives and fixing times in maintaining liver tissue integrity and quality. Our hypothesis is that: Short-term fixation (up to 24 hours) using NBF and Zenker's fixatives results in higher histological quality than prolonged fixation intervals. Bouin's fluid will produce the best results for 12- and 24-hour fixation durations. Carnoy's and Clarke's fixatives are most successful for short-term fixation (6 and 12 hours), but their efficiency degrades with longer fixation intervals. By addressing these possibilities, we hope to improve our understanding of fixative performance and provide evidence-based suggestions for histology procedures.

## MATERIALS AND METHODS

In total, 18 rat liver samples that were solely taken from healthy animals were used in this investigation.

### Sample Preparation and Fixation Protocols

Adult rats' liver tissue specimens were taken, each measuring around 0.5 cm in length. Sample Preparation and Fixation Protocols. Adult rats' liver tissue specimens were taken, each measuring around 0.5 cm in length. The samples were immediately immersed and distributed among the following fixatives, five samples for each fixative:10% neutral buffered formaldehyde (NBF), Bouin's, Carnoy's, Zenker's, and Clarke's solutions. The fixatives were made following conventional protocols: 10% neutral buffered formaldehyde (NBF) requires 100 mL of 37–40% formaldehyde, 900 mL of distilled water, and phosphate buffer. Bouin's fluid contains picric acid, formaldehyde, and acetic acid. Carnoy's solution contains ethanol, chloroform, and acetic acid. Zenker's solution contains mercuric chloride, potassium dichromate, sodium sulfate, and glacial acetic acid. Clarke's solution contains ethanol and acetic acid. The tissues were fixed for 6, 12, 24, 48, and 72 hours, with each time point consisting of three tissue specimens per fixative. Parallel processing of unfixed tissues was used as a control method to ensure comparable results. Specimens of tissue measuring about 0.5 cm in length from each sample were represented by six duplicate parts, and each component was placed in its container with one of the fixatives. In our investigation, we used five fixatives: 10% neutral buffered formaldehyde (NBF), Bouin's, Carony's, Zinker, and Clarke's fixatives (Table 1). Each sample received a container of the suitable form and size, as well as a suitable quantity of fixative in well-sealed and precisely labeled containers with a tissue volume to fixative volume ratio of more than 1:20. Histological sectioning of liver specimens was done at four separate times: 6, 12, 24, 48, and 72 hours after fixation. The storage was done in the designated laboratory at room temperature between 22 and 26 °C. Standard histological processing protocols were followed for the remainder of the other processing. In summary, fixation was followed by dehydration (which was accomplished by increasing the concentration of alcohol) and tissue-clearing incubation in xylene. Following that, the tissue blocks were impregnat

Table 1. Summarizes the tissue fixatives employed in the study.

| Fixative fluid | Composition | pH | Mechanism of action |
|---|---|---|---|
| NBF (10%) | Formaldehyde, methanol, sodium phosphate monobasic, sodium phosphate dibasic, and deionized water | 7.0-7.4 | Cross-linking |
| Bouin's Fluid | Formaldehyde, acetic acid, and picric acid | < 2 | Cross-linking/ denaturing |
| Carony,s Solution | Ethyl alcohol, chloroform, acetic acid glacial | - | denaturing |
| Zinker Solution | Distal water, Mercuric chloride, Potassium dichromate, and Sodium sulfate | | Cross-linking/ denaturing |
| Clarke,s Solution | Absolute alcohol, Glacial acetic acid | | denaturing |

NBF = 10% neutral buffered formalin; pH = hydrogen potential, (Bancroft and Gamble, 2019).

ed with paraffin. Finally, tissue slices measuring 5 microns thick from each block were cut and stained on a glass slide with hematoxylin and eosin (Bancroft and Gamble, 2019). After fixation, the tissue samples were processed according to normal histological methods. They were dehydrated in a graded ethanol series, then cleaned with xylene and embedded in paraffin wax. A rotary microtome was used to cut sections measuring 5 µm thick. For a routine histological investigation, the slices were stained with hematoxylin and eosin (H and E).

### Hematoxylin staining

Sections were soaked in Harris hematoxylin for 5 minutes, then rinsed in tap water, differentiated in 1% acid alcohol, and blued using Scott's tap water substitution. After hematoxylin staining, slices were stained with eosin for 2 minutes, then dehydrated, cleared, and mounted. The quality of the histological sections was evaluated using Image J software. The evaluation criteria included tissue shape, staining intensity, and the preservation of both cellular and extracellular components. Each segment was graded on a scale of 1 to 5 for these characteristics, with 1 indicating poor quality and 5 signifying excellent. A histologist evaluated representative tissue sections stained with hematoxylin and eosin under the microscope. By using the Image J program for quantitative and qualitative estimation of pigments and cellular morphology of tissue, no abnormal state was identified in any of the samples tested.

### Scoring

The following categories were used to statistically evaluate the data:

1. Sectioning standards: It was evaluated based on the presence or absence of two factors: being hard to cut and crumbling.
2. The specialists in histology observers scored the slides under a light microscope at 10 and 40X magnification on a scale of 0 to 5 (with 3 being the minimum score required for an acceptable outcome). The factors they used to evaluate the staining were nuclear and cytoplasmic staining.
3. Microscopic information: It was assessed using a light microscope at a magnification of 40X, scoring the slides from 0 to 5 (score 3 was maintained as the minimum score for an acceptable outcome), and using the Image J program to measure nuclear and cytoplasmic shrinkage, swelling, and distortion. A score (0–5) represents the following terms: (0 very poor), (1 poor), (2 average), (3 good), (4 very good), and (5 excellent). The Department of Histology and Anatomy at the University of Basrah's Veterinary Medicine College authorized the study.

The data were evaluated statistically to compare the efficiency of each fixative over time. The mean scores for each fixative and time point were obtained, and significant differences between groups were determined using ANOVA and Tukey's Honest Significant Difference (HSD) test. A p-value of <0.05 indicated statistical significance.

## RESULTS

The practical value of our findings stems from their immediate applicability to improved histology techniques. This study's identification of the most effective fixatives and optimal fixation periods provides important advice for selecting acceptable fixation protocols customized to specific histology requirements. The findings show that 10% neutral buffered formaldehyde (NBF) and Zenker's fixatives are particularly good for short-term fixation (up to 24 hours), which is consistent with the study's goals of maximizing histological quality over different periods.

A total of 45 tissue samples were divided and fixed with NBF, Bouin's, Carony,s, Zenker,s, and Clark's fixatives. Three replicates were included for each fixative, and each replicate included three samples and a comparison of the quality of the histological section and turnaround time.

### Hard To Cut

As the time interval increases (76 hr.), in all fixatives (NBF, Boun,s, Caronoy,s, Zanker,s, and Clarke's), hard-to cut and crumbling frequency of specimens increases. The NBF and Zanker,s fixative showed the best cutting during 6, 12, and


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**Table 2.** The overall histological score shows the staining score and histological deformity (shrinkage, swelling, and distortion/dissolution) of the nuclear and cytoplasmic as a comparison of various fixatives and intervals of time with alteration of procedure for standard fixation criteria.

| (Average Score) Parameters | | Fixatives | 6hrs. | | 12hrs. | | 24hrs. | | 48hrs. | | 72hrs. | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Nuclear | Cytoplasmic | Nuclear | Cytoplasmic | Nuclear | Cytoplasmic | Nuclear | Cytoplasmic | Nuclear | Cytoplasmic |
| **Staining** | | NBF (10%) | 3 | 3 | 4 | 4 | 4 | 4 | 3 | 3 | 2 | 1 |
| | | Bouin | 2 | 2 | 2 | 3 | 4 | 3 | 2 | 1 | - | - |
| | | Carony | 3 | 3 | 2 | 2 | 1 | 1 | 1 | 2 | - | - |
| | | Zenker | 3 | 3 | 4 | 4 | 4 | 4 | 3 | 3 | 1 | 1 |
| | | Clarke | 3 | 2 | 2 | 3 | 2 | 3 | 2 | 2 | 1 | 1 |
| Histological deformity | NBF (10%) | Shrinkage | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 2 | 3 |
| | | Swelling | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | Distortion/dissolution | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 2 | 2 |
| | Bouin's | Shrinkage | 0 | 0 | 1 | 1 | 2 | 1 | 2 | 1 | 3 | 1 |
| | | Swelling | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | Distortion/dissolution | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 2 | 1 | 3 |
| | Carnoy,s | Shrinkage | 0 | 0 | 0 | 0 | 1 | 0 | 2 | 1 | 2 | 1 |
| | | Swelling | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | Distortion/dissolution | 0 | 0 | 0 | 0 | 2 | 1 | 2 | 2 | 2 | 2 |
| Zenker,s | | Shrinkage | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 2 | 2 | 3 |
| | | Swelling | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 |
| | | Distortion/dissolution | 0 | 0 | 0 | 0 | 1 | 1 | 2 | 2 | 2 | 2 |
| Clarke,s | | Shrinkage | 0 | 0 | 0 | 0 | 1 | 1 | 2 | 1 | 2 | 3 |
| | | Swelling | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 1 |
| | | Distortion/dissolution | 0 | 0 | 0 | 0 | 1 | 1 | 2 | 2 | 3 | 3 |

Staining: The score (0-5) denotes: (0) poorer, (1) poor, (2) average, (3) good, (4) very good, and (5) excellent, while the histological deformity that: The score (0-5) denotes: (0) very poor, (1) poor, (2) average, (3) high, (4) sharp, and (5) very sharp, as well as (-) not results.

24 hours, while after 48 hours onwards, the specimens became hard to cut. Bouin's fluid showed the best cutting during (12 and 24 hr), whereas from 48 and onwards specimens in Bouin's fluid became hard to cut during sectioning by microtome, which was statistically significant. While Carony,s and Clarke's solutions recorded the best cutting during 6 and 12 hours, they did not show good results, as hardness and cracking of the samples were observed during cutting during the other specified experimental periods (Figure. 1-a).

## Crumbling

The specimens in Clarke's fixative showed maximum crumbling at 48 and 76 hours ($p<0.05$), whereas specimens in NBF and Bouin's fluid showed minimum crumbling at 24 and 12 hours, respectively. As time increased (48 and 76 hours), the maximum frequency of crumbling ($p<0.05$) was observed in specimens in NBF and Bouin's fluid (Figure. 1-b).

## Nuclear stains

It was observed that the sample of tissues in 10% formaldehyde (NBF) and Zenker,s fixatives displayed good staining and normal cellular structure results at 6, 12, and 24 hours. This deterioration in pigmentation quality was evident at 48 and 72 hours, whereas those in Bouin's fluid showed excellent results (stinging and histological deformity) at 12 and 24 hours, compared to the other time intervals. While Carolyn's and Clarke's fixative exhibited positive results at 12 hours, the quality dropped (Table 2).

## Cytoplasmic stains

Samples of the liver in 10% formaldehyde (NBF) and Zenker,s solution displayed good staining and normal cellular structure results at 6, 12, and 24 hours and decreased in quality thereafter at 48 and 72 hours. Those in Bouin's fluid showed excellent results at 12, and 24 hours, while this de-

**Table 3.** Quantification by use of the image J analysis program of the cellular nuclear and cytoplasmic areas of the main liver section area as a comparison of various fixatives and intervals of time with modification of the methodology for conventional fixation time criterion.

| Fixative type | (Area%) of total Area (784641 pixel) | | | | | |
|---|---|---|---|---|---|---|
| | **Cell structure area (%)** | **6hr.** | **12hr.** | **24hr.** | **48hr.** | **72hr.** |
| NBF | Nuclear | 10.31 ± 0.44a | 15.29 ± 1.21b | 16.63 ± 1.87b | 12.33 ± 0.99c | 10.87 ± 0.59ca |
| | Cytoplasmic | 42.98 ± 4.81a | 60.45 ± 3.98b* | 54.65 ± 4.97bc | 48.73 ± 4.84c | 45.76 ± 5.77ac |
| Bouin's | Nuclear | 13.61 ± 2.33c | 17.86 ± 1.11d* | 16.75 ± 1.34d* | 10.55 v 0.61b | 6.98 ± 0.32ca |
| | Cytoplasmic | 52.33 ± 3.22ab* | 58.71 ± 5.33c | 60.43 ± 3.88c* | 54.54 ± 2.42b | 47.83 ± 4.99a* |
| Carnoy's | Nuclear | 15.51 ± 1.21d* | 14.71 ± 1.41d | 12.99 ± 1.88c* | 9.31 ± 0.77b | 6.04 ± 0.32a |
| | Cytoplasmic | 46.9 ± 3.61b | 48.45 ± 2.18b* | 43.43 ± 3.89a* | 40.96 ± 4.42a* | 42.33 ± 3.97a |
| Zenker's | Nuclear | 13.41 ± 1.77a | 15.88 ± 1.97b | 16.11 ± 1.07b | 15.19 ± 1.33b* | 12.31 ± 1.04a* |
| | Cytoplasmic | 44.98 ± 1.78a | 47.99 ± 4.91b | 49.78 ± 1.42c | 46.51 ± 1.31b | 42.21 ± 3.94a |
| Clarke's | Nuclear | 12.66 ± 1.34a | 11.76 ± 1.62a | 11.87 ± 1.11a | 10.98 ± 0.65a | 8.99 ± 0.98b |
| | Cytoplasmic | 42.51 ± 6.91a | 47.17 ± 4.99b | 45.87 ± 4.75b | 41.87 ± 1.98a | 40.77 ± 2.91a |

n= number of images analyzed by Image J Microsoft program. each value= mean ±SD of image analyzed. Small letters represent a significant difference between of times p≤0.05.* represent a significant difference p≤0.05 between fixatives.

terioration in pigmentation quality was evident at 48 and 72 hours. Carony,s and Clarke's fixatives showed good results at 6 and 12 hours while showing a decrease in quality thereafter (Table 2).

### Histological deformity

Histological sections in 10% (NBF) and Zenker,s solution showed high shrinkage and distortion / dissolution at extended time intervals of 48 and 72 hr. (The higher the score poorer the quality of the slide), while Bouin's fluid showed poor, average and high shrinkage and distortion/dissolution gradually increased at 24, 48 and 72hr., respectively, on the other hand, the Carony,s and Clarke's fixative was not appeared histological deformity at 6 and 12 hr. , while 24, 48 and 72 hr. were shown poor and average shrinkage and distortion/dissolution respectively, This deterioration in pigmentation quality and texture clarity gradually increased over time, respectively (Table 2). Our findings are consistent with prior research showing the efficiency of formaldehyde-based fixatives in preserving tissue integrity (Titford, 2001; Bancroft and Gamble, 2019). However, this study expands on previous research by carefully analyzing numerous fixatives at different time points. Unlike Fox *et al.* (1985), who concentrated on long-term fixation effects, our findings highlight the crucial role of short-term fixation in retaining tissue shape and staining quality. Furthermore, the fact that Bouin's fluid is optimal for 12 and 24-hour fixation periods supports the findings of Park *et al.* (2016), who found it effective in preserving cellular features for intermediate fixation durations.

### Software analysis of histological images (by Image J Program)

Using Image J software, a digital analysis of histological images representing formaldehyde, Bouin's fluid, and Carnoy,s solution was carried out with varying fixation times. Some histological features, represented by the % area of nuclei and cytoplasm of cells, were calculated from the total area of the tissue, using 30 replications for each treatment. At 48 and 72 hours, specimens preserved in 10% formaldehyde and Bouin revealed significant nuclear and cellular shrinkage, deformation, and dissolution. This was observed through the histological examination of the sections, as well as through what was recorded as a decrease in area percentage, representing the area of nuclei and cellular cytoplasmic from the total area of the liver histological sections, compared to other times, whereas Carnoy's showed significant nuclear and cellular shrinkage, distortion, and dissolution at 24, 48, and 72 hours, while the Microsoft analysis program (Image J) recorded a decrease in area percentage, representing the area of nuclei, cytoplasm, and sinusoids from the total area of the liver histological sections, compared to 12 hr. Generally, Table 3 and Figure 2. (a, b, c, d, e, f, h, j, k, l, m, n, o, p, q) illustrate the effects of various fixatives at different time intervals.

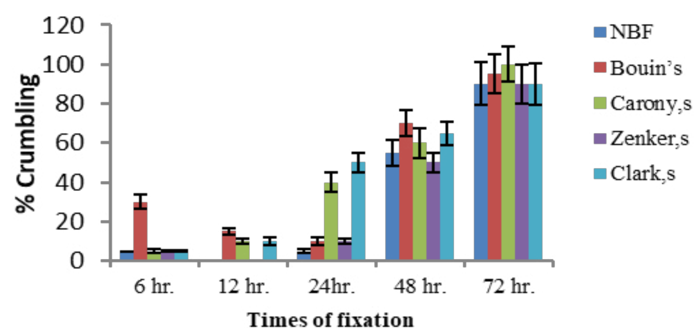


**Figure 1.** shows the percentage % of hard and cut (a) and crumbling sections of samples according to an alteration in fixation protocol (different fixations and time).



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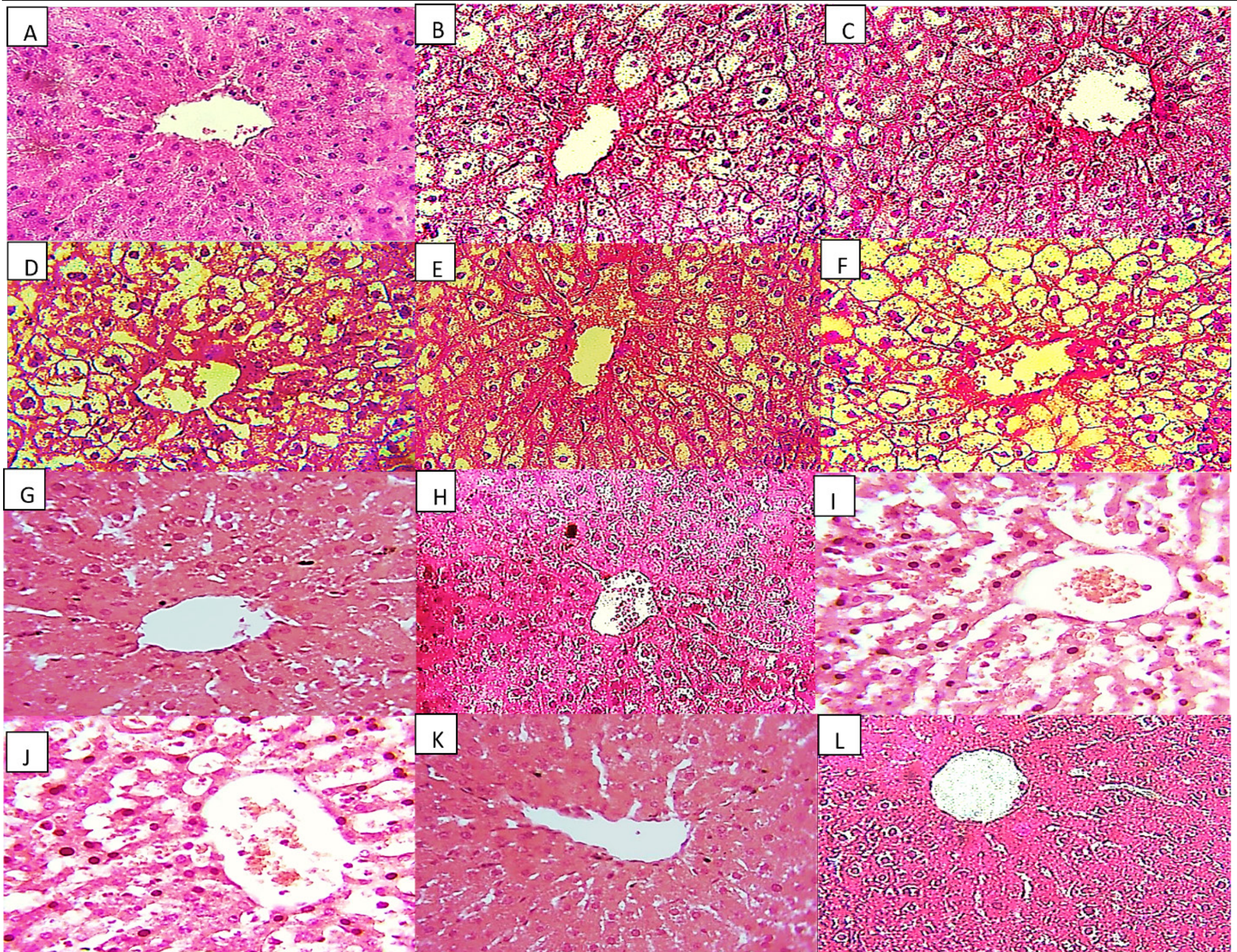


**Figure 2.** Showed histological liver specimens were fixed in NBF (10%) for 6 hr. (A), 12 hr. (B), and 24 hr. (C). Fixed in Bouin's for 6 hours (D), 12 hours (E), and 24 hours (F). Fixed in Carony,s for 6 hours (G) and 12 hours (H). In Zenker,s 12 hours (I) and 24 hours (J). In Clarke's 6 hours (K) and 12 hours (L), staining of H and E 400X.

## DISCUSSION

Fixation is the most critical stage in tissue preparation. Errors in fixing will irreversibly ruin the tissue specimen, and all morphological, histological, and histopathological information obtainable from the specimen will be degraded, no matter how much care is taken subsequently in tissue processing. Fixation attempts to prevent putrefaction and autolysis that occur due to the cutoff of blood supply to the tissue. The ideal fixative, however, should retain the cellular and tissue structures in as lifelike a state as possible yet still enable them to undertake further preparatory processes without modification. The coefficient of diffusivity of the fixative and the rate of its reaction with the constituent parts of the tissue modulate fixation (Leong *et al*., 1996). Generally, the fixative is better the higher the coefficient of diffusivity. Principles and practices for fixing tissues are critical to creating quality slides. Generally, 10% buffered formaldehyde is used as a fixative in most normal paraffin-embedded sections; the use of additional fixatives is also growing due to their ease of use and accessibility. The fixation time is, therefore, essential to achieving useful stainability to allow for adequate histological, histopathological, and immunohistochemistry studies. Formaldehyde binding to tissue slices increases over time at room temperature and achieves saturation after 12 to 24 hours at 25 °C, according to (Fox *et al*., 1985).

Biological Significance and Implications In the present study, specimens fixed for 24, 48, and 72 hours in Carnoy's and Clarke's solutions were the most complex and most troublesome to cut; they did not hold together but crumbled. Formaldehyde 10% Zenker's and Bouin's fluid were most accessible to cut at 6, 12, and 24 hours of fixation; however, they seemed the hardest to cut and crumble after 48 and 72 hours of fixation. These results are entirely from (Stickland, 1975). This could have been due to alcohol-fixed tissues being more fragile sometimes compared


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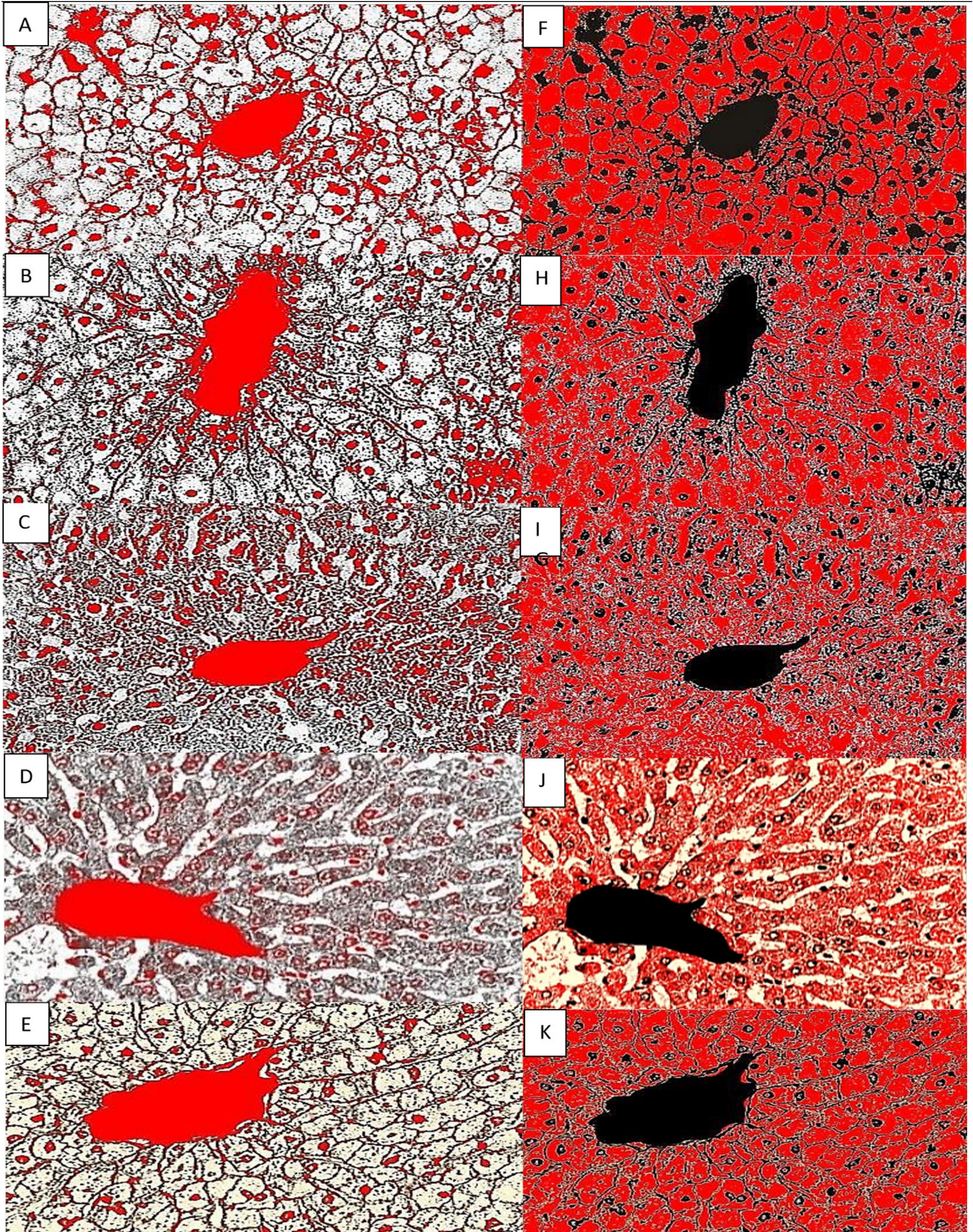


**Figure 3.** Photomicrographs (400X) of liver tissue specimens were image-analyzed using Image J software as a percentage of quantification for nuclei and cell cytoplasm (a, b, c, d, e, and f). The samples were fixed in formaldehyde for 12, 24, and 48 hours. While (g, h, I, j, k, and l) were fixed in Bouin's fluid for 12, 24, and 48 hours, and (m, n, p, and q) were fixed in Carony,s fluid for 12 and 24 hours.


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to formalin-fixed tissues. In comparison with the specimens processed for a shorter duration in the same fixative, hard-to-cut criteria at 48 and 72 hours, it occurred in more specimens fixed in 10% formaldehyde. This could be because proteins continue cross-linking for an extended period following infiltration of the tissue by a fixative during formalin fixation of a longer duration. According to Troiano *et al*., (2009), this can result in excessive hardening of tissues. Common disadvantages of formaldehyde concern its disastrous effects on nuclear and nucleic acids. The detrimental effects of formaldehyde are affected by several factors, such as chemical composition and fixation time. Moreover, the longer a tissue stays fixative, the more poor-quality DNA becomes available.

### Potential mechanisms

Bouin's fluid is a rapid fixative that shows very minimal shrinkage in tissues. Tissue samples treated with this are best suited for trichrome staining and demonstrating glycogen. Nevertheless, the major drawback is that picric acid rather significantly stains yellow; hence, this excess yellow color has to be washed out by laborious rinses in alcohol.

Since Bouin's is a non-coagulate picrate solution, it cannot be used for long-term preservation because of picric acid-induced shrinkage, as noted by (Lihui *et al*., 2011). Its well-preserved nuclear information, however, enables good evaluation of detailed histopathology, thus helping narrow the broad differentials between inflammation and malignancy, as pointed out by (Bostwick *et al*., 1994).

Practical Implications for Histological Practices Small and delicate tissues are well-preserved in Bouin's solution due to their superiority in protecting the architecture of the nucleus, as demonstrated in the present study at 24 hours. For Carnoy's and Clarke's solution, the ethyl alcohol present could have caused the collapse of protein structure, as water induces significant swelling of proteins followed by shrinkage in absolute alcohol. Exposure time should thus be limited to 12 hours or less. Using nuclear and cytoplasmic staining criteria, our results were compared with those presented by (Cox *et al*., 2006). In contrast, Bultitude *et al*., (2011) reported that Bouin's solution better preserved nuclear detail than formaldehyde did at 10%. Future Research Suggestions: Currently applied limitations include using only one model of specimens within laboratory animals, such as the liver. Further investigation is needed regarding additional verification for generalization in a broader range of applications within laboratories from these findings and their corresponding interpretations. Future studies are supposed to have variable and larger sample sizes, additional criteria, and human pathological tissues that validate and generalize these results.

## CONCLUSIONS

Although 10% buffered formaldehyde should still be used as a standard fixative, other fixatives may be used in its place if it is unavailable or because of potential health risks to humans, such as its noxious vapor's ability to trigger allergic reactions and damage to the respiratory epithelium. Other fixatives have lower toxicities and are simpler to prepare in laboratories. The histologist and pathologist should be conversant with the histologic and morphological alterations of over-fixed tissue with various fixatives, which reduces accurate diagnostic value.

## NOVELTY STATEMENT

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## AUTHOR'S CONTRIBUTION

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## ACKNOWLEDGMENTS

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### Conflict of Interest

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