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Preliminary Study on Long Fixation in Histological Preparations of Internal Organs of Sprague Dawley Rats

Galang Prahanarendra[1], Devy Ariany [2]*, Nurlaely Mida Rachmawati [3], Luluk Hermawati [4], Ghea Farmaning Thias Putri [5]

*Correspondence:
devyariany.patologi@gmail.com

[1]*Medical Study Program, Faculty of Medicine, Syarif Hidayatullah State Islamic University, Jakarta, Indonesia.*
[2]*Medical Study Program, Faculty of Medicine, Universitas Islam Negeri Syarif Hidayatullah Jakarta, South Tangerang, Indonesia.*
[3]*Department of Biochemistry, Faculty of Medicine, Syarif Hidayatullah State Islamic University, Jakarta, Indonesia.*
[4]*Department of Medical Biology, Faculty of Medicine, Sultan Ageng Tirtayasa University, Banten, Indonesia.*
[5]*Department of Medical Biology, Faculty of Medicine, Sultan Ageng Tirtayasa University, Banten, Indonesia.*

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ABSTRACT

Background: Histotechnology is a series of processes ranging from tissue handling to the preparation of slides that can be observed under a microscope. One crucial stage in this process is fixation, which serves to preserve the structure and morphology of the tissue as close as possible to its original physiological state. However, prolonged fixation duration may lead to tissue hardening, dissolution, and structural damage. **Objective:** This study aims to obtain supporting data for the development of a standard operating procedure (SOP) in histotechnology that can be applied in the animal house and histology laboratories of the Faculty of Medicine, Syarif Hidayatullah State Islamic University Jakarta. **Results:** The results showed that fixation for three weeks caused morphological damage to the kidney, liver, and pancreas of Sprague Dawley rats. The findings included tissue perforation in all three organs, endothelial nuclear damage in the kidney, central vein wall damage in the liver, and cellular disintegration in the pancreatic islets of Langerhans. **Conclusion:** Based on these findings, it can be concluded that a fixation duration of three weeks does not produce optimal histological images and therefore cannot be used as a reference for establishing a standard histotechnology SOP in the laboratory of the Faculty of Medicine, Syarif Hidayatullah State Islamic University Jakarta.

Keywords: histotechnology, prolonged fixation, kidney, liver, pancreas, Sprague Dawley.

INTRODUCTION

Histotechnology is a series of laboratory procedures designed to process biological tissues for microscopic examination. The main stages of this process include tissue grossing, fixation, dehydration, clearing, embedding, thin sectioning (microtomy), and staining using dyes such as hematoxylin and eosin[1]. Through histotechnological techniques, cellular and tissue structures can be observed in detail, allowing the identification of pathological changes that support disease diagnosis [2,3].

Among these stages, fixation plays a particularly crucial role. The purpose of fixation is to preserve tissue morphology as close as possible to its normal physiological state by halting autolysis and enzymatic activities that can damage the tissue [2,4]. The success of fixation is influenced by various technical factors, including the type of fixative solution, pH and buffering system, the ratio of fixative volume to tissue (ideally ≥20:1), tissue thickness, as well as fixation duration and temperature [5]. Failure to optimize one of these factors such as excessive tissue thickness or insufficient fixative volume may hinder reagent penetration, leading to suboptimal fixation in the deeper tissue layers [6].

However, excessive fixation duration (over-fixation) can also produce undesirable artifacts. This condition may cause tissue hardening, shrinkage, loss of antigenic reactivity, and damage to delicate tissue elements, ultimately reducing staining quality and microscopic interpretation [4,5]. A study by [19] on cat ovarian tissues demonstrated that variations in fixation time affected both tissue morphology and immunohistochemical staining sensitivity. These findings highlight that, in addition to the type of fixative, fixation duration must also be optimized based on the tissue type and analytical objectives. Nevertheless, most histotechnology protocols emphasize short fixation periods, while evidence on prolonged fixation remains limited. In educational laboratories, tissues are often stored in fixative for extended durations due to logistical constraints, including fixation periods of up to three weeks, yet the histological impact of such practices has not been clearly documented.

In the context of medical education, accredited laboratories are required to establish standardized and validated Standard Operating Procedures (SOPs) for all technical processes, including histotechnology. A well-developed SOP should be supported by adequate laboratory infrastructure, competent technical personnel, up-to-date scientific references, and a consistent quality control system [4,18]. At the Faculty of Medicine and Health Sciences (FKIK), Syarif Hidayatullah State Islamic University Jakarta, there is currently no standardized SOP specifically regulating tissue fixation procedures, despite the increasing number of student research projects involving tissue and animal models.

Therefore, the formulation and preliminary validation of histotechnology procedures are essential, including determining the optimal fixation duration for internal organs such as the kidney, liver, and pancreas of Sprague Dawley rats. In this study, a three-week fixation duration was selected to represent an extended yet practically relevant timeframe commonly encountered in student-based laboratory research. With robust empirical data, the resulting SOP is expected to enhance the consistency and validity of histological preparations in the animal house and histology laboratories of FKIK, Syarif Hidayatullah State Islamic University Jakarta. This study aims to evaluate the effect of fixation duration on tissue quality as a foundation for developing histotechnology SOP recommendations at the institution.

MATERIALS AND METHODS

This descriptive laboratory study aimed to evaluate the effects of long-term fixation on the histological quality of the kidney, liver, and pancreas of Sprague Dawley rats. One male Sprague Dawley rat aged 80 days with an average body weight of 180–200 g was obtained from the Animal House of the Faculty of Medicine and Health Sciences, Syarif Hidayatullah State Islamic University Jakarta. All procedures followed ethical guidelines for animal research. After euthanasia, the organs were collected through necropsy and processed for histological examination. Each organ was divided into three tissue sections (A, B, and C), which were fixed in 10% formalin and processed for histological evaluation after the first, second, and third weeks of fixation, respectively.

Tissues were fixed in 10% formalin for three weeks at room temperature with a fixative-to-tissue ratio of 20:1. After fixation, samples were dehydrated through graded ethanol, cleared with xylene, embedded in paraffin, and sectioned at 4–5 µm thickness using a microtome. Sections were mounted on glass slides, dried, and stained with Hematoxylin and Eosin (H&E). The stained slides were examined under a light microscope at 40× and 400× magnifications to assess tissue integrity, cell boundaries, nuclear condition, and signs of autolysis or shrinkage. Slide evaluation was performed by a single trained examiner under the direct supervision of an experienced faculty

member with expertise in histopathology. Observations were described qualitatively and supported by photomicrographic documentation.

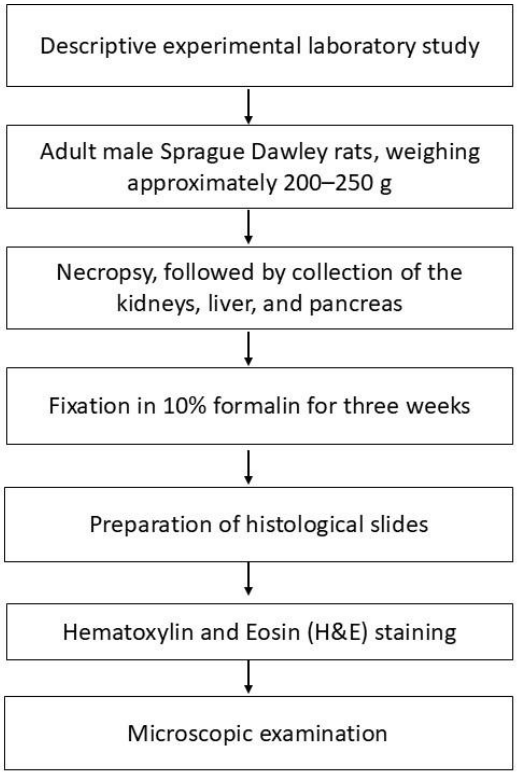

Figure 1. Study Methodology Flowchart

RESULTS

Macroscopic Findings

After prolonged fixation in 10% formalin, macroscopic changes were observed in the kidney, liver, and pancreas of Sprague Dawley rats. All tissues became progressively firmer and denser over the fixation period, indicating protein denaturation and excessive cross-link formation induced by formaldehyde exposure. The fixative solution, initially clear, gradually became turbid and contained small tissue fragments, suggesting partial protein dissolution and structural degradation during extended fixation. These findings are consistent with previous reports indicating that prolonged formalin fixation may result in excessive tissue hardening and solution turbidity due to the release of protein components into the fixative medium [2,7].

Organ-specific differences were observed during macroscopic examination. The pancreas appeared softer and more fragile compared to the kidney and liver throughout the fixation period. In contrast, the kidney and liver exhibited progressively paler surfaces and increased tissue firmness with longer fixation duration. These differences may be attributed to the higher proteolytic enzyme content of pancreatic tissue, which predisposes it to faster degradation, whereas prolonged formalin exposure in the kidney and liver promotes water withdrawal and excessive protein cross-linking, resulting in increased tissue rigidity [8].

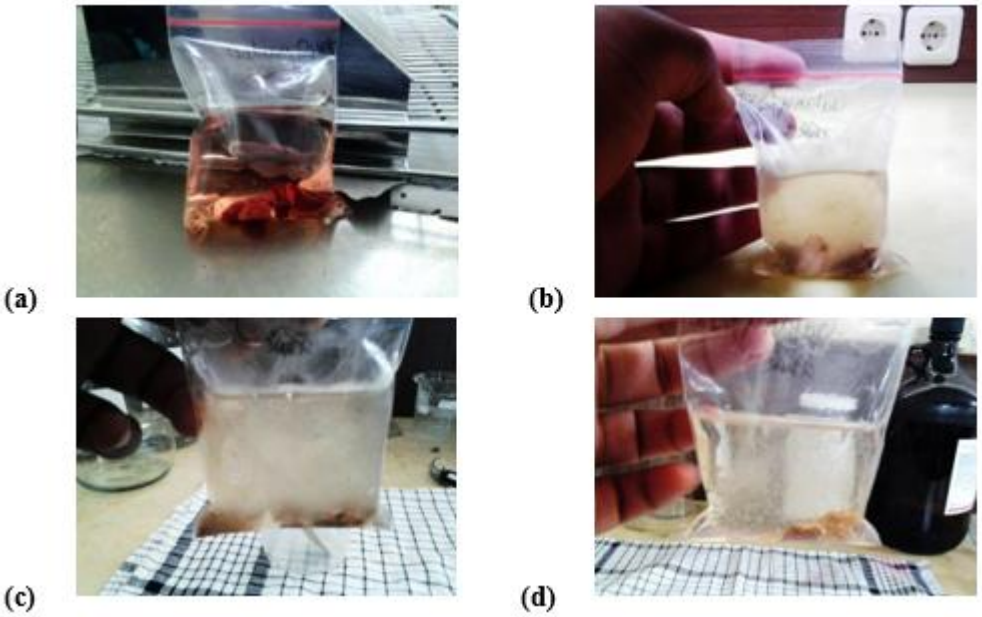

Figure 2. Tissue samples immersed in 10% formalin fixative at 2–8°C: (a) after necropsy, (b) after the first week, (c) after the second week, and (d) after the third week.

Organ-Specific Observations

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Table 1. Week Fixation Results

No.	Organ Code	Consistency with Theory	General Appearance	Structural Integrity	Intercellular Space	Cell Shrinkage	Cell Autolysis
1	Kidney A	C	D	V	L	√	Sg
2	Kidney B	C	D	V	L	√	NSg
3	Liver A	C	D	UI	L	√	NSg
4	Liver B	C	D	UI	L	√	NSg
5	Liver C	C	D	V	L	√	Sg
6	Pancreas A	C	D	V	L	√	Sg
7	Pancreas B	C	D	V	L	√	Sg
8	Pancreas C	C	D	V	L	√	Sg

Notes: C = consistent; I = inconsistent; G = good; D = damaged; V = visible; UI = unclear / unidentifiable; O = orderly; L = loose; Sg = significant;NSg = not significant.

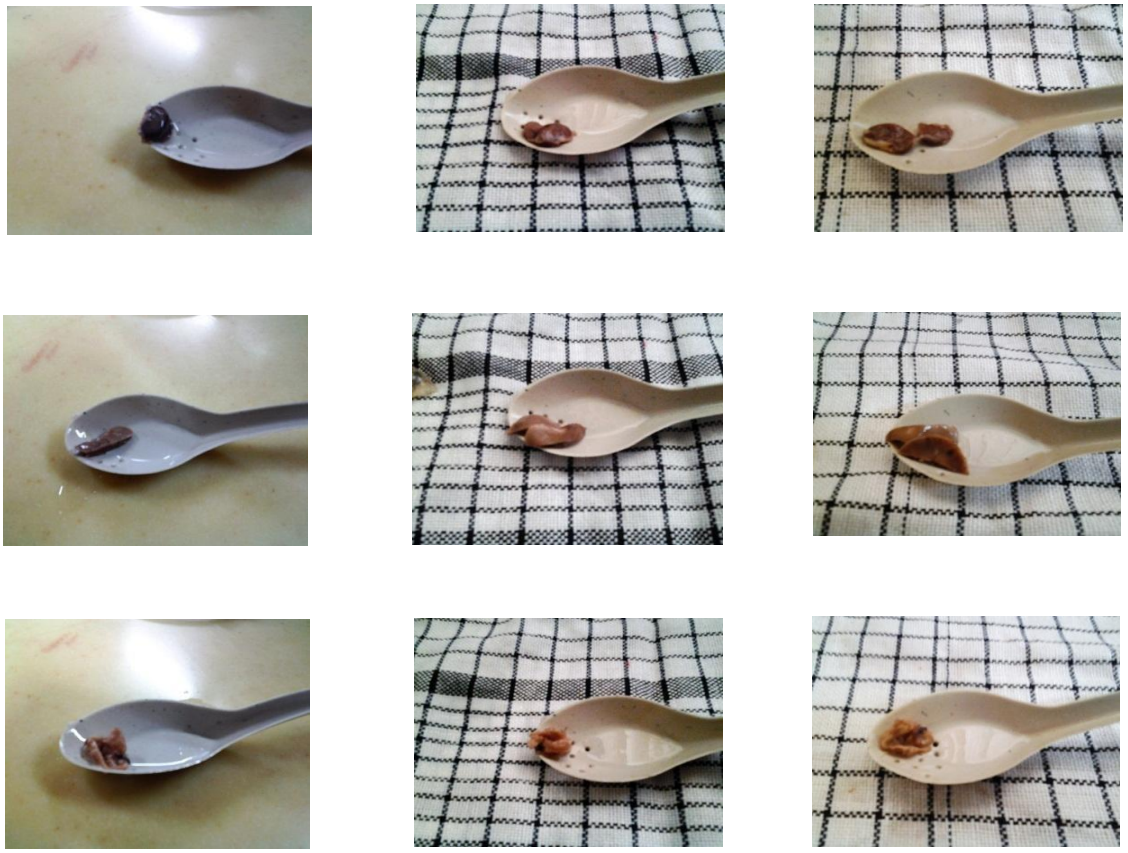

Figure 3. Tissue sections of the kidney, liver, and pancreas fixed for (a) one week, (b) two weeks, and (c) three weeks.

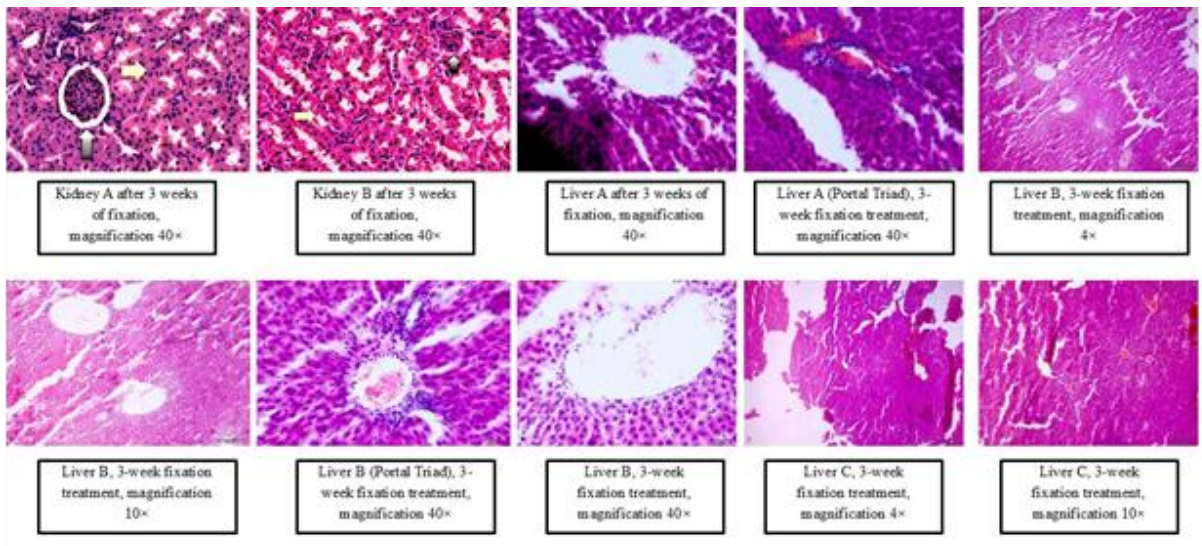

Figure 4. Representative histopathological images of the kidney, liver, and pancreas observed under different magnifications following a 3-week fixation period.

DISCUSSION

Fixation is a crucial initial step in the success of histotechnological processing, serving to preserve tissue morphology as close as possible to its original physiological condition [2]. In this study, tissue samples from Sprague Dawley rats were fixed in 10% formalin for three weeks to evaluate the effects of prolonged fixation duration on histological quality. The findings demonstrated that fixation time had a noticeable impact on both macroscopic and microscopic tissue characteristics. Macroscopically, tissues appeared firmer and more rigid, while microscopically, structural damage and loss of cellular integrity were evident.

During the fixation process, tissues undergo dehydration due to the hypertonic nature of formalin, which draws water out of the cells and replaces it with the fixative solution[10]. This leads to tissue hardening and paler coloration [11]. The fixative gradually became turbid due to the dissolution of blood residues and tissue fragments [12]. These findings are consistent with previous studies reporting that prolonged exposure to formalin can cause color alteration and increased tissue stiffness due to excessive protein cross-linking [7,13]. Additionally, the progressive turbidity of the fixative indicates ongoing dissolution of tissue proteins and lipids during fixation. This phenomenon aligns with the principle of over-fixation, in which formalin continuously reacts with amino and carboxyl groups in proteins, resulting in excessively hard and brittle tissues that are difficult to section microtomically [9].

Microscopic evaluation revealed that three weeks of fixation caused cellular degradation in all examined organs. In the kidney, loss of glomerular endothelial integrity and widening of intercellular spaces were observed. The liver exhibited damage to the central vein wall and loss of the characteristic radial hepatocyte arrangement, while the pancreas showed cytoplasmic disintegration and indistinct cellular boundaries, particularly in the islets of Langerhans [14,15,16].These alterations suggest that prolonged fixation disrupts the osmotic balance within tissues. The reactive nature of formalin toward proteins and enzymes induces excessive denaturation and subcellular damage [19]. This observation is consistent[19] who reported that fixation exceeding 48 hours may alter tissue antigenicity and reduce hematoxylin-eosin staining quality.

Variations in tissue response reflect differences in histological composition and enzymatic content among organs. The pancreas exhibited more rapid degradation than the kidney and liver due to its high content of proteolytic enzymes, which accelerate autolysis if enzymatic activity is not fully inhibited during fixation [17]. Conversely, the kidney and liver, which have denser cellular structures, appeared more resistant to early degradation, although significant structural deterioration was also observed by the third week. These findings align with [20], who emphasized that the optimal fixation duration for soft tissues ranges between 12 and 24 hours, depending on organ size and type. Fixation exceeding this duration promotes excessive protein cross-linking, impairs dye penetration, and results in non-representative histological morphology.

Based on this study, fixation for three weeks did not preserve tissue quality optimally. The observed microscopic structural damage indicates that this duration is excessive and unsuitable for use as a reference in developing histotechnology SOPs for animal house and histology laboratories. Therefore, further research is required to compare shorter fixation periods (e.g., 12, 24, and 48 hours) to determine the ideal fixation time for each organ type. Moreover, SOP validation should also consider other technical parameters such as fixative-to-tissue ratio, tissue thickness, and

environmental conditions (temperature and pH). Optimization of these factors is expected to produce representative histological preparations with uniform staining and well-preserved tissue architecture.

CONCLUSION

This study demonstrated that three weeks of fixation in 10% formalin resulted in significant macroscopic and microscopic deterioration of kidney, liver, and pancreatic tissues in Sprague Dawley rats, including increased tissue hardness, loss of cellular integrity, and signs of autolysis. These findings indicate that prolonged fixation leads to over-fixation and compromises histological quality. Therefore, a three-week fixation period is not suitable as a reference for histotechnology SOP development, and and shorter fixation durations, particularly within the 12–48 h range, are recommended for further investigation to determine optimal histological preservation.

Competing Interests

The authors declare that they have no financial or non-financial conflicts of interest that could have influenced the content or outcomes of this article. They are solely responsible for the accuracy, integrity, and presentation of the data and interpretations provided, and no funding or external affiliations affected the research.

Authors' contributions

All authors contributed equally to the conceptualization, literature review, data acquisition and analysis, manuscript drafting, and critical revisions. All authors have reviewed, approved the final manuscript, and agreed to be accountable for all aspects of the work, ensuring its accuracy and integrity.

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