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Evaluation of the Use of White Vinegar as an Alternative Agent in the Histological Detection of Digestive System Organs

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Short Title: White Vinegar as a Tissue Fixative

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Abstract

Traditional formaldehyde fixation poses severe health and environmental risks, necessitating the investigation of eco-friendly alternatives. This study aimed to evaluate the efficacy of natural white vinegar (an acetic acid-based bio-fixative) as a sustainable, cost-effective, and low-toxicity alternative to formalin in routine histotechnological processes. Liver, pancreas, and ileum tissues obtained from 18 male Wistar Albino rats (8 weeks old) were randomly assigned to three fixation groups: Control (10% Neutral Buffered Formalin [NBF]), Experiment 1 (Pure White Vinegar), and Experiment 2 (Formalin + White Vinegar combination). Following routine tissue processing, sections were stained with hematoxylin-eosin (H&E), Masson's trichrome, and toluidine blue. Semi-quantitative scoring was performed blindly by six independent histologists based on tissue integrity, cellular morphology, and staining homogeneity. Digital image analysis was conducted via optical density quantification using ImageJ software, and statistical evaluations were performed using SPSS ($p < 0.05$). Optimal morphological preservation was observed in the Control group. In tissues fixed solely with white vinegar (Experiment 1), the cellular architecture was largely preserved, with only minor focal disorganizations detected in the hepatic cords. The combination group (Experiment 2) exhibited

prominent structural irregularities, particularly in the mucosal villi of the ileum. Inter-observer reliability among the six academicians was remarkably high, with a significant inter-group difference emerging in the scoring of only one observer ($p > 0.05$ overall). ImageJ densitometric analysis demonstrated that color and optical density distributions were statistically similar across all groups ($p > 0.05$), confirming that vinegar fixation did not adversely affect dye-binding capacity. Histological and digital densitometric analyses demonstrate that white vinegar successfully preserves tissue and cellular integrity at a level comparable to formalin. These findings suggest that white vinegar-based formulations hold strong potential as sustainable, bio-safe, and cost-effective alternative fixatives in routine histotechnology.

1. Introduction

Histological and histopathological analyses constitute the cornerstone of biomedical research and clinical diagnostics, enabling the microscopic evaluation of tissue architecture and cellular morphology at high resolution (Gökçimen, 2006).

The quality of these analyses directly depends on the success of the fixation process, which aims to preserve the *in vivo* structure of biological tissues by preventing post-mortem autolysis and bacterial degeneration (Eltoum et al., 2001; Fox et al., 1985; Grizzle, 2009)

Currently, the most prevalent fixative accepted as the gold standard in histology laboratories is formaldehyde, which has been widely utilized since its initial discovery by Ferdinand Blum in 1893 (Puchtler & Meioan, 1985).

Formaldehyde stabilizes tissue by establishing methylene bridges (cross-linking) between the functional groups of amino acids; owing to its low cost and long-term success in tissue archiving, it has been globally recognized as an irreplaceable agent (Zanini et al., 2012).

However, this pervasive utilization of formaldehyde poses severe acute and chronic toxicity along with carcinogenic risks to laboratory personnel (International Agency for Research on Cancer - IARC Group 1), while its volatile nature presents critical threats to biological wastewater treatment systems and environmental health (Binetti, 2006; Costa et al., 2008; John D. Bancroft, 2013) Furthermore, the neutralization and recycling processes of formaldehyde waste—encompassing chemical deamination reactions, exothermic risks, and toxic by-product management—impose a heavy technical and economic burden on laboratories (John D. Bancroft, 2013).

These distinct disadvantages necessitate the development of biologically safe, eco-friendly, and biodegradable alternative fixatives within the framework of the modern "green laboratory" concept.

In recent years, alcohol-based solutions, natural organic acids, and plant-derived bio-fixatives have attracted considerable attention as alternatives to conventional fixatives. Vinegar, a natural product obtained through fermentation, is a bioactive matrix characterized by high antimicrobial and antioxidant capacities, and is rich in various organic acids (primarily acetic acid), phenolic compounds, and amino acids (Karabiyikli, 2017; M. Plessi, 2003)

Acetic acid, the major component of vinegar, is well-known in histology (as seen in Carnoy's or Bouin's solutions) for its ability to coagulate proteins, precipitate nucleoproteins, and accelerate fixation. It is hypothesized that naturally fermented white vinegar, by virtue of its standardized acetic acid concentration, can trigger protein denaturation in a controlled manner and preserve tissue morphology. Nonetheless, the structural preservation efficacy of white vinegar alone—as a non-standard bio-fixative—in routine histological tissue fixation has not yet been thoroughly and systematically investigated in the literature in comparison with formaldehyde.

The aim of this study is to evaluate the availability of white vinegar, a natural bio-component, as a potential alternative fixative for histological tissue preservation. To this end, the qualitative and quantitative efficacy of white vinegar fixation in liver, pancreas, and ileum tissues—which exhibit distinct metabolic and structural characteristics—was comprehensively investigated in comparison with the gold standard, traditional 10% neutral buffered formalin (10% NBF). The data obtained are intended to contribute to the development of eco-friendly, sustainable, and safe alternative protocols that will reduce the utilization of toxic fixatives in pathology and histology laboratories.

2. Methods

2.1. Ethical Approval

This experimental study was approved by the Local Ethics Committee for Animal Experiments at Aydın Adnan Menderes University (Decision No: 64583101/2025/001, dated 09.01.2025) with no ethical concerns identified (Supplementary Material 1).

2.2. Experimental Animals

A total of 18 healthy male Wistar albino rats (8 weeks old, weighing 200–220 g) were obtained from the Laboratory of Experimental Animals at Aydın Adnan Menderes University. Throughout the study, the rats were housed in transparent polycarbonate cages under a controlled 12-hour light/12-hour dark cycle, with free (*ad libitum*) access to standard laboratory chow and water. Following an acclimation period, the rats were deeply anesthetized via an intraperitoneal injection of a ketamine (100 mg/kg) and xylazine (5 mg/kg) cocktail, and subsequently euthanized by cervical dislocation. Immediately after euthanasia, tissue samples from the liver, pancreas, and ileum were harvested. To ensure high comparability between analysis groups and to optimize fixative penetration, the dimensions of the dissected tissues were strictly standardized. Accordingly, the liver samples were sectioned into 1 x 1 cm² pieces, pancreas samples into 0.5 x 0.5 cm² pieces, and ileum segments were cut to lengths of 1 x 0.3 cm².

2.3. Chemicals Used

For the tissue fixation procedures, 10% neutral buffered formalin and commercial food-grade white vinegar were utilized. For subsequent histological evaluations, the paraffin-embedded tissue sections were subjected to hematoxylin and eosin (H&E), Masson's trichrome, and toluidine blue staining protocols.

Tissue samples from the liver, pancreas, and ileum were systematically allocated into three distinct fixation protocols (n = 6 rats per group, yielding 18 tissue samples per group):

- Control Group: Tissue samples fixed traditionally in 10% neutral buffered formalin (NBF).
- Experimental Group 1: Tissue samples fixed solely in commercial white vinegar.
- Experimental Group 2: Tissue samples fixed in a combination mixture of white vinegar and 10% NBF (1:1) .

In each group, one sample of each organ was harvested from 6 individual rats, resulting in 18 samples per group. A total of 54 tissue specimens were successfully processed and evaluated throughout the study.

2.4. Preparation of Fixatives

The fixative solutions utilized in this study were prepared according to standard formulations and brand specifications as follows:

- 10% Formalin Solution: Prepared by mixing 100 mL of formaldehyde (37% stock solution) with 900 mL of distilled water, following the standard protocol described by Hopwood (D. Hopwood, 1990).
- White Vinegar: Commercial food-grade white vinegar containing an acetic acid concentration of 4% - 7% was obtained from a regional supplier (Yeni Kavaklıdere, Kavaklıdere Sarapları A.S., Ankara, Turkey) and used without further dilution.
- Formalin-Vinegar Combination (for Experimental Group 2): Prepared fresh before application by mixing equal volumes of the 10% formalin solution and the commercial white vinegar at a 1:1 (v/v) ratio.

All components were measured volumetrically and combined sequentially at room temperature to ensure complete homogeneity prior to the tissue fixation process.

2.5. Tissue Tracking and Embedding

Routine Tissue Processing Following the completion of the fixation protocols, all tissue specimens were subjected to a routine automated tissue processing procedure. The sequential steps utilized for dehydration, clearing, and paraffin infiltration to prepare the samples for light microscopy examination are detailed in Table 1.

Step	Control Group: 10% Formalin	Experimental Group 1: White Vinegar (4–7% Acetic Acid)	Experimental Group 2: White Vinegar and 10% Formalin Mixture (1:1)
Fixation	4 days	4 days	4 days
Leaching	Under running water for 24 hours	Under running water for 24 hours	Under running water for 24 hours
Dehydration	50%, 70%, 80%, 90%, 96% (1 hour in each alcohol concentration)	50%, 70%, 80%, 90%, 96% (1 hour in each alcohol concentration)	50%, 70%, 80%, 90%, 96% (1 hour in each alcohol concentration)
Clearing	Xylol (30 minutes) Xylol (30 minutes)	Xylol (30 minutes) Xylol (30 minutes)	Xylol (30 minutes) Xylol (30 minutes)
Paraffin Embedding	Paraffin at 58–60°C for 2 hours	Paraffin at 58–60°C for 2 hours	Paraffin at 58–60°C for 2 hours
Paraffin Blocking	After embedding, blocks were left in the freezer overnight before sectioning.	After embedding, blocks were left in the freezer overnight before sectioning.	After embedding, blocks were left in the freezer overnight before sectioning.

Table 1. Routine tissue processing schedule for light microscopy (Bancroft, 2008; Carson, 1997; Suvarna, 2019)

Following the completion of fixation, washing, dehydration, and clearing stages, the tissue specimens were infiltrated in liquid paraffin wax within an incubator at 58–60°C for 2 hours. Subsequently, the

processing cassettes were transferred to a tissue embedding station. The specimens were carefully oriented and placed into brass base molds using heated forceps to ensure the optimal sectioning plane. Molten paraffin wax maintained at 58–60°C was then poured over the tissues to form solid blocks, ensuring that the predetermined orientation of each specimen was preserved. Each paraffin block was systematically labeled according to its respective fixation group. The finalized paraffin blocks were allowed to solidify at room temperature and were subsequently stored in a refrigerator overnight to achieve optimal block hardness prior to microtomy.

2.6. Cross-Section

Following the completion of the embedding process, paraffin blocks were sectioned at a thickness of 5 micrometer using a rotary microtome (Leica RM2125 RTS, Leica Biosystems, Wetzlar, Germany). The obtained sections were floated in a distilled water bath maintained at 37°C to ensure proper expansion and elimination of wrinkles, and were subsequently mounted onto clean glass slides. To prepare the tissue sections for downstream histological staining protocols, the slides were placed in an incubator at 60°C overnight to melt the excess paraffin, achieve complete deparaffinization, and ensure optimal tissue adherence to the slides.

2.7. Histological Staining Protocols

Three distinct histological staining procedures were performed on the tissue sections to enable the evaluation of different structural characteristics. Hematoxylin and eosin (H&E) staining was utilized for the assessment of general tissue morphology. Masson's Trichrome staining method was preferred to determine the distribution of connective tissue and collagen fibers. Additionally, toluidine blue staining was applied to enhance the visibility of the mucosal architecture and acidic components (Bancroft, 2008; Carson, 2015; Suvarna, 2019).

The histological staining of the liver, pancreas, and ileum sections was performed systematically using three distinct techniques. Hematoxylin and Eosin (H&E) staining was carried out in accordance with the sequential protocol detailed in Table 2. For the evaluation of connective tissue architecture, Masson's Trichrome staining was applied using a commercial kit protocol based on the classic method described in the literature (Table 3) (Bancroft, 2008). Additionally, Toluidine Blue staining was performed to emphasize specific mucosal components according to the procedure outlined in Table 4. Following the completion of each respective staining protocol, all sections were subjected to identical final processing stages: they were thoroughly dehydrated through an ascending ethanol series, cleared in xylene to achieve optimal transparency, and subsequently mounted with a synthetic mounting

medium (Entellan™, Merck, Darmstadt, Germany) using clean coverslips for subsequent light microscopy examination.

Stage	Procedure	Duration
Deparaffinization Stage	Xylene	5 min
	Xylene	5 min
Rehydration Stage	100% Alcohol	2 min
	96% Alcohol	2 min
	80% Alcohol	2 min
	70% Alcohol	2 min
	Distilled Water	5 min
Hematoxylin and Eosin Staining Stage	Hematoxylin	5 min
	Running Water (Wash)	2 min
	Tap Water	1 min
	Eosin	2 min
Dehydration Stage	70% Alcohol	2 min
	80% Alcohol	2 min
	96% Alcohol	2 min
	100% Alcohol	2 min
Clearing Stage	Xylene	5 min
	Xylene	5 min

Table 2. Hematoxylin–Eosin Staining Protocol (Suvarna, 2019)

Stage	Procedure	Duration
Deparaffinization Stage	Xylene	5 min
	Xylene	5 min
Rehydration Stage	100% Alcohol	2 min
	96% Alcohol	2 min
	80% Alcohol	2 min
	70% Alcohol	2 min
	Distilled Water	5 min
Masson Trichrome Staining Stage	Solution A + Solution B	10 min
	Rinse under running tap water	3 min
	Solution D	10 min
	Distilled Water	Rinse
	Solution E	15 min
	Rinse until blue color disappears	Until reagent runs clear
	Solution F	10 min
	Distilled Water	Rinse
Dehydration Stage	95% Alcohol	2 min
	95% Alcohol	2 min
Clearing Stage	Xylene	2 min
	Xylene	2 min

Table 3. Masson trichrome staining protocol

Stage	Procedure	Duration
Deparaffinization Stage	Xylene	5 min
	Xylene	5 min
Rehydration Stage	100% Alcohol	2 min
	96% Alcohol	2 min
	80% Alcohol	2 min
	70% Alcohol	2 min
	Distilled Water	5 min
Toluidine Blue Staining Stage	1% Toluidine Blue Solution	15 min
	Rinse with Distilled Water	2 min
Dehydration Stage	70% Alcohol	2 min
	80% Alcohol	2 min
	96% Alcohol	2 min
	100% Alcohol	2 min
Clearing Stage	Xylene	5 min
	Xylene	5 min

Table 4. Toluidine blue staining protocol (Suvarna, 2019)

2.8. Preparation of Experimental Staining

For each target organ (liver, pancreas, and ileum), tissue sections representing the control, Experimental 1 (white vinegar), and Experimental 2 (formalin + white vinegar) groups were prepared and stained with Hematoxylin and Eosin (H&E), Masson's Trichrome, and toluidine blue dyes (Table 5).

Tissues	Stains Used	Experimental Groups
Liver	Hematoxylin and Eosin	Control (formol) Experiment 1 (white vinegar) Experiment 2 (formol+white vinegar)
	Masson Trichrome	Control (formol) Experiment 1 (white vinegar) Experiment 2 (formol+white vinegar)
	Toluidine Blue	Control (formol) Experiment 1 (white vinegar) Experiment 2 (formol+white vinegar)
Pancreas	Hematoxylin and Eosin	Control (formol) Experiment 1 (white vinegar) Experiment 2 (formol+white vinegar)
	Masson Trichrome	Control (formol) Experiment 1 (white vinegar) Experiment 2 (formol+white vinegar)
	Toluidine Blue	Control (formol) Experiment 1 (white vinegar) Experiment 2 (formol+white vinegar)
Ileum	Hematoxylin and Eosin	Control (formol) Experiment 1 (white vinegar) Experiment 2 (formol+white vinegar)
	Masson Trichrome	Control (formol) Experiment 1 (white vinegar) Experiment 2 (formol+white vinegar)
	Toluidine Blue	Control (formol) Experiment 1 (white vinegar) Experiment 2 (formol+white vinegar)

Table 5. Experimental Staining Groups

2.9. Imaging and Analysis

For each experimental group, the histological sections of the liver, pancreas, and ileum tissues were comprehensively examined using a Zeiss Axio Vert.A1 inverted light microscope (Carl Zeiss Microscopy GmbH, Jena, Germany) to assess fixation-related histological findings based on the specific staining techniques applied. Representative photomicrographs were captured from selected areas of interest. To eliminate bias, the microscopic evaluation of the tissue sections was

performed using a blinded method. Each tissue section was systematically analyzed at 20x and 40x objective magnifications across randomly selected, non-overlapping fields.

The histopathological evaluation focused on key structural parameters, including structural tissue integrity, cellular architecture, morphological alterations, staining quality, nuclear morphology, clarity of cytoplasmic structures, and connective tissue (extracellular matrix) organization. Based on these parameters, a semi-quantitative scoring system ranging from 1 to 5 was established for each staining technique (Table 6).

1 (Poor Quality)	Severe disruption of tissue architecture; cellular boundaries are extensively lost; widespread structural disintegration and pronounced artifacts in staining quality.
2 (Fair Quality / Inadequate Fixation)	Major irregularities in the architectural pattern; tissue integrity is partially compromised; heterogeneous staining intensity.
3 (Moderate Quality / Partially Deficient Fixation)	Tissue architecture is partially preserved with localized areas of structural disruption; moderate but acceptable staining quality.
4 (Good Quality)	Tissue architecture is substantially preserved; minimal localized alterations; mostly homogeneous staining distribution.
5 (Excellent Quality / Optimal Fixation)	Tissue architecture and cellular integrity are normal or nearly identical to the ideal reference morphology; sharp, distinct, and fully homogeneous staining.

Table 6. Semi-quantitative scoring criteria and histological quality grading scales used for the histopathological evaluation of tissue sections

The photomicrographs captured via light microscopy were quantitatively analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Within the predetermined regions of interest (ROIs), mean gray value measurements and area calculations were systematically performed. This densitometric and morphometric analysis allowed for the generation of objective, quantitative data representing the staining intensity and structural alterations across all experimental groups and staining techniques.

Statistical Analysis

For each target organ (liver, pancreas, and ileum), histological sections were systematically sampled across the experimental groups. To capture the heterogeneous tissue distribution and prevent localized artifacts from biasing the measurements, quantitative morphometric assessments were performed on captured micrographs using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Within each representative section, mean gray value measurements and area calculations were conducted across randomly selected, non-overlapping regions of interest (ROIs) at 20x magnification. Numerical data were generated for each staining technique and experimental cohort, and the average values per section were utilized for downstream statistical analyses, evaluating a comprehensive pool of 81 microscopic fields per staining method.

In addition, to evaluate overall histological preservation and fixation quality, a semi-quantitative image assessment protocol was established. A total of 27 representative micrographs—comprising one selected image per animal across all groups—were evaluated. Microscopic analysis was conducted concurrently at 20x and 40x magnifications. To ensure objectivity, a blinded evaluation method was employed. Each specimen was scored on a scale from 1 to 5 based on pre-established criteria, including structural tissue integrity, cellular architecture, morphological alterations, nuclear details, clarity of cytoplasmic structures, and connective tissue (extracellular matrix) organization.

Statistical analyses of the acquired quantitative and semi-quantitative datasets were performed using SPSS software (v26.0; IBM Corp., Armonk, NY, USA). The normality of data distribution was assessed using the Kolmogorov–Smirnov test, and the homogeneity of variances was verified via Levene’s test. For normally distributed parametric data displaying homogenous variances, a one-way analysis of variance (ANOVA) was performed, followed by Tukey’s Honestly Significant Difference (HSD) post-hoc test for multiple comparisons. Non-parametrically distributed continuous data were analyzed using the Kruskal–Wallis test, and intergroup differences were identified via the Dunn-Bonferroni post-hoc test.

Semi-quantitative scores were treated as ordinal variables and analyzed using the Kruskal–Wallis test. Upon detecting a statistically significant global variance, the Mann-Whitney U test was implemented as a post-hoc procedure to isolate specific pairwise differences between groups. For all statistical tests, the level of significance was set a priori at $p < 0.05$. Continuous data are reported as mean $\pm$ standard error (mean $\pm$ SE) or as median with interquartile range (25th–75th percentiles) depending on the respective distribution profile.

3. Results

In this study, histological sections obtained from the liver, pancreas, and ileum tissues were comprehensively evaluated following different fixation protocols using hematoxylin and eosin (H&E), Masson's Trichrome, and toluidine blue staining techniques. The sections for each organ were examined under a light microscope and analyzed in terms of their staining characteristics, morphological integrity, and cellular details; the resulting qualitative and quantitative findings are presented under separate subheadings for each organ.

During the histological evaluations, certain variations in the sectioning process were observed depending on the fixation methods utilized. Specifically, in some specimens from the liver tissue of Experimental Group 1 (fixed solely with white vinegar) and the ileum tissue of Experimental Group 2 (fixed with the formalin + white vinegar combination), technical difficulties such as tissue friability and minor fragmentation were encountered during microtomy, making the preservation of structural integrity challenging. Nevertheless, the sections selected for final histopathological and morphometric evaluation were meticulously chosen from premium-quality preparations that exhibited sufficient morphological features and structural preservation to allow for robust histological analysis.

3.1. Histopathological Findings

3.1.1. Histopathological Evaluation of Liver Tissue

Comprehensive histopathological evaluation of the liver architecture was conducted utilizing hematoxylin and eosin, Masson's Trichrome, and toluidine blue staining techniques across all experimental cohorts. In the H&E-stained sections of the control group (fixed with 10% formalin), the hematoxylin-stained nuclei were distinct, while the eosin-stained cytoplasm exhibited a uniform, homogeneous appearance alongside a well-preserved, identifiable classical lobular organization, which was further verified under high power with distinct hepatocyte nuclei and homogeneous cytoplasmic delineation (Figure 1A, B).

Similarly, the H&E-stained sections of Experimental Group 2 (fixed with the white vinegar and 10% formalin mixture) demonstrated prominent hepatocyte nuclei and a highly homogeneous distribution of cytoplasmic staining (Figure 1C, D); the classical lobular organization was easily discernible, indicating that the characteristic nuclear distinctiveness and cytoplasmic staining properties observed in the control group were successfully preserved in this combination cohort.

Conversely, H&E-stained liver sections from Experimental Group 1 revealed pronounced structural variations in tissue architecture compared to the control group; localized irregularities in the classical lobular organization were observed at low power, while high-power examinations highlighted disruptions in the linear cords of hepatocytes, expansions within the sinusoidal spaces, and a prominent heterogeneity in cytoplasmic staining intensity (Figure 1E, F).

Under MT staining, low-power evaluation of the control group confirmed that the lobular framework was structurally intact, demonstrating regularly arranged hepatocyte cords, distinct and continuous sinusoidal lines, and physiological distributions of connective tissue elements and collagen fibers within normal boundaries around the portal areas and central veins (Figure 1G).

For the MT-stained sections of Experimental Group 2, the hepatocyte cytoplasm stained in characteristic red-purple hues, the lobular architecture remained predominantly stable with clear sinusoidal tracks and regularly distributed hepatocyte cords, and despite a slight prominence of blue-green stained collagen fibers around the central vein and within perisinusoidal spaces compared to the control group, high-power analysis revealed only minor localized alterations in radial alignment without any evidence of diffuse fibrotic banding or advanced collagen deposition, thereby indicating well-preserved structural integrity (Figure 1H, I).

In Experimental Group 1, MT staining demonstrated that the classic lobular framework was majorly sustained with distinguishable central veins, radiating hepatocyte cords, and apparent sinusoidal gaps; at higher magnification, hepatocytes retained their polygonal morphology with acidophilic cytoplasm and centrally located euchromatic nuclei, and despite localized cytoplasmic vacuolation-like appearances in certain fields, the cellular boundaries remained distinct, and the characteristic blue-green staining of collagen fibers within the central vein wall and perisinusoidal areas was successfully achieved, confirming that white vinegar fixation preserves the staining properties of connective tissue elements (Figure 1J, K).

Furthermore, high-contrast TB staining in the control group revealed an entirely intact parenchymal architecture and classical lobular organization with highly resolved nuclear and cytoplasmic details, orderly hepatocyte cords, and clearly visible sinusoids (Figure 1L).

In Experimental Group 2, TB-stained specimens retained structural integrity comparable to the control group, yet exhibited a pronounced increase in dye affinity toward tissue components, yielding a more intense and darker metachromatic reaction across cellular structures (Figure 1M).

Lastly, TB-stained sections from Experimental Group 1 allowed for the identification of the general histological architecture, but the typical cordonal arrangement of hepatocytes was indistinct; the staining intensity presented localized heterogeneity that compromised the clarity of cellular boundaries and tissue framework, and although nuclear structures were partially recognizable in some regions, the limited staining contrast ultimately reduced the distinctness of fine cellular details (Figure 1N).

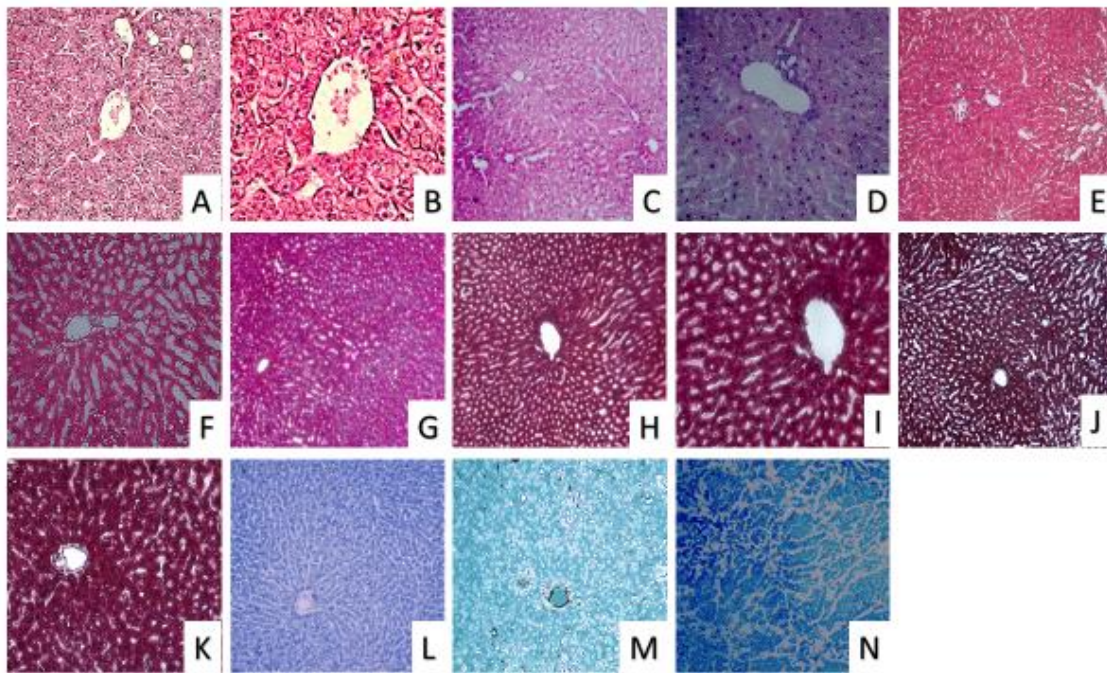


Figure 1. Representative light micrographs of rat liver tissue sections across different fixation and staining cohorts. (A, B) Control group (10% formalin) stained with H&E, showing well-preserved classical lobular architecture and homogeneous cytoplasmic features at 20x and 40x magnifications. (C, D) Experimental Group 2 (formalin + white vinegar) stained with H&E, demonstrating optimal preservation of nuclear distinctiveness and cellular boundaries. (E, F) Experimental Group 1 (white vinegar) stained with H&E, exhibiting localized disruptions in hepatocyte cords and sinusoidal dilatations. (G) Control group stained with MT, showing physiological collagen distribution. (H, I) Experimental Group 2 stained with MT, displaying distinct perisinusoidal collagen fibers without diffuse fibrosis. (J, K) Experimental Group 1 stained with MT, demonstrating preserved connective tissue staining and localized vacuolation. (L) Control group with high-contrast TB staining. (M) Experimental Group 2 showing intense metachromatic TB affinity. (N) Experimental Group 1 illustrating heterogeneous TB staining intensity and reduced cellular clarity.

3.1.2. Histopathological Evaluation of Pancreatic Tissue

Comprehensive light microscopic evaluation of pancreatic tissue architecture was performed across all experimental cohorts utilizing hematoxylin and eosin (H&E), Masson's Trichrome, and toluidine blue staining techniques. In the H&E-stained sections of the control group (fixed with 10% formalin), the tissue exhibited optimal structural integrity and well-preserved classical histomorphological landmarks; low-power examination revealed regular parenchymal architecture organized into distinct lobules by thin connective tissue septa, with clearly delineated serous acinar borders (Figure 2A), while high-power analysis highlighted characteristic cellular polarization in pyramidal acinar cells—demonstrating prominent basal basophilia and apical acidophilia due to zymogen granules—alongside healthy vesicular nuclei, well-circumscribed, lightly stained islets of Langerhans, and morphologically intact interlobular ductal and vascular components (Figure 2B).

Similarly, the H&E-stained sections of Experimental Group 2 (fixed with the formalin + white vinegar mixture) demonstrated excellent preservation of overall tissue integrity and staining quality; low-power assessment confirmed a regular lobular pattern and distinct interlobular septa consistent with the control group (Figure 2C), while high-power magnification revealed distinct acinar cell polarization, well-preserved nuclear morphology without distortion, and a high degree of contrast enabling sharp discrimination between the pale-stained endocrine islets of Langerhans and the surrounding exocrine parenchyma (Figure 2D).

Conversely, in Experimental Group 1 (fixed solely with white vinegar), H&E-stained sections maintained the general tissue framework and lobular configurations, yet low-power views revealed localized interlobular and interacinar spaces resulting from retraction artifacts (Figure 2E); high-power evaluation showed that while the pyramidal acinar morphology and basal-apical staining polarization remained identifiable, localized irregularities in cellular boundaries and minor cytoplasmic vacuolation-like clearing were evident in selected fields (Figure 2F).

Under MT staining, the control group exhibited a well-preserved structural organization; low-power views demonstrated densely arranged exocrine acini with deep basal basophilia and bright acidophilic (red/fuchsia) apical zymogen granules, separated by light-blue/turquoise collagen-rich loose connective tissue septa and flanked by intact vascular/ductal bundles composed of red-stained smooth muscle and blue-stained collagenous walls, confirming a healthy morphomorphological profile (Figure 2G).

For the MT-stained sections of Experimental Group 2, the pancreatic tissue displayed stable lobular organization and pronounced staining contrast, wherein collagen fibers within interlobular septa, periductal, and perivascular areas presented a regular and bright blue distribution at low power (Figure 2H); furthermore, high-power analysis demonstrated that the endocrine islets of Langerhans were easily distinguished by their characteristic pale staining relative to the dense exocrine matrix, showing that this hybrid fixation protocol provides superior contrast for parenchymal-stromal differentiation (Figure 2I).

In Experimental Group 1, MT staining confirmed that the overall morphological integrity and classical lobular architecture were sharply maintained, showing densely packed exocrine acinar units partitioned by thin interlobular connective tissue septa rich in turquoise-blue collagen fibers (Figure 2J); high-power detailed analysis verified distinct acinar cell polarization—characterized by dark basophilic bases and lighter acidophilic (purple) zymogen-rich apices—alongside structurally intact intralobular ducts and vascular elements exhibiting red smooth muscle components and blue peripheral connective tissue walls, thereby presenting a physiological distribution of stromal and parenchymal tissue elements (Figure 2K).

Furthermore, low-power evaluation of the TB-stained control group demonstrated an excellently preserved parenchymal framework with distinct, well-isolated interlobular connective tissue septa (Figure 2L).

In Experimental Group 2, TB-stained specimens retained structural integrity fully comparable to the control group but exhibited a pronounced increase in dye affinity across tissue components; specifically, the nucleoprotein-rich basal regions of the exocrine acinar cells displayed a highly intense, deep blue metachromatic reaction, indicating that the acetic acid content within the white vinegar enhanced cellular dye susceptibility and maximized micro-visual contrast (Figure 2M).

Lastly, TB-stained sections from Experimental Group 1 illustrated a well-preserved general histological framework with clearly identifiable lobular patterns captured across distinct representative microscopic fields (Figure 2N, O); the pyramidal acinar cells within the exocrine compartments exhibited prominent basal basophilia driven by rich ribosomal content alongside granular, lighter-stained apical zymogen zones, while the embedded islets of Langerhans were easily distinguished by their circular/oval cell clustering and pale staining features, with both interlobular and intralobular ductal and vascular networks maintaining complete structural integrity.

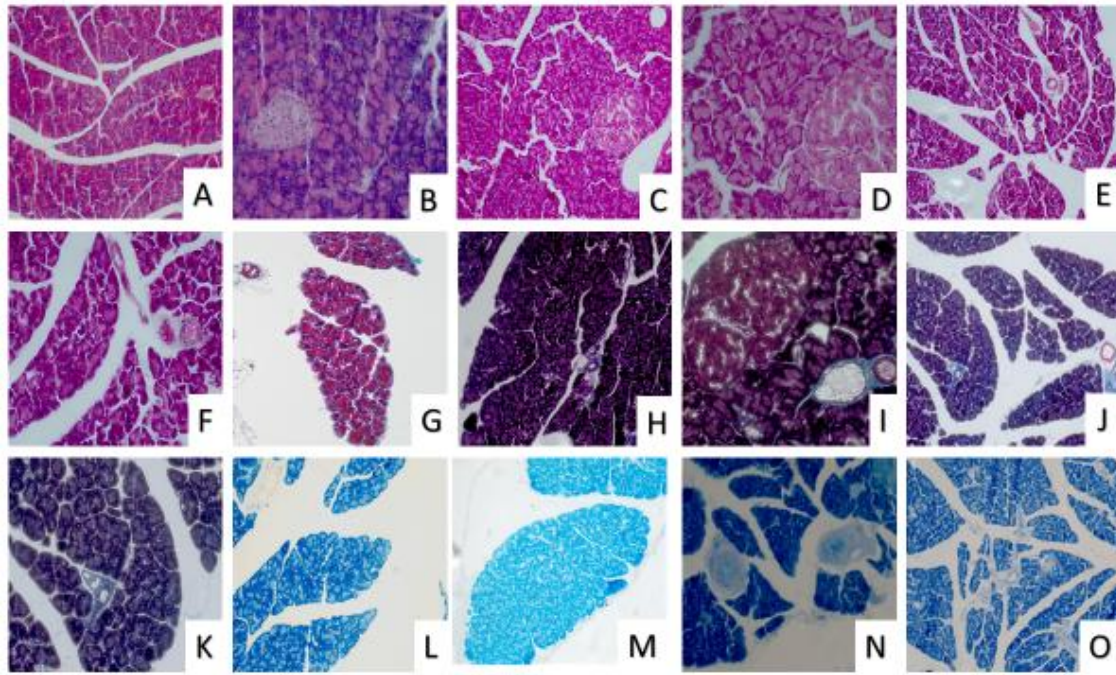


Figure 2. Representative light micrographs of rat pancreatic tissue sections across different fixation and staining cohorts. (A, B) Control group (10% formalin) stained with H&E, showing well-preserved pancreatic acini and lobular landmarks at 20x and 40x magnifications. (C, D) Experimental Group 2 (formalin + white vinegar) stained with H&E at 20x and 40x magnifications, demonstrating optimal cellular boundary and nuclear preservation. (E, F) Experimental Group 1 (white vinegar) stained with H&E at 20x and 40x magnifications, illustrating general architecture alongside localized interlobular retraction spaces. (G) Control group stained with MT (20x), showing physiological collagen distribution within interlobular septa. (H, I) Experimental Group 2 stained with MT at 20x and 40x magnifications, displaying high-contrast differentiation of connective tissue and peripheral framework. (J, K) Experimental Group 1 stained with MT at 20x and 40x magnifications, showing packed acinar units partitioned by thin fibrovascular septa. (L) Control group with high-contrast TB staining (20x). (M) Experimental Group 2 stained with TB 20x, exhibiting a pronounced increase in metachromatic dye affinity. (N, O) Experimental Group 1 stained with TB (20x, separate representative fields), illustrating structural maintenance, distinct ribosomal basal basophilia, and intralobular ductal integrity.

3.1.3. Histopathological Evaluation of Ileum Tissue

Comprehensive light microscopic examination of the ileum architecture was executed across all experimental groups using hematoxylin and eosin (H&E), Masson's Trichrome, and toluidine blue staining methodologies.

In the H&E-stained sections of the control group (fixed with 10% formalin), the characteristic histological strata of the small intestine—namely the tunica mucosa, submucosa, and tunica muscularis—fully retained their structural integrity; low-power overview revealed a highly organized alignment of parallel-arranged, finger-like villous projections lined by morphologically pristine simple columnar enterocytes, alongside regular tubuloalveolar Lieberkühn crypts extending down to the muscularis mucosae (Figure 3A), which was further confirmed under high-power magnification by the precise basal positioning of enterocyte nuclei and the complete absence of tissue-processing artifacts (Figure 3B).

Conversely, the H&E-stained sections of Experimental Group 2 (fixed with the formalin + white vinegar mixture) revealed marked structural deformations and advanced degenerative alterations compared to the control group; low-power assessment highlighted compromised mucosal integrity characterized by significant villous shortening, apical blunting, severe epithelial desquamation, and dense inflammatory cell infiltration coupled with vascular congestion in the lamina propria (Figure 3C), while high-power detailed examination highlighted prominent luminal dilation and architectural distortion within the Lieberkühn crypts, extensive loss of the enterocytic striated brush borders, marked depletion of goblet cells, edematous widening between smooth muscle fibers in the tunica muscularis, and profound perivascular cell cuffing within the dilated submucosal vessels (Figure 3D).

In Experimental Group 1 (fixed solely with white vinegar), H&E-stained sections demonstrated substantial maintenance of the general tissue framework, though early-stage degenerative focal facets were discernible; low-power views illustrated structurally intact villi with only mild localized blunting at the tips and minor epithelial irregularities overlying a lamina propria exhibiting low-grade edema and mononuclear cell infiltration (Figure 3E), while high-power analysis verified well-preserved crypt morphology paired with focal goblet cell hyperplasia or hypersecretion, alongside minor cellular stress indicators such as localized nuclear displacement from the basal pole and cytoplasmic vacuolation within enterocytes, indicating that while solo vinegar fixation induces mild-to-moderate cellular stress, it successfully averts full-thickness mucosal sloughing (Figure 3F).

Under MT staining, low-power evaluation of the control group validated an entirely intact intestinal wall framework and a physiological distribution of stromal components; the smooth muscle strands within the tunica muscularis and muscularis mucosae stained in characteristic fuchsia-red hues, whereas the collagen fibers within the loose connective tissue of the submucosa and lamina propria presented a regular, light-blue network without any pathologic fibrotic expansions across different representative fields (Figure 3G, H).

For the MT-stained sections of Experimental Group 2, a prominent structural degradation was concurrent with a pathological expansion of fibrous tissue; a severe increase in blue-stained collagen density was observed within the expanded submucosa and the core of the distorted villi across distinct areas evaluated at low power, reflecting marked fibrotic remodeling alongside disrupted muscularis mucosae boundaries and extensive transmural structural distortion (Figure 3I, J).

In Experimental Group 1, MT-stained specimens retained clearly distinguishable tissue layers with moderate structural alterations localized to the mucosa and submucosa; low-power inspection revealed overall stable villous contours with localized apical blunting and a moderately intensified distribution of blue-stained collagen fibers within the lamina propria and submucosa compared to controls (Figure 3K), while high-power interrogation confirmed a mild connective tissue accentuation around the Lieberkühn crypts and perivascular fields, with the tunica muscularis maintaining its fuchsia-red structural alignment despite focal edematous spaces at the submucosal transition line (Figure 3L). Furthermore, low-power analysis of the TB-stained control group illustrated a perfectly preserved parenchymal framework, characterized by orderly, finger-like villous projections devoid of surface erosion or mucosal sloughing (Figure 3M); high-power evaluation revealed distinct basal basophilia within both surface enterocytes and crypt cells, complete continuity of the apical brush borders, and distinct granular compartments inside the pristine goblet cell populations (Figure 3N).

In Experimental Group 2, TB-stained sections presented noticeable structural deviations within the mucosa; low-power views demonstrated localized villous disorganization, focal thickening, and moderate shortening, coupled with an increased cellular density within the stroma that did not, however, obliterate the baseline tissue architecture (Figure 3O), while high-power imaging identified localized crypt disorganization, minor enterocytic vacuolation, and a significantly reduced definition of the microvillous brush border relative to control benchmarks (Figure 3P).

Lastly, TB-stained sections from Experimental Group 1 illustrated a majorly sustained histological framework and regular configurations; low-power tracking confirmed normal morphological contours

in the majority of villi despite minor apical blunting and focal epithelial misalignment over a slightly cellular lamina propria (Figure 3Q), while high-power details verified intact Lieberkühn crypt patterns, typical goblet cell morphology, and well-aligned smooth muscle bundles within the tunica muscularis, proving that the basic tissue organization remained steady under this protocol (Figure 3R).

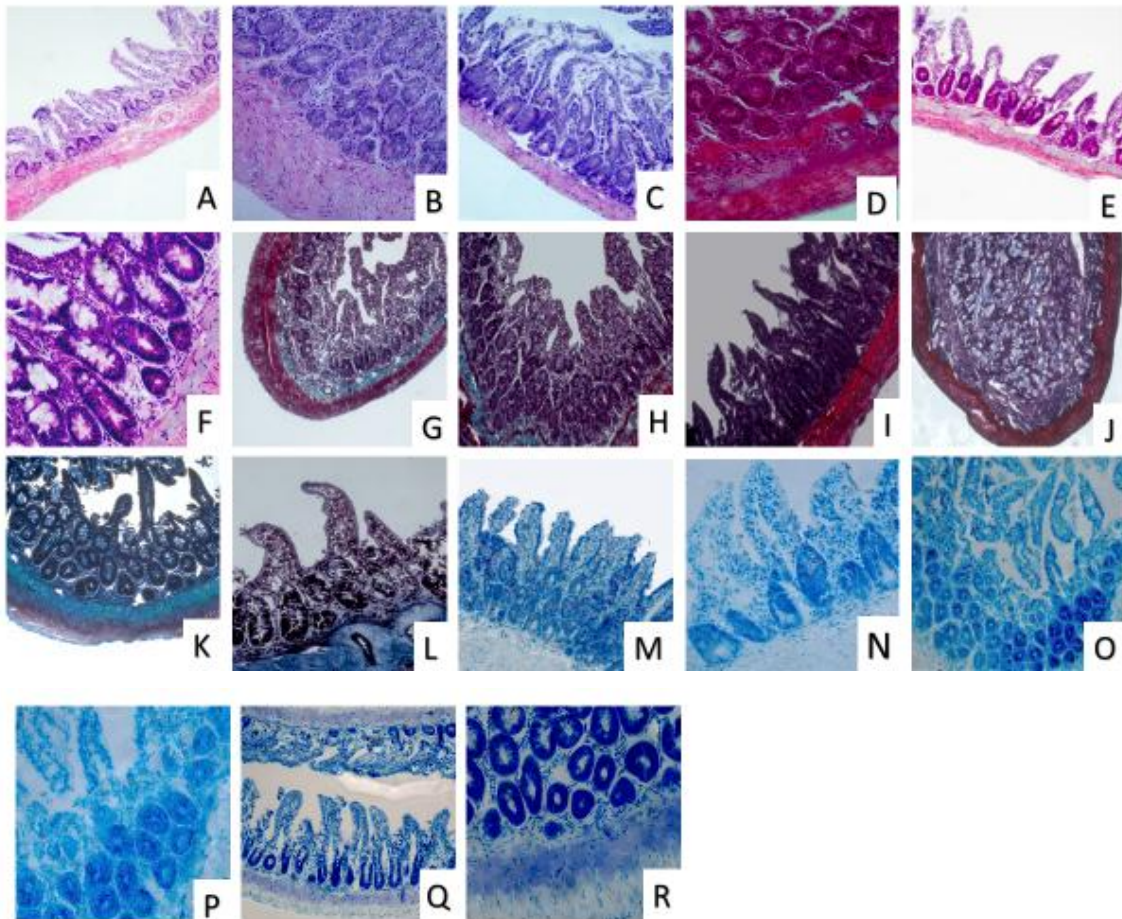


Figure 3. Representative light micrographs of rat ileum tissue sections across different fixation and staining cohorts. (A, B) Control group (10% formalin) stained with H&E, showing well-preserved mucosal layers, intact villi, and regular Lieberkühn crypts at 20x and 40x magnifications. (C, D) Experimental Group 2 (formalin + white vinegar) stained with H&E at 20x and 40x magnifications, demonstrating severe mucosal deformation, villous atrophy, and epithelial desquamation. (E, F) Experimental Group 1 (white vinegar) stained with H&E at 20x and 40x magnifications, illustrating moderate structural preservation with mild apical blunting. (G, H) Control group stained with MT (20x, separate representative fields), exhibiting physiological distribution of blue-stained collagen networks and fuchsia-red smooth muscle fibers. (I, J) Experimental Group 2 stained with MT (20x, separate representative fields), showing significant mucosal degradation accompanied by a pronounced fibrovascular expansion and increased collagen deposition within the lamina propria and submucosa.

(K, L) Experimental Group 1 stained with MT at 20x and 40x magnifications, showing identifiable intestinal strata with moderate, localized collagen intensity around the crypt-vessel junctions. (M, N) Control group stained with TB at 20x and 40x magnifications, revealing distinct basal basophilia and complete brush borders. (O, P) Experimental Group 2 stained with TB at 20x and 40x magnifications, demonstrating localized villous irregularities, blunted mucosal fringes, and reduced microvillous definition. (Q, R) Experimental Group 1 stained with TB at 20x and 40x magnifications, illustrating a well-preserved parenchymal framework with mild, focal inflammatory infiltration.

3.1.4. Semi-Quantitative Histological Evaluation Results

Semi-quantitative evaluation of the histological images was executed in alignment with the predetermined morphological and staining criteria. Within this framework, tissue sections of the liver, pancreas, and ileum were systematically assessed regarding tissue structural integrity, cellular architecture, morphological alterations, staining quality, nuclear morphology, distinctness of cytoplasmic structures, and connective tissue (extracellular matrix) profile.

Inter-observer reliability for the semi-quantitative histological assessments was analyzed using the Intraclass Correlation Coefficient (ICC), based on the scores assigned to the histological images by six independent observers experienced in the field of Histology and Embryology, using a structured 27-item evaluation form. A two-way random-effects model and an absolute agreement approach were utilized for the analysis. The calculated ICC value for the average measurements was 0.768 (95% confidence interval [CI]: 0.603–0.880; $p < 0.001$), demonstrating a good level of inter-observer reliability (Table 7).

Measurement Type	ICC	95% Confidence Interval	F	p
Average Measures	0.768	0.603 – 0.880	4.415	<0.001

Table 7. Results of intraclass correlation coefficient (ICC) analysis for inter-observer reliability in the evaluation of histological images.

Following confirmation of good interobserver agreement, the Kruskal–Wallis test was performed to evaluate the distribution of semi-quantitative scores among the experimental groups. According to the Kruskal–Wallis analysis, no statistically significant differences were observed among the Control, Experimental Group 1, and Experimental Group 2 for Scores 1, 2, 3, 4, and 6, which represent the evaluations assigned to the histological images by the six independent observers ($p > 0.05$).

In contrast, a statistically significant difference was detected among the groups for Score 5 (the score assigned by the fifth observer) ($\chi^2 = 8.176$, $df = 2$, $p = 0.017$). Examination of the mean rank values revealed that the Control group had the highest mean rank, Experimental Group 1 had the lowest, and Experimental Group 2 exhibited an intermediate mean rank (Table 8).

Evaluator (Score)	Control (Mean Rank)	Experimental Group 1 (Mean Rank)	Experimental Group 2 (Mean Rank)	χ^2	df	p
Score 1	16.72	10.00	15.94	4.288	2	0.117
Score 2	17.17	11.45	13.63	2.666	2	0.264
Score 3	14.06	12.95	15.25	0.415	2	0.813
Score 4	14.17	12.50	15.69	0.809	2	0.667
Score 5	19.50	9.80	13.06	8.176	2	0.017
Score 6	16.67	10.10	15.88	4.442	2	0.109

Table 8. Kruskal–Wallis test results of semi-quantitative evaluation scores according to experimental groups.

To provide a more detailed overview of the distributions across statistically significant and non-significant groups, the median (min–max) values of the semi-quantitative scores assigned by six independent observers are presented by organ in Table 9. To visually compare the median values and distributions, boxplot graphs for the liver, pancreas, and ileum tissues are shown in Figures 4, 5 and 6 respectively.

Table 9 and the corresponding boxplots revealed that the median scores of the Experiment 1 group in liver tissue were lower than those of the control group. In pancreas tissue, the median values remained

similar across all groups, whereas in ileum tissue, both Experiment 1 and Experiment 2 groups exhibited lower median scores compared to the control group.

Tissues	Group	Median	Min	Max
Liver	Control	3	1	5
	Experimental Group 1	2	1	5
	Experimental Group 2	3	3	5
Pancreas	Control	3	2	5
	Experimental Group 1	3	2	5
	Experimental Group 2	3	1	4
Ileum	Control	3	1	5
	Experimental Group 1	2	1	3
	Experimental Group 2	2	1	5

Table 9. Median (min–max) semi-quantitative scores of the experimental groups, stratified by tissue

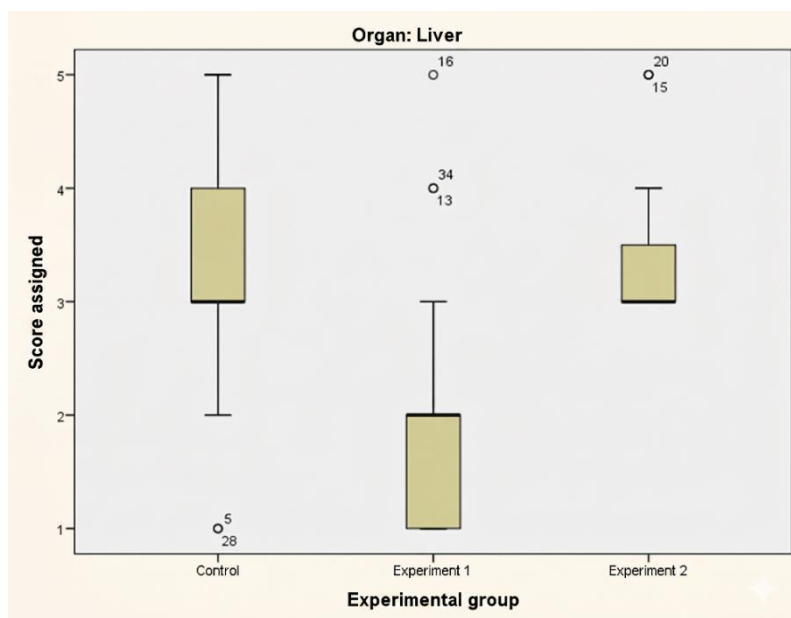


Figure 4. Boxplot illustration showing the distribution of semi-quantitative scores in liver tissue across different experimental groups

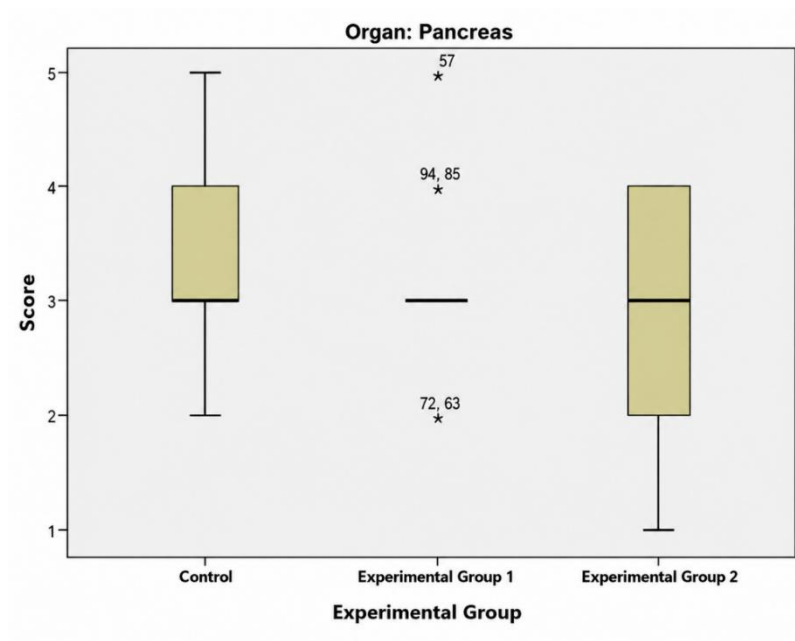


Figure 5. Boxplot showing the distribution of semi-quantitative scores in pancreatic tissue among the experimental groups

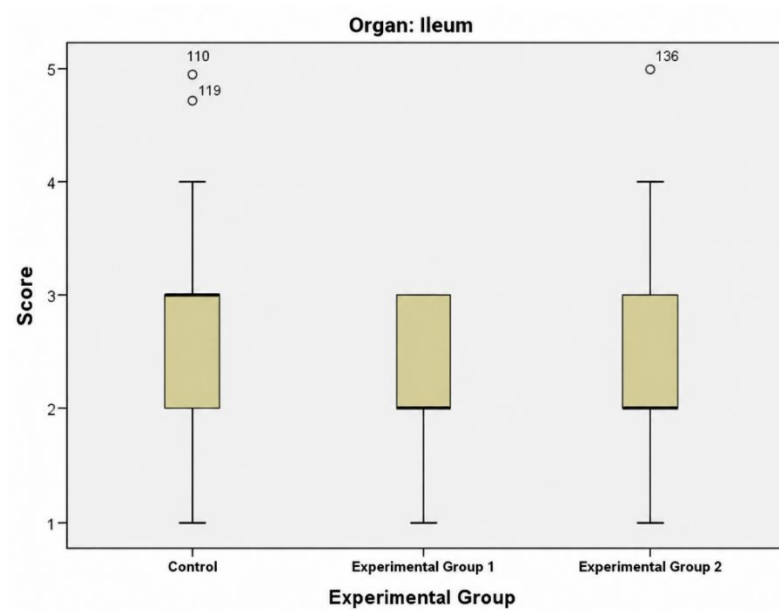


Figure 6. Boxplot showing the distribution of semi-quantitative scores in ileal tissue among the experimental groups

Following the detection of a statistically significant difference among groups in the Score 5 parameter (the score assigned to the images by the 5th observer) via the Kruskal–Wallis test, the Mann–Whitney U test was performed for pairwise comparisons to determine the source of this variance. According to the Mann–Whitney U test results, a statistically significant difference was observed between the control and Experiment 1 groups ($U = 14.50$; $Z = -2.626$; $p = 0.009$). Similarly, a statistically significant difference was identified between the control and Experiment 2 groups ($U = 17.00$; $Z = -2.015$; $p = 0.044$). In contrast, no statistically significant difference was found between the Experiment 1 and Experiment 2 groups ($p > 0.05$) (Figure 7).

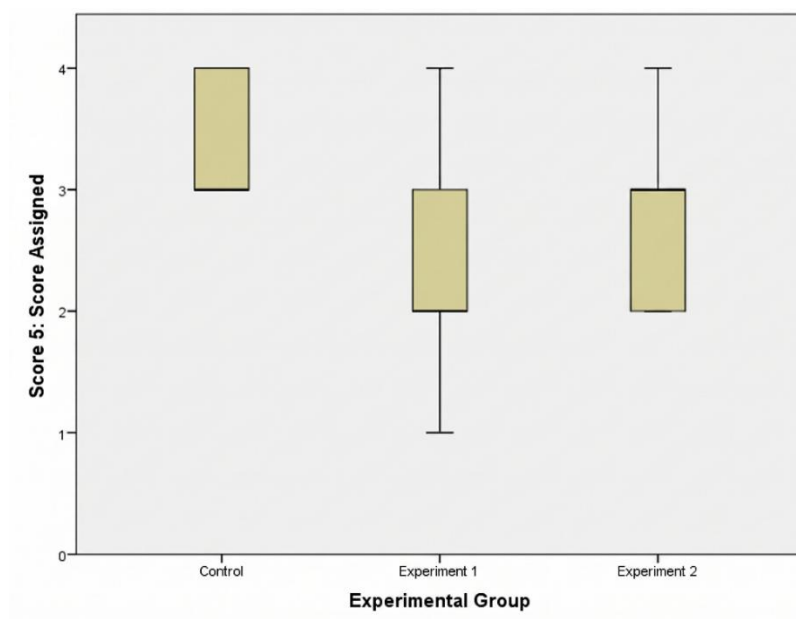


Figure 7. Intergroup boxplot for the "Score 5" variable

3.1.5. Results of Quantitative Image Analysis Using ImageJ

3.1.5.1. Optical Density Analysis of Liver H&E-Stained Sections

Optical density analysis of Hematoxylin and Eosin (H&E)-stained liver sections was performed on digital images captured at 20× objective magnification using ImageJ software (NIH, Bethesda, MD, USA). Pixel intensity was measured via the 'mean gray value' method on an 8-bit grayscale ranging from 0 (maximum opacity) to 255 (maximum brightness). Five independent regions of interest (ROIs) were evaluated per sample, and the mean value was calculated for statistical analysis.

The mean optical density values for the control, Experiment 1, and Experiment 2 groups in H&E-stained liver tissues were 155.99 ± 8.27 , 156.07 ± 20.98 , and 168.41 ± 20.72 , respectively. The normality of

the mean intensity values obtained from H&E staining was assessed using the Shapiro–Wilk test. The test results indicated a normal distribution across all groups ($p > 0.05$); thus, parametric tests were employed for subsequent statistical analysis. Homogeneity of variances was confirmed by Levene's test ($p = 0.304$). One-way ANOVA revealed no statistically significant differences among the groups ($F(2,6) = 0.490$, $p = 0.635$). Tukey's HSD post-hoc test further confirmed the absence of significant differences in all pairwise group comparisons ($p > 0.05$). Figure 8 graphically illustrates the mean gray value intensities across the experimental groups.

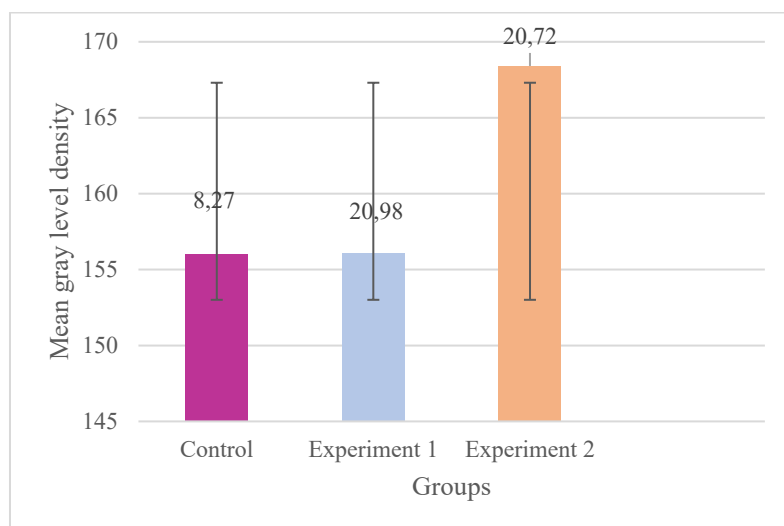


Figure 8. Density of groups in liver HE staining (mean $\pm$ SD)

3.1.5.2. Optical Density Analysis of Liver Masson's Trichrome-Stained Sections

Optical density analysis of Masson's Trichrome-stained liver sections was conducted using the identical ImageJ processing parameters and ROI evaluation workflow described for the H&E analysis. The mean optical density values for the control, Experiment 1, and Experiment 2 groups in MT-stained liver tissues were 143.59 ± 16.18 , 122.63 ± 21.18 , and 123.39 ± 3.67 , respectively.

The normality of the datasets was verified via the Shapiro–Wilk test ($p > 0.05$ for all groups), justifying the use of parametric analysis. Levene's test demonstrated homogeneity of variances ($p = 0.197$). One-way ANOVA revealed no statistically significant differences among the experimental groups ($F(2,6) = 1.757$, $p = 0.251$). Tukey's HSD post-hoc analysis further confirmed that none of the pairwise comparisons reached statistical significance ($p > 0.05$). Figure 9 graphically represents the mean gray-level intensity values across the cohorts.

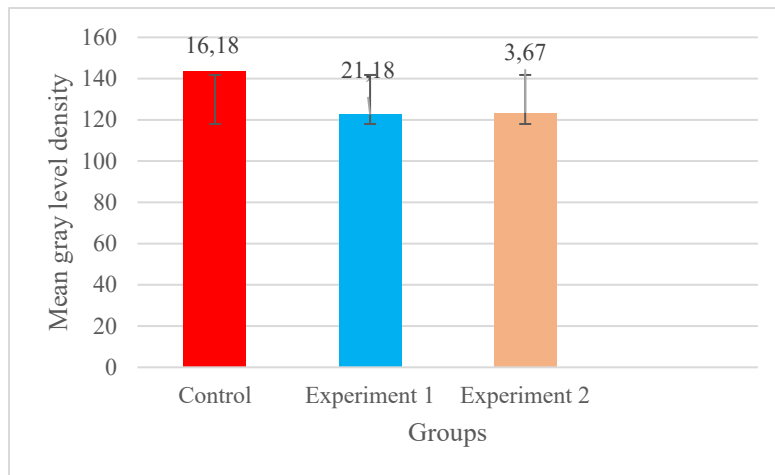


Figure 9. Density of groups (mean $\pm$ SD) in liver Masson trichrome staining

3.1.5.3. Optical Density Analysis of Liver Toluidine Blue-Stained Sections

Optical density analysis of Toluidine Blue (TB)-stained liver sections was similarly performed using the established ImageJ quantification workflow and ROI evaluation parameters. The mean intensity values for the control, Experiment 1, and Experiment 2 groups in TB-stained liver tissues were 184.00 ± 24.94 , 187.67 ± 19.87 , and 191.29 ± 38.06 , respectively.

Normality of the TB staining intensity datasets was confirmed by the Shapiro–Wilk test ($p > 0.05$ for all groups), meeting the assumptions for parametric analysis. Levene's test indicated homogeneity of variances ($p = 0.538$). One-way ANOVA revealed no statistically significant variance among the experimental cohorts ($F(2,6) = 0.048$, $p = 0.953$). Tukey's HSD post-hoc test corroborated that all inter-group comparisons were non-significant ($p > 0.05$). Figure 10 depicts the graphical distribution of the mean gray-level intensities across the groups.

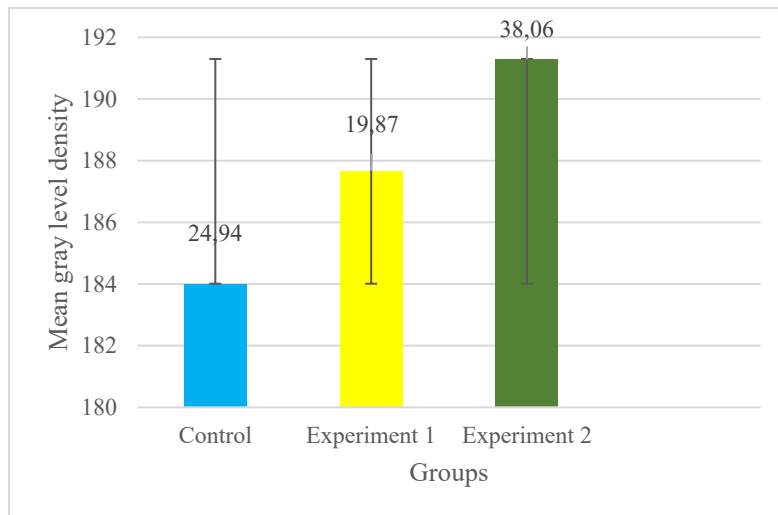


Figure 10. Density of groups in liver toluidine blue staining (mean $\pm$ SD)

3.1.5.4. Optical Density Analysis of HE-Stained Pancreatic Specimens

The dataset conformed to a normal distribution (Shapiro-Wilk test, $p > 0.05$) with homogenous variances across all experimental groups (Levene's test, $p = 0.199$). One-way ANOVA revealed no statistically significant differences in the mean gray value intensities of H&E-stained pancreatic tissues among the Control, Experiment 1, and Experiment 2 groups, $F(2, 6) = 0.132$, $p = 0.879$. Furthermore, pairwise comparisons using Tukey's HSD post-hoc test confirmed the absence of significant differences between any of the groups ($p > 0.05$). The average gray-level intensity values across all groups are visually represented in Figure 11.

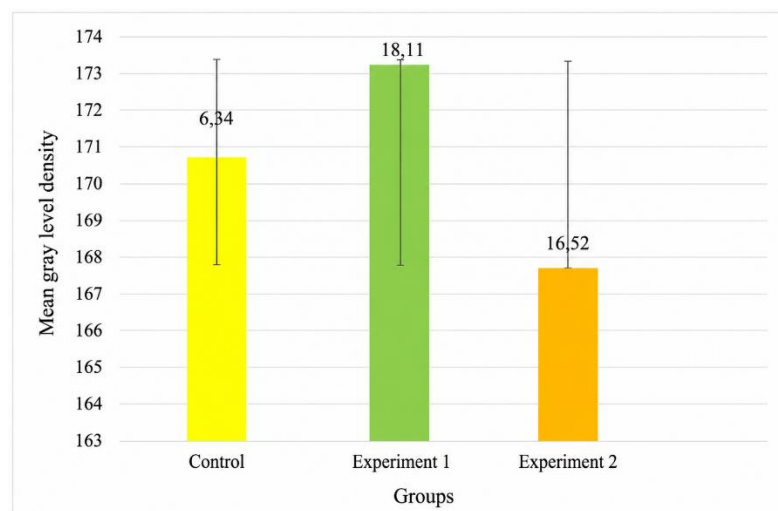


Figure 11. Density of the groups in HE staining of the pancreas (mean $\pm$ SD)

3.1.5.5. Optical Density Analysis of Masson's Trichrome-Stained Pancreatic Sections

The optical density data exhibited a normal distribution (Shapiro-Wilk test, $p > 0.05$) with homogenous variances across all experimental groups (Levene's test, $p = 0.547$). One-way ANOVA revealed a statistically significant difference in the mean gray value intensities of Masson's trichrome-stained pancreatic tissues among the groups, $F(2, 6) = 9.097$, $p = 0.015$.

Subsequent pairwise comparisons using Tukey's HSD post-hoc test demonstrated that this significant difference was driven exclusively by the comparison between the Control group and the Experiment 2 group ($p < 0.05$). Conversely, the Experiment 1 group exhibited intermediate intensity values, showing no statistically significant differences when compared to either the Control or the Experiment 2 groups ($p > 0.05$). The statistical distributions and variations in mean gray-level intensities among the groups are graphically illustrated in Figure 12.

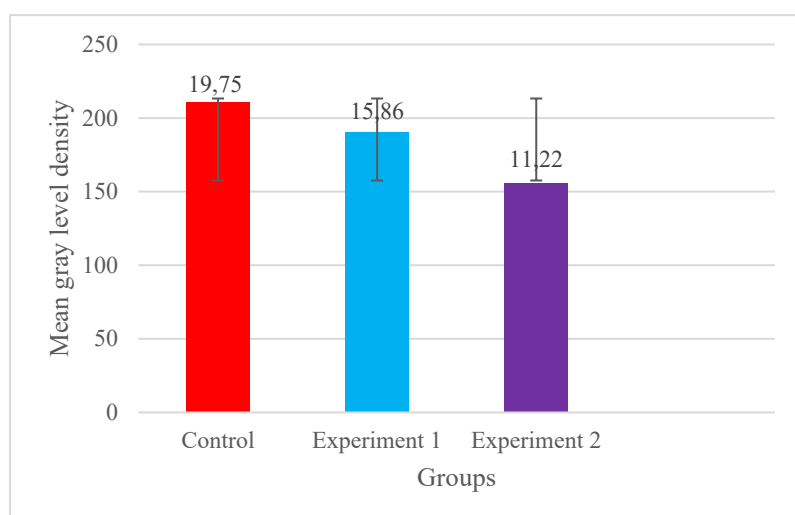


Figure 12. Density of groups in Masson's trichrome staining of the pancreas (mean $\pm$ SD)

3.1.5.6. Optical Density Analysis of Pancreas Specimens Stained with Toluidine Blue

The dataset satisfied the assumption of normality across all experimental groups (Shapiro-Wilk test, $p > 0.05$); however, Levene's test revealed a lack of homogeneity of variances, $p = 0.046$. Despite the heterogeneity of variances, one-way ANOVA demonstrated no statistically significant differences in the mean gray value intensities of Toluidine Blue-stained pancreatic tissues among the Control, Experiment 1, and Experiment 2 groups, $F(2, 6) = 2.672$, $p = 0.148$.

Furthermore, pairwise comparisons using Tukey's HSD post-hoc test confirmed the absence of significant differences between any of the groups ($p > 0.05$). The average gray-level intensity values and their distributions across all experimental groups are visually illustrated in Figure 13.

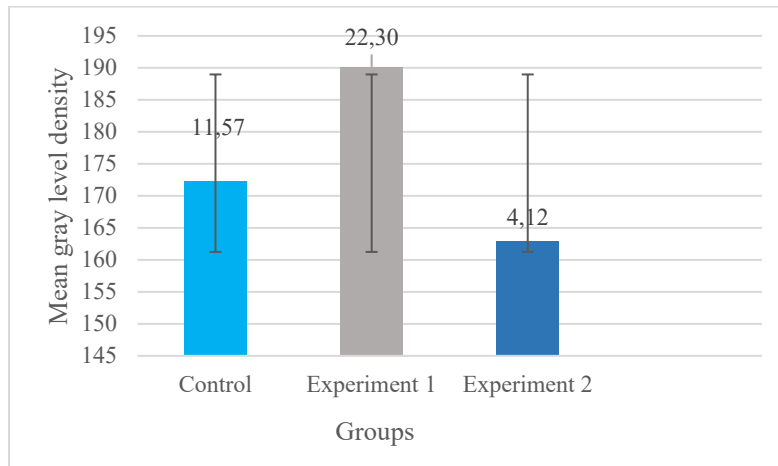


Figure 13. Group density in toluidine blue staining of the pancreas (mean $\pm$ SD)

3.1.5.7. Optical Density Analysis of HE-Stained Ileum Specimens

The dataset conformed to a normal distribution (Shapiro-Wilk test, $p > 0.05$) with homogenous variances across all experimental groups (Levene's test, $p = 0.919$). One-way ANOVA demonstrated no statistically significant differences in the mean gray value intensities of H&E-stained ileum tissues among the Control, Experiment 1, and Experiment 2 groups, $F(2, 6) = 2.225$, $p = 0.189$.

Furthermore, pairwise comparisons using Tukey's HSD post-hoc test confirmed the absence of significant differences between any of the groups ($p > 0.05$). The average gray-level intensity values and their distributions across all groups are graphically illustrated in Figure 14.

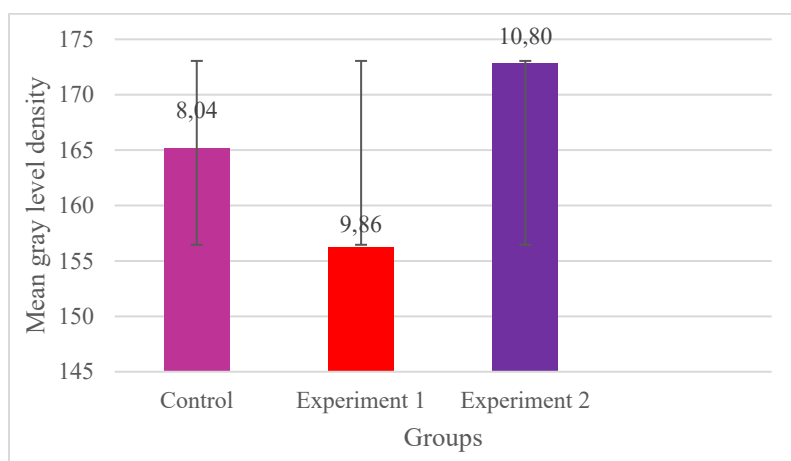


Figure 14. Density of the groups in HE staining of the ileum (mean $\pm$ SD)

3.1.5.8. Optical Density Analysis of Masson's Trichrome-Stained Ileum Preparations

Assessment of data distribution via the Shapiro-Wilk test revealed a deviation from normality within the Experiment 2 group ($p < 0.05$), although the remaining groups conformed to a normal distribution ($p > 0.05$). Nevertheless, Levene's test confirmed that the assumption of homogeneity of variances was strictly satisfied across all experimental groups, $p = 0.949$. One-way ANOVA demonstrated no statistically significant differences in the mean gray value intensities of Masson's trichrome-stained ileum tissues among the Control, Experiment 1, and Experiment 2 groups, $F(2, 6) = 0.748$, $p = 0.513$.

Furthermore, pairwise comparisons using Tukey's HSD post-hoc test confirmed the absence of significant differences between any of the groups ($p > 0.05$). The average gray-level intensity values and their variations across all experimental groups are graphically illustrated in Figure 15.

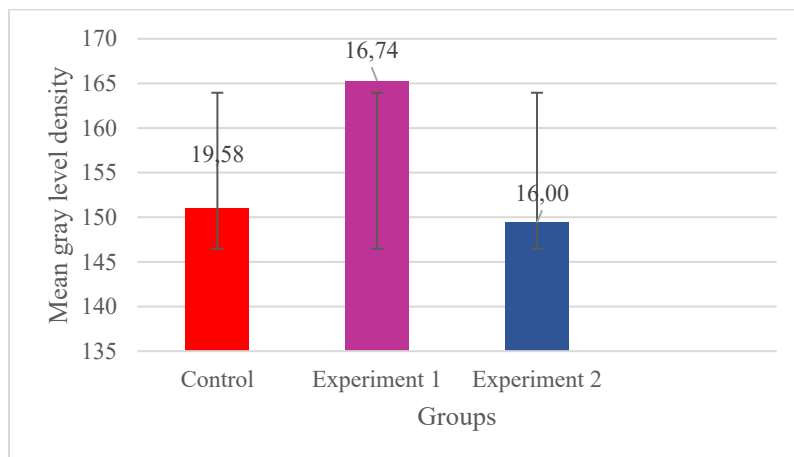


Figure 15. Density of groups in Masson's trichrome staining of the ileum (mean $\pm$ SD)

3.1.5.9. Optical Density Analysis of Toluidine Blue-Stained Ileum Preparations

The dataset conformed to a normal distribution (Shapiro-Wilk test, $p > 0.05$) with homogenous variances across all experimental groups (Levene's test, $p = 0.098$). One-way ANOVA demonstrated no statistically significant differences in the mean gray value intensities of Toluidine Blue-stained ileum tissues among the Control, Experiment 1, and Experiment 2 groups, $F(2, 6) = 1.896$, $p = 0.230$.

Furthermore, pairwise comparisons using Tukey's HSD post-hoc test confirmed the absence of significant differences between any of the groups ($p > 0.05$). The average gray-level intensity values across all groups are graphically illustrated in Figure 16.

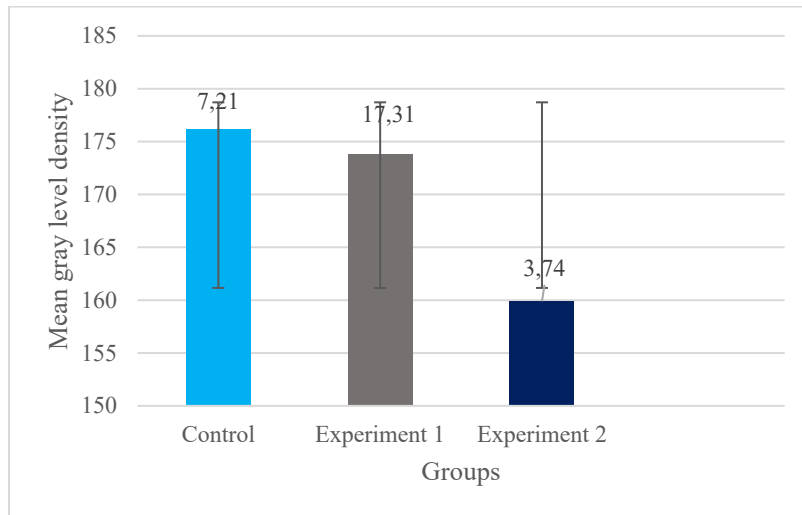


Figure 16. Density of groups in toluidine blue staining of the ileum (mean $\pm$ SD)

4. Discussion

Histological fixation is a critical and irreversible stage aimed at preserving the morphological structure, molecular composition, and cellular details of tissue in its viable state (Bancroft & Gamble, 2008; International Agency for Research on Cancer [IARC], 2012). An ideal fixative must arrest autolysis, prevent bacterial decomposition, and render the tissue resilient against mechanical stresses during subsequent sectioning and staining processes (Carson, 2015). Although 10% formalin has been utilized as the gold standard for many years, safer alternatives are being actively investigated due to its genotoxic, carcinogenic, and environmental risks (Eltoum et al., 2001; Baker, 1958; Karabiyıklı & Şengün, 2017).

In this study, the histomorphological integrity of liver, pancreas, and ileum tissues fixed with formalin (control), white vinegar (Experiment 1), and a formalin/white vinegar mixture (Experiment 2) was evaluated using semi-quantitative and digital (ImageJ) analyses (Binetti et al., 2006). Among the evaluations of six independent observers, only the fifth observer showed significant differences in certain groups, demonstrating that observer-dependent variation is an expected outcome (Eltoum et al., 2001; Kiernan, 2008). In multi-observer evaluations, such individual differences are defined as

"interobserver variability" in the literature and are considered expected due to the nature of subjective evaluation methods. Based on median values and group rankings, it was observed that individual observer variations did not significantly influence the overall trend of the results.

The morphological integrity of the tissues was examined using Hematoxylin and Eosin (HE), Masson's Trichrome (MT), and Toluidine Blue (TB) stainings; in MT, the connective tissue was observed in blue tones and the parenchyma in red tones, while in TB, basal basophilic and metachromatic reactions were noticed. Depending on the type of fixative, staining intensity and contrast exhibited slight differences, which created variation in the evaluations of some observers. It is recommended that staining quality be evaluated using quantitative parameters in the future (Latendresse et al., 2002; McLaughlin, 2003; Plessi, 1985).

Since the Experiment 2 mixture contains formalin and acetic acid (white vinegar)—similar to Bouin's fixative—it was able to penetrate tissues rapidly and yielded results consistent with reported mechanisms suggesting that it may have coagulative effects on proteins (McLaughlin, 2003; Plessi, 1985; Puchtler & Meloan, 1985). Acetic acid contributes to the preservation of the nuclear structure by ensuring the coagulation of nucleic acids, whereas formaldehyde stabilizes cytoplasmic and extracellular proteins (Crookham & Dapson, 1991; Bancroft & Gamble, 2013). This combination facilitated a detailed examination of connective tissue and collagen fibers, particularly through MT and TB stainings (Bancroft & Gamble, 2008; Latendresse et al., 2002). Furthermore, the rapid penetration and protein-shrinking effect of acetic acid supported the histomorphological integrity of the tissues by enhancing contrast and color intensity during staining (Hewitson & Darby, 2010; Humason, 2002; Sridharan & Shankar, 2012; Suvarna et al., 2019).

When evaluated in an organ-specific manner, a decrease in the distinctness of cellular boundaries and heterogeneity in tissue integrity were observed in liver tissue specimens fixed with white vinegar. Conversely, in pancreatic tissue, morphological integrity was largely preserved due to the maintenance of acinar structures and connective tissue elements. In the ileum tissue, more sensitive responses to the chemical effects of fixatives were observed due to the delicate structure of the mucosal epithelium, and partial irregularities in villus integrity were noted in the Experiment 2 group (Zanini et al., 2012). These findings demonstrate that the effects of fixatives on histological tissues can vary depending on the structural and cellular characteristics of the respective organ.

During histological evaluations, certain differences were observed in the sectioning process depending on the methods used for tissue fixation. In the liver tissues of the Experiment 1 group fixed with white

vinegar and the ileum tissues of the Experiment 2 group fixed with the mixture method (formalin + white vinegar), difficulties were encountered in maintaining tissue integrity during the sectioning stage using a microtome in some specimens. This situation suggests that the distinct chemical action mechanisms of fixatives on proteins may affect the mechanical resilience of the tissue.

Due to the ordinal nature of the semi-quantitative scores, non-parametric statistical methods (Kruskal–Wallis test) were preferred and the analyses were conducted within this scope. ImageJ-based quantitative analyses, on the other hand, were utilized as a complementary tool to the semi-quantitative observational evaluations; while they revealed general trends and prominent differences, they reflected structural changes at the micro-level only to a limited extent (Eltoum et al., 2001).

Prior studies support the potential of alternative or complementary fixatives to formalin. Öztürk Gürgen and Yıldırım (2016) compared the histological efficacy of 3.7% formaldehyde, Carnoy, and Hollande solutions in spleen and kidney tissues, reporting that 3.7% formaldehyde was more effective, whereas Carnoy and Hollande solutions could be particularly useful in neoplastic and lymphoid tissues. Özdemir (2016) demonstrated the limited fixation effect of a saturated saline solution, while reporting that its temporary combined use with formalin is possible. Demiraslan (2024), on the other hand, stated that a solution containing honey, ethanol, and citric acid could preserve the histomorphological integrity of tissues in HE, MT, and TB stainings, and that a solution containing 40% honey might stand out among alternative fixatives if standardized.

Karaman (2011) emphasized that formalin-free fixatives such as Glyo-Fixx®, Cell block®, and FineFIX® yielded results close to or better than formalin at the histomorphological and molecular levels, but required optimization in immunohistochemical applications. These findings support that compound and alternative fixatives can be effective in preserving the histomorphological integrity of tissues and should be evaluated more comprehensively through comparative quantitative analyses with formalin (Hopwood et al., 1990; Altman, 1991; Fox et al., 1985; Gökçimen, 2006; Grizzle, 2009).

In conclusion, white vinegar and the Experiment 2 mixture can be considered as alternative or complementary fixatives to formalin with appropriate optimizations and tissue-specific approaches; particularly, the Experiment 2 compound fixative was effective in preserving the histomorphological integrity of tissues via MT and TB stainings (Humason, 2002; Latendresse et al., 2002; Sridharan & Shankar, 2012).

5. Conclusion

This study successfully evaluated and compared the histomorphological effects of alternative bio-fixatives—white vinegar (Experiment 1) and a formalin/white vinegar mixture (Experiment 2)—against standard formalin using multi-observer semi-quantitative criteria and digital ImageJ analysis. Our findings indicate that white vinegar preserves morphological integrity remarkably well in organs with compact parenchymal organization, such as the pancreas, though it may introduce structural heterogeneity and altered cellular clarity in the liver and delicate mucosal tissues like the ileum.

Notably, the compound fixative (Experiment 2) demonstrated a performance closely rivaling formalin, offering distinct advantages in visualizing connective tissue architecture and collagen fibers via specialized Masson's Trichrome and Toluidine Blue stainings, while maintaining high consistency across independent evaluations.

These variations are heavily driven by the distinct chemical modalities of the fixatives, where white vinegar acts through protein coagulation and formalin operates via molecular cross-linking. In conclusion, while tissue-specific optimizations remain essential, both white vinegar and its formalin blend emerge as viable, eco-friendly, and safer alternatives to traditional formaldehydes, marking a significant step forward for green laboratory practices in modern histology.

Limitations

Although this study provides valuable insights into the potential use of white vinegar as an eco-friendly alternative bio-fixative, several limitations must be acknowledged. First, the evaluation was primarily restricted to structural and histomorphological aspects utilizing routine histochemical stains (H&E, Masson's Trichrome, and Toluidine Blue). Advanced molecular analyses, such as immunohistochemistry (IHC) to assess antigen preservation or nucleic acid extraction to determine DNA/RNA integrity, were not performed, which are essential to fully validate this bio-fixative for modern molecular pathology. Second, during the microtomy phase, certain tissue specimens fixed with pure white vinegar or the combination mixture exhibited technical challenges, including increased tissue friability and minor fragmentation during sectioning. This indicates that further procedural adjustments or chemical modifiers are needed to optimize tissue elasticity and mechanical resilience. Third, this study did not evaluate the long-term stability and preservation capacity of vinegar-fixed tissues over extended archiving periods, leaving its compatibility with long-term paraffin block storage undetermined. Lastly, the experimental scope was restricted to a subset of digestive system organs (liver, pancreas, and ileum) harvested from a specific rodent model. Future comprehensive investigations encompassing wider tissue diverse profiles (e.g., central nervous system tissues, lungs,

and kidneys) and human clinical bioptic specimens are required to generalize these green laboratory applications.

Declarations

Ethical Approval

This study was approved by the Local Ethics Committee for Animal Experiments at Aydın Adnan Menderes University (Decision No: 64583101/2025/001, dated 09.01.2025). All experimental procedures involving animals were conducted in strict compliance with institutional and international guidelines for the care and use of laboratory animals.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contributions

All authors contributed significantly to the current work. **N.E.** and **A.G.** conceived and designed the study. **N.E.** and **N.S.K.** performed the experimental animal procedures, tissue processing, and histological stainings. **A.G.**, **N.E.**, and **N.S.K.** conducted the semi-quantitative scoring, digital ImageJ image analysis, and statistical evaluations. **N.S.K.** and **N.E.** drafted the manuscript. **A.G.** critically revised the manuscript for intellectual content. All authors have read and approved the final version of the manuscript.

Data Availability Statement

The datasets generated and analyzed during the current study, including raw ImageJ densitometric outputs and semi-quantitative evaluation forms, are available from the corresponding author upon reasonable request.

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