UNIVERSITI PUTRA MALAYSIA

EFFECTS OF DIFFERENT FIXATIVE AGENTS IN PROLONGED FIXATION ON HISTOLOGICAL STRUCTURES OF MURINE RENAL TISSUE

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FACULTY OF VETERINARY MEDICINE

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A project paper submitted to the

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In partial fulfilment of the requirement for the

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Serdang, Selangor Darul Ehsan

CERTIFICATION

It is hereby certified that I have read this project paper entitled "Effects of Different Fivative Agents in Prolonged Fixation on Histological Strutures of Murine Renal Tissue", by Mastura binti Samsuddin and in my opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4999 - Project.

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LIST OF ABBREVIATION

NBF	Neutral Bufferd Solution
PFA	Paraformaldehyde
EtOH	Ethanol
PBS	Phosphate Buffered Saline
H&E	Hematoxylin & Eosin
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
EMA	A combination of ethanol, methanol & acetic acid
PCT	Proximal convoluted tubule
DCT	Distal convoluted tubule
LoH	Loop of Henle
CD	Collecting duct
SD	Standard Deviation
p	Significance value

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek.

KESAN FIKSATIF YANG BERBEZA DALAM FIKSASI YANG LAMA KE ATAS STRUKTUR HISTOLOGI TISU GINJAL MURIN

oleh

Mastura binti Samsuddin

2023

Penyelia: Dr. Mohd Akmal Mohd Noor

Penilaian ginjal pada peringkat sel diperlukan untuk menentukan status kesihatan ginjal. Oleh itu, penyediaan ginjal untuk pemeriksaan histologi adalah penting. Langkah fiksasi adalah sangat penting kerana fiksatif yang digunakan dan tempoh fiksasi menentukan kualiti tisu yang diperiksa semasa proses pemerhatian. Disebabkan longgokan kes berlaku di makmal histopatologi, banyak projek penyelidikan memerlukan fiksasi yang berpanjangan untuk pemeliharaan spesimen. Oleh itu, kajian terkini bertujuan untuk mengkaji dan membandingkan kesan fiksatif yang berbeza terhadap penilaian histologi ginjal murin di bawah fiksasi yang berpanjangan. Bahagian tisu ginjal diambil dari sampel murin bebas. Fiksatif berikut digunakan dalam kajian ini: 10% Neutral-Buffered Formalin (NBF), 10% Formalin, 4% Paraformaldehyde (PFA), 10% NBF diikuti oleh 70% Etanol (EtOH), dan 10% NBF diikuti oleh larutan Phosphate-Buffered (PBS). Hematoksilin dan Eosin (H&E) digunakan untuk pemeriksaan mikroskopik tisu yang diproses. Struktur histologi yang

diperhatikan adalah korpuskel ginjal, tubulus konvolus proximal, tubulus konvolus distal, lingkaran Henle, dan saluran pengumpul. Di bawah pembesaran 40X, histologi ginjal dinilai oleh peratusan sel yang mengalami autolisis. Bahagian yang difiks dengan 10% NBF diikuti oleh 70% Etanol menyediakan pemeliharaan morfologi tisu yang baik kerana menghasilkan peratusan sel autolisis terendah (38%) di bawah fiksasi yang berpanjangan. Bahagian yang difiks dengan 10% Formalin mempunyai peratusan autolisis tertinggi (48%). Bahagian lain yang difiks dengan 10% NBF, 10% NBF diikuti oleh PBS, dan 4% PFA masing-masing mempunyai 40%, 44%, dan 47% autolisis. Secara keseluruhan, 10% NBF diikuti oleh 70% EtOH terbukti sebagai fiksatif terbaik untuk fiksasi yang berpanjangan kerana ia memberikan kesan paling kurang terhadap histologi sel berbanding dengan fiksatif yang lain.

Kata kunci: fiksatif, fiksasi yang berpanjangan, struktur histologi, tisu ginjal murin, autolisis.

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4999- Project.

EFFECTS OF DIFFERENT FIXATIVE AGENTS IN PROLONGED FIXATION ON HISTOLOGICAL STRUCTURE OF MURINE RENAL TISSUE

by

Mastura binti Samsuddin

2023

Supervisor: Dr. Mohd Akmal Mohd Noor

The assessment of the kidney at the cellular level is required to determine the kidney's health status. Thus, preparing the kidney for histological examination is critical. The fixation step is extremely important since the solutions used and fixation durations determine the tissue quality examined during the viewing step. Due to the large caseload in the histopathology laboratory, many research projects require prolonged fixation and preservation of specimens. Thus, the current study intends to examine and compare the effects of different fixative agents on murine renal histological evaluation under prolonged fixation. The sections of renal tissue were taken from archived samples of the specific pathogen-free murine. The following fixatives were used: 10% Neutral-Buffered Formalin (NBF), 10% Formalin, 4% Paraformaldehyde (PFA), 10% NBF followed by 70% Ethanol (EtOH), and 10% NBF followed by Phosphate-Buffered solution (PBS). Hematoxylin and eosin (H&E) were used for microscopic examination of the processed tissues. Histological structures

observed were renal corpuscles, proximal convoluted tubule, distal convoluted tubule, loop of Henle, and collecting duct. Under 40X magnification, renal histology was evaluated by the percentage of autolysed cells. Sections fixed with 10% NBF followed by 70% Ethanol provided acceptable preservation of tissue morphology as they had the lowest percentage of autolysed cells (38%) under prolonged fixation. Sections fixed with 10% Formalin had the highest percentage of autolyses (48%). The other sections fixed with 10% NBF, 10% NBF followed by PBS, and 4% PFA have 40%, 44%, and 47% of autolyses respectively. Overall, 10% NBF followed by 70% EtOH proved to be the best fixative for prolonged fixation as it resulted in the least effects on cellular histology compared with other aldehyde-based fixatives.

Keywords: fixative agents, prolonged fixation, histological structures, murine renal tissue, autolysis

1.0 INTRODUCTION

Fixation is a physiochemical process that involves chemically fixing cells or tissues. It is assumed that an ideal fixative imparts mechanical toughness to tissue, enabling it to withstand damage from subsequent processing stages. It prevents tissue autolysis, putrefaction, and destruction of tissue components. Fixation has to be able to maintain the tissue architecture and cellular structure in a life-like manner (Singh *et al*., 2019) . Fixatives can be placed into two categories: cross-linking and coagulative fixatives (Howat & Wilson, 2014). Aldehyde-based fixatives are an example of cross-linking fixatives, whereas alcohol-based fixatives are an example of coagulative fixatives (Eltoum *et al*., 2001).

Formaldehyde is the fixative that has been investigated the most due to its method of action, which involves cross-linking proteins. A 37% formaldehyde solution is a common form of formaldehyde, which is a small molecule that exists as a gas and is produced by bubbling formaldehyde gas through water until it reaches saturation. In histology labs, formaldehyde is most frequently found in 10% solutions, which equal to 4% formaldehyde. It is either diluted in water (formalin) or in a buffered solution (neutral buffered formalin, NBF) (Howat & Wilson, 2014). On the other hand, Paraformaldehyde (PFA) is a polymerized form of formaldehyde.

As for alcohol-based fixatives, it is a protein precipitating agent. By removing water from the free carboxyl, hydroxyl, amino, amido, and imino groups of the proteins, the alcohol presence in the solution acts to produce protein denaturation, which causes tissue shrinkage and protein coagulation (Howat & Wilson, 2014) . The example of alcohol-based fixatives that are readily available in laboratory are methanol, ethanol, or, EMA (a combination of ethanol, methanol and acetic acid = 3:1:1).

According to Singh *et al.* (2019), there are several factors affecting fixation such as the length and temperature of fixation, concentration of the fixative, and the

thickness of the tissue sample. In terms of fixation duration, longer fixation times lead to over-cross-linking and brittle samples. While there won't be enough tissue penetration and cross-linking mechanism if the fixation time is too short.

For the past decades, animal study as a diseased model has flourished significantly, and the consequences of this trend resulted in a high caseload in the histopathology laboratory. This has forced the researchers to preserve the samples in the fixative agents slightly longer due to the long queue and process of tissue fixation. It may take one week or more for the tissue to be processed, while the optimum fixation period is approximately 24 hours. Thus, the effect of different fixative agents on a prolonged fixation of murine renal histology remains elusive and only based on anecdotal sources. Hence, this study aims to evaluate and compare the effects of different fixative agents in prolonged fixation on murine renal histological evaluation. The null hypothesis (H_0) posits that different fixative agents in prolonged fixation do not alters the histomorphology of the murine renal microscopic architecture. Conversely, the alternative hypothesis (H_A) suggests that different fixative agents in prolonged fixation alters the histomorphology of the murine renal microscopic architecture.

2.0 LITERATURE REVIEW

2.1 Introduction to Fixative Agents

Fixation primarily aims to preserve the superior morphological features of tissues by reducing the enzymatic destruction of cellular and extracellular molecules, preserving macromolecular structures and protecting tissues from microbial degradation. It prevents the destruction of cellular components like peptides, proteins, lipids, mRNA, and DNA, and minimize the loss of macromolecular structures like nuclear membranes, lysosomes, and cytoplasmic membranes (Eltoum *et al.*, 2001). Over the past century, numerous methods of fixation and varieties of fixatives have been developed and evaluated. Fixatives work by hardening and preserving tissues by a variety of mechanisms, which can include heat effects, cross-linkers, dehydrants, acid effects and the combinations of these categories. Every fixative has advantages as well as disadvantages. Each fixative and tissue processing technique preserves some molecular and macromolecular characteristics of the tissue more well than other fixative-processing combinations. Currently, no universal or ideal fixative has been established; fixatives are chosen based on the tissue biopsy colledted to demonstrate a specific feature of a tissue (Eltoum *et al.*, 2001).

Tissue fixation can be executed physically or chemically. Heating, microwaving, and freeze-drying are examples of physical methods. The majority of fixation procedures used in tissue processing for medical or veterinary diagnostics rely on chemical fixation using liquid fixatives. Fixation methods include the use of chemicals that produce covalent cross-links between proteins, specific protein moieties, and nucleic acids and proteins. Formaldehyde and glutaraldehyde are the examples of cross-linking fixatives. Another biochemical method to protein fixation is the use of chemicals that remove free water from tissues, causing proteins to precipitate and

coagulate. Dehydrants such as methanol, ethanol, and acetone are examples of such fixatives. Other fixatives may be based on precipitating proteins and nucleic acids via pH shifts or the production of salts. Acetic acid, trichloroacetic acid, and zinc acetate are examples of such fixatives. Some complex fixatives are blends of various fixatives. Alcoholic formalin, for example, acts to fix tissues by cross-linking as well as dehydration (Eltoum *et al.*, 2001).

2.2 Cross-linking Fixatives

Chemical fixatives are classified into two types: coagulant fixatives and non-coagulant (cross-linking) fixatives. The most common used cross-linking fixatives are aldehyde-based fixatives, such as formaldehyde, glutaraldehyde, and paraformaldehyde. The mechanism of action of formaldehyde occurs through the formation of intra- and intermolecular cross-links. The primary cross-links form between the side chain amino groups of lysine, resulting in the formation of methylene bridges over time. However, cross-linking between aminomethylol groups and phenol, indole, and imidazole side chains may also occur. As a result, formaldehyde affects a wide range of amino acids, including lysine, arginine, tyrosine, asparagine, histidine, glutamine, and serine (Howat & Wilson, 2014).

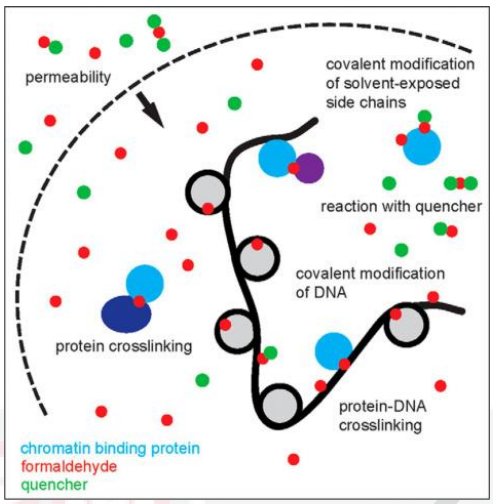

Figure 1. Formaldehyde reactivity in cells. Figure courtesy of Hoffman *et al.*

The basic characteristics of formaldehyde reactivity in cells are depicted in Figure 1. The dashed arc symbolizes cell or nuclear membranes, which are thought to be extremely permeable to formaldehyde (red circles). DNA is represented by the thick black curved line, which is depicted assembled as nucleosomes (light gray circle). A chromatin-interacting factor is represented in cyan, whereas additional partner proteins are represented in dark blue and purple. Green circles represent small molecules such as glycine and Tris that react with formaldehyde and hence quench reactivity with biological components. Formaldehyde can crosslink macromolecules and alter exposed groups on macromolecules, resulting in a product species that may be stabilized by reaction with a quencher (Hoffman *et al.*, 2015).

According to a study conducted by Singh *et al.,* (2019), formaldehyde produces less tissue shrinkage than other fixatives, with the exceptions of acetone and ethanol. When compared to other fixatives, formaldehyde appears to harden tissue more. However, although lipids are preserved, formaldehyde does not fix carbohydrates. Singh *et al.,* also stated that formalin, which comprise of 37 to 40% formaldehyde and 60 to 63% water by weight, produce accumulations of white deposits in the solution after prolonged storage. These are the precipitates of paraformaldehyde. In

accordance with study conducted by Singh *et al.,* (2020), Although formalin fixation followed by paraffin embedding is the oldest and gold standard procedure for tissue preservation, the main disadvantage is the strong cross-linking property of formaldehyde, which affects the effective retrieval of genetic material due to chemical modifications and degradation of DNA and RNA. As strong covalent bonds are established by formalin during fixation methods, nucleic acid fragmentation occurs during the extraction procedure.

Paraformaldehyde is widely used in histochemistry and immunohistochemistry because of the rapid penetration and produce minimal shrinkage of the tissue. Paraformaldehyde is dissociated into formaldehyde during fixing. Paraformaldehyde and formalin results should be comparable after prolonged fixation. In an experiment conducted by Ma *et al.,* (2002), X-Gal staining results with 4% paraformaldehyde in PBS and 10% NB formalin were similar.

2.3 Coagulating Fixatives

The most frequently used coagulating fixatives are dehydrants such as alcohols and acetone. The alcohol in the solution causes protein denaturation by removing water from the free carboxyl, hydroxyl, amino, amido, and imino groups of the proteins, resulting in protein coagulation and tissue shrinkage (Howat & Wilson, 2014). Tissue proteins may be affected in a number of ways by the removal and replacement of free water. Figure 2 depicted the denaturation process of proteins after fixation with coagulant fixative such as alcohol.

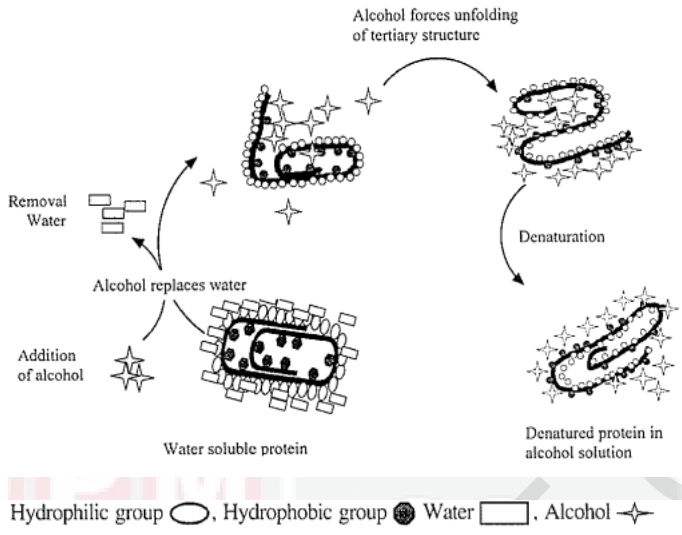

Figure 2. Denaturation of proteins after a coagulant fixative. Figure adapted from The Journal of Histotechnology, Vol. 24, No. 3, September 2001

In an aqueous environment, water surrounds the hydrophobic parts of proteins and pushes these hydrophobic elements closer together, stabilizing their bonding. Removing water has the opposite effect, which disrupt these bonds as hydrophobic areas are no longer repelled by water and can spread out more. Water is also involved in hydrogen bonding in the hydrophilic areas of proteins. Removing water destabilizes these hydrogen bonds in hydrophilic regions. These combined changes disturb the tertiary structure of proteins.

Proteins that typically dissolve in aqueous environment have hydrophilic groups on their outer surface. If alcohols or acetone replace water, they attract the hydrophobic parts of peptides/proteins, causing a potential reversal in the protein structure. This reversed structure, where hydrophobic groups are on the outside, is less ordered than the original water-soluble state. Once the tertiary structure of a soluble protein is disrupted, it will remain insoluble even if returned to an aqueous environment (Eltoum *et al.,* 2001).

According to a study conducted by Chung *et al*. , the histomorphological features of tissue fixed in buffered ethanol with H&E staining are similar to those of 70% ethanol-fixed tissue but slightly different from NBF-fixed tissue. In NBF-fixed tissue, the intensity of the H&E staining in the cytoplasm began to lessen at 1 week of fixation and subsequently declined to 6 months of fixation, showing a distinctive loss of eosinophilic staining in the cytoplasm. Furthermore, the cellular and nuclear membrane outlines became blurred, and the chromatin became hazy. Conversely, the intensity of hematoxylin and eosin staining in buffered ethanol-fixed tissue was not affected by fixation duration.

2.4 Factors affecting Fixation

According to Singh *et. al.* (2019), there are several factors affecting fixation such as the length of fixation, temperature of fixation, concentration of the fixative agents, thickness of the tissue sample, and the osmolarity between the tissue and the fixatives used. Concerning the duration of fixation, extended fixation periods result in excessive cross-linking, causing the samples to become overly rigid and brittle. On the contrary, if the fixation time is too brief, there won't be sufficient penetration into the tissue or activation of the cross-linking mechanism.

In a study conducted by Chung *et al.,* (2017), with regard to the comparison of crosslinking fixatives and coagulative fixatives, coagulative fixatives not only fix the tissue faster, but the tissue tissue can dwell in the fixative longer. Tissue is stable for lengthy periods of time with ethanol-based fixatives. As for NBF, the current clinical recommendation for fixation is 24 hours, plus or minus 8 hours. Although shorter fixation durations have typically resulted in sufficient histopathology, immunohistochemical results have been impaired when attempted with NBF.

3.0 METHODOLOGY

3.1 Specimen and Fixation

An archive sample of Sprauge Dawley rat was acquired from Animal Research Facility Unit (ARF), Faculty of Veterinary Medicine, UPM. The rat was housed and euthanized in accordance to the guidelines for the care and use of laboratory animals. The kidneys were excised within 5 min of euthanasia. The kidneys were cut into 5 equal pieces and were fixed individually in the following fixatives: 10% NBF (Control group), 10% Formalin (Group 1), 4% PFA (Group 2), 10% NBF followed by 70% Ethanol (EtoH) (Group 3), and 10% NBF followed by Phosphate Buffered Saline (PBS) (Group 4). Tissues were fixed for 7 days (Control group, Group 1 and Group 2). Whereas tissues were fixed in 10% NBF for 1 day, followed by 6 days of fixation in 70% EtoH in Group 3. For Group 4, tissues were fixed in 10% NBF for 1 day, followed by 6 days of fixation in PBS. In addition, fixative volume used were at least 30-50 times greater than the tissue volume. All fixed tissues were stored at room temperature. After a week of fixation, the tissues were processed through a routine histological tissue processing protocol and subsequently subjected to Hematoxylin and Eosin (H&E) staining (see Appendix A). This was followed by impregnation of the tissue blocks with paraffin. Finally, 4 µm thick tissue sections were cut from each block, placed on a glass slide, and stained with H&E.

3.2 Renal Histological Evaluation

Renal histological characteristics were evaluated by the percentage of autolysed cells and overall staining characteristics (Figure 3). Under X20 and X40 magnification, ten regions of the cortex and medulla were examined. Histological structures in cortical parenchyma that were observed include renal corpuscles, proximal convoluted tubule (PCT), and distal convoluted tubule (DCT). Whereas in medullary parenchyma, histological structures of the Loop of Henle (LoH) and collecting duct (CD) were observed. All slides were digitised using an image analyzer and stored in Tag Image File (.tif) format.

3.3 Data Analysis

Data was collected, arranged, and analysed using Microsoft Excel version 2010. Statistical analysis was performed with IBM SPSS version 26 using Kruskal-Wallis H Test and Bonferroni Post-hoc Test. Significant value for each group were calculated to determine the significance of reduced autolysed cells in comparison to the control group. Values of $p<0.05$ were considered statistically significant.

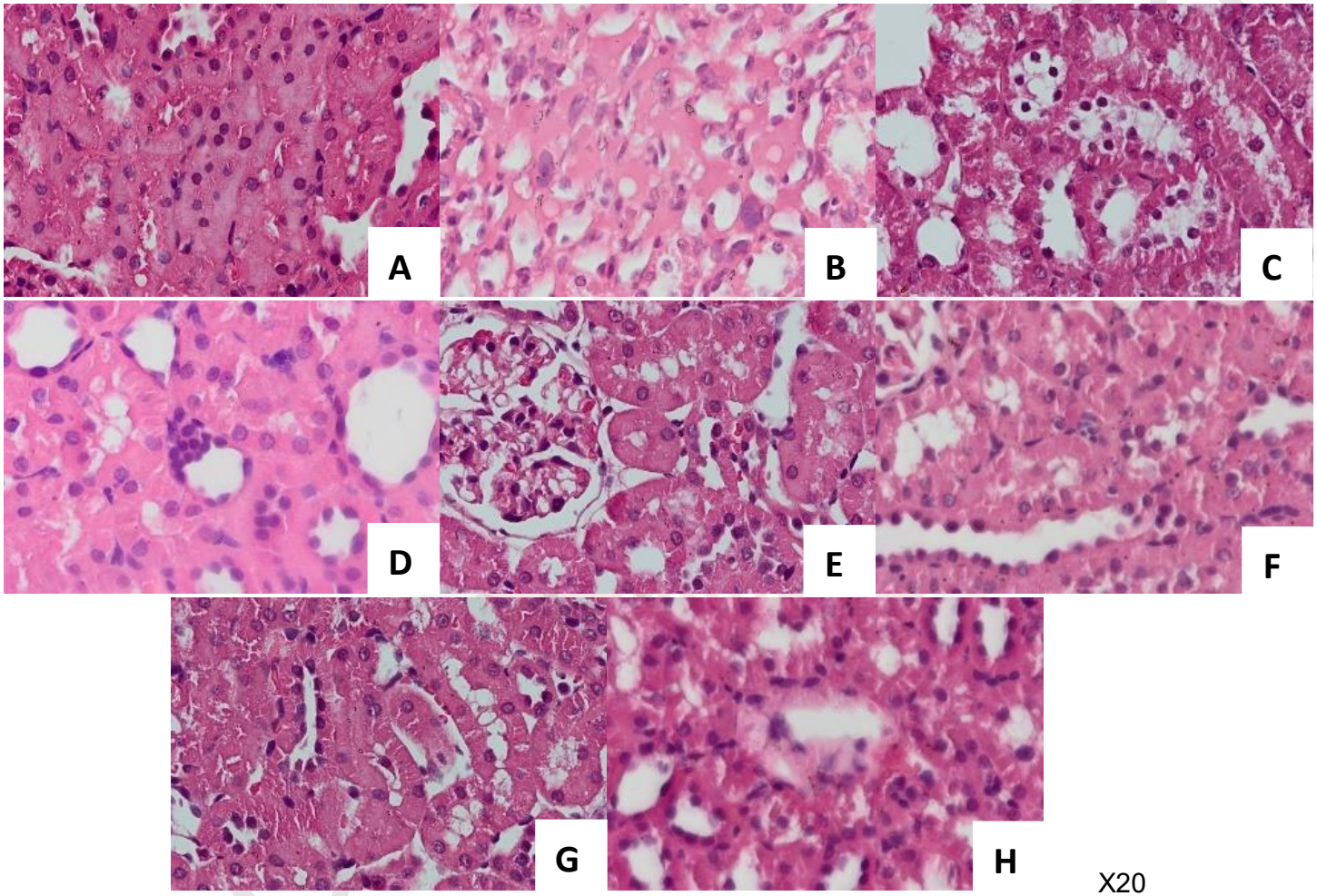

Figure 3. Histological structures with autolysed cells. (A) Cytoplasmic swelling, (B) Cytoplasmic vacuolation, (C) Desquamation, (D) Pyknosis, (E) Karyolysis, (F) Karyorrhexis, (G) Cytoplasmic shrinkage, (H) Zoning

4.0 RESULTS

4.1 Data Tabulation & Histological Evaluation

Representative of typical morphology for each of the fixatives are shown in figure 4 – 6. Figure 4 demonstrates the histomorphologic features of the glomerulus of murine renal tissue comparing all group of fixative agents. Following a 7-day fixation period, tissue sections fixed in 10% Formalin demonstrated the highest percentage of autolysed cells (45%). This autolytic process is characterized by cytoplasmic swelling, pyknosis, desquamation and karyolysis, consistently observed across ten consecutive sections of the renal corpuscle. As the tissue sections fixed in 4% PFA marks the lowest percentage of autolysed cells (25%). In reference to the Control group, the tissue sections fixed in 10% NBF exhibit notable manifestations of autolysis, comprising cytoplasmic swelling, desquamation, karyolysis, and pyknosis, collectively accounting for 40% of the observed alterations.

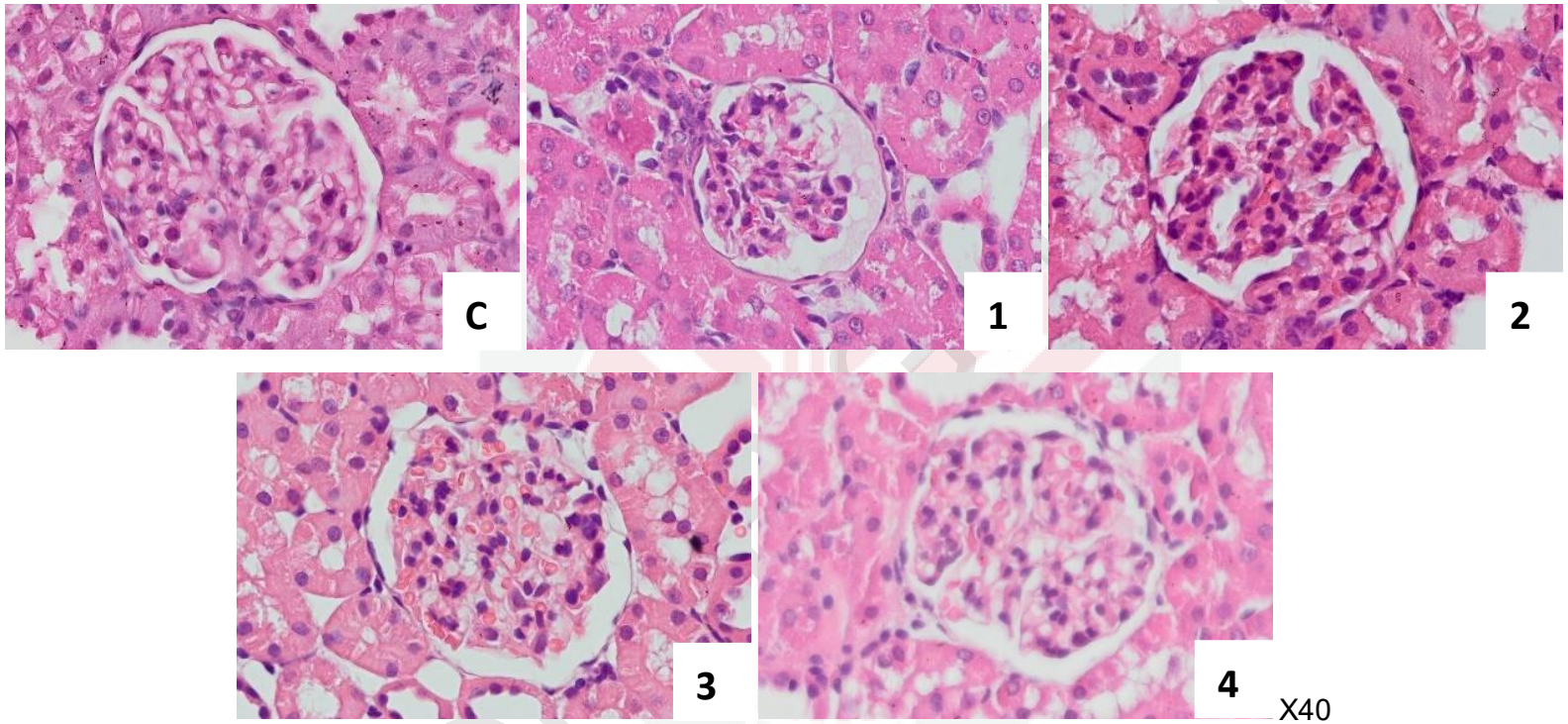

Figure 4. Representative histomorphology of the glomerulus in renal cortex. Tissue section was stained with H&E after 10% NBF (C), 10% Formalin (1), 4% PFA (2), 10% NBF followed by 70% EtoH (3), and 10% NBF followed by PBS (4) fixation

Figure 5 illustrates the histomorphologic features of both proximal and distal convoluted tubules. Tissue sections subjected to fixation in 10% formalin, 4% paraformaldehyde (PFA), and 10% neutral buffered formalin (NBF) followed by phosphate-buffered saline (PBS) exhibit an autolysis rate of 50% in the proximal convoluted tubules. Notably, the percentage of autolysis in the proximal convoluted tubules fixed with 10% NBF is comparable to those fixed with 10% NBF followed by 70% ethanol (45%).

Conversely, in the distal convoluted tubules, 10% formalin fixation records the highest autolysis percentage (42%), with consistent presence of desquamation and cytoplasmic shrinkage observed across all examined regions. In contrast, tissue sections fixed in 10% NBF followed by 70% ethanol exhibit the lowest autolysis percentage, comparatively similar to the Control group (26% and 25%, respectively).

Figure 6 illustrates the histomorphologic features of the renal medullary parenchyma, including the Loop of Henle and collecting duct. Tissue fixed in the Control group, 10% NBF shows autolysis percentages of 40% in the Loop of Henle and 47% in the collecting duct. Notably, 4% PFA-fixed tissue exhibits the highest autolysis rates, reaching 50% for both structures in the renal medulla. In Group 2, 50% of autolysis rate was also noted in the collecting duct.

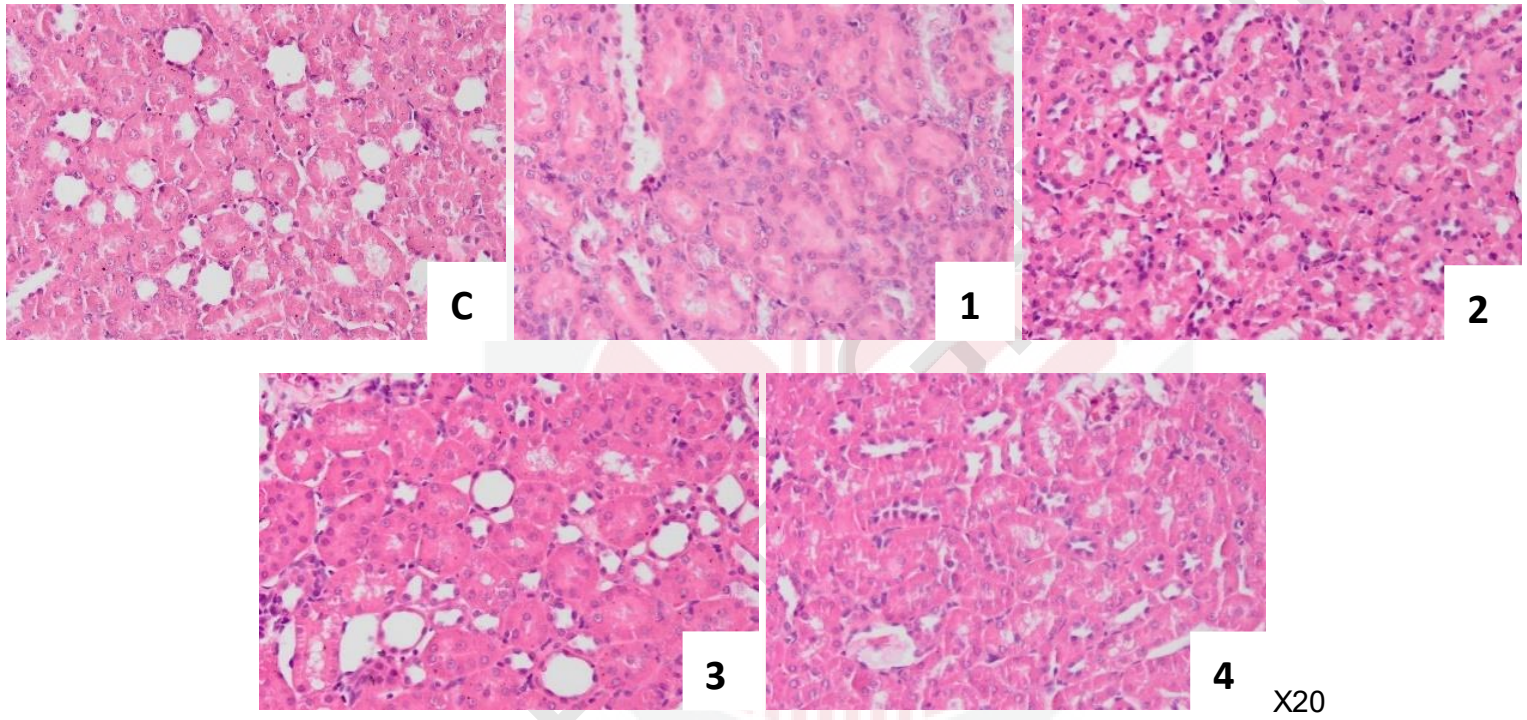

Figure 5. Representative histomorphology of the PCT and DCT in renal cortex. Tissue section stained with H&E after 10% NBF (C), 10% Formalin (1), 4% PFA (2), 10% NBF followed by 70% EtoH (3), and 10% NBF followed by PBS (4) fixation

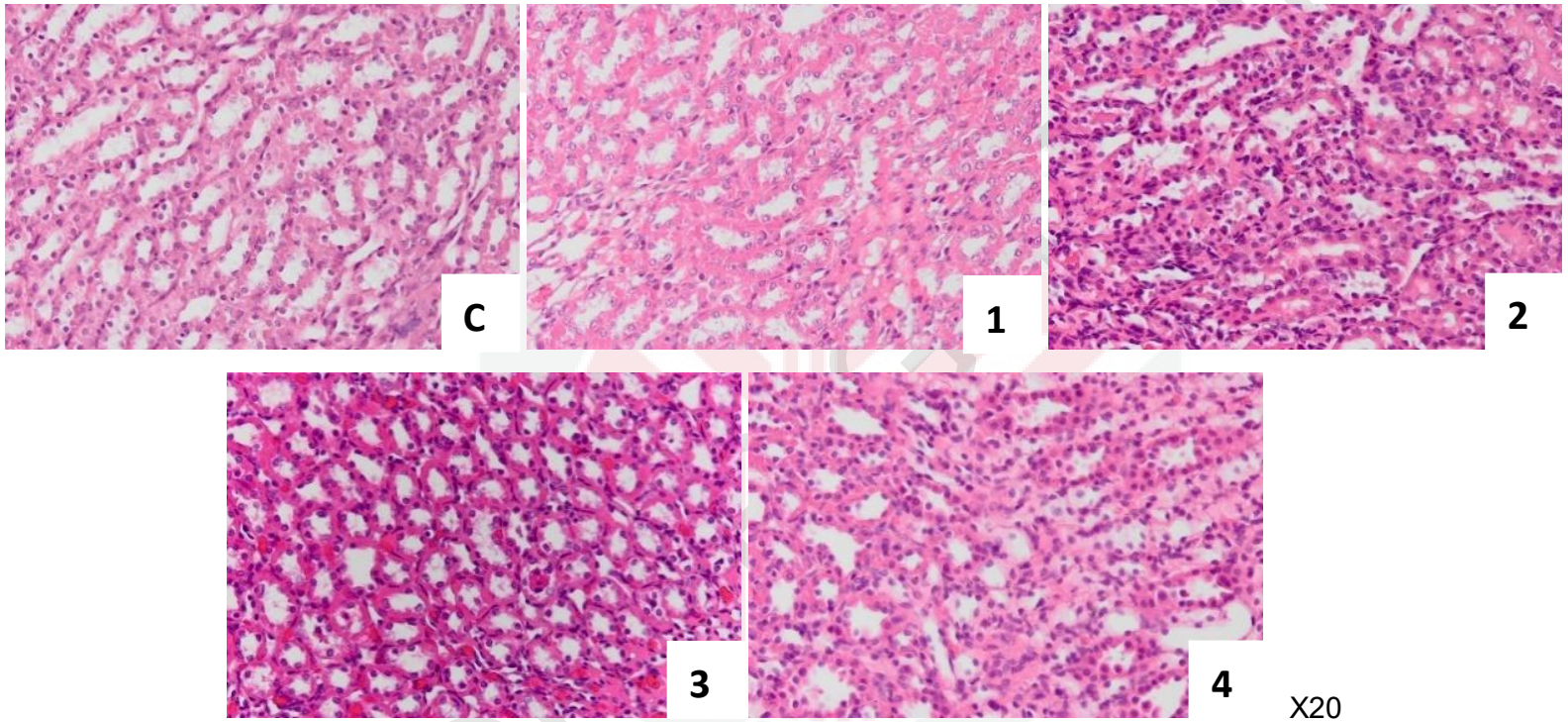

Figure 6. Representative histomorphology of the LoH and CD in renal medulla. Tissue section stained with H&E after 10% NBF (C), 10% Formalin (1), 4% PFA (2), 10% NBF followed by 70% EtoH (3), and 10% NBF followed by PBS (4) fixation

In general, the tissue sections fixed in 10% formalin exhibit the highest autolysis percentage, as illustrated in Table 1. Conversely, tissue fixed in 10% neutral buffered formalin (NBF) followed by 70% EtOH demonstrates the lowest autolysis percentage (38%), even surpassing the Control group (40%).

Table 1. Percentage of autolysis (%) for each histological structures observed in the renal parenchyma

Fixatives	Percentage of Autolysis (%)					Total Autolysis (%)	SD
	Glomerulus	PCT	DCT	LoH	CD		
Control	40	46	26	40	47	40	16.78
Group 1	45	50	42	50	50	48	7.64
Group 2	25	50	40	50	50	47	14.39
Group 3	30	46	25	38	45	38	18.16
Group 4	35	50	36	43	47	44	13.46

4.2 Statistical Analysis

Table 2 illustrates that the Kruskal-Wallis test produced a statistically significant result, with a p-value of 0.038. As depicted in Table 3, the significant value of Group 3 is 0.011 as compared to the Control group. It shows that only in Group 3, where the tissue sections were fixed in 10% neutral buffered formalin (NBF) followed by 70% EtOH, a statistically significant (s) reduction in autolysed cells is observed in comparison to the Control group (Figure 7).

Table 2. Independent-Samples Kruskal-Wallis Test Summary

Total N	25
Test Statistic	10.118
Degree of freedom	4
Asymptomatic Sig. (2-sided test)	0.038

Table 3. Pairwise Comparison of Groups

Control-Group	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.
Control-Group 1	-5.900	4.647	-1.270	0.204	1.000
Control-Group 2	5.200	4.647	1.119	0.263	1.000
Control-Group 3	11.800	4.647	2.539	0.011	0.111
Control-Group 4	-2.100	4.647	-0.452	0.651	1.000

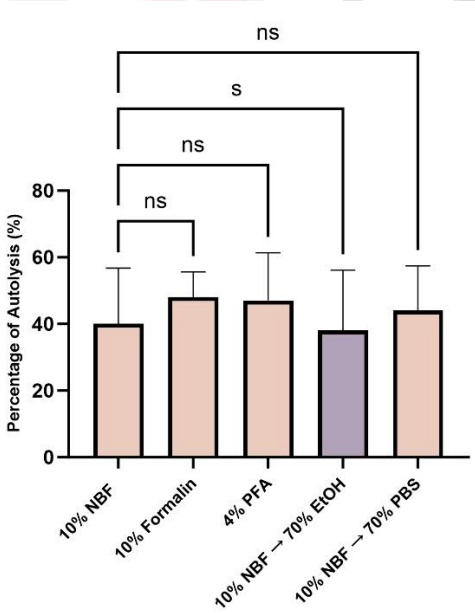

Figure 7. Statistically significant reduction in autolysed cells observed in Group 3

In Figure 8 the histomorphological characteristics, as revealed by H&E staining, are presented. Notably, the intensity of staining in the tissue sections subjected to fixation in 10% NBF followed by PBS exhibited a noticeable reduction, clearly differing from the staining characteristics observed in other experimental groups.

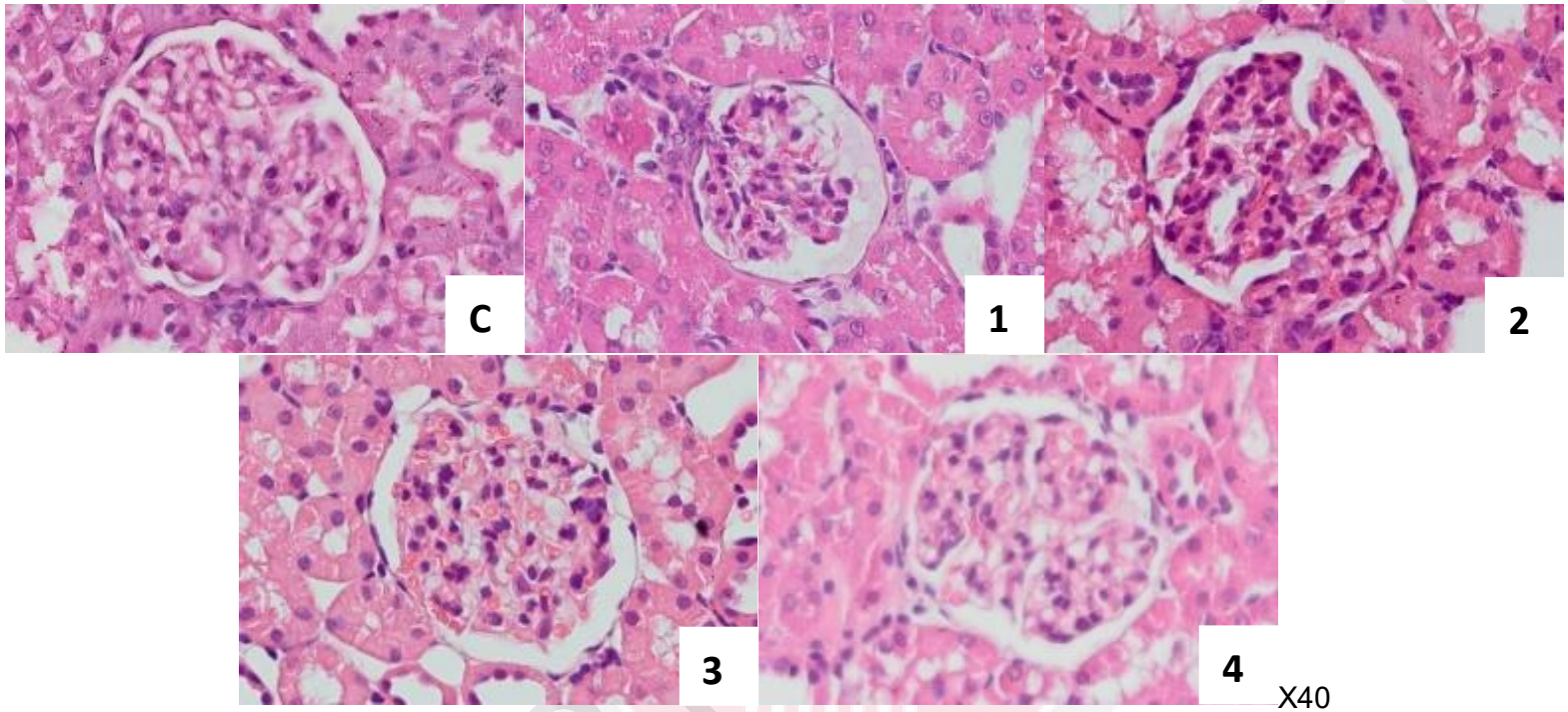

Figure 8. The intensity of H&E staining in Group 4 was reduced after 7 days of fixation

5.0 DISCUSSION

The primary aim of this study was to identify an optimal fixative that would comprehensively achieve an ideal equilibrium in preserving tissue morphology. The assessment of morphological features associated with each fixative involved evaluating the percentage of autolysis present. Aldehyde-based fixatives were employed in the Control group, Group1, Group 2, and Group 4. Conversely, fixatives utilized in Group 3 comprised a combination of aldehyde-based fixative and alcohol-based fixative, herein referred to as buffered ethanol fixative. The findings indicate that tissue sections fixed in Group 4, utilizing 10% NBF followed by 70% EtOH as fixatives, exhibited preferable histomorphological architecture, evidenced by the lowest percentage of autolysis and a significantly reduced rate of autolysis compared to the Control group.

In accordance with a study by Singh *et al.* (2020), the formation of intra- and intermolecular cross-links within the first 24 – 48 hours is a reversible process. However, over a span of 30 days, irreversible methylene bonds are formed, rendering these covalent bonds resistant to breakage, leading to DNA fragmentation. Notably, tissue sections fixed with aldehyde-based fixatives in this study recorded the highest percentage of autolysis, potentially attributable to prolonged fixation times (7 days), surpassing the optimum fixation period recommended in clinical practice, which typically advises a 24-hour duration for fixation in NBF (Chung *et al.,* 2017).

The optimal morphology of renal histological structures in this study was observed in tissue fixed with a combination of aldehyde-based fixative and alcohol-based fixative (Group 3). Tissue sections fixed in 10% NBF for one day, followed by 70% ethanol, mitigated some of the adverse effects associated with pure ethanol fixatives. As cross-linking effects were established within the initial 24 hours, the presence of alcohol in the solution, serving to remove water from proteins, countered

the effects of tissue shrinkage and hardening. This aligns with the findings of Gillespie *et al.* (2002), where tissues fixed in alcohol were deemed to offer superior morphology compared to 10% NBF based on nuclear morphology, cellular morphology, tissue architecture, and staining characteristics. A study by Chung *et al.* (2017) also compared the histological features of tissue specimens using both buffered ethanol and NBF. Notably, the intensity of H&E staining in the cytoplasm began to weaken after one week of fixation and gradually diminished up to six months in NBF-fixed tissues (Chung *et al.,* 2017). However, the staining intensity of hematoxylin or eosin in buffered ethanol-fixed tissue was not altered by fixation time.

As for Group 4, tissue sections fixed in 10% NBF for one day were sufficient to establish the cross-linking effects of formalin, ensuring adequate structural integrity. Subsequent fixation in PBS for the following days resulted in a reduction in the intensity of staining, as PBS is considered as holding agents rather than fixatives, which do not chemically alter the tissues. By maintaining equilibrium in osmotic pressure, PBS prevents swelling or shrinkage of the tissue and over-fixation (Cox *et al*., 2005). However, it does not facilitate H&E staining uptake, leading to a marked reduction in staining intensity within this group.

6.0 CONCLUSION

In conclusion, the choice of fixative, whether employing cross-linking or coagulating mechanisms, introduces certain compromises to tissue morphology. Consequently, the selection of a fixation regime can be customized based on the desired outcome. The findings of this study highlight that the incorporation of both cross-linking and coagulative mechanisms in prolonged tissue fixation show a broader time spectrum for histomorphological features compared to the limitations observed with aldehyde-based fixatives. Therefore, the alternative hypothesis is accepted, indicating that the distinct fixative agents, particularly during prolonged fixation periods, contribute to alterations in the properties of murine renal microscopic architecture.

7.0 RECOMMENDATION

In offering recommendations for future research, it is advised to conduct histological evaluations at distinct time intervals, specifically after 24 hours and 1 week of fixation. This approach enables a thorough examination of the distinctions that arise when utilizing different fixative agents over an extended fixation period. Additionally, it is highly recommended to conduct thorough analyses to differentiate the diverse impacts of various fixative agents in a range of assessments. Such analyses may include evaluations using immunohistochemistry or examinations of RNA and DNA quantity and quality. These approaches would enhance our understanding of the complex dynamics involved in fixation processes.

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9.0 APPENDICES

Appendix A

Harris' Hematoxylin and Eosin Staining Procedure

1. Submerge slides in Xylene for 5 mins
2. Submerge slides in 70% Alcohol for 5 mins
3. Rinse with tap water
4. Submerge slides in Hematoxylin for 5 mins
5. Rinse with tap water for 3-5 times
6. Dip slides in 1% Acid Alcohol for 3 seconds
7. Rinse with running tap water for 5 mins
8. Submerge slides in Eosin for 1 min
9. Spray slides with 95% alcohol
10. Rinse slides in running tap water for 5-10 seconds
11. Spray slides with 95% alcohol,
12. Clean and leave to dry
13. Mount with DPX
14. Ready for viewing