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Delayed Tissue Fixation for as Short as Five Minutes Can Distort Tissue Architecture and Falsify Research Results

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ABSTRACT

Timely fixation is crucial to the preservation of the cellular components of tissues and prevent autolysis and putrefaction. Delayed tissue fixation can adversely affect tissues and engender false negative histopathological results. This study aimed to assess the histology of liver and kidney tissues following time-dependent delays in fixation. Ten male Wistar rats weighing 150-170g were used for this study. Animals were acclimatized for two weeks under standard conditions and divided into 5 groups of 2 rats each (A, B, C, D & E). They were sacrificed by cervical dislocation and the liver and kidneys harvested from each group. The tissues for the control group were fixed immediately. For the tissues from rats in groups B, C, D & E, they were allowed delay periods of 5mins, 10mins, 30 mins and 45 mins respectively. All tissue fixation was in 10% formalin. Tissues were processed using the H & E method. The results showed that delay of fixation for as short as 5 minutes produced adverse histological effects, which were more pronounced in the liver tissue than in the kidney tissue. It was concluded from this study that delayed tissue fixation produced a time-dependent adverse effect on the liver and kidney tissues, but the effect was more pronounced in the liver tissue.

Keywords: Delayed Fixation, Mitotic Figures, Histopathology, Pyknosis, Necrosis, Fixative.

Introduction

Putrefaction is the fifth stage of death, following pallor mortis, algor mortis, rigour mortis, and livor mortis.[1] It can be viewed more broadly as the decomposition of proteins, the eventual breakdown of the cohesiveness between tissues, and the liquefaction of most organs.[2] This is caused by the decomposition of organic matter by bacterial or fungal digestion, which causes the release of gases that infiltrate the body's tissues and lead to the deterioration of the tissues and organs.[3] The approximate time it takes for putrefaction to occur depends on various factors. Putrefaction may occur due to a population explosion of foreign chemical compounds in the body, as different organisms compete for the available energy the decomposing tissues represent. In putrefaction, bacteria build up causes the production of gases and other metabolic products which leads to some specific odour change, colour change and bloating, which are universally recognized as the hallmark of a decaying body.[4] This process causes gradual and sometimes complete loss of structural integrity and makes tissues unrecognizable and also the ultimate reduction of the tissues into their component molecules, atoms and molecular fractions.[4]Autolysis on the other hand refers to the destruction of a cell through the action of its enzymes.[25]

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It may also refer to the digestion of an enzyme by another molecule of the same enzyme.[4] Autolysis is an aseptic or sterile event or phenomenon that is caused by the release and subsequent uncontrolled activity of the intracellular enzymes that break down cellular constituents hydrolytically, and these constituents may serve as substrates for catalysis.[5] When these enzymes are released from their subcellular locations, it serves as the beginning of an irrecoverable process that will eventually cause a complete reduction of the newly dead organism to the residue of decomposition and this process exists in every cell as a result of the presence of the biochemical system necessary to process nutrients, degrade toxic breeds or species and even recycle structural and functional molecules and the entire cellular organelles.[6] According to Dawkins,[7] during the process of autolysis under anaerobic conditions, the glycogen disappears due to the operation of the normal metabolic sequence or pathway. Autolysis of normal tissues postmortem can be differentiated from autolysis in the living body because the one occurring postmortem is not localized while the one occurring in the living body is localized.[89] Fixation is a biochemical process by which biological tissue is preserved from damage or decay due to autolysis and/or putrefaction. It terminates any ongoing biochemical reactions and may also increase the treated tissues' mechanical strength or stability.[10] Tissue fixation is a critical step in the preparation of histological sections, its broad objective being to preserve cells and tissue components and to do this in such a way as to allow for the preparation of thin, stained sections. [1011] When biochemical tissues are immersed in a fixative fluid, autolysis and bacterial growth which will cause putrefaction are prevented. Formalin-based fixatives are mostly used. Formaldehyde reacts with proteins and peptides and forms stable methylene bridges while acetone is most often used as a fixative for cell-surface markers.[11] The purpose of this study is to determine the extent of autolysis and putrefaction following a delay in fixating the liver and kidney of Wistar rats. Fixation preserves a sample of biological material (tissue or cells) as close to its natural state as possible. A fixative usually acts to disable intrinsic biomolecules

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particularly proteolytic enzymes which otherwise digest or damage the sample. Second, a fixative typically protects a sample from extrinsic damage. Fixatives are toxic to most common microorganisms (bacteria in particular) that might exist in a tissue sample or which might otherwise colonize the fixed tissue. In addition, many fixatives chemically alter the fixed material to make it less palatable (either indigestible or toxic) to opportunistic microorganisms. Fixatives often alter the cells or tissues on a molecular level to increase their mechanical strength or stability. This increased strength and rigidity can help preserve the morphology (shape and structure) of the sample as it is processed for further analysis.[12] The effect of delay in fixation on the number of mitotic figures in tissues has received little attention, and previous studies have reached differing conclusions. In a study, the numbers of mitotic figures in the normal mucosa of six colectomy specimens were counted with delays in fixation of 30 minutes, one hour, two hours, three hours and six hours for samples from each specimen. The numbers of mitotic figures were counted in 50 whole crypts in each specimen by two observers. All phases of mitosis were counted. The number of observable mitotic figures declined by about 30% with a delay in fixation of two hours and by 50% with a delay of six hours. This observation has important implications for the handling of surgical specimens.[1314] The liver is a gland and plays a major role in metabolism with numerous functions in the human body. It is an accessory digestive gland and produces bile, an alkaline compound which aids in digestion via the emulsification of lipids.[15] It is located in the upper right quadrant of the abdomen, below the diaphragm.[16] The liver has a wide range of functions, including detoxification of various metabolites, protein synthesis, and the production of biochemicals necessary for digestion. It also plays a role in metabolism, regulation of glycogen storage, decomposition of red blood cells and hormone production.[17]The kidneys are two reddish-brown bean-shaped organs found in vertebrates.[18] They receive blood from the paired renal arteries; blood exits into the paired renal veins. Each renal artery branches into segmental arteries, dividing further into interloper arteries, which penetrate the renal capsule and extend through the renal columns between the renal pyramids. The interlobar arteries then supply blood to the arcuate arteries that run through the boundary of the cortex and the medulla. Each arcuate artery supplies several interlobular arteries that feed into the afferent arterioles that supply the glomeruli.[19]

Materials and Methods

Ethical approval

Ethical approval was obtained from the ethical committee, Faculty of Basic Medical Sciences, Nnamdi Azikiwe University, Nnewi Campus with the number (NAU/CHS/NC/FBMS/631). All the principles of laboratory animal care were followed in accordance with animal handling regulations of the Faculty of Basic Medical Sciences, Nnamdi Azikiwe University.

Methods

Ten (10) male Wistar rats weighing approximately 150 – 170g were procured from a private farm in Nnewi, Anambra state, Nigeria and acclimatized in iron cages with sawdust bedding at the animal house of the Department of Anatomy, College of Health Sciences, Nnamdi Azikiwe University, Nnewi campus for two weeks under standard laboratory and photoperiodic conditions. They were fed standard feed (growers mash) manufactured by Eastern Premier Feed mills Ltd, a subsidiary of Premier Feeds Mills company limited, Plateau state, Nigeria) *ad libitum* throughout the studies. After acclimatization, the animals were weighed using digital weighing balance (model WT6000GT manufactured by WANT balance instrument company limited, China) and divided into five (5) groups of two rats each. After the period of acclimatization, the animals were sacrificed by cervical dislocation and the organs for the study were harvested and fixed thus; Group A: Rat tissues (liver and kidney) were harvested and fixed immediately in 10% formalin; Group B: Liver and kidney tissues were left unfixed for 5 minutes, followed by fixation in 10% formalin; Group C: Liver and kidney tissues were left unfixed for 10 minutes and afterwards fixed in 10% formalin; Group D: tissues were fixed in 10% formalin after 30 minutes delayed fixation; Group E: Tissues were fixed in 10% formalin after 45 minutes.

Histological procedure

Small portions of the tissues were cut and fixed in 10% formalin. Tissues were subjected to standard H&E processing after 72 hours according to the method of Carleton et al. [20] Tissues were placed in ascending grades of alcohol (60%, 70%, 80%, 90%) for one hour each and in absolute (100%) twice, one hour each. Tissues were immersed in two changes of xylene for one hour each, infiltrated in four changes of molten paraffin wax at constant temperatures of 36-40ºC in an oven of paraffin bath for one hour each and embedded with Metal blocks. Thin sections were made at 5µm using a rotary microtome, and picked up with prelabelled glass slide made sticky using egg albumin. Haematoxylin and eosin staining was done according to the procedure described by Carleton et al., (1967) and mounted in distrene plasticizer xylene (DPX) using clean glass cover slide. Micrographs were taken using Amscope 14MP digital microscope camera fitted on NOVEX compound microscope with Hi-PLAN objectives. Slides were micrographed at X100 combined magnification.

Results and Discussion

Physical changes

Physical changes were observed in the liver and kidney. When the liver and kidney were delayed for 10 minutes before fixing, the colour gradually changed from reddish brown to dark red. The colour changes occurred in both organs (Liver and Kidneys). Early signs of tissue shrinking were also observed on both the liver and kidney when exposed for 30 and 45 minutes.

Histological findings in the liver

Photomicrographs of rats' liver and kidney tissues show structural and cellular distortions following delays in fixation. The severity of the distortions increased as the time of delay elongated with those delayed for 45 minutes showing several signs of tissue and cellular degeneration compared to those delayed for 30 minutes, 10 minutes and 5 minutes. Photomicrographs of rat liver in the Control group A (Plate 1A & 1B) shows normal liver architecture with a prominent central vein, normal hepatocyte and normal liver sinusoids. Photomicrographs of rat liver in group B (plate 2A & 2B) whose fixation was delayed for five minutes shows a non-distinct central vein outline with signs of atrophy in the hepatocytes and sinusoids. Plate 3A & 3B shows photomicrographs of rat liver in group C delayed for ten minutes before fixation. Signs of mild pyknosis were observed on the hepatocyte. There was also aggregation of inflammatory cells. Plate 4A & 4B for rat liver in group D delayed for thirty minutes before fixation showed shrinkage and mild aggregate of inflammatory cell. Representative photomicrographs of rat liver in group E (plate 5A & 5B) delayed for forty-five minutes before fixation showed severely perfused hepatic tissue and necrotic hepatic tissue. Autolysis begins within minutes of death and represents the process of self-digestion from the activity of endogenous enzymes.[2122] In this study, immediate fixation of the liver and kidney tissues provided the best preservation for histology studies using H & E. The progression of autolysis in tissues is species-specific and depends on the organs and the environment in which the organ was harvested.[5] The liver and kidney are among the most common organs of research interest due to their significant importance in the functioning of the organism. The observation in the micrographs of rat liver in Group B; a non-distinct central vein with atrophied sinusoids, corroborates the work done by Khoury (2012)[23], on delayed formalin fixation for breast carcinoma.[23] These are normally reported when a toxicant is administered to an animal. But in this case the animals were relatively healthy at the time of this study. This implies that wrong methodologies can create wrong inferences for histological results, leading to false or misleading interpretation of outcomes and erroneous conclusions. Khoury (2012)[23] however opined that breast cancer tissues fixed before one hour did not show significant interference with the result as compared to those fixed immediately. When the liver and kidney were delayed for 10 minutes

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before fixing, physical observation showed a gradual colour change from reddish brown to dark red. The liver tissue showed degeneration of hepatic cells with mild pyknotic appearance of hepatocytes and mild aggregation of inflammatory cells. The work done by Tomit et al., reported that ultrastructural changes occurred earlier in the kidneys compared to the liver while morphological degeneration was observed later in the liver than in the kidneys.[24] The result of Group D liver delayed for 30 minutes before fixation showed perfused degeneration of hepatic cells. Group E in which fixation was delayed for 45 minutes shows severe degeneration of hepatic cell. Hepatic necrosis multiplied and evidence of tissue inflammation was seen.

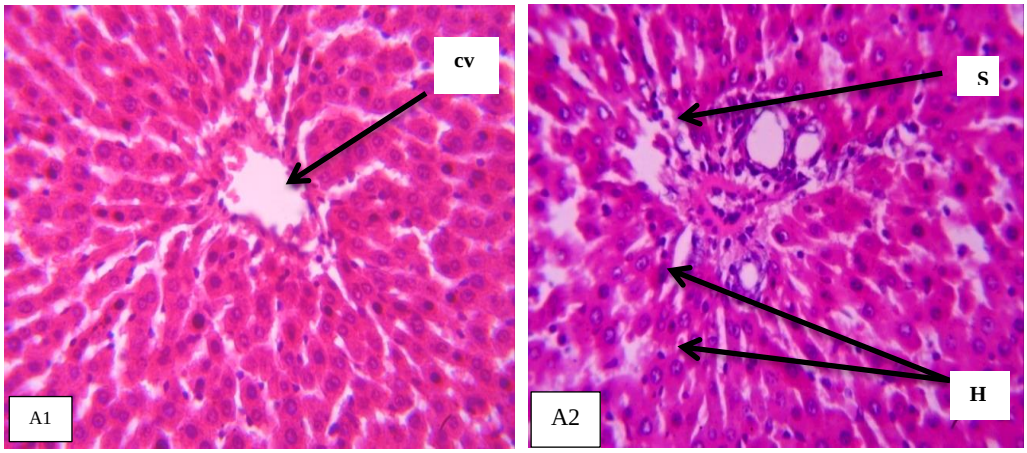

Plate 1: Representative photomicrographs of rat liver in group A (Control). Plate 1A shows normal liver architecture with a prominent central vein (CV). Plate 1B shows normal hepatocyte (H) and normal sinusoids (S).

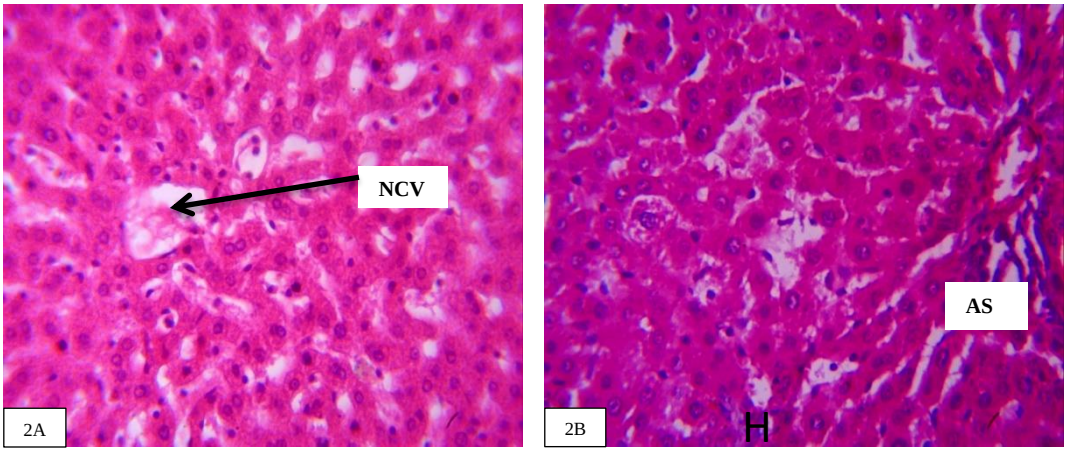

Plate 2: Representative photomicrographs of rat liver in (group B) delayed for five minutes before fixation. Plate 2A shows a non-distinct central vein (NCV). Plate 2B shows hepatocyte (H) with atrophied sinusoids (AS).

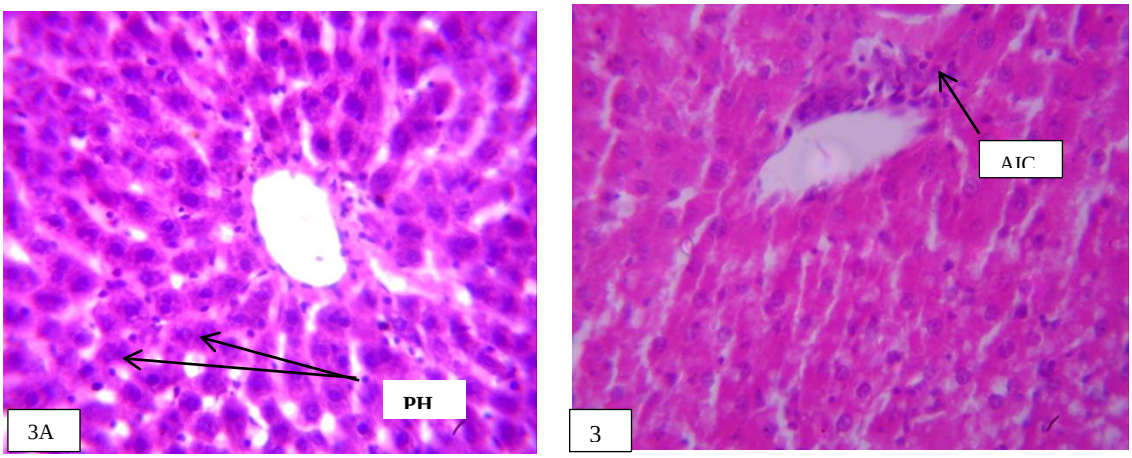

Plate 3: Representative photomicrograph of rat liver in (group C) delayed for ten minutes before fixation. Plate 3A shows mild pyknotic appearance of the hepatocyte (PH); Plate 3B shows mild aggregate of inflammatory cell (AIC).

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These are not supposed to be seen in healthy rats. However, delay in the fixation of the rats created those distortions, which ordinarily the researcher would have attributed to an agent administered if such was the case. In this study however, no agent was administered, yet these distortions were seen on the tissues. Tomit et al.,[24] in their study reported ultrastructural changes in organelles of various organs following death.[24] In their report, even at 23^0C, ultrastructural changes were observed; mitochondrial amorphous dense deposits in pancreatic acinar cells 1 hour after death, irregular microvilli in the proximal tubules 3 hours after death, resemblance to apoptotic change in the distal tubule 5 hours after death, amorphous dense deposits in the mitochondria of the distal tubule 10 hours after death. However, in our study, the longest period of delay was 45 minutes and there was no refrigeration of tissues.

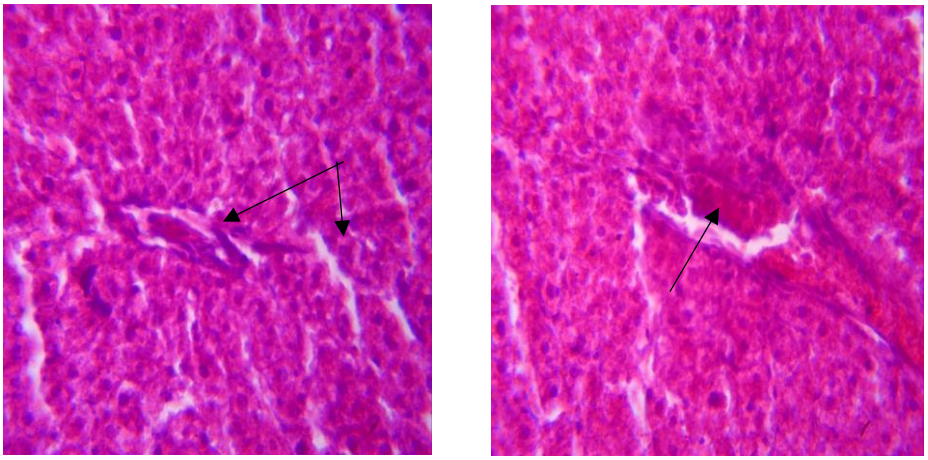

Plate 4: Representative photomicrographs of rat liver in (group D) delayed for thirty minutes before fixation. Plate 4A shows tissue shrinkage (arrow). Plate 4B shows mild aggregate of inflammatory cell (arrow).

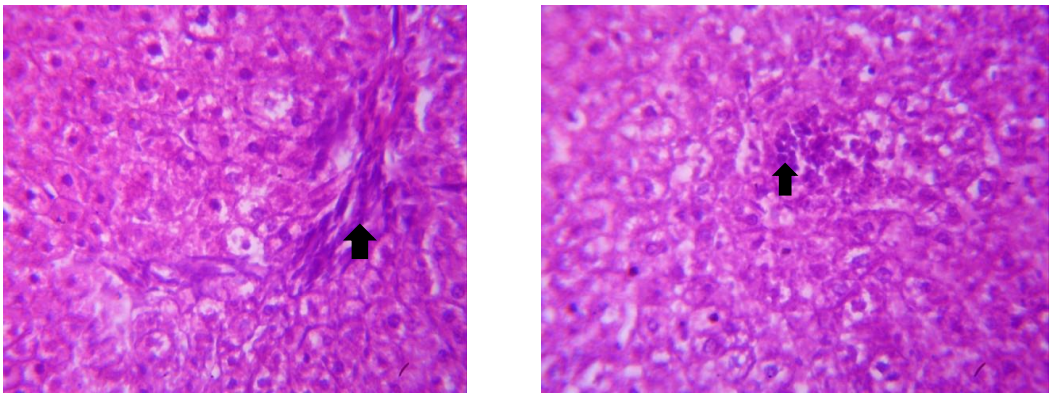

Plate 5: Representative photomicrographs of rat liver in (group E) delayed for forty-five minutes before fixation. Plate 5A and Plate 5B shows sever perfused hepatic tissue and necrotic hepatic tissue (arrowheads).

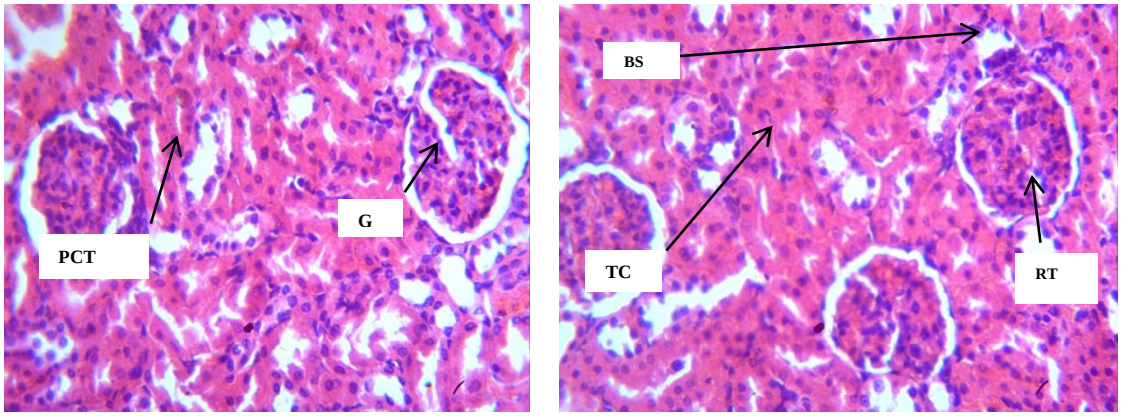

Plate 6: Representative photomicrograph of the rat kidney in group A (Control). Plate 6A shows normal renal architecture with glomeruli (G), proximal convoluted tubule. Plate 6B shows normal bowman space (BS), renal tubules (RT), sand tubular cell (TC).

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Histological findings in the kidney

Photomicrographs of the rat kidney in the experimental group B (plate 7A and plate 7B) delayed for five (5) minutes before fixation. showed poorly outlined Bowmann's space but otherwise relatively normal renal tissue with well outlined tubular cells. This shows that within as short as 5 minutes of delay, signs of autolysis and putrefaction set gradually set in. Reasons for delay in tissue fixation includes lack of adequate preparation prior to animal sacrifice and organ harvesting, shortage of adequate man power during the procedure, delay in fixative preparation or arrival, other processes like weighing organs etc. Plates 8A & 8B shows representative photomicrographs of rat kidneys in group C delayed for ten minutes before fixation. The micrographs show mild eosinophilic appearance of the cytoplasm and distorted renal tissue with signs of tissue inflammation (arrows). Plates 9A & 9B are photomicrographs of the rat kidney in group D. delayed for thirty (30) minutes before fixation. The figures show poorly perfused renal tissue

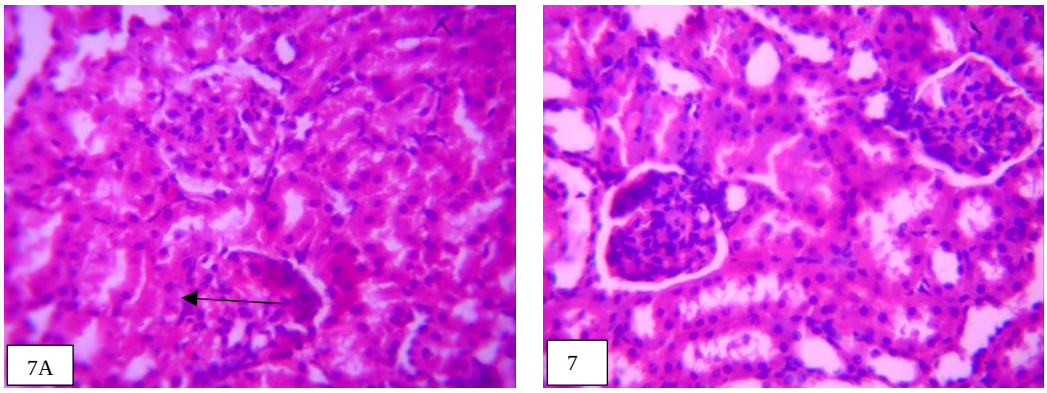

Plate 7: Representative photomicrograph of rat kidney in (group B) in plate 7A and plate 7B delayed for five (5) minutes before fixation. 7A show poorly outlined Bowmann's space. Bowman's capsule poorly outlined. 7B shows relatively normal renal tissue with well outlined tubular cells.

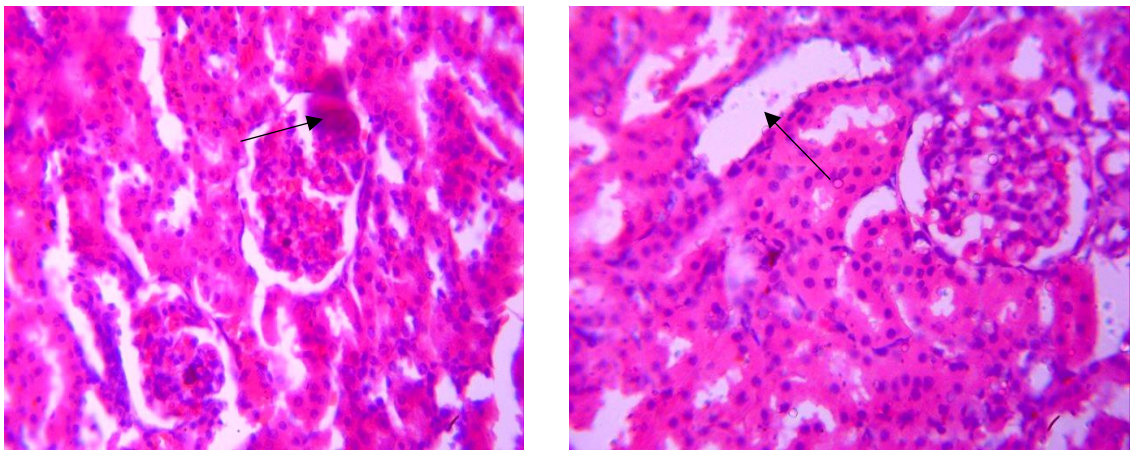

Plate 8: Representative photomicrograph of the rat kidney in group C (Plate 8A and Plate 8B delayed for ten minutes before fixation) shows mild eosinophilic appearance of the cytoplasm and distorted renal tissue with signs of tissue inflammation (arrows).

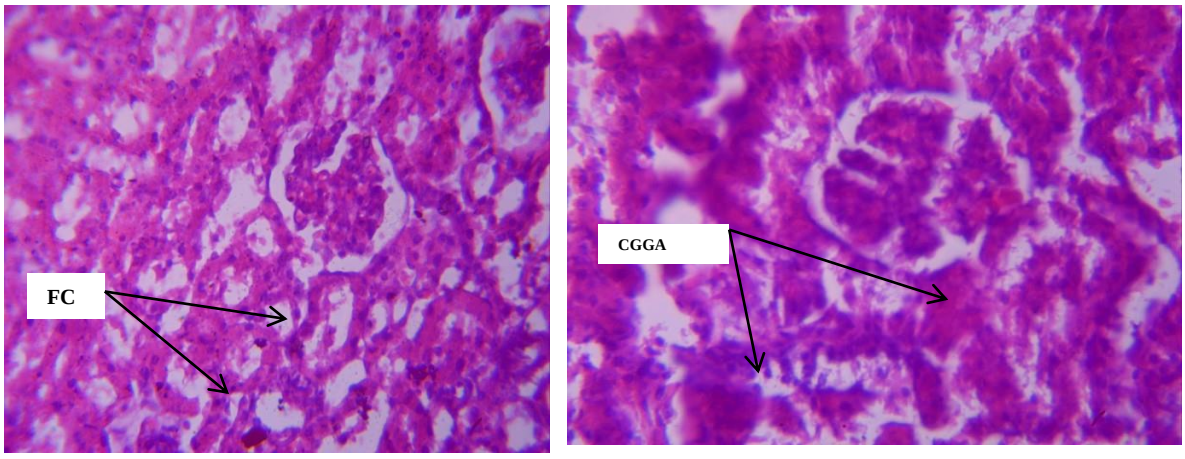

Plate 9: Representative photomicrograph of the rat kidney in (group D) Plate 9A (x100) and Plate 9B (x400) delayed for thirty (30) minutes before fixation. Shows poorly perfused renal tissue with cytoplasmic ground glass appearance (CGGA) and fatty change (FC) the overall features show necrotic renal tissue.

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with cytoplasmic ground glass appearance (CGGA) and fatty changes (FC). The overall features show necrotic renal tissue.Plates 10A 10B for rats in group E delayed for forty-five minutes before fixation shows severe degeneration with coagulative necrosis of the renal tubules. The kidneys are some of the earliest organs to suffer post-mortem structural changes.[24] In the present study, changes in the kidneys were the earliest seen in the examined tissues. Tomita *et al.*, reported that these changes were related to the release of lysosome enzymes.[24] In Group B delayed for 5 minutes before fixation, the kidneys still showed normal renal architecture, but in the distal tubule epithelium, two forms of nuclear change were seen; one was the disappearance of organelles resembling necrotic change, and the other was cell shrinkage with peripheral chromatin condensation resembling apoptotic change. This agrees with the assertion of Tomita et al. [24] Group C delayed for 10 minutes before fixation shows a mild appearance of cytoplasm infractions and renal tissue distortions. Group D, delayed for 30 minutes before fixation shows perfused degeneration of renal tissue with cytoplasmic ground glass appearance and fatty change. This is in line with Tomita *et al*, who reported that tissues delayed for 25-30 minutes, show degeneration of renal tissue.[24]

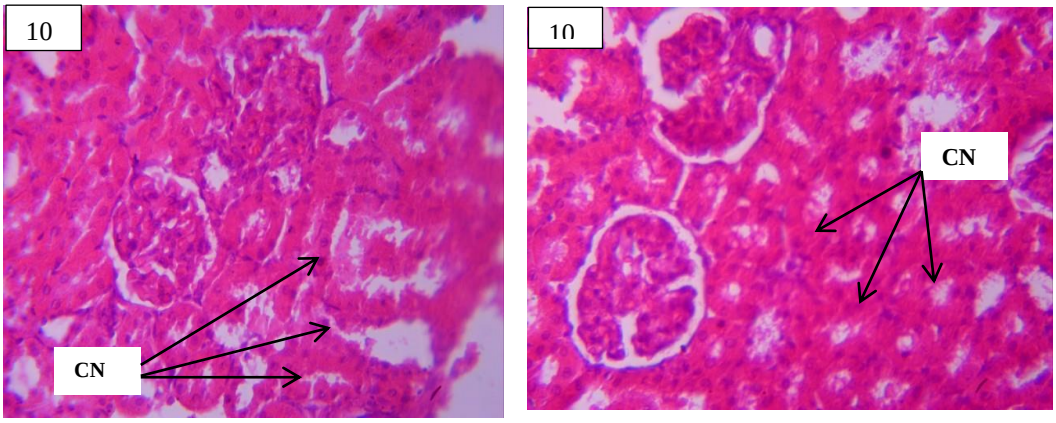

Plate 10: Representative photomicrograph of the rat kidney in (group E). Plate 10A and Plate 10B (delayed for forty-five 45 minutes before fixation). Shows sever degeneration with coagulative necrosis of the renal tubules.

Reports has shown that the onset of autolysis is more rapid in tissues with a high content of hydrolytic enzymes, such as the pancreas and gastric mucosa, whereas it is intermediate in the heart, liver, and kidney, and slow in fibroblasts, which have relatively few lysosomes and a low level of hydrolytic enzymes.[2526] In this research however, theses processes were not considered slow since signs of necrosis were present even after five minutes of delay.-The kidneys from Group E rats delayed for 45 minutes before fixation showed severe degeneration with necrosis of the renal tubules. This is in line with the work done by Apple *et al.* who stated that tissues delayed for 40 minutes to 1hr show severe degeneration with necrosis of the renal tubules.[26] Part of the problem is related to the fact that there is no specific assay or parameter, with the exception of morphological and histomorphometric observations, which allows the unequivocal distinction between apoptosis and necrosis. In addition, some aspects of the signaling pathways are similar.[27] Most of the studies available were done on cadavers and were concerned with body/organ preservation in the embalming or pathological procedure. This study focused on histological tissue processing using the H&E method.

Conclusion

Fixation is considered a key step in histological tissue processing. The observations in this study show that organs collected and fixed immediately after animal sacrifice gave optimal preservation of tissues for histology using H&E. Delay in tissue fixation as short as 5 minutes and upwards showed adverse effects on the histoarchitecture of the liver and kidney in a time-dependent manner, which by extension falsifies any inference drawn from such histological results. This study outlines the exact extent of the histological distortion with each stage of delay in fixation in the liver and kidney tissues.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

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