# RATE OF AUTOLYTIC CHANGES IN DIFFERENT UNFIXED TISSUES AT DIFFERENT TIME INTERVAL

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**Aim and Background**: Fixation hardens tissues and stabilizes the protein skeleton of the cell by giving the cell some structural support to resist deformation or crushing which may occur in the tissue processing sequence. Since fixation is essential for optimum tissue preparation for sectioning, it is worthwhile to study the autolytic changes in tissues which may affect this stage of specimen handling.
**Method**: A healthy six-month old Wister rat was anaesthetized with chloroform, killed and dissected to aseptically harvest the brain, lung and liver. These organs were cut into 10 sections, each of 2×2×3mm, transferred into sterile universal containers from where sections were removed and fixed in 10% formal saline at a two hour intervals from 0 hours to 18 hours. Haematoxylin and Eosin and Gram's Iodine stain were used to stain all the sections obtained from tissue blocks, Periodic acid Schiff's (PAS) for the section of liver tissue, Verhoff elastic fibre stain for lungs section and Bielschowsky's stain for brain tissue section.
**Results**: Putrefaction in the brain was noticeable within 8 hours, in the liver it started at the 14th hour while putrefaction was absent in the lungs.
**Conclusion**: Optimal staining reaction in brain and liver tissues would be unrewarding if the tissue is not fixed within 4 hours while the lung biopsy must be fixed within 6 hours.
**Keywords**: Putrefaction, Autolysis, Fixation, Tissue

## INTRODUCTION

Hystomorphologically, the autolysis represents the intravital or post mortal disintegration of living structures, and biochemically corresponds to a loss in the system of metabolic balance with demotion of the metabolic substance which results in energy and material loss. Autolysis matches with the activity of certain enzymes, called autolytical enzymes, found in lysosomes of living cells, which after death lead to the destruction of their own cell components. Those enzymes disintegrate intracellular material, including organelles very quickly, so the cytoplasm becomes of homogenic looks and intensively eosinophilic, which culminates with a loss of cell details and tissue architecture (Zdravković *et al.,* 2006).

Research have been carried out on the rate of autolysis in unfixed tissues but inadequate data is available on the effect of autolysis on factors such as; hardening, staining reactions, optical difference and preservation of chemical components. Even though much effort has been put into educating clinicians and surgeons on the importance of fixation, the local experience in most laboratories have shown change to ideal practice is still slow.

Tissues fixed in concentrated and over diluted formalin solution are frequently encountered as well as normal saline and chlorhexidine-fixed tissues. Therefore, determination of these factors is important, especially in rural areas of our community where access to adequate fixation for tissues is often unavailable or not given proper attention and yet such samples need to be salvaged for histopathological diagnosis.

Fixation is the basic, most important step of pathology due to its role in preventing autolysis and degradation of cells and tissue components. It enables accurate observation of sections both anatomically and microscopically following sectioning (Howat, 2014). Temperature at which the sample is stored plays a vital role in in the rate at which tissues are fixed. This is due to the basic chemical principles which states that the speed of any reaction can be increased with heat, and then slowed when cooled (Fox, 1985).

There are several types of fixatives; however 10% neutral buffered formalin was used due to its advantages. Formaldehyde solution contains methylene glycol as its active ingredient which is responsible for the fast rate of penetration and but relative slow rate of fixation (Fox, 1985)

The mechanism of action of formaldehyde occurs through the formation of intra and inter-molecular cross-links which occur through side chain amino group of lysine which over time results in the formation of methylene bridges (Gustavson, 1956)

The process of putrefaction is described following the end stage of autolysis; however, the two processes can occur simultaneously within different regions of a body. Temperature, both physiologically and environmentally, has the most vital impact on the decomposition process. With respect to the chemical processes of autolysis and putrefaction, it is generally reported that heat increases the decomposition rate and cold reduces it (Forbes 2017).

This study aims to determine the effect of delayed fixation on tissue architectural preservation, staining reaction and viability of different tissues at different time intervals after excision. Data derived will assist the histoscientist in knowing which tissue is salvageable for histological analysis as well as assist pathologists in adequately interpreting the sections.

## MATERIALS AND METHODS

The brain, liver and lung of sacrificed 6-month old Wister rat was aseptically harvested and were then cut into 10 sections, each measuring approximately 2×2×3mm and transferred into sterile universal containers. A section was then taken every 2 hours from 0 hour to 18$^{th}$ hour and fixed in 10% buffered formalin. Tissues were dehydrated in ascending grades of alcohol (50%, 70%, 90% and 100%) for 3 hours and cleared in two changes of xylene (clearing agent) for 3hours. The tissues were then infiltrated and embedded in paraffin wax (Sigma Aldrich), cut with a rotary microtome (Leica, RM-2125 RTS) and stained. Staining properties were assessed with Haematoxylin and Eosin and Gram's Iodine stain on all sections from the tissue blocks (Avwioro, 2011); Periodic acid schiff's (PAS) for the section of liver tissue (Avwioro, 2011); Verhoff elastic fibre stain for lungs (Bancroft 2008) and Bielschowsky's stain for brain tissue (Bancroft 2008). These were graded as:

Haematoxylin and Eosin:

i) Good staining: nuclei are bluish purple and cytoplasm light pink. (+)
ii) Poor staining: nuclei are blue-black and cytoplasm light pink. (1-)
iii) Moderately poor staining: nuclei are blackish and cytoplasm bright pink. (2-)
iv) Extremely poor staining: nuclei and cytoplasm are completely pink. (3-)

Gram's Iodine-

i) Positive: when there is presence of bacteria (+)
ii) Negative: when there is absence of bacteria (-)

Periodic acid Schiff's stain-

i) Positive : when the cytoplasm is magenta (+)
ii) Negative: when there is loss of cytoplasmic magenta (-)

Verhoff Van Geison elastic fibre stain-

i) Positive: the elastic fibres are well differentiated and stained brown-black (+)
ii) Negative: when the whole tissue is diffusely brown- black (-)

Bielschowsky Stain for brain

i) Positive : when axons and dendrites are stained black (+)
ii) Negative ; when there is loss of axons and dendrites (-)

Architectural properties were assessed on the H&E stained slides as follows:

i) Well preserved architecture: nuclear and cytoplasmic borders are preserved (+)
ii) Mildly unpreserved: karyopyknosis, tissue still recognizable (1-)
iii) Moderately unpreserved: karyorrhexis, tissue almost unrecognizable (2-)
iv) Extremely unpreserved: No nuclear or cytoplasmic preservation and tissue is completely unpreserved (3-)

## RESULTS

The effect of delay in fixation on tissue architecture and tinctural properties are represented in table 1 and Figures 1 to 16.

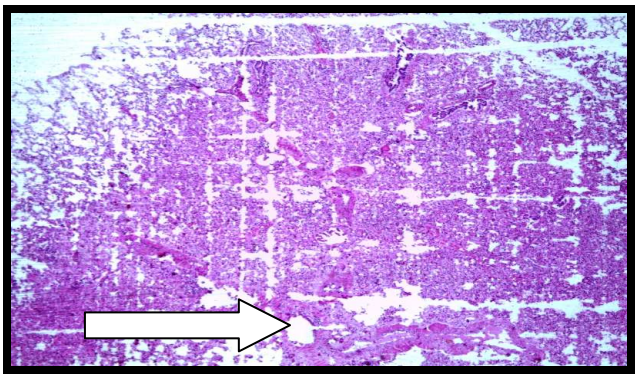
In the lung karyopyknosis started from the $6^{th}$ hour, karyorrhexis, which is the fragmentation of cell nucleus (as shown in fig. 3) began at the $12^{th}$ hour and karyolysis, which is the dissolution of the cell nucleus (as shown in fig. 4) from the $16^{th}$ hour. Poor staining (1-) reaction after 4 hour delay was seen, moderately poor staining ( 2-) by the $10^{th}$ hour (as shown in fig. 1) and extremely poor staining (3-) by the $14^{th}$ hour delay (as shown in fig. 2). Putrefaction which was assessed using the Gram stain was absent. The loss of elastic fibres which was assessed using the Verhoef Van Gieson stain started at the $6^{th}$ hour (as shown in fig. 5) to reach its maximum at the $18^{th}$ hour.

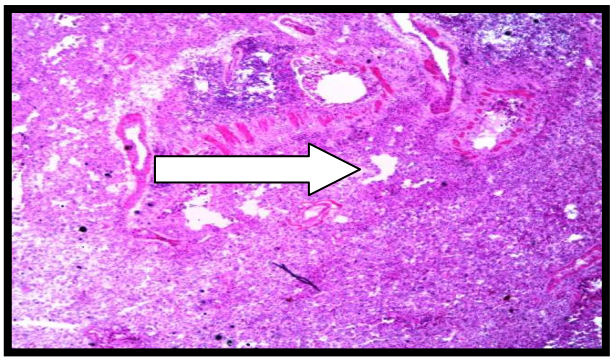
In the liver karyopyknosis (as shown in fig. 12) started at the $6^{th}$ hour, karyorhexis was absent while karyolysis (as shown in fig. 13) started after 10 hours of delay, to reach its maximum after 18 hours delay in fixation. Staining reaction which was assessed using the heamatoxylin and eosin was found to be at 1- (poor staining) at the $10^{th}$ hour up to the $18^{th}$ hours (as shown in fig. 11). Putrefaction (as shown in 14) which was assessed using the Gram stain started at the $14^{th}$ hour following delay in fixation. The loss of carbohydrates (as shown in16) which was assessed using PAS stain started after the $4^{th}$ hours delay to attain its maximum at the $18^{th}$ hour.

In the brain, karyopyknosis was absent, karyorhexis (as shown in fig 8) had become obvious by the $2^{nd}$ hour and by the $4^{th}$ hours karyolysis (as shown in fig 9) was well established. Staining reaction was already moderately poor (2-) (see fig. 6) by the $2^{nd}$ hour and by the $4^{th}$ hour it was extremely poor (3-) (as shown in fig. 7). Putrefaction in brain (as shown in fig. 15) started at the $6^{th}$ hours and loss of staining for axons and dendrites was easily assessable by the $4^{th}$ hour (as shown in fig.10). The overall results are summarized in table 1.

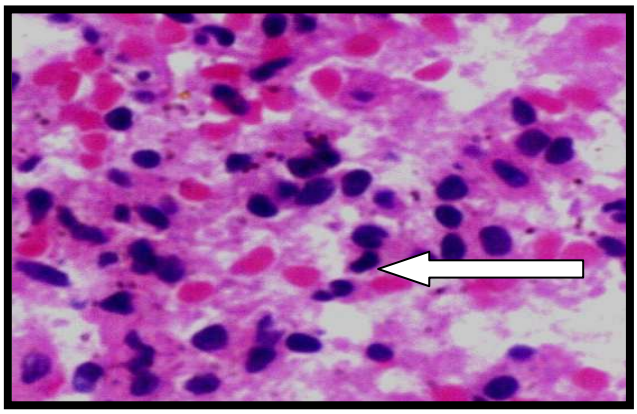
**TABLE 1:** shows the pattern of architectural changes and staining reaction in the Lung, liver, & brain.

| | **Commencement of loss of architectural pattern (hour)** | | | | **Beginning of loss of staining intensity** | | | | **Special stains** | |
|---|---|---|---|---|---|---|---|---|---|---|
| Tissue | **Normal** | **Pyk** | **Rhexis** | **Lysis** | **Normal** | **1-** | **2-** | **3-** | **Gram,** | **VVG, PAS, Bielschowsky** |
| Lung | $4^{th}$ | $6^{th}$ | $12^{th}$ | $18^{th}$ | $2^{nd}$ | $4^{th}$ | $10^{th}$ | $14^{th}$ | Nil | $6^{th}$ |
| Liver | $4^{th}$ | $6^{th}$ | Nil | $10^{th}$ | $8^{th}$ | $10^{th}$ | - | - | $16^{th}$ | $4^{th}$ |
| Brain | $4^{th}$ | $6^{th}$ | $2^{nd}$ | $4^{th}$ | $8^{th}$ | $10^{th}$ | $2^{nd}$ | $8^{th}$ | $6^{th}$ | $4^{th}$ |





Figures 1 and 2 show Lung tissue exhibiting progressive loss of architecture and staining property, with increased tissue eosinophilia and loss of basophilia at 14 and 18 hours.H&E x 20.



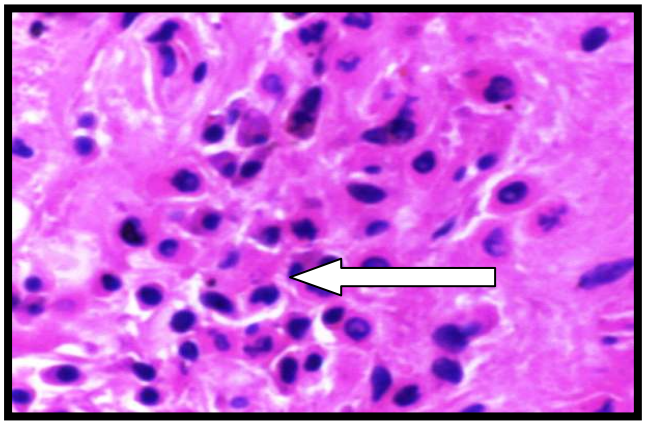


Figure 3 and 4 show lung tissue exhibiting karyorrhexic and karyolitic nuclear architecture at 12 and 18 hours respectively. H&E X100.

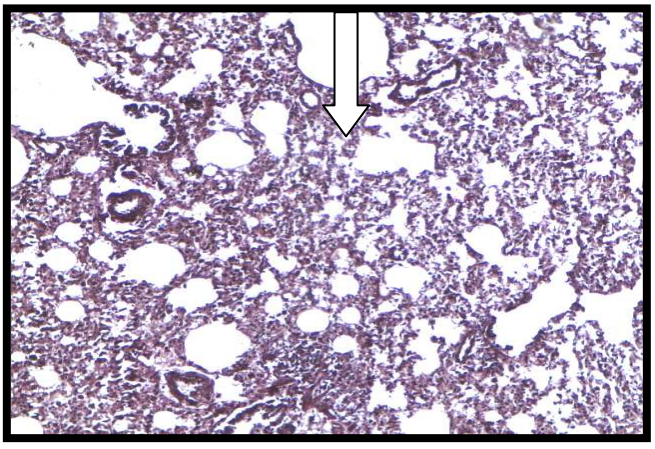
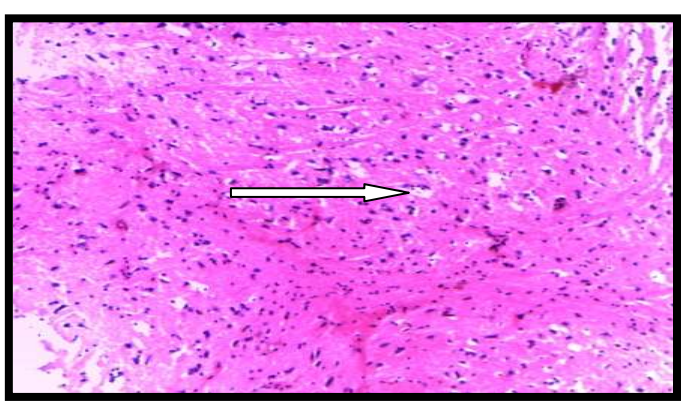
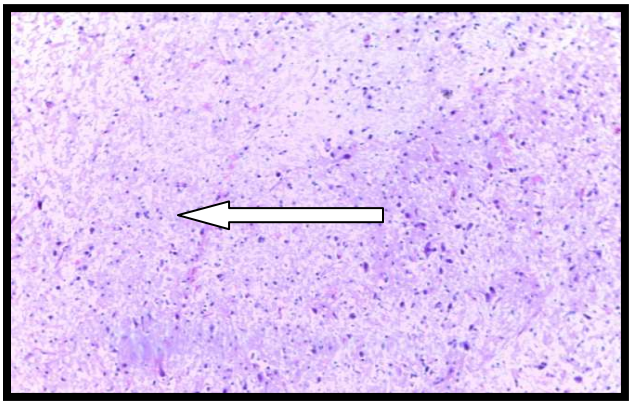
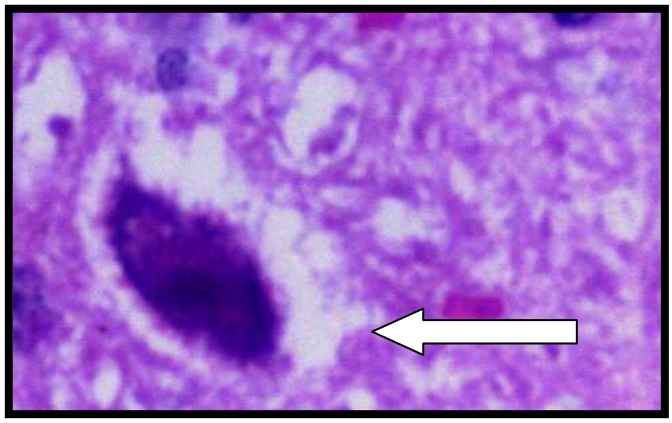
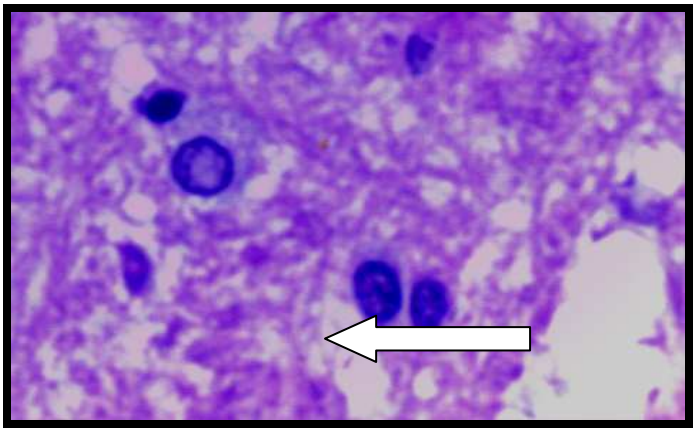


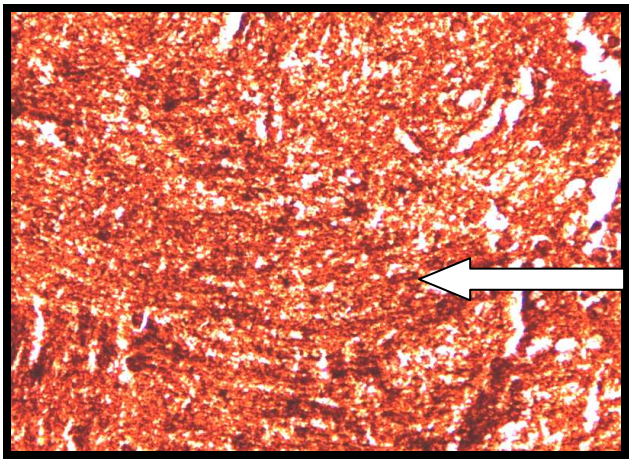
Figure 5 shows loss of staining reaction in the unfixed lung tissue at 6 hours . Verhoeffstain x20.

Figures 6 and 7 show brain tissue exhibiting progressive loss of architecture and staining reaction at 2 and16 hours respectively. H&E x 20

Figures 8 and 9 show nuclear architectural changes in nuclei of axons in brain tissue at 2 and 16 hours respectively. H&E x100.



Figures 10 shows unfixed brain tissue at 2 hours exhibiting loss of staining reactions for axons and dendrites . Bielschowski stain x20

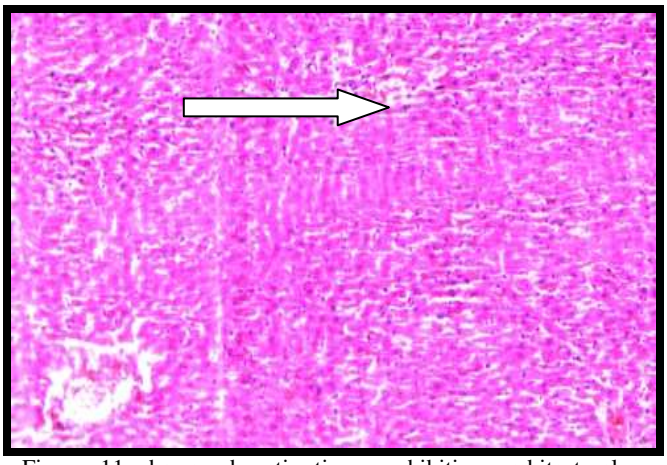
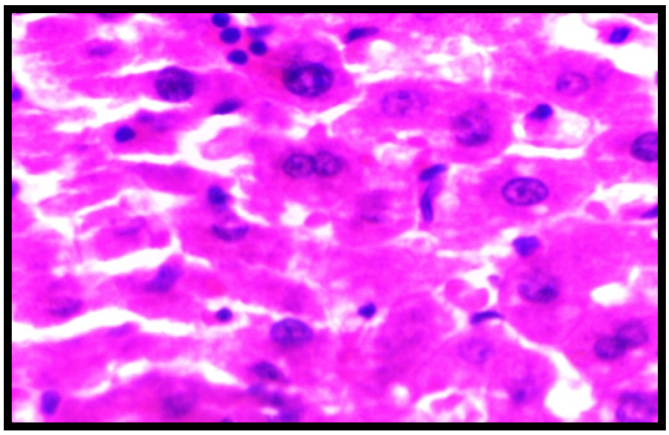
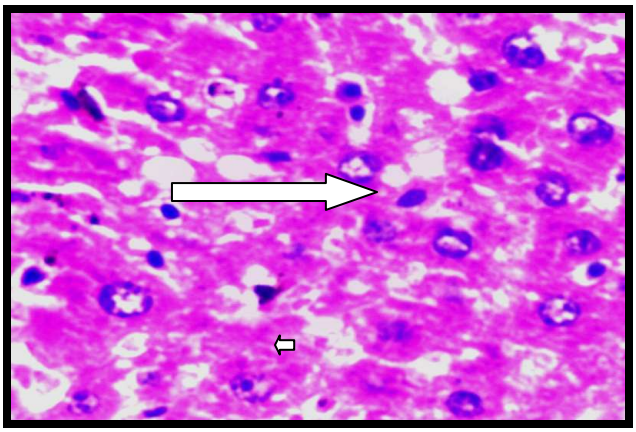


Figure 11 shows hepatic tissue exhibiting architectural distortion and loss of basophilia at 10 hours . H&E x2



Figures 12 and 13 show architectural nuclear changes in hepatocytes of the Liver. They exhibit karyorrhexis and karyolysis at 8 and 12 hours respectively. H&E x100



Figures 12 and 13 show architectural nuclear changes in hepatocytes of the Liver. They exhibit karyorrhexis and karyolysis at 8 and 12 hours respectively. H&E x100

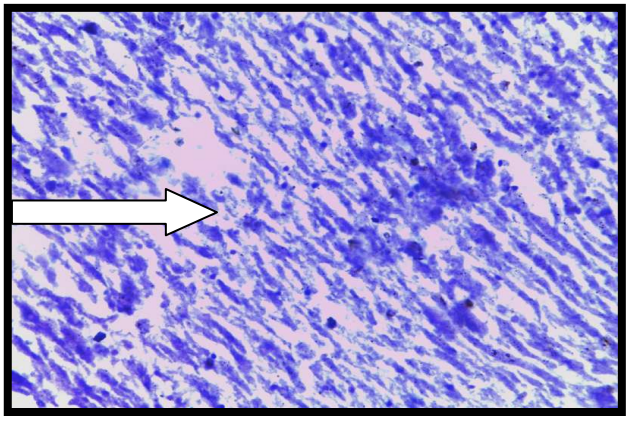


Figure 14 shows growth of Gram – cocci on unfixed hepatic tissue at 14 hours.

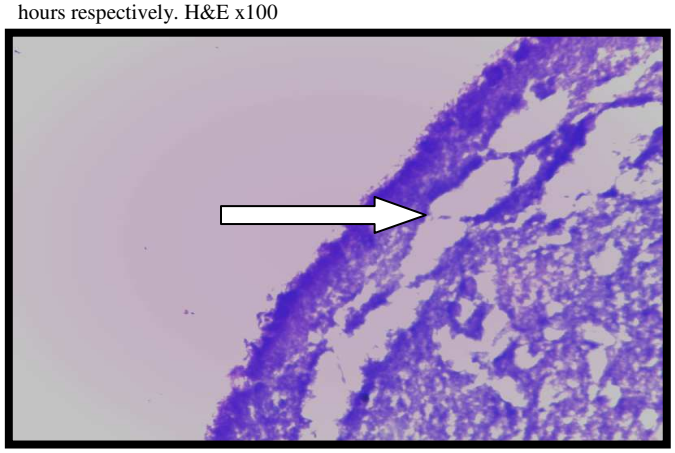
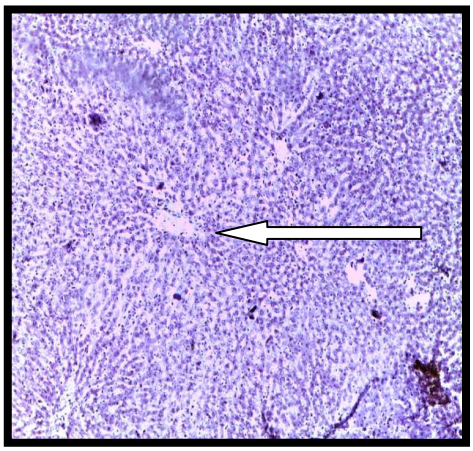


Figure 15 shows fuzzy growth of Gram- bacteria on the pouter surface of brain tissue at 6 hours.



Figures 16 shows unfixed hepatic tissue at 4 hours exhibiting loss of staining reaction. PAS x 20.

## DISCUSSION

This study shows that commencement in loss of tissue architecture was first noted in the brain tissue from 2hours. This was followed by liver and lung tissues which both commenced showing architectural deformities at 6 hours respectively.

The quick change in the brain architecture is due to the fact that the brain is a soft tissue and highly susceptible to hypoxia (Moore, 2010). The reason for taking longer in the liver may be attributable to the fact that it is richer in nutrients such as glycogen than other tissues and thus takes longer before ATP depletion may start to occur (Pradeep *et al.,* 2013). In the case of lung tissue, the longer time before architectural changes became noticeable may reflect the higher oxygen concentration present in lungs than other tissues, thus maintaining the cells for longer (Kumar, 2010).

Putrefaction in the brain was observed after 8 hours delay in fixation; in the liver it started at the 14th hour while putrefaction was absent in the lungs. The high oxygen concentration in the lungs may retard growth of anaerobes often responsible for putrefaction (Oyewole 2014). It may also be a reflection of the relatively low humidity of the environment in Kano (40% to 70%) (Oyewole, 2014). This is another factor that could affect bacterial growth.

The loss of Bielschowsky staining reaction in the brain tissue shows that assessing for dendrites from brain tissues in the diagnosis of brain lesions would be unrewarding if the tissue is not fixed within 4 hours.(As shown in fig 10)

In assessing liver tissues for intracellular accumulations and where PAS has been shown to be an invaluable ancillary test, for optimum evaluation of this compound the liver biopsy must be fixed within 4 hours of biopsy. The PAS stains carbohydrates with the 1-2 glycol configurations (OH group on carbons 3 and 4 of the hexose sugar). The periodic acid component oxidizes these OH groups to produce aldehyde groups with which the basic Schiff reagent reacts (Kiernan, 1999). As hepatocytes are dying the cells begin to breakdown the glycogen to generate ATP, thus making these molecules available for staining by PAS. Powerful hydrolases released by the degenerating hepatocytes will degrade the hexose structure thus rendering the PAS stain ineffective.

For elastic fibre detection using Verhoef VanGieson is used to identify the presence or absence of elastic fibers in tissues and to demonstrate loss of elastic tissue in the lung in patients with emphysema, and thinning and loss of elastic fibers in blood vessels of patients with arteriosclerosis (Kazlouskaya, 2013). In support of the index study, Khoury *et al* found out that there was no significant differences in the extent or intensity of immunostaining with a fixation delay of less than 8 h while Piccinin *et al* (2018) observed deterioration in intensity of immunohistochemistry staining from 24 h of delay. However, lung biopsy must be fixed within 6 hours.

## CONCLUSION

In conclusion this study shows that for optimum evaluation of the architectural preservation and staining reaction, fixation of brain tissue must not be delayed beyond 2 hours. For liver and lungs it must not be delayed beyond 4 and 6 hours respectively.

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