

Original Research Article

Periodic Acid Schiff reactions and general tissue morphology of conventionally-processed versus two rapid microwave-processed tissues

ABSTRACT

Aims: To process tissues using the microwave techniques, to establish a processing schedule that eliminates xylene. To check the effect of microwave processing techniques on glycogen reactions as well as tissue morphology.

Study design: Animal acquisition, sacrifice, harvesting and fixation of tissues. Grossing into triplicates, processing using three different techniques. Staining using the PAS-Diastase as well as Haematoxylin and eosin staining techniques. Assessment and grading of morphology and staining characteristics.

Place and Duration of Study: Apparently healthy rabbits from the animal house, National Veterinary research Institute, Vom, Jos, Nigeria, between August and December, 2014.

Methodology: Two apparently healthy rabbits were sacrificed and the , Lungs, Heart, Liver, Kidney, Stomach, and the Spleen were harvested and fixed in 10% buffered formalin for three days. They were grossed into triplicates, labeled and processed using the conventional, microwave without vacuum and microwave with vacuum respectively. They were sectioned and stained simultaneously using the PAS-Diastase as well as haematoxylin & eosin staining techniques. They were graded as excellent if they permitted microscopy, fair if not very good but can permit microscopy and poor if they cannot permit microscopy at all

Results: Processing cycle as well as quantity of reagent used per cycle is greatly reduced in the microwave techniques. Tissue morphology, special features as well as glycogen were clearly demonstrated without significant differences. Dye uptake as well as section thickness were comparable among the three techniques. Block durability is comparable among the three techniques after 64 weeks of storage.

Conclusion: Same-day turn-around is possible in histology, with reduced reagent consumption and elimination of xylene, without compromising section quality, dye uptake or the reactions of glycogen in the PAS-Diastase technique. This will result to quick diagnosis hence quick intervention at a cheaper rate to both laboratories and clients. It is a good innovation in forensic and diagnostic histopathology and should be encouraged. Its compatibility with other histochemical techniques, immunohistochemical and molecular techniques should be evaluated to give a wider application.

Keywords: *Tissue processing, Duration, reagent consumption, Glycogen storage disease, PAS-Diastase, Tissue morphology, microwave, vacuum*

1. INTRODUCTION

The foundation of all good histological preparations is adequate fixation and good tissue processing. Sections cut from poorly processed tissue blocks show poor resistance to the rigors of antigen retrieval techniques and are easily lost. Morphology is impaired and antigens may be lost or become diffused. For almost 100 years, the steps followed to prepare tissues for microscopic review have remained practically unchanged. Substantial shortcoming associated with that practice include at least a 1- day delay in providing the diagnosis, reagent toxicity, and degradation of nucleic acids [1]. Although, microwave radiation seems to overcome some of the problems [2]. Microwavebased processing methods have not gained widespread acceptance. [3]. The basic aim in any field of life sciences is to utilize eco-friendly chemicals which are nontoxic, less bio-hazardous, and economical. Various substitutes of xylene are available [4]. Analytical accuracy, health and safety and cost-efficiency are major concerns in modern pathology laboratories. In histopathology, traditionally, xylene has been used in tissue processing applications; however, this chemical is particularly toxic and hazardous. In response, laboratories are increasingly investing in methods that do not involve the use of xylene. Isopropanol is less toxic than xylene. The use of isopropanol during dehydration and paraffinization of tissues simplifies and accelerates the process of histological processing. It excludes treatment with intermediate solvents of paraffin (chloroform, xylene, and benzene), which

reduces tissue condensation, provides its greater plasticity in cutting and allows obtaining thin serial sections [5]. Periodic acid–Schiff (PAS) is a staining method used to detect polysaccharides such as glycogen, and mucosubstances such as glycoproteins, glycolipids and mucins in tissues. The reaction of periodic acid oxidizes the vicinal diols in these sugars, usually breaking up the bond between two adjacent carbons not involved in the glycosidic linkage or ring closure in the ring of the monosaccharide units that are parts of the long polysaccharides, and creating a pair of aldehydes at the two free tips of each broken monosaccharide ring. The oxidation condition has to be sufficiently regulated so as to not oxidize the aldehydes further. These aldehydes then react with the Schiff reagent to give a purple-magenta color. A suitable basic stain is often used as a counterstain. PAS diastase stain (PAS-D) is PAS stain used in combination with diastase, an enzyme that breaks down glycogen. Alcian blue/periodic acid–Schiff (AB/PAS or AB-PAS) uses alcian blue before the PAS step [6]. PAS staining is mainly used for staining structures containing a high proportion of carbohydrate macromolecules (glycogen, glycoprotein, proteoglycans), typically found in e.g. connective tissues, mucus, the glycocalyx, and basal laminae. PAS staining can be used to assist in the diagnosis of several medical conditions:

- Glycogen storage disease (versus other storage disorders).
 - Adenocarcinomas, which often secrete neutral mucins.
 - Paget disease of the breast.
 - Alveolar soft part sarcoma.
 - Staining macrophages in Whipple's disease.
 - It can be used to diagnose α 1-antitrypsin deficiency if periportal liver hepatocytes stain positive.
 - Aggregates of PAS positive lymphocytes are present in epidermis in Mycosis fungoides and Sezary syndrome, called Pautrier microabscesses.
 - Ewing Sarcoma
 - Erythroleukemia, leukemia of immature red blood cells. These cells stain a bright fuchsia.
 - Pulmonary alveolar proteinosis.
 - Fungal infection, the cell walls of fungi stain magenta; this only works on living fungi. In contrast, Grocott's methenamine silver stain (GMS) will stain both living and dead fungal organisms.
 - It is used to identify glycogen in lung biopsy specimens of infants with pulmonary interstitial glycogenosis (PIG).
 - It can be use to highlight super cross-linked lipids inclusions in ceroid lipofuscinosis (NCL).
- Presence of glycogen can be confirmed on a section of tissue by using diastase to digest the glycogen from a section, then comparing a diastase digested PAS section with a normal PAS section. The diastase negative slide will show a magenta staining where glycogen is present within a section of tissue. The slide that has been treated with diastase will lack any positive PAS staining in those locations on the slide PAS staining is also used for staining cellulose. One example would be looking for implanted medical devices composed of nonoxidized cellulose. If the PAS stain will be performed on tissue, the recommended fixative is 10% neutral-buffered formalin or Bouin solution. For blood smears, the recommended fixative is methanol. Glutaraldehyde is not recommended because free aldehyde groups may be available to react with the Schiff reagent, which may result in false positive staining.

2. MATERIAL AND METHODS / EXPERIMENTAL DETAILS / METHODOLOGY

Two apparently healthy rabbits [7] aged 8 weeks (weaner) and 48 weeks (adult rabbit) respectively, were sacrificed after chloroform anesthesia [8]. Dissection was performed via one median and two transverse (behind the rib arc) incisions of the soft abdominal wall to expose the viscera in the thoracic and extrathoracic parts [9]. The following tissues were immediately harvested (after a careful observation of their gross morphology in order to rule out any macroscopic abnormality) from each of the rabbits and fixed in 10% buffered formalin for three days: Liver, Kidney, stomach, Heart, lungs and spleen.

2.1 Tissue Grossing

-The fixed tissues were transferred to the fume cabinet and observed for any macroscopic abnormality.

-The tissues were each cut in triplicates using a sharp scalpel blade in a one stroke action, to sizes of 10mm X 5mm X 2mm and labeled 'C', 'W' and 'V'.
 -Each of the individual group tissues were placed in tissue cassettes and labeled appropriately
 -Each group (C, W and V) were placed in 10% buffered formalin, labeled appropriately and kept ready for processing.

2.2 Tissue Processing

Tissues in the C category were placed in running tap water for five minutes, dehydrated (by passing through ascending grades of ethanol as follows: 70%, 80%, 90%, 95%, Absolute ethanol I, II and III, cleared (in two changes of Xylene) and infiltrated (in two paraffin wax ovens placed at 4°C above the melting point of the paraffin wax used). The SPIN tissue processor, STP 120 (Thermoscientific) was used in which the tissues were subjected to each stage for two hours making a total of twenty two hours. The 'W' category of tissues were processed using the TissueWave™ 2 microwave processor (Thermo Scientific®, Kalamazoo, MI) processor in which they were first washed with tap water for five minutes and then dehydrated in two changes of absolute ethanol at 67°C for 15 minutes. [10]. The tissues were then passed through two changes of isopropanol at 67°C for 15 minutes each. They were then transferred to preheated paraffin wax and infiltrated at 70°C in the wax for 30 minutes, all at atmospheric pressure, making a total of one hour, thirty minutes. The 'V' category of tissues was processed in the same manner with those for W except that the infiltration was done in a vacuum at a pressure of 20Hg for 20 minutes making a total of one hour, twenty minutes. Reagents used in the microwave processor were filtered using Whatman No.1 filter paper and reused as the need arises.

2.3 Tissue Embedding

At the end of each procedure, the tissues were embedded using embedding cassettes on a tissue Tek Embedding Centre (SLEE MPS/P2), and cooled rapidly on the cooling component as follows:

- tissues were removed from tissue cassettes and placed on the embedding chamber
- Molten paraffin wax was dispensed to full capacity into Tissue Tek embedding mould by pressing the tap backward.
- A Tissue cassette was labeled appropriately with the tissue label being prepared for embedding.
- Using a preheated forceps, the each tissue was picked and orientated in the moulten paraffin wax in the mould.
- The cover of the labeled cassette is removed and the reverse side of the cassette is placed on the mould containing the tissue embedded in paraffin wax.
- The embedded mould is now placed on the cooling chamber and allowed to cool and solidify.
- This was repeated for individual tissues until all tissues were embedded.
- After a period of 10 minutes, the block is detached from the mould (now containing the tissue embedded in the solidified wax).
- Excess wax was trimmed using a scalpel blade.
- They were observed for retraction.

2.4 Tissue Sectioning

- Tissue blocks were attached to the block holder of a rotary microtome microtome (MICROM HM340E ThermoScientific) and trimmed to expose the tissue.
- Tissues were simultaneously placed on ice (tissue side downward) and left for 10 minutes.
- Tissue blocks were each returned to the block holder of the microtome and sectioned at three micromes (3µm).
- Each section obtained was placed on 20% ethanol to flatten before floating on a floating-out bath.
- They were each picked using albuminised slides, placed at 90° and picked at 45°.
- Sections were dried by placing them vertically and when dry, they were placed on the hot plate (section side uppermost) set at a temperature of 4°C above the melting point of the paraffin wax used, to fix.

The smoothness and ease of sectioning was accessed. The paraffin blocks were stored in a polythene bag and observed for retraction of the tissue daily, for a period of thirty days.

2.5 Tissue Staining

Triplicate sections (one from each of the three processing methods used) of each tissue were simultaneously stained by the Haematoxylin and eosin method for general tissue structure and the Periodic acid Schiff (with diastase technique) [11].

142 2.6 General Criteria for Evaluation of Quality of Sections

143 The following criteria were used [12,13] to evaluate the three processes:

144 **2.6.1 Section preparation;** Cutting texture of blocks, Uniformity of blocks, Cohesiveness
145 of blocks, Ribboning & compression during cutting.

146 2.6.2 Microscopic Assessment;

147 **i. Physical quality of section (excludes stain quality):** this was checked to assess
148 disruption, adhesion, cracking and section thickness [14].

149 **ii. Quality of tissue preservation: this was used to assess nuclear and cytoplasmic**
150 **details,** special features, (kidney-basement membrane definition, liver-sinusoidal
151 endothelium definition), extracellular components and muscle (collagen, elastin),
152 uniformity of preservation (includes zonal fixation) [15].

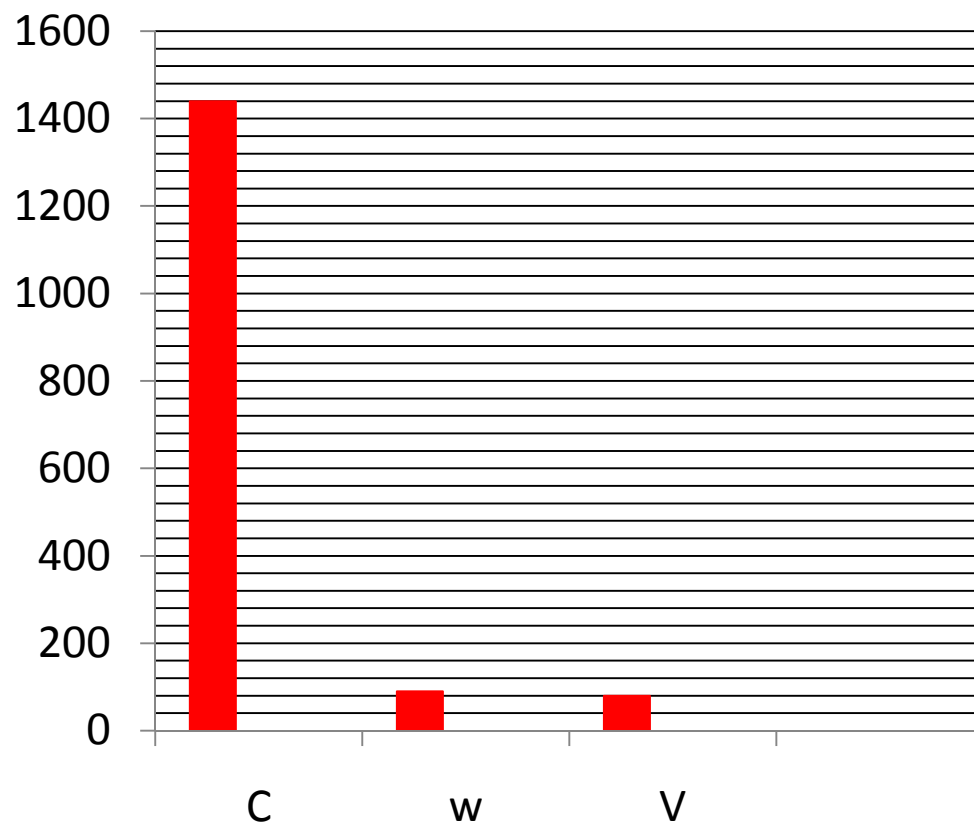
153 **iii. Quality of staining (chemical):** uniformity, nuclear and cytoplasmic details, as well as
154 extracellular components & muscle (collagen, elastin) were checked and graded. For
155 cellular morphology evaluation, greater eosinophilia of cytoplasm producing
156 enhancement of the nuclear-cytoplasmic contrast, good stroma, whether secretory
157 products are appreciable, red cell lysis absent, whether differentiation can be made
158 between cells and other components of the tissue [16,17].

159 The following grading was done:

- 160 i. Excellent: Tissue clearly demonstrated.
- 161 ii. Fair: Tissues not very well demonstrated, but can be used for microscopy.
- 162 iii. Poor: Tissue not clearly demonstrated. Not good for microscopy.

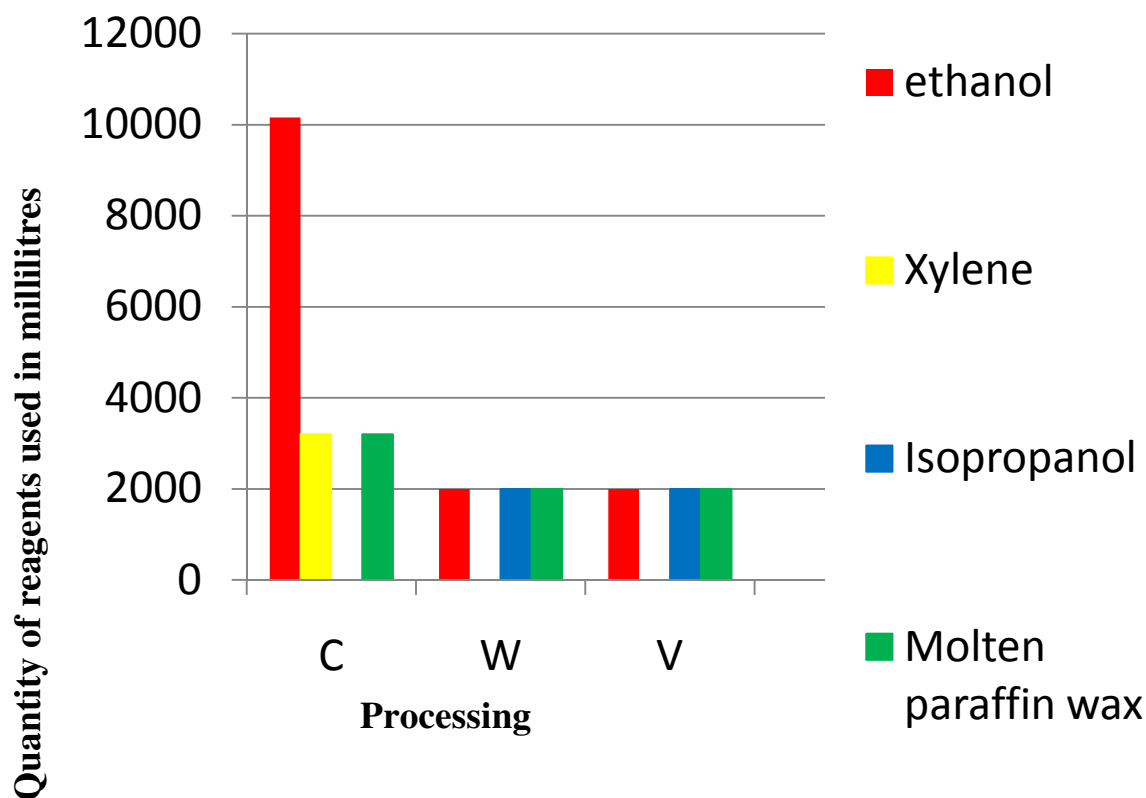
163 3. RESULTS AND DISCUSSION

164 Duration per cycle was observed to be drastically reduced in the microwave tissue techniques as it
165 took an average of 85 minutes while the conventional method took one thousand, four hundred and
166 forty minutes (Figure 1). Reagent consumption, which is the major determinant for the cost of
167 processing, is seen to be low in the microwave techniques-while xylene is completely eliminated in
168 the microwave techniques, the conventional method consumes over 3000mL of xylene, only 2000mL
169 of ethanol was used per cycle while the conventional method used over 10,000mL of ethanol (Figure
170 2). Isopropanol was used for dehydration in the microwave techniques. Isopropanol has the ability to
171 complete dehydration if not completely achieved in the ethanol stage. Blocks from the three
172 techniques show no retraction after 64weeks of observation. There was no discrimination in tissue
173 texture as evident with smooth and uniform sectioning in the three techniques.



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Processing techniques used techniques. The figure shows that the conventional technique (C) takes one thousand and forty (1440) minutes for a complete cycle, the microwave without vacuum (W) takes ninety (90) minutes while the microwave with vacuum (V) takes eighty (80) minutes.



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181 Figure 2: An evaluation of the quantity of reagents used among the three processing techniques.
 182 C=conventional method, W= microwave without vacuum method. V= microwave with vacuum
 183 method.

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185 Sections adhere to glass slides without difficulty and there was no section detachment during
 186 staining. The degree of glycogen digestion with diastase as well as the periodic acid Schiff reaction
 187 (without diastase) was comparable among the three techniques. This has shown that the three
 188 techniques do not have a negative chemical effect to glycogen hence can all be use for the study of
 189 glycogen-storage diseases. General tissue morphology, staining intensity and demonstration of other
 190 substances like basement membrane was also comparable for the three techniques. There was
 191 uniformity in the quality of both nuclear and cytoplasmic staining as demonstrated in the plates as no
 192 portion picked the stain more than others either within the section or between one technique and the
 193 others. Stained and mounted slides showed no colour change after 64weeks of storage hence hence
 194 the result of this works shows that there is no change in chemical composition of the tissues in
 195 relation to the commonly used mountant, D.P.X (Destrene, Plasticize and Xylene)

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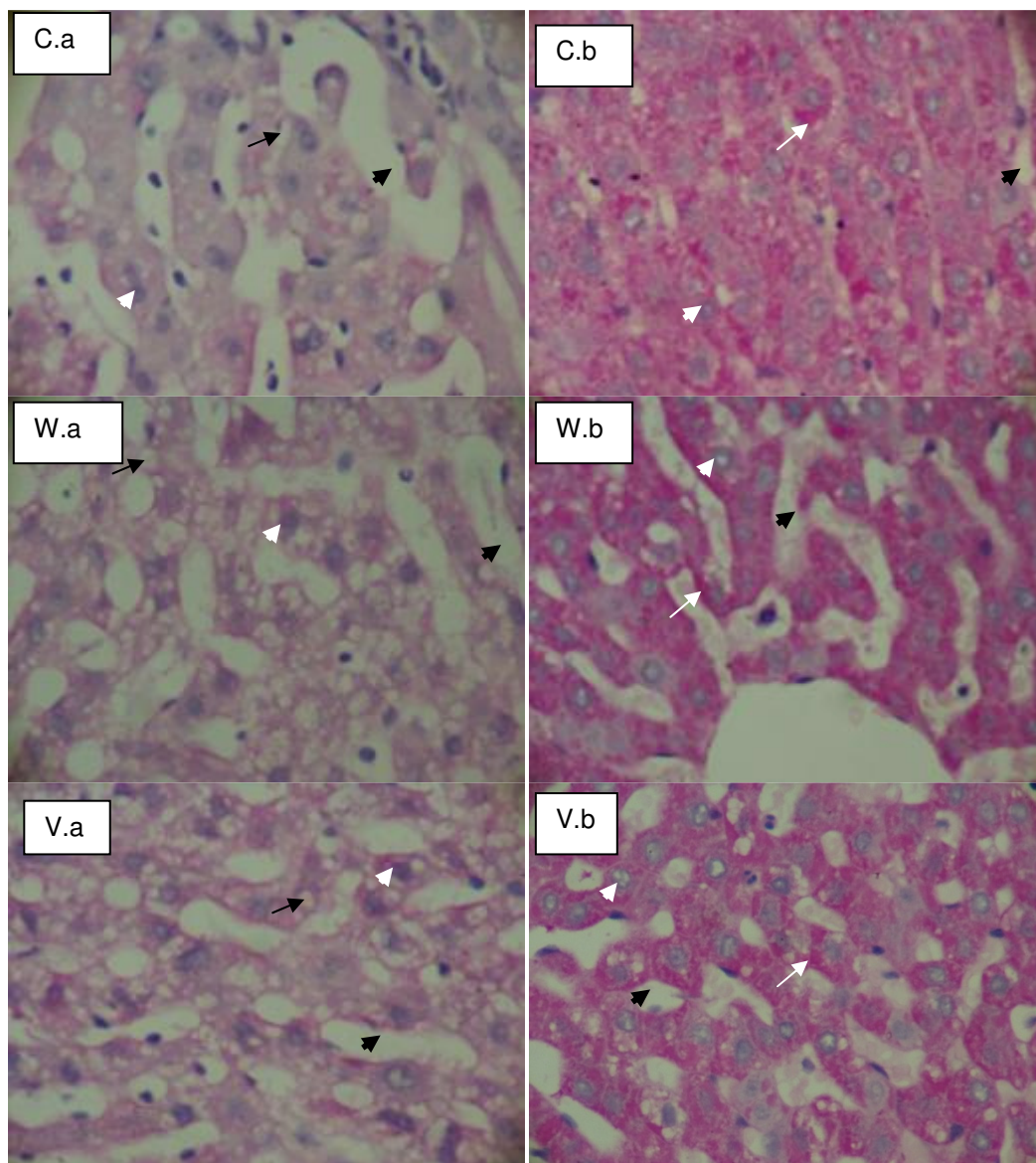
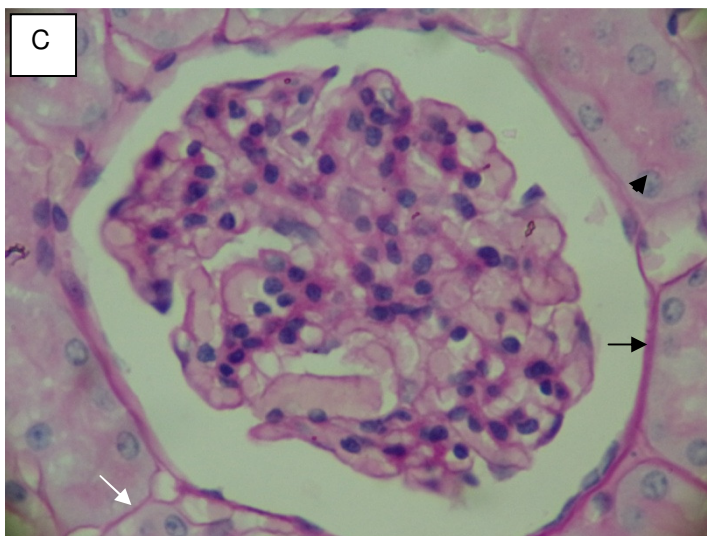
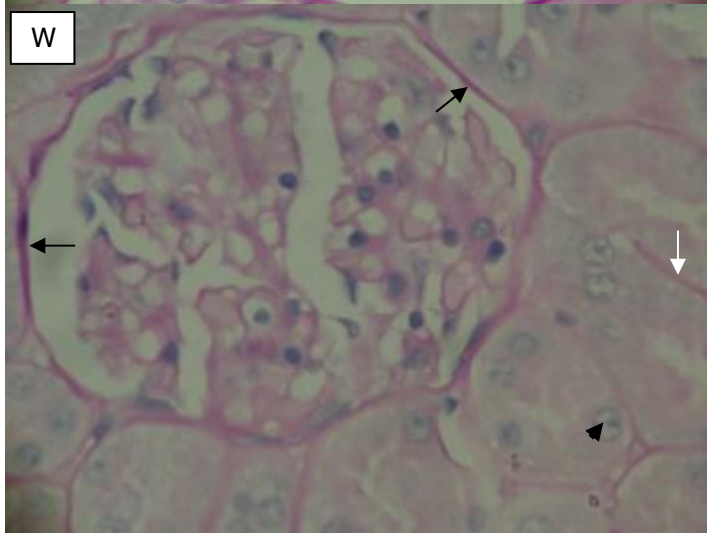


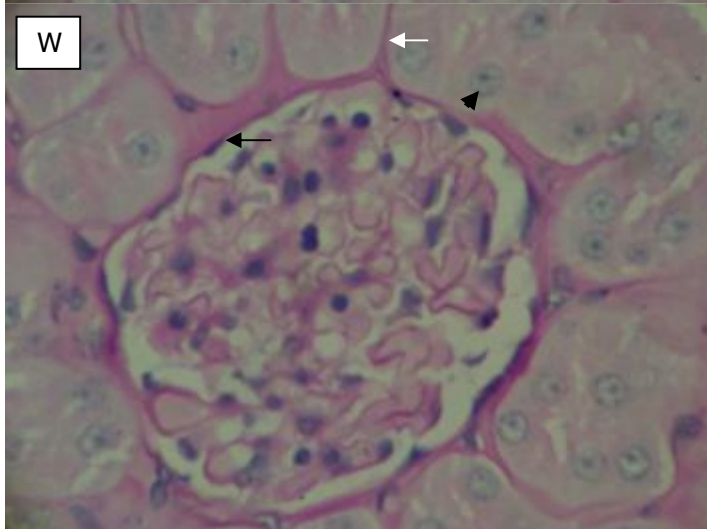
Plate 1. Liver of a weaner processed using the three techniques and stained using the Periodic acid Schiff reaction with diastase (PAS-D) (Plate 1Ca, Wa and Va) and Periodic acid Schiff reaction without diastase (Plate 1Cb, 1Wb and 1Vb). Glycogen deposits are observed to be digested and the space appears empty in PAS-Diastase (black arrows) while glycogen deposits are clearly demonstrated (white arrows) in PAS without Diastase. Nuclei (white arrowheads) and sinusoids (black arrowhead) are clearly demonstrated in the three techniques. PAS-D. X400. C=Conventional, W=Microwave without vacuum, V=Microwave with vacuum.



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Plate 2 Kidney glomerulus of adult rabbit processed using the three techniques. Glomerular capsule (black arrows) and basement membranes (white arrows) and glycogen pick their characteristic pinkish colour in which this technique is known for. The conventional method presented a sharper staining characteristic. Nuclei (black arrowhead) stain bluish. PAS 400X.
C=Conventional, W=Microwave without vacuum, V=Microwave with vacuum.

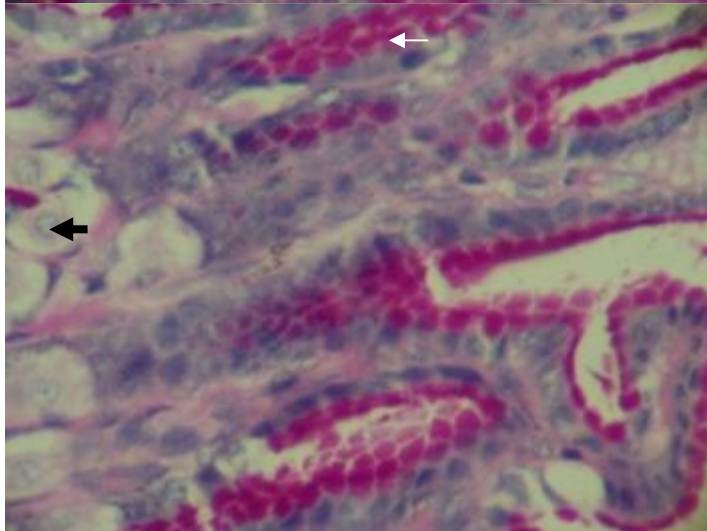
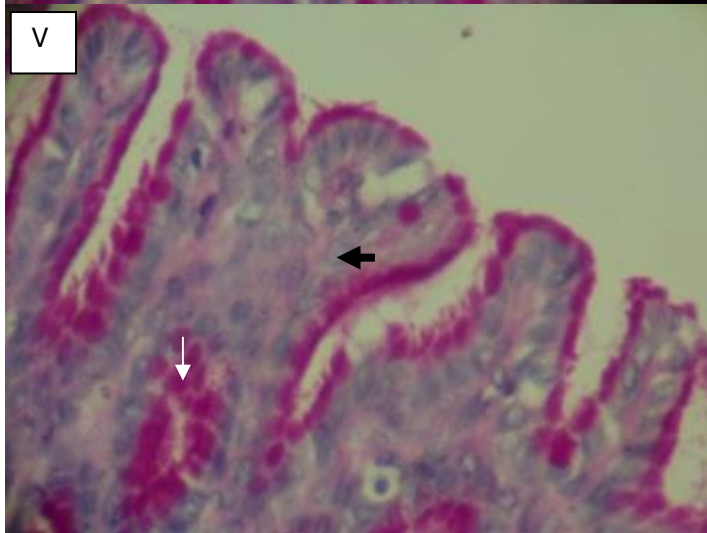
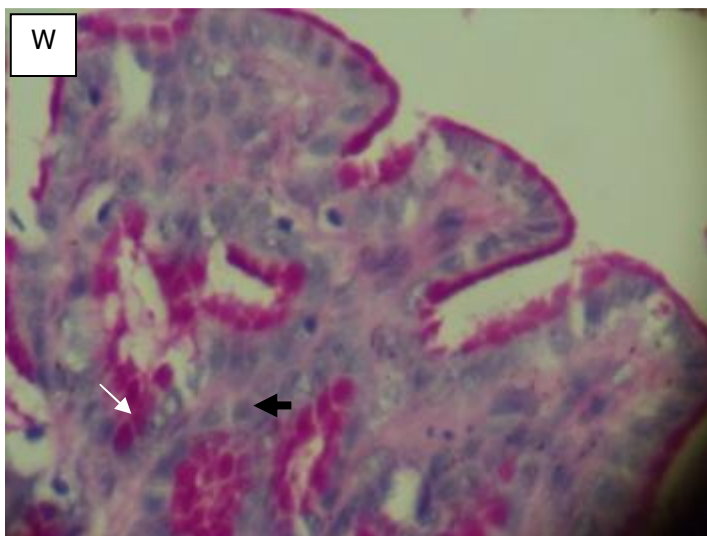


Plate 3 Stomach Gastric mucosa of a weaner demonstrating the mucus-secreting cells (white arrows). Mucus-secreting cells are special features that pick magenta with PAS stain. Nuclei (black arrows). This characteristic is retained in the three techniques as seen above. PAS without diastase taining Technique. X400.

C=Conventional, W=Microwave without vacuum, V=Microwave with vacuum.

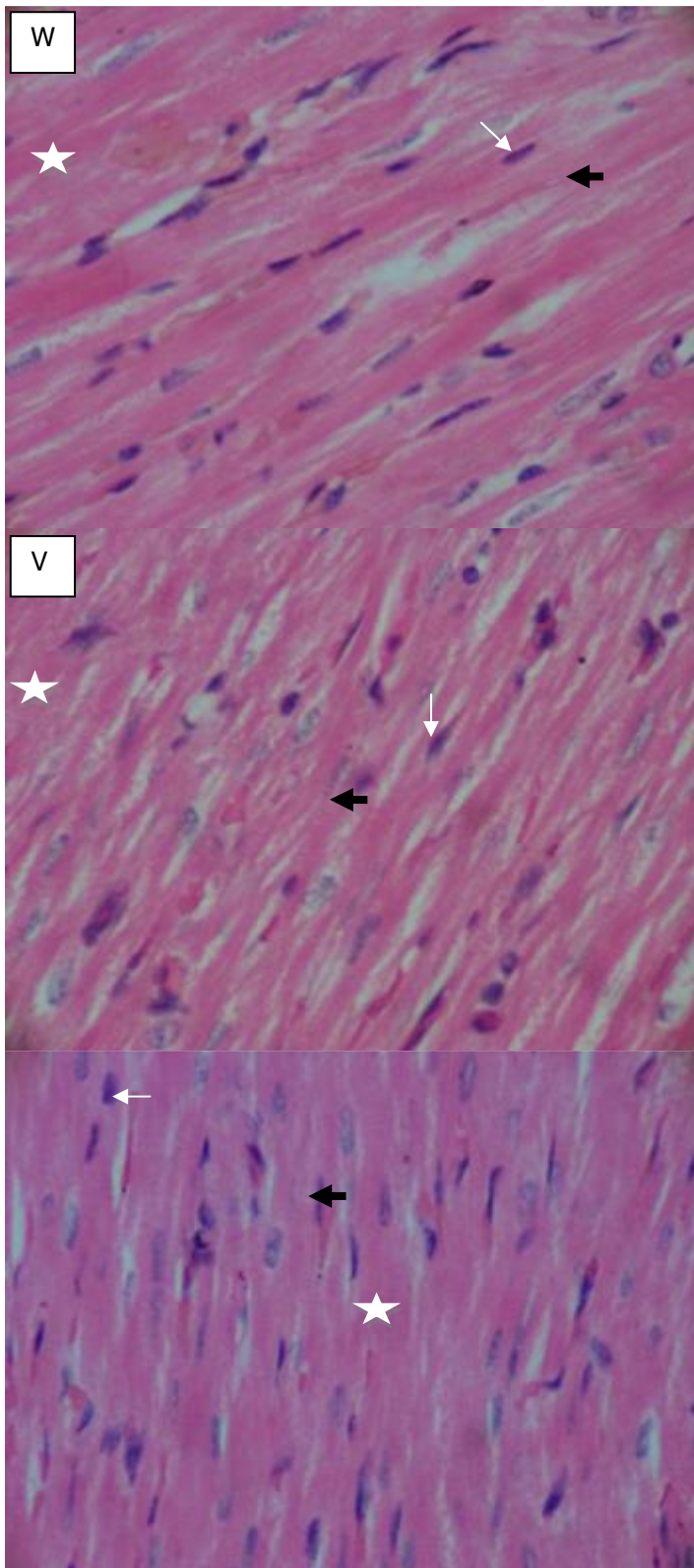
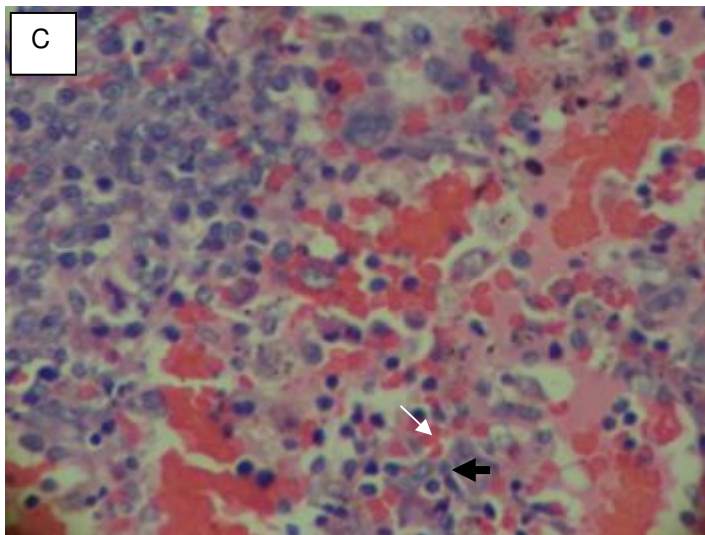
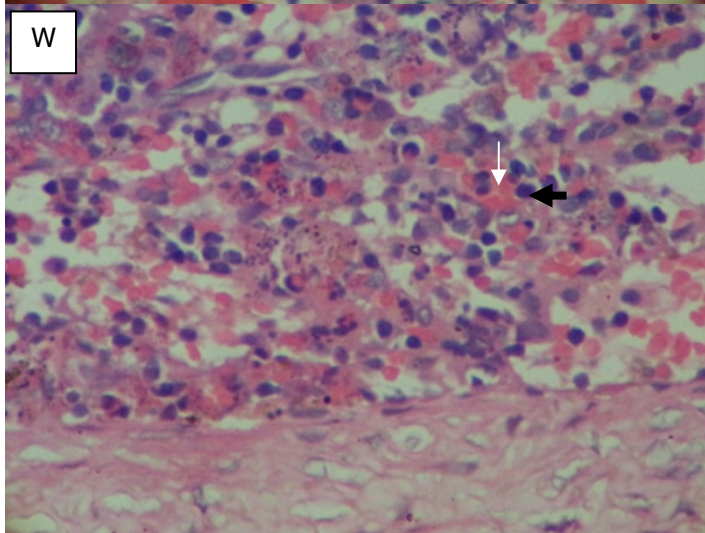


Plate 4 Heart of a weaner processed by the three techniques. Characteristic pinkish staining of cardiac muscle (white stars) and bluish staining of the nuclei (white arrows) is demonstrated clearly in the three techniques. Black arrows= muscle striation. H&E. X400.
C=Conventional, W=Microwave without vacuum, V=Microwave with vacuum.

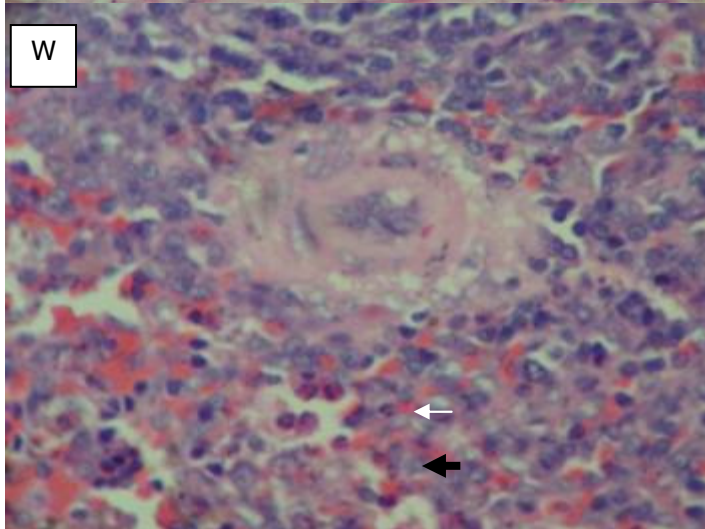
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Plate 5. Spleen of an adult rabbit processed by the three techniques showing intact splenic cells with comparable staining characteristic among the three techniques. . Splenic cords in red pulp (black arrows) are seen interspersed by red blood cells (white arrows) H&E. X400. C=Conventional, W=Microwave without vacuum, V=Microwave with vacuum.

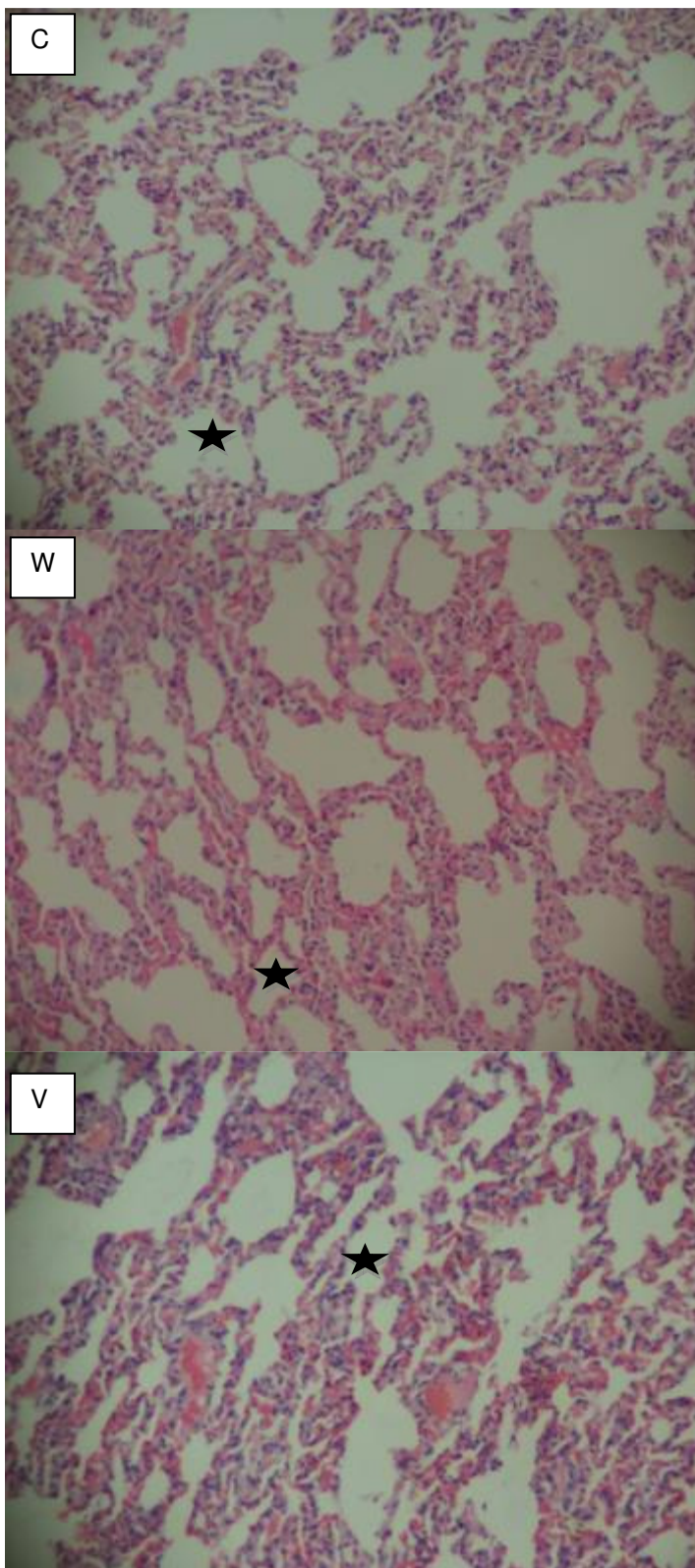


Plate 6. Adult rabbit lung tissue Processed by the three techniques. The characteristic bluish staining of the nuclei and pinkish staining of the cytoplasm is clearly demonstrated in the three techniques. Black stars= alveolar spaces. H&E X100.
C=Conventional, W=Microwave without vacuum, V=Microwave with vacuum.

CONCLUSION

The aim of establishing a quicker method of tissue processing is to save time and create an avenue for faster interventions to the treatment or management of diseases. However, a good tissue processing technique must be seen to be compatible with the chemical composition of the tissue. The microwave techniques have been proven in this experiment to be faster than the conventional method and retain the ability to maintain the chemical composition of PAS positive substances. Since the microwave has also been used for fixation as well as staining of tissues, a fresh tissue brought to histology laboratory can therefore, be processed in less than 100 minutes, stained, mounted, read and results released same day. It has also been established that tissue processing schedules are possible without the use of xylene hence the deleterious effect of xylene, both on the tissue and the laboratorian is now eliminated. The quantity of alcohol used for dehydration is also reduced in the microwave techniques. Isopropanol, which is miscible with both paraffin wax and ethanol and has the ability to complete dehydration, can now be properly utilized in tissue processing. Very long processing schedules have been known to have deleterious effect on the tissue as it results to tissue shrinkage, excessive hardening that results to crumbling of tissues during sectioning as well as retraction of tissues stored in the blocks [18,19]. The microwave techniques retain the tissue for a very short time as compared to the conventional methods hence these negative effects are now eliminated.

This work shows that same day turn-around is possible in histology laboratory without compromising tissue morphology, or the reaction of PAS positive substances. The microwave techniques are also cheaper than the conventional method hence patient care can be made cheaper if this technique is utilized. This work agrees with other authors [10,14]. This technique can be utilized in institutions where electricity supply is erratic as tissue processing can be carried out within a very short time. Packaging of histology samples for further investigations like forensic investigation, immunohistochemistry and molecular analysis can be made faster. Tissue processed using the microwave techniques should be subjected to these advance techniques so as to have a wider application.

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