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Histochemical and Morphological Evaluation of the Conventional Versus Two Rapid Microwave Tissue Processing Techniques

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Authors' contributions

This work was carried out in collaboration between all authors. Author TPPC designed the first draft of the experiment. Authors AAN and JOA criticized the first draft and made adjustment to the experimental design. Authors BND, CD and LDD made literature search. Authors TPPC, KPS and RDP prepared the reagents and carried out the experiment. Authors SIO, PRK and TPPC read and graded the slides. Authors TPPC, CD and LDD drafted the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To utilize the laboratory microwave as a processing instrument in place of the automatic tissue processor, to replace xylene with isopropanol in the clearing stage of tissue processing and check its effect on the chemical reactivity and tissue integrity, to establish a rapid method of tissue processing.

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Study Design: Fixation and grossing of tissues into triplicates. Processing, sectioning and histochemical demonstration of cells and extracellular components. Scoring of blocks and section, statistical evaluation of grades.
Place and Duration of Study: The study was carried out at the central diagnostic Division, National. Veterinary Research Institute, Vom, Jos, Nigeria, for 8 months between October, 2014 and May, 2015.
Methodology: The Trachea, Lungs, Heart, Liver, Kidney, Stomach, Skin, Brain, and the Spleen were harvested from each of two apparently healthy rabbits after scientific ally sacrificing them and fixed in 10% buffered formalin for 3 days. They were grossed into triplicates, labeled and processed using the conventional, microwave without vacuum and microwave with vacuum respectively. They were sectioned and subjected to some histochemical methods as well as silver impregnation, graded and average scores subjected to one way Analysis of Variance (ANOVA) at 95% confidence interval. P-values less than 0.05 were considered statistically significant.
Results: Reagents and time consumption were severely reduced in the microwave techniques when compared with the conventional method even as xylene was replaced with isopropanol, a cheaper and more user-friendly reagent than xylene. There was no statistically significant difference among the three techniques in terms of section preparation (*P*=.22), details of microscopic assessment/quality of tissue preservation (*P*=.74) and details of microscopic assessment/quality of staining (*P*=.90). Microscopic assessment/physical quality is also comparable among the three techniques (*P*=.74).
Conclusion: A cheaper and faster method of tissue processing as presented in this work shows that histology results can be results in less than three hours of sample arrival. Xylene, with its deleterious effects on both the tissue and the laboratory personnel can now be eliminated from the processing schedule, with no negative effects of tissue morphology nor dye uptake. This was will encourage speed and accuracy in histopathology thereby improving patient care and management. Tissues processed using the microwave techniques can be assessed immunohistochemically since the microwave itself is used for antigen retrieval.

Keywords: Tissue processing; time consumption; cost of processing; reagent toxicity; microwave; histochemistry; immunohistochemistry; morphology.

1. INTRODUCTION

Tissue processing is a sequence of procedures, aimed at providing an internal as well as an external support to tissues. A good processing technique should also retain the histochemical reactivity of the tissues as well as their components. The paraffin wax method which is compatible with a wide variety of histochemical techniques utilizes paraffin wax as the supporting medium for the tissue so as to present it for sectioning and subsequently, for staining. The aim of tissue processing is to place the tissue in a solid medium, firm enough to support the tissue and to give it sufficient rigidity [1]. The physico-chemical basis of tissue processing is the diffusion of reagents to the substance of the tissue to be processed [2]. The processing preserves microscopic anatomy of the tissue and makes them hard, so that very thin section (4-5 µ) can be made. After staining, the section should depict the tissue as close to the living state as possible. Although the conventional paraffin wax method of tissue processing continues to serve histology laboratories well, it has a number of shortcomings, such as a 1-day delay of diagnosis, the need to batch specimens, the relatively large volumes and toxicity of reagents used, and the extent of RNA degradation [3]. Patient anxiety, the delay to plan or institute treatment, and other adverse aspects related to the delay between surgery and diagnosis are consequences of constraints imposed by the time required to prepare tissue for microscopy. There is therefore, a need, to develop techniques that are faster and cheaper than the conventional method, but they will be more valid if they have no deleterious effect on the chemistry as well as morphology of the tissue. Several investigators [4-6] have pioneered the use of microwaves to expedite fixation and/or processing of tissue for microscopy. Because dielectric solutions and tissue sections absorb microwave energy, it is translated into heat, both in the solutions and within the tissues immersed in those solutions. The resulting effect is accelerated diffusibility of the solutions and denaturation of proteins; both phenomena lead to shortened processing time. This reduction was accomplished primarily by

combining vacuum and microwave energy and using mixtures of reagents different from those used in conventional methods. The haematoxylin and eosin staining techniques has been utilized over the years for the study of tissue morphology. However, it is limited in terms of adequate differentiation between different tissue structures and extracellular components like collagen. It is also not very suitable for the identification of organisms in the tissue. Therefore, the chemical reactivity of a tissue is better evaluated using different histochemical techniques. There are a limited number of reagents that can be used for fixation as they must possess particular properties that make them suitable for this purpose. For example tissue components must retain some chemical reactivity so that specific staining techniques can be applied subsequently [7]. It has been estimated that tissues shrink as much as 20% or more by the time they are infiltrated with wax. Notwithstanding these effects, sections prepared from optimally processed tissues will consistently show excellent morphological detail which allows comparisons to be made between specimens and accurate histopathological diagnoses to be determined. There have been many attempts in recent years to introduce alternative methods of tissue fixation and processing [8]. Most have impractical aspects for routine laboratory use; e.g. the requirement for fixation and/or processing at or below -4℃, or the lack of potential for high throughput automation. With few exceptions, these methods and reagents have not been evaluated or validated for tissue. It is therefore, important to evaluate the histochemical behavior of any method before it can be accepted. This assessment should cover a wide range of histochemical techniques rather than relying on the Haematoxylin and eosin method alone since it has limitations as far as the demonstration of some substances is concerned. The success of histochemical methods is dependent on a lot of factors, some of which include potency and concentration of reagents, staining duration, P^H, temperature and the skills of the personnel. The use of controls also helps in validating results as it serves a quality control measure hence it should never be discarded. When all these are put in place, then the principle of histochemistry will be presented. These include: specificity, ability to retain the substance of interest in its original position, production of coloured substances and the retraention of tissue morphology.

2. MATERIALS AND METHODS

2.1 Sample Acquisition

Two apparently healthy rabbits [9] aged 8 weeks (weaner) and 48 weeks (adult rabbit) respectively, were sacrificed scientifically [10]. 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 thoracal and extrathoracal parts [11-14]. 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, Trachea, Lungs, Heart, Kidney, Stomach, Skin, Brain, and the Spleen. Each of the tissue samples was appropriately labeled and grossed in triplicates of 10 mm X 5 mm X 2 mm. The triplicates were labeled as 'C', 'W' and 'V' respectively.

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℃ above the melting point of the paraffin wax used). The SPIN tissue processor, STP 120 (Thermo scientific) 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 Tissue Wave™ 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℃ for 15 minutes [12-14]. The tissues were then passed through two changes of isopropanol at 67℃ for 15 minutes each. They were then transferred to preheated paraffin wax and infiltrated at 70℃ 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 20 Hg 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 molten 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 (3 µm).
- 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℃ 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 240 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, Masson's Trichrome staining technique for muscle fibres, keratin and collagen, Periodic acid Schiff Diastase for glycogen, Haematoxylin and Van Gieson to differentiate between collagen and smooth muscle, Gordon And Sweet's Method to demonstrate reticular fibers, and Phosphotungstic Acid-Hematoxylin for muscle cross-striations, collagen and fibrin [15,16].

2.6 General Criteria for Evaluation of Quality of Sections

Slides were distributed among three experienced observers for a blind assessment using the following criteria [4,14] to evaluate the three processes:

2.6.1 Section preparation

Cutting texture of blocks (very smooth, smooth or rough), Uniformity of blocks, Cohesiveness of blocks, Ribboning & compression during cutting.

2.6.2 Mounting of sections unto slides

Dehydration & clearing (appearance of xylene after inserting sections for dewaxing), cohesiveness, and flattening of sections were assessed.

2.6.3 Block storage: Block stability on storage

(1 month, 2 months, 4 months, and 6months) to check for specimen shrinkage and opacity.

2.6.4 Microscopic assessment

2.6.4.1 Physical quality of section (excludes stain quality)

This was checked to assess disruption, adhesion, cracking and section thickness [14].

2.6.4.2 Quality of tissue preservation

This was used to assess nuclear and cytoplasmic details, special features, (kidney-basement membrane definition, liver-sinusoidal endothelium definition), extracellular components and muscle (collagen, elastin), uniformity of preservation (includes zonal fixation) c.

2.6.4.3 Quality of staining (chemical)

Uniformity, nuclear and cytoplasmic details, as well as extracellular components & muscle (collagen, elastin) were checked and graded. For cellular morphology evaluation, greater eosinophilia of cytoplasm producing enhancement of the nuclear-cytoplasmic contrast, good stroma, whether secretory products are appreciable, red cell lysis absent, whether differentiation can be made between cells and other components of the tissue [17,18].

2.6.5 Scoring criteria

The slides were randomly numbered and circulated among three experienced observers (Observers A, B and C) for blind study and scoring (using a three point scale) as seen below:

i. Excellent: Tissue clearly demonstrated. Most suitable for microscopy (Numerical Value 2)
ii. Fair: Tissues not very well demonstrated, but can be used for microscopy (Numerical Value 1).
iii. Poor: Tissue not clearly demonstrated. Not good for microscopy (Numerical Value 0).

Average scores obtained from the three observers were subjected to one way Analysis of Variance (ANOVA) at 95% confidence interval (H_0: $C = W = V$) to determine whether there is a significant difference among the three methods of processing. *P*-values less than 0.05 were considered significant [12,13].

3. RESULTS

Paraffin blocks obtained from the three techniques show neither retraction nor colour change after storage for up to 6 months. Sections obtained from the three techniques came out in good ribbons and attached to slides without difficulties and were not washed out during staining. While the conventional method took an approximate of 24 hours to complete, the microwave techniques took an average of 85 hours to complete. The conventional method consumed a total of 10.3 Ltrs of ethanol while the microwave methods consumed 2 Ltrs of ethanol. While xylene was completely eliminated in the microwave methods, 3.2 Ltrs of xylene was used in the conventional method. This has shown that the microwave techniques are not only faster and more convenient than the conventional method, but they are also cheaper. There was no statistically significant difference in section preparation (*P*=.22, see Table1), details of microscopic assessment/quality of tissue preservation (*P*=.74, Table 3) and details of microscopic assessment/quality of staining (*P*=.90, Table 4) among the three techniques. Microscopic assessment/physical quality is also comparable among the three techniques (*P*=.74, Table 2). Extracellular components like collagen (Plates 1, 2 and 3) and red blood cells (Plates 2 and 6) were clearly demonstrated according to the different histochemical technique used. Silver reduction was observed to be comparable among the three techniques (Plate 4). The results have also shown that there is no disparity among the different organs processed from the same rabbit, neither is there evidence of differences in morphology/ histochemistry between the tender tissues of the weaner and the tougher tissues of the adult rabbit. It was expected that where there is overprocessing, the tissues will crack and where there is underprocessing, the tissues will be difficult to cut but these deformities were not observed in any of the sections produced.

3.1 DISCUSSION

The constituent part of cell and intercellular materials are usually transparent after fixation and processing. Very little details can be viewed microscopically [19]. Thus it needs to be stained with coloured agents, such as dyes. The specificity of the different histochemical methods with the tissue components has shown that chemical reactivity is retained in the three techniques. Plastic cassettes were exclusively used for the microwave processing, as metallic cassettes could cause a risk of sparking and even explosion [20,21]. Section thickness, as evidenced by uniformity in staining intensity was comparable among the three techniques and within the same section (sees plates). Tissue components pick stains according to their reactivity with the different staining techniques and according to the reactivity of the components with the dye. The specificity of reaction was evident by the use of controls where substances were removed in some instances to see if

reactions will still occur or not (Plate 5). This work agrees with other research works [3,14,22] in terms of red cell preservation, ribonning and the relationship of epithelia cells to basement membranes [22-25]. It has also covered a wider number of organs as well as histochemical techniques than other research works. No crisp (brittle) ethyl alcohol patterns of nuclear features were seen as observed by other researchers [12]. This may be attributed to the use of potent reagents especially ethanol and isopropanol.

Table 1. Average scores for section preparation using a simple three point scale for each parameter (2, 1, or 0, with zero being a fail)

Parameter				Group C	Group W	Group V
Texture of block				1.3	2	1.7
Uniformity of block				1.7	1.3	2
Cohessiveness of block				2	1.7	2
Ribboning & compression during cutting				1.7	1.7	2
Dehydration & clearing				1.3	1.3	1.7
Cohessiveness during mounting				2	1.7	1.7
Flattening				1	1.7	1.7
ANOVA						
Source of variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between groups	0.2552	2	0.1276	1.6374	0.2221	3.5545
Within groups	1.4028	18	0.0779			
Total	1.6580	20				

Table 2. Average scores for details of microscopic assessment/physical quality of section using a simple three point scale for each parameter (2, 1, or 0, with zero being a fail)

Parameter			Group C	Group W	Group V	
Disrubtion X4			2	1.3	2	
Adhession X4			1.7	1.7	1.7	
cracking (coarse-crazy paving) X4			2	2	2	
Cracking (fine) X40			1.3	1.3	1.3	
Section thickness			2	2	2	
ANOVA						
Source of variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between groups	0.065	2	0.0327	0.3131	0.737	3.8852
Within groups	1.252	12	0.1043			
Total	1.317	14				

Table 3. Average scores for details of microscopic assessment/quality of tissue preservation using a simple three point scale for each parameter (2, 1, or 0, with zero being a fail)

Parametre			Group C	Group W	Group V	
Nuclear detail			1.3	1.7	1.7	
Cytoplasmic detail			1.3	1.7	1.3	
Special features			1.7	1	1.3	
Extracellular components and muscle			1.3	1	1.7	
Uniformity of preservation (includes zonal fixation)			2	2	2	
Appearance of intercellular spaces			1.7	1.7	2	
ANOVA						
Source of variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between groups	0.0744	2	0.037	0.31	0.738	3.6823
Within groups	1.7967	15	0.12			
Total	1.8711	17				

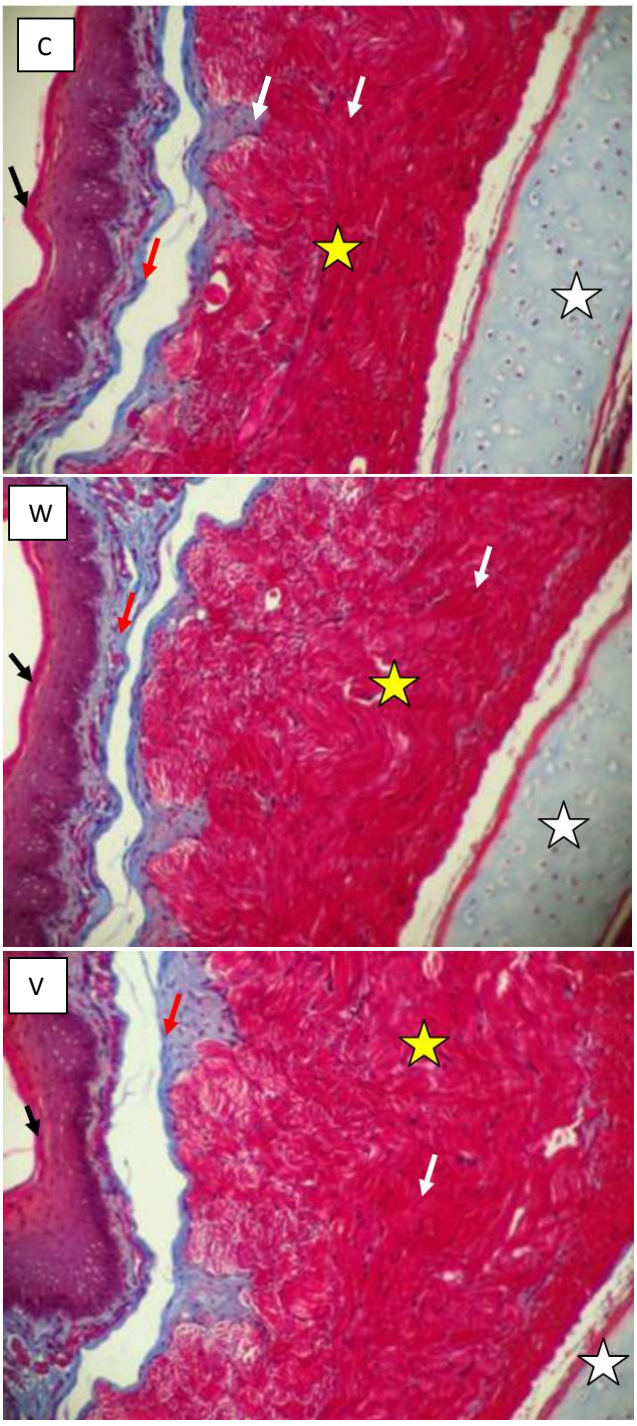

Plate 1. Oesophagus of a weaner processed by the three techniques and stained by the Masson Trichrome staining method. This histochemical stain shows the reactivity of the different tissue components to the different components of the stain. Muscle fibre (yellow arrows) presented their characteristic red colour while collagen fibres present their characteristic blue colour. The stratified squamous epithelial lining (black arrows) of the oesophagus show their red to brown stain as nuclei (white arrows). Masson Trichrome. 400X; C=conventional method, W=microwave technique without vacuum, V= microwave with vacuum

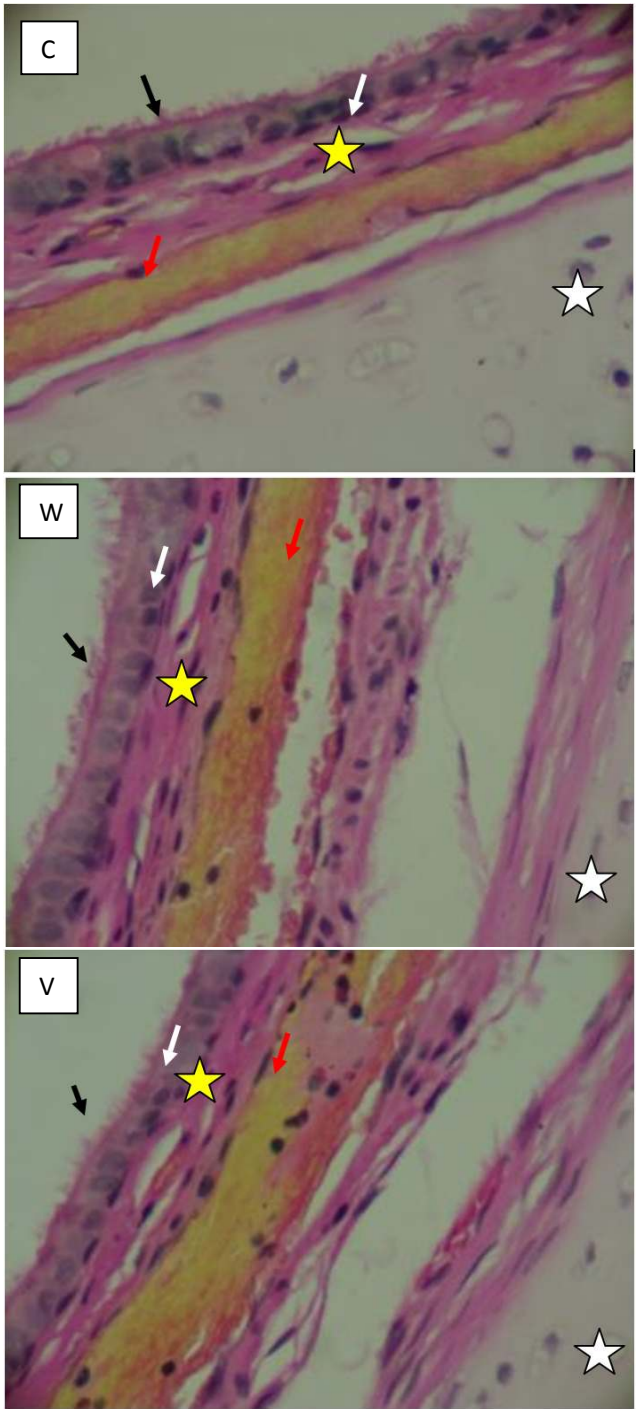

Plate 2. Trachea of a weaner processed by the three techniques and stained by the haematoxylin and Van Gieson histochemical method. Ciliated epithelial lining of the trachea are clearly demonstrated in the three techniques. Black arrows= cilia, white arrows=pseudostratified columna epithelium, yellow star= collagen, red arrows=red blood cells, white stars= cartilage. The relationship between the different layers of the trachea is clearly retained while the characteristic colour update for the different components is maintained. HVG 400X; C=conventional method, W=microwave technique without vacuum, V= microwave with vacuum

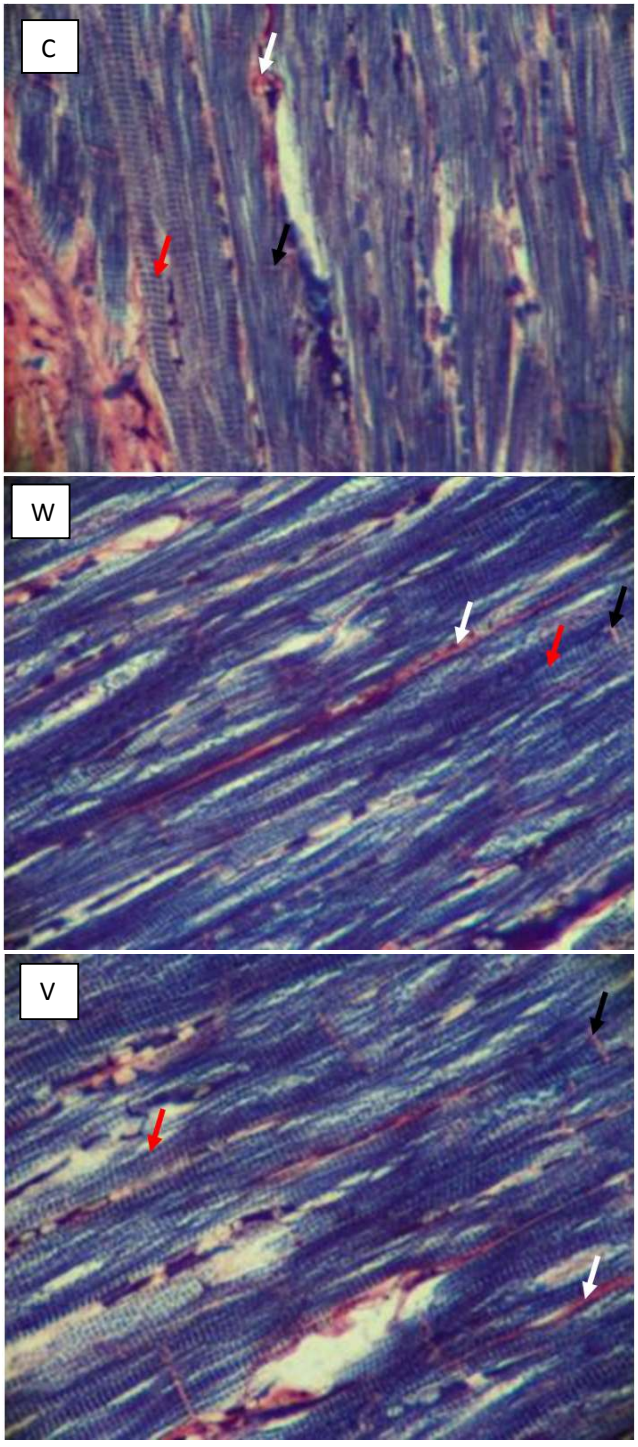

Plate 3. Heart muscle of an adult rabbit processed using the three techniques and stained using the Phosphotungstic acid Haematoxylin (PTAH) method. Muscle striations (red arrows), intercalated discs (black arrows) and collagen (white arrows) are well demonstrated in the three techniques without any major discrimination in terms of colour intensity as well as differentiation among the different components. PTAH 400X; C=conventional method, W=microwave technique without vacuum, V= microwave with vacuum

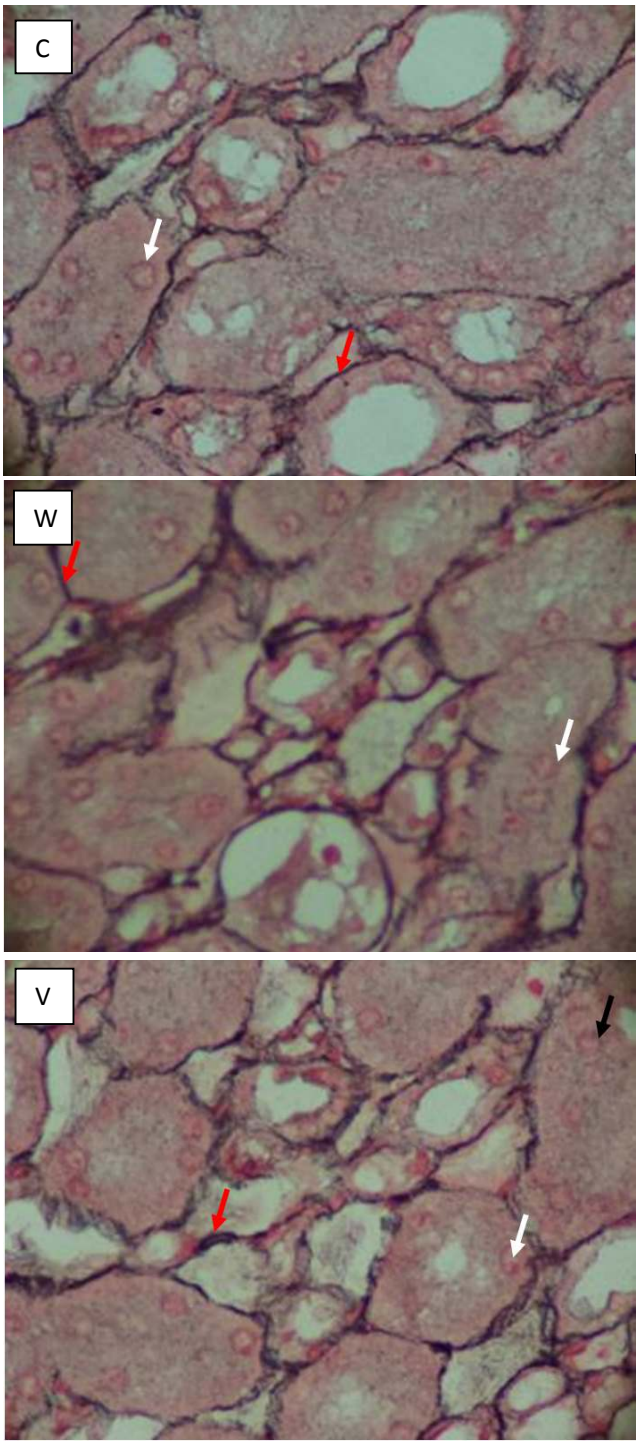

Plate 4. Transverse section through the kidney medulla of an adult rabbit demonstrating reticular fibres. The ability of reticular fibres (red arrows) to reduce silver to its metallic form and present a black colour was demonstrated well in the three processing methods. White arrows=cell nuclei. This can be used for studies on kidney, liver and spleen integrity. Gordon and Sweet's Staining Technique. 400X; C=conventional method, W=microwave technique without vacuum, V= microwave with vacuum

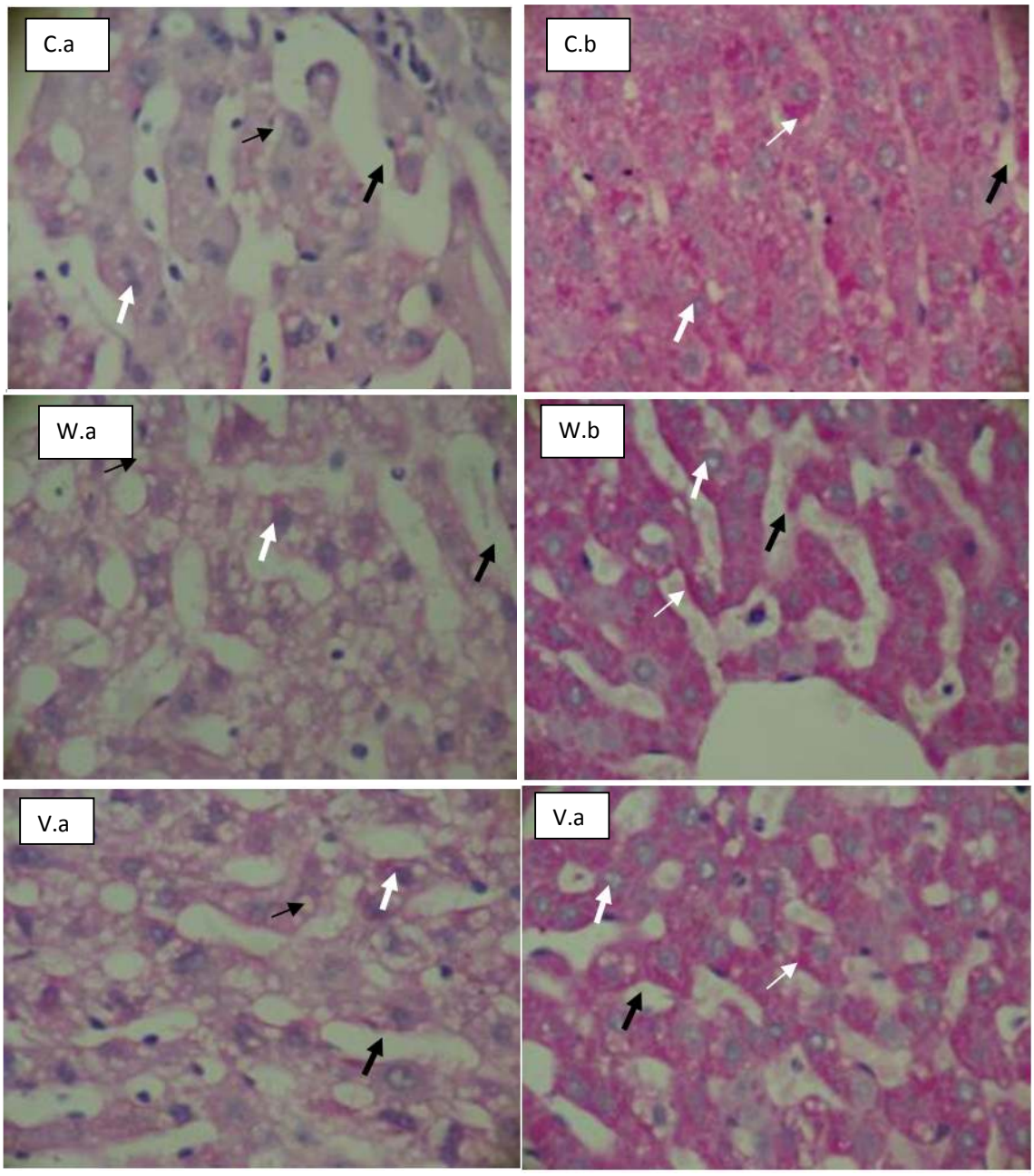

Plate 5. Liver of a weaner processed using the three techniques and stained using the Periodic acid Schiff reaction with diastase (PAS-D) (Plate 1Ca, Wa and Va) and Periodic acid Schiff reaction without diastase (Plate 1Cb, 1Wb and 1Vb). Glycogen deposits are observed to be digested and the space appears 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

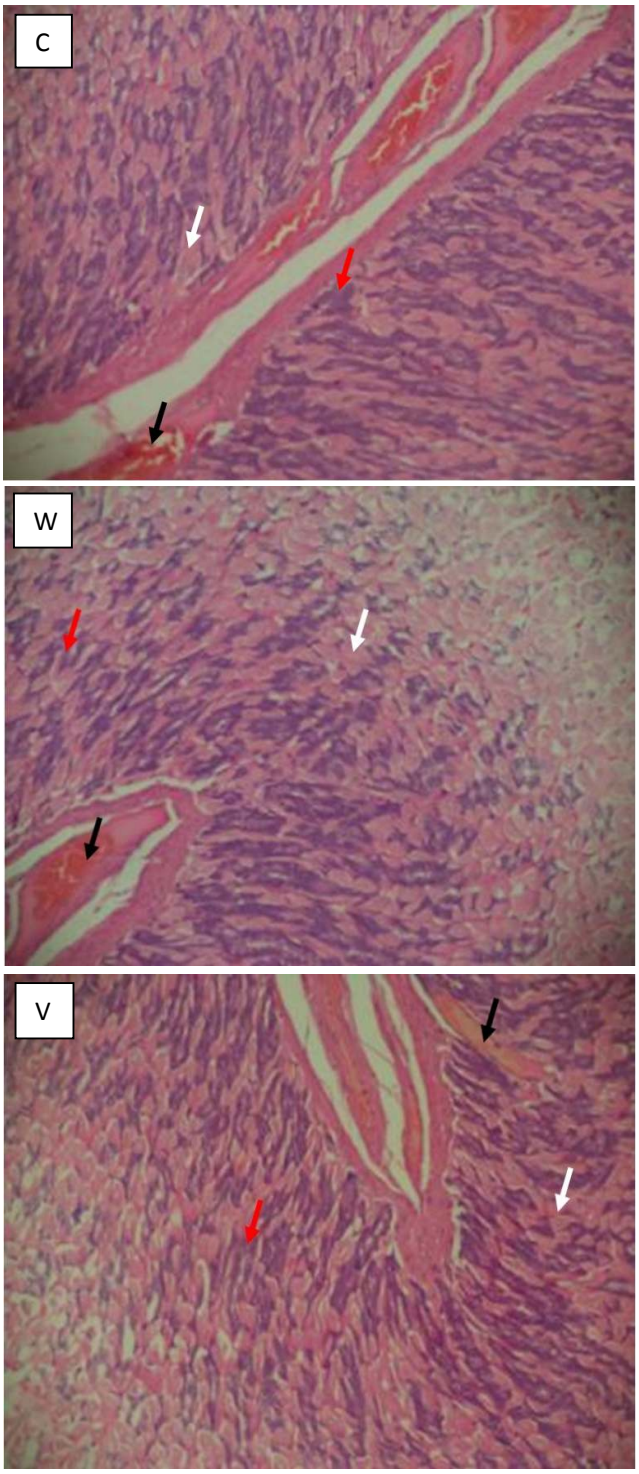

Plate 6. Stomach gastric glands of a weaner. Parietal cells (white arrows) are clearly differenciated from the Chief (zymogen) cells (red arrows). The characteristic coarse granules of the chief cells are comparably demonstrated in the three techniques. Red blood cells= black arrows. H&E. 100X; C=Conventional, W= Microwave without vacuum, V Microwave with vacuum

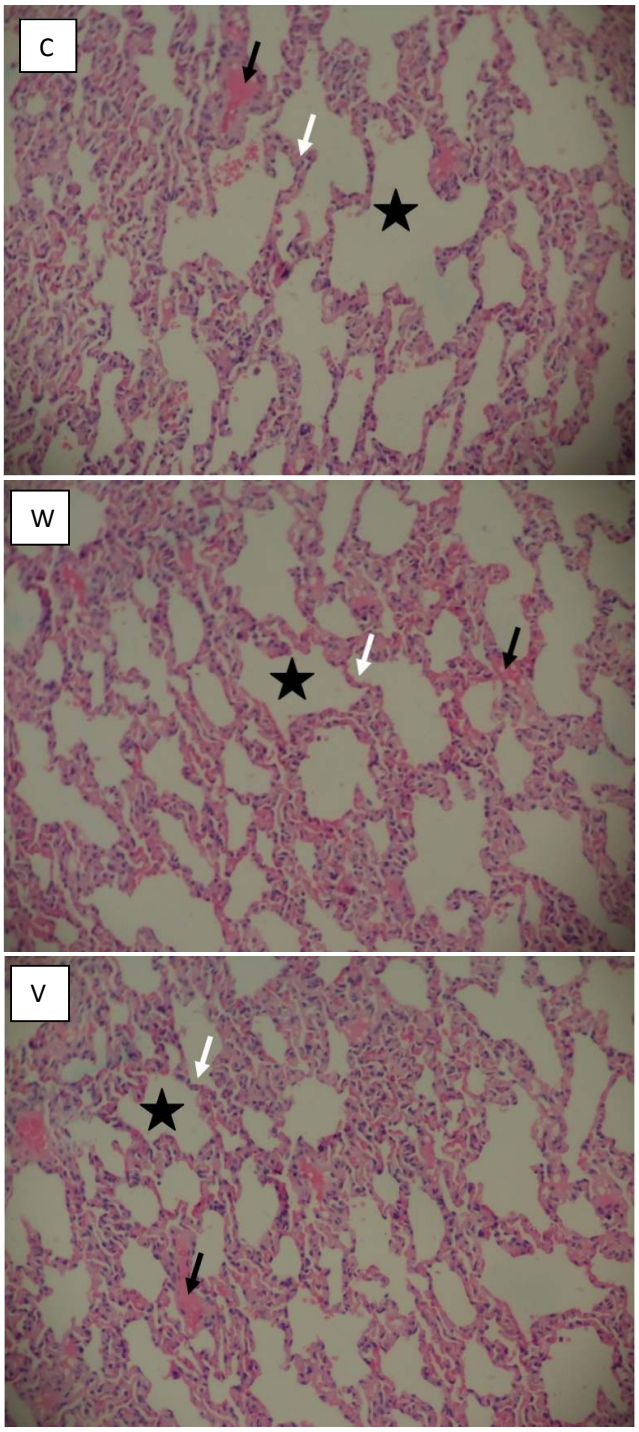

Plate 7. Lung tissue of adult rabbit showing alveolar spaces (black stars) and the characteristic simple squamous epithelial cells (white arrows). Red blood cells (black arrows) retain their normal morphology and are either within the epithelial cells or free within the alveolar spaces. The characteristic blue staining of nuclei and pinkish cytoplasm is demonstrated well in the three techniques. H&E. 100X; C=Conventional, W= Microwave without vacuum, V Microwave with vacuum

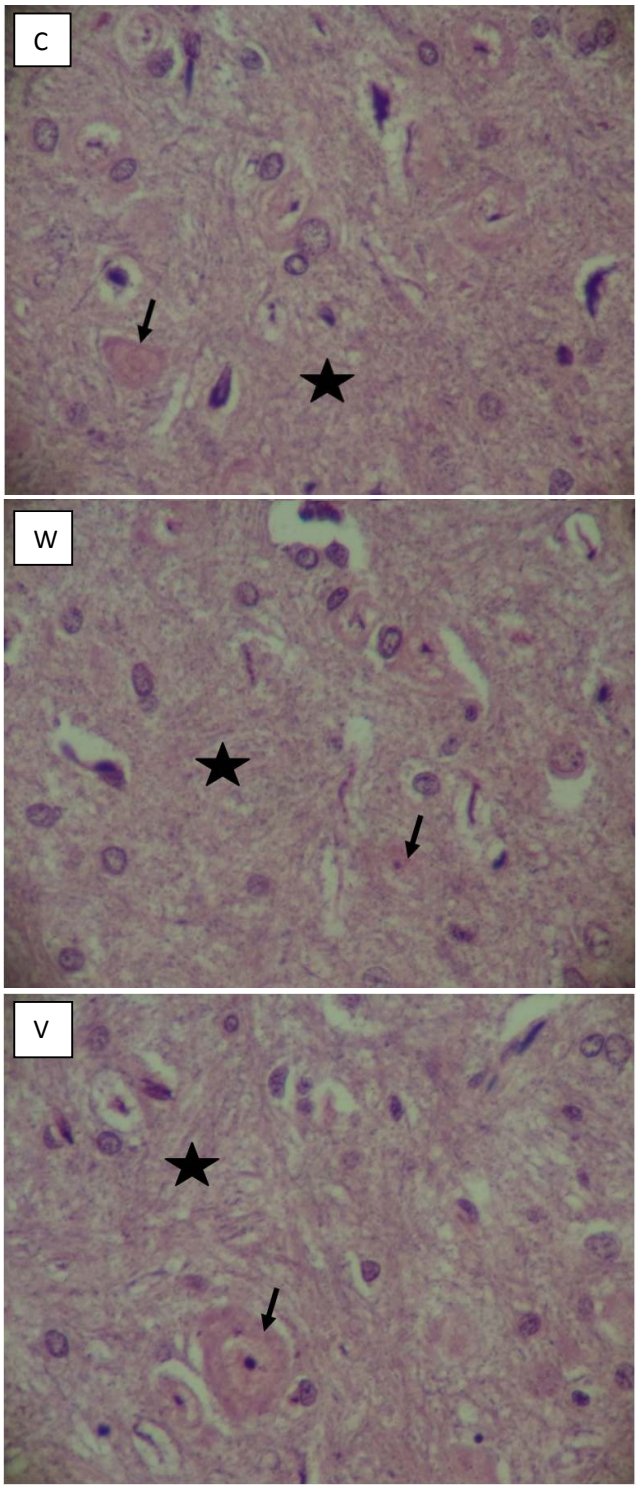

Plate 8. Brain of weaner processed using the three techniques. Neurons (black arrows) are seen within the neurophil (composed of nerve cell processes as well as supporting cells) (black stars). The brain tissue morphology remains intact in the three techniques. PTAH. 100X; C=Conventional, W= Microwave without vacuum, V Microwave with vacuum

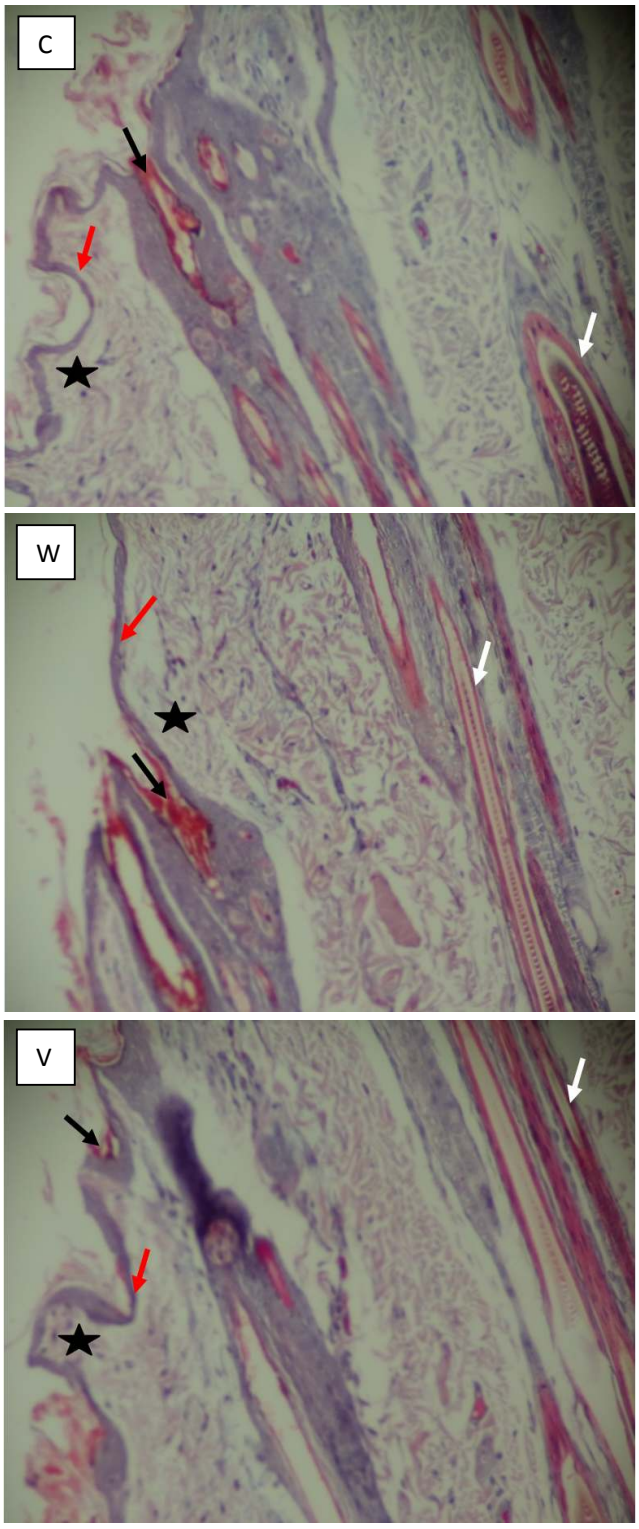

Plate 9. Section of the skin of adult rabbit showing the hair follicles (white arrows), stratified squamous epithelial cells (red arrows) and keratin (black arrows). Papillary layer= black stars. PTAH. 100X; C=Conventional, W= Microwave without vacuum, V Microwave with vacuum

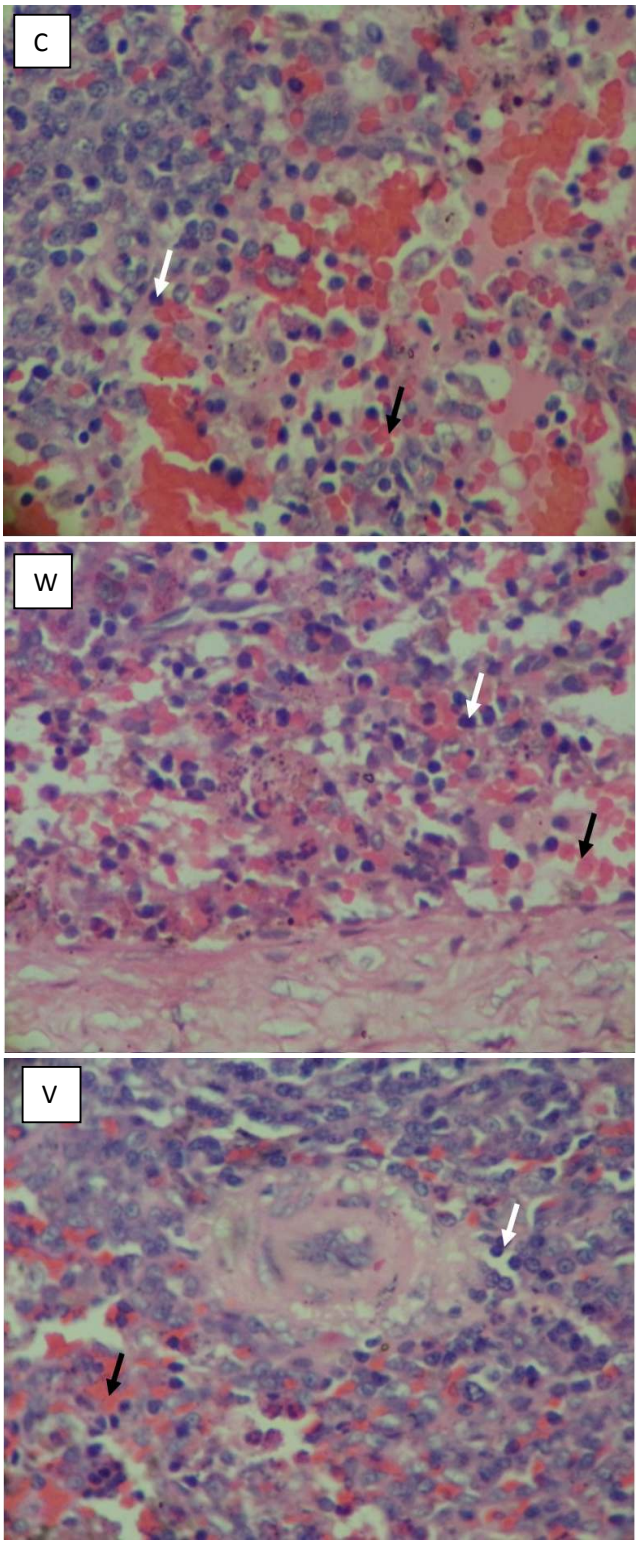

Plate 10. Parenchyma of the spleen of adult rabbit processed using the three techniques. Reticular cells= white arrows, red blood cells = black arrows. H&E 400X; C=Conventional, W= Microwave without vacuum, V Microwave with vacuum

Table 4. Average scores for details of microscopic assessment/quality of staining using a simple three point scale for each parameter (2, 1, or 0, with zero being a fail)

Parametre	Group C	Group W	Group V
Uniformity of staining	2	2	2
Nuclear stain	1.3	1.3	1.3
Cytoplasmic stain	2	2	2
Extracellular components & muscle (collagen, elastin)	1.3	1.7	1.7

ANOVA

Source of variation	SS	df	MS	F	P-value	F crit
Between groups	0.0267	2	0.0133	0.1043	0.902	4.2565
Total	1.1767	11				

Apart from the time consumed in the conventional method of tissue processing, this method has retained the use of toxic chemicals, that are not only harmful to the tissues being processed, but to the user too. This is not good in patient management as speed and accuracy are a major determinant to its success. When processing fluids are heated, the movement of molecules is increased as a result of increase in kinetic energy, thereby enhancing their penetration into the tissue [22]. The heat generated by the microwave oven is evenly distributed among the contents of the oven (in this case, tissue samples) by the action of the agitator. With this, the effect of uneven distribution of heat which results to inferior quality sections as obtained when tissues are heated in the manual method is averted. The preservation of tissue morphology observed in the different tissues processed from the two animals where one is tender (weaner) and the other is tough (adult rabbit) is an evidence that tissues of different textures such as the tender tissues of the central nervous system like the brain and spinal cord can now be processed in the same schedule with tissues of tougher textures like the skin, kidney and liver. Comparing the two microwave techniques, this work shows that there is no comparable difference hence it shows that provided a microwave is functioning well, good results are obtained with or without vacuum.

4. CONCLUSION

This work has shown that same day turn-around is feasible in histopathology without compromising tissue morphology and/or chemical composition. It has also revealed that histopathology samples can be processed at a cheaper and convenient rate than the conventional method. Elimination of xylene from the processing schedule has been demonstrated in this work without any negative consequence on tissue morphology, silver impregnation or reaction with different stains. We filtered the ethanol and isopropanol through Whatman filter paper No. 1 and used for over three rounds of processing in the microwave technique thereby increasing the economic advantage over the conventional method of tissue processing. Organs covering almost all the systems in the body were used and two animals, one with a tender tissue texture and the other with a harder tissue texture were used in this experiment and there was no discrimination in relationship to over or under processing as sectioning was done without difficulties in any category of the section and fine ribbons were obtained regardless of the animal's age or type of tissue. This has further revealed that samples can now be easily processed in one batch regardless of their texture, unlike in the conventional method where tender tissue like those of the central nervous system are usually processed in a shorter cycle than other tissues of the body. This work should be replicated using tissues from different animal species so as to increase its scope. Moreso, tissues processed using the microwave method should be subjected to immunohistochemical techniques as well as molecular techniques so as to check their effect in relation to antigen-antibody reactions as well as to see their behavior at the molecular level. This will give the microwave processing technique a wider application.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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