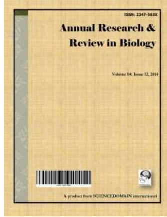

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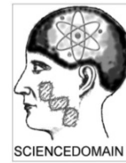

Assessment of the Conventional versus Two Rapid Microwave Processing Methods Using the Phosphotungstic Acid Haematoxylin Technique

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Authors' contributions

This work was carried out in collaboration between all authors. Authors TPPC and AAN designed the experiment. Authors SIO and LPU prepared the reagents for the experiment. Authors MUA and PNZ carried out literature researches. Authors TPPC, EIV and GID carried out the experiment. Authors TPPC and PRK read and interpreted the slides. Authors CEM and TPPC studied the reviewers' comments and made the required corrections on text, figures and plates as well as search for more literature. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To process tissues using the rapid microwave techniques and check their reactivity with the phosphotungstic acid haematoxylin. To establish a very fast and cheap method of tissue processing and evaluate its effects on tissue morphology and dye uptake. To eliminate xylene from the processing schedule. To check for additional information that can be obtained from the

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phosphotungstic acid haematoxylin technique so as to encourage its use in the laboratory.
Study Design: Harvesting and fixation of tissues. Grossing into triplicates, processing using three different techniques. Staining and grading of sections.
Place and Duration of Study: Human samples were obtained from the Anatomy Department, Nnamdi Azikiwe University, Nnewi Campus while animal samples were obtained from the Animal experimental station, National veterinary Research institute, Vom, Nigeria. The work was done between August and December, 2014.
Methodology: Heart, skin, brain, kidney, lungs, and liver tissues were harvested from an apparently healthy rabbit after sacrificing it scientifically. Ethical clearance as well as human cervix, fibroid, prostate and testis were obtained. All samples were fixed in 10% buffered formalin, cut into triplicates and processed using the conventional as well as two rapid microwave techniques respectively. Sections were made from each technique and stained simultaneously using the Phosphotungstic Acid Haematoxylin technique. The Masson Trichrome methos as well as the Haematoxylin and Eosin Techniques were also used to assess morphology They were each evaluated for quality of block, ribboning, nuclear, cytoplasmic and extracellular components, collagen and muscle fibre. Section thickness, uniformity and staining intensities were all checked.
Results: Processing cycle as well as quantity of reagents used was drastically reduced in the microwave techniques. Paraffin blocks obtained, ribbonning as well as flattening on 20% alcohol were comparable in the three techniques. Tissue morphology, integrity of tissue as well as tissue histochemistry to the phosphotungstic acid haematoxylin were comparable among the three techniques. The stroma of the cervix is better retained in the microwave with vacuum technique.
Conclusion: Samples received in histology laboratory can be processed and slides produced within the same day without compromising tissue morphology or histochemistry. Xylene can be eliminated from the paraffin wax processing cycle with quality paraffin block and ribbon production. The cost and duration of tissue processing can be drastically reduced with the production of quality sections. This work can be replicated using other histochemical techniques to give a wider application.

Keywords: Paraffin wax method; processing cycle; reagent toxicity; microwave; isopropanol; histochemistry; phosphotungstic acid haematoxylin.

1. INTRODUCTION

The paraffin wax method of tissue processing is the most utilized method of processing in histopathology. This is because of the good ribboning, coupled with its compatibility with a wide range of fixation as well as staining techniques. The durability of blocks is another great advantage. Despite these advantages, the method has continued to be characterized by delay is tissue processing, use of toxic substances like xylene as well as use of a large volume of reagents per cycle. Histology laboratories supporting toxicology testing are routinely tasked with production of very large numbers of histologic specimens from standard laboratory animal species. Because of this, minimizing slide preparation time is an ongoing challenge [1]. Most tissues had slightly better morphology when microwave fixation/microwave processing protocols were followed. One should note that both tissue trimming and fixation times need to be optimized for microwave methods [2]. Microwave causes heating within a material by exciting molecules to rotate. The rotation produces energy in the form of heat. Heat reduces the viscosity of liquids, thereby increasing the rate of diffusion of reagents into and out of the tissue. Unlike conventional heating, the effect occurs simultaneously throughout the whole material being microwaved ('internal heating') [3]. This resulted in substantial reduction in each of the basic steps of histoprocessing, thereby reducing turnaround times and permitting same day diagnosis for a variety of types of tissue biopsy specimens. Elimination of xylene from tissue processing cuts costs, saves time, and improves the laboratory environment [4,5]. Vacuum applied during dehydration, clearing and infiltration stages improve the quality of processing. Tissues, particularly lung, are de-aerated, and the solvent boiling point is reduced, thus facilitating evaporation of the reagent from the molten infiltration medium. Duration of wax infiltration is dependent upon viscosity and is not reduced by the application of vacuum. “Staining mechanisms” imply accounts of molecular processes involved in selective uptake of dyes into biological specimens during biological staining In histological sections, cellular structures are not significantly different to one

another. Hence, dyes are used whenever defined intra- or extracellular elements have to be displayed [6]. In trying to evaluate a new technique, it is therefore, necessary; to study its effect on tissue histochemistry as a very cheap and fast technique will lose its value if tissue histochemistry is altered as a result of its application. Histochemical methods usually produce insoluble colored or electron-dense compounds that enable the localization of specific substances by means of light or electron [7]. The phosphotungstic acid hematoxylin method (PTAH) gives a polychrome effect with cytoplasm stained shades of blue and connective tissue stained yellow to brick red while red blood cells stain bleuish. This stain, though occasionally used has very wide applications in histopathology; it is especially suitable for the demonstration of muscle striations, intercalated discs, nervous tissue, and fibrin. It is used for the staining of astrocytes, myoglia and fibroglia tissues. It is use in staining striated muscle fibres and mitochondria, also to reveal some specific disease processes in the central nervous system. It used to check for tumours in skeletal muscle. Phosphotungstic acid haematoxylin (PTAH) is a mix of haematoxylin with phosphotungstic acid, used in histology for staining. It stains some tissue in contrasting colors in a way similar to haematoxylin and eosin stain, as phosphotungstic acid binds to tissue proteins. It is used to show gliosis in the central nervous system, tumours of skeletal muscles, and fibrin deposits in lesions. Muscle is stained blue-black to dark brown, connective tissue is pale orange-pink to brownish red, fibrin and neuroglia stain deep blue, coarse elastic fibers show as purple, and bone and cartilage obtain yellowish to brownish red color. PTAH is ideal for demonstrating striated muscle fibers and mitochondria, often without a counterstain. As such, it is used to identify contraction bands, as seen in contraction band necrosis [8]. This experiment is aimed at reducing both cost and duration of tissue processing without altering the tissue morphology and reactivity with the phosphotungstic acid haematoxylene technique. It is also aimed at eliminating xylene from the processing schedule, owing to its deleterious nature on tissues and harmful effect on the laboratory personnel.

2. MATERIALS AND METHODS

2.1 Sample Acquisition

Ethical clearance was obtained from the Anatomy department, Faculty of Health Science and Technology, Nnamdi Azikiwe Uiversity, Nnewi Campus, Nigeria, followed by the acquisition of Formalin-fixed (10% buffered) human autopsy samples of the Testis, prostate, fibroid and cervix, from the same department. An apparently healthy rabbit was sacrificed after chloroform anesthesia [9]. 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 [10,11]. The heart, brain, liver, kidney, lungs and skin were harvested and fixed in 10% buffered formalin for three days. Each of the ten tissue sample 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 (ThermoScientific) was used in which the tissues were subjected to each stage for two hours making a total of twenty two hours. On the average, a processing station consumes 1.6 Ltrs of the reagent hence the conentional method consumed an average of 10.3 Ltr. The 'W' category of tissues were processed using the TissueWave™ 2 microwave processor (ThermoScientific®, 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]. 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. On the average, each processing station in the microwave consumes 2Ltrs of the reagent hence 2 Ltrs each of alcohol, xylene and paraffin wax was used for the microwave schedule. 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 was detached from the mould (now containing the tissue embedded in the solidified wax). Excess wax was trimmed using a scalpel blade and 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. They 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 ɥm). Each section obtained was placed on 20% ethanol to flatten before floating on a floating-out bath, picked using albuminised slides, placed at 90° and picked at 45° drained 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 thirty days.

2.5 Tissue Staining: Phosphotungstic Acid-Hematoxylin, Mallory's Staining Technique

Sections were dewaxed and hydrated, placed in Zenker's fixative, microwave Hi power, for 45 seconds, allowed to stand for 5 minutes, washed with tap water, placed in Lugol’s iodine for 15 minutes, immerse in 95% ethanol until the colour of the iodine is completely lost (for 1 hour), rinsed in distilled water, oxidised in 0.25% potassium permanganate for 5minutes and rinsed in distilled water. Sections were bleached in 5% Oxalic acid for 5 minutes, washed well in tap water for 5 minutes and stained in PTAH solution at 56℃ for 1 hour. Sections were dehydrated very rapidly through 95% alcohol and absolute alcohol, cleared in xylene, mounted in DPX and coverslipped. The Masson trichrome method as well as the Haematoxylin and eosin staining techniques were also used to assess the morphology as well as the chemical behavior of the tissues.

2.6 General Criteria for Evaluation of Quality of Sections

The following criteria were used [5,13] to evaluate the three processes:

2.6.1 Section preparation

Cutting texture of blocks**,** Uniformity of blocks, Cohesiveness of blocks, Ribboning & compression during cutting.

2.6.2 Microscopic assessment

I. **Physical quality of section (excludes stain quality):** This was checked to assess disruption, adhesion, cracking and section thickness [13].

II. **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 [14]. If most features were present, then it was called distinct and if there was granularity of cytoplasm, focal condensation of stroma, cellular outline blurred [13], red blood cells lysed (focal or generalized), [14] and no differentiation could be made between cells then it was classified as indistinct [13].

III. **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 [15,16]. Staining of tissues was evaluated as poor, satisfactory, and good. Poor indicates that the tissue failed to take up stain adequately, stained unevenly or had artifacts in processing or staining. Satisfactory indicates that details were not visualized up to the mark, but slide was suitable to give diagnosis. Good means good contrast between the nucleus and cytoplasm, and visibility of details along with brilliance of staining. The overall architecture of the epithelial tissue and connective tissue was assessed as per the above-mentioned criteria.

IV. Evaluation of slides for nuclear morphology was done on the basis of chromatin condensation, prominent nuclear membrane, and crisp staining of the nucleus and mitotic activity, if appreciable. [14]. It was distinct if all features were appreciated and indistinct if smudging and pyknosis of nuclei were seen.

V. **Grading and scoring:** The slides were distributed among three experienced observers and graded on a three pointer scale with zero (0) is no meaningful details can be obtained (unsuitable for diagnosis), one (1) is not satisfactory but can be used for diagnosis and two (2) details are clearly defined and can be used for diagnosis. Average scores were used to construct bar charts used for comparison among the three techniques.

3. RESULTS AND DISCUSSION

The conventional method took an average of one thousand, four hundred and forty minutes to complete while the rapid microwave techniques took an average of ninety five minutes. This makes the rapid microwave techniques time friendly and faster hence provides an avenue for same day turn around. Besides being faster than the conventional method, the rapid microwave methods consume less quantity of reagents. The basic effect of microwave irradiation is stimulation of diffusion and enhancement of reaction rates with internal heating being the key element in the process [17]. Heating by conventionalmeans can also be used for tissue processing, but the results obtained are markedly inferior to that in microwave processing. The suggested reason is that in conventional method of heating, the heat might not be uniformly distributed throughout the tissue [12]. Xylene is eliminated in the rapid microwave techniques thereby making it more user-friendly as the toxic effects of xylene are boycotted. Isopropanol, which is used for clearing in the microwave techniques also, has an advantage of completing dehydration if it is not completed in the initial stage of dehydration. The paraffin blocks produced from the three methods were all good with neither retraction nor change in colour. Ribbons produced were of good quality and adhesion. Uniformity of sections was a common feature among the three. Red cells were well preserved in the three techniques showing that it is compatible with studies on diseases that can cause blood cell destruction as well as demonstrate blood cells in their primary locations either within or outside the blood vessels. There was no noticeable crack in the sections produced from the three techniques (Plates 1, 3 4 and 6). Red cells, epithelial cells, nuclear and cytoplasmic membranes were observed to be well preserved in the three techniques (Plate 6). The characteristic red-brown colour of collagen is well demonstrated, while smooth muscles take their characteristic blue colour (Plates 1, 2 4 and 5) showing that tissue histochemistry is well retained in the three techniques. The brain tissue and its components are all well demonstrated (Plate 3) as different components showed their reactivity with the technique. This research work has proved that tender tissues like the brain can be processed in the same cycle with tissues of harder textures and variation of texture like the skin. This will encourage a single cycle of processing of tissues as tissues of different textures can now be processed in a single cycle. The stroma of the cervix is better retained in the microwave with vacuum technique than in the other two techniques. This work has shown that despite all the advantages achieved by the rapid microwave, tissue histochemistry and morphology are not altered. Differentiation between cellular components and extracellular components as very good in the three techniques. A comparison on structural and histochemical presentations among the three techniques (Tables 1 and 2) shows that there is no significant variation in their behavior (Figs. 1 and 2). While there is no comparable difference in terms of section thickness among the three techniques (Fig. 2), the microwave methods show lesser degree of cracking as compared to the conventional method. The conventional method, with the microwave without vacuum, however, show better cohesiveness than the microwave with vacuum technique (Fig. 1).

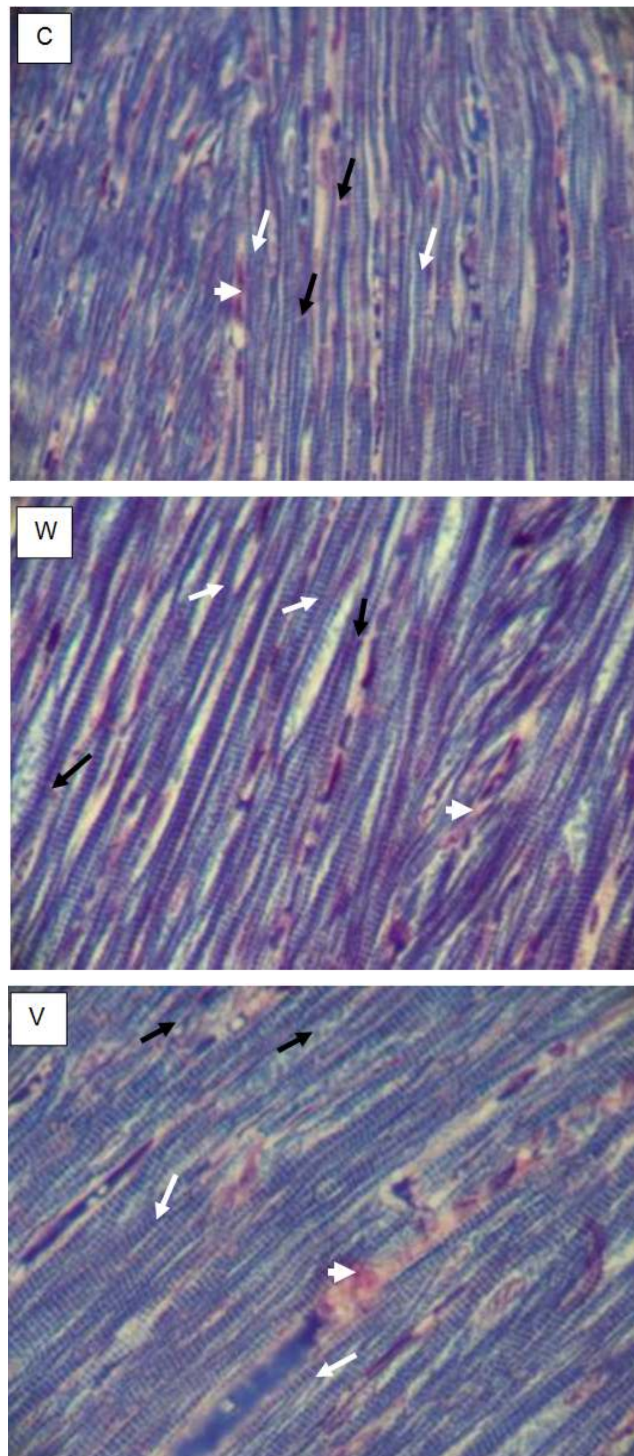

Plate 1. Heart muscle of a rabbit showing noticeable striations of the muscles, clearly distinct from collagen fibres. Muscle striations (white arrows) are clearly demonstrated in the three techniques. Intercalated discs (black arrows) as well as collagen (white arrow-head) are well demonstrated in the three techniques. Phosphotungstic Acid Haematoxylin stain. 400X

C=conventional method, W= microwave without vacuum method. V= microwave with vacuum method

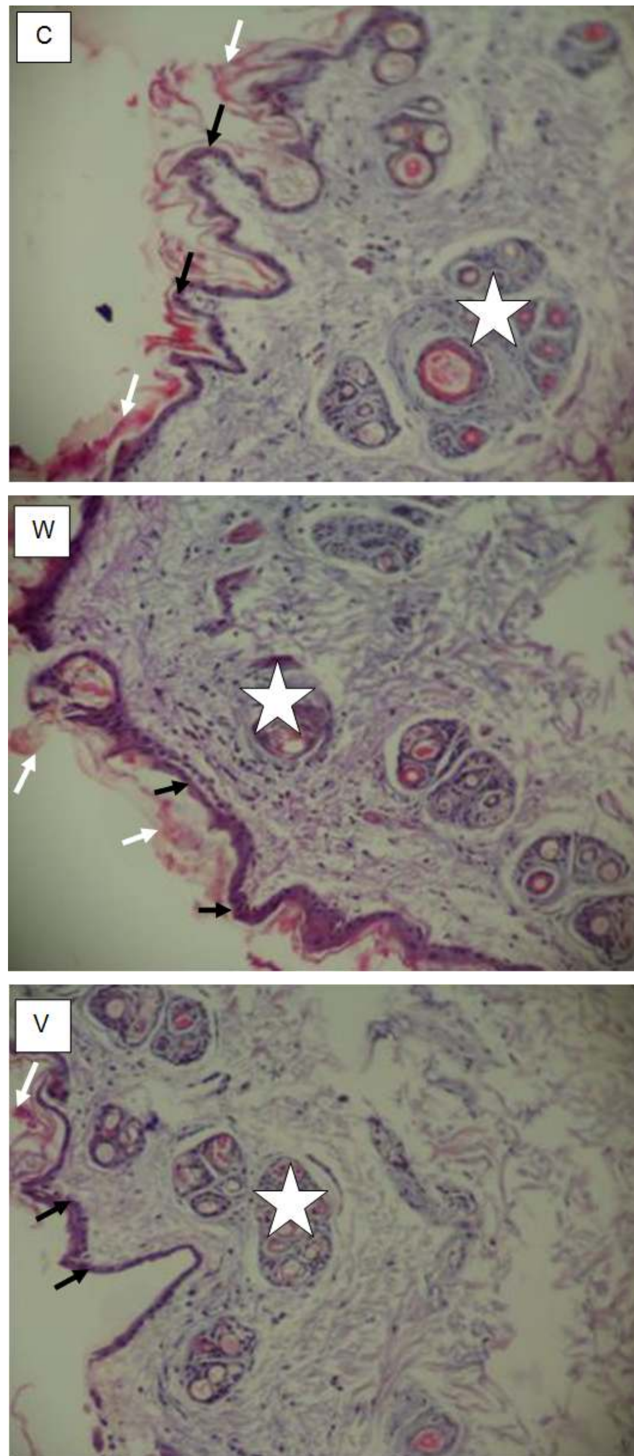

Plate 2. Rabbit skin showing the different skin layers and components well preserved and each component picking its characteristic colouration. White stars= hair follicles, black arrows= stratified squamous epithelium, white arrows= keratin. Phosphotungstic Acid Haematoxylin stain. 100X

C=conventional method, W= microwave without vacuum method. V= microwave with vacuum method

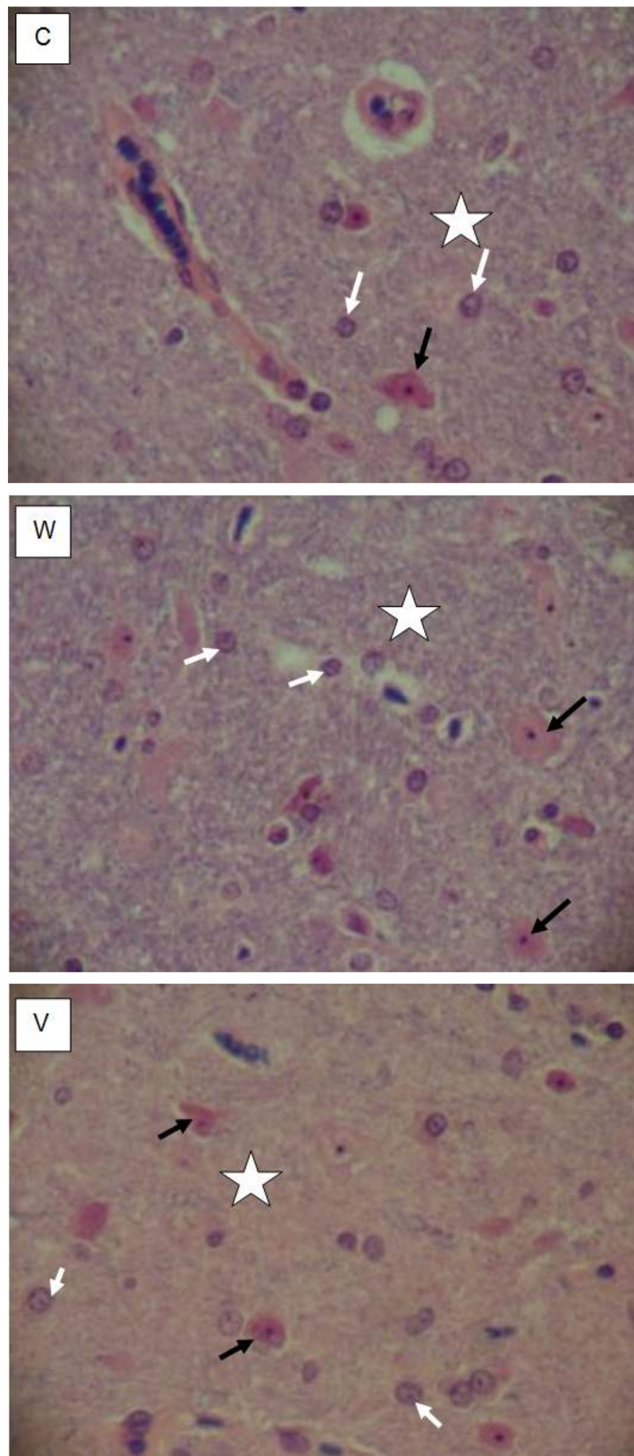

Plate 3. Brain of a rabbit histochemically stained to demonstrate the various components of the brain tissue. This histochemical technique demonstrates in details, the different components of the brain tissue hence is compatible with brain studies. White stars= neurophil, black arrows= neurons, white arrows= neuroglia cells. Phosphotungstic Acid Haematoxylin stain. 400X

C=conventional method, W= microwave without vacuum method. V= microwave with vacuum method

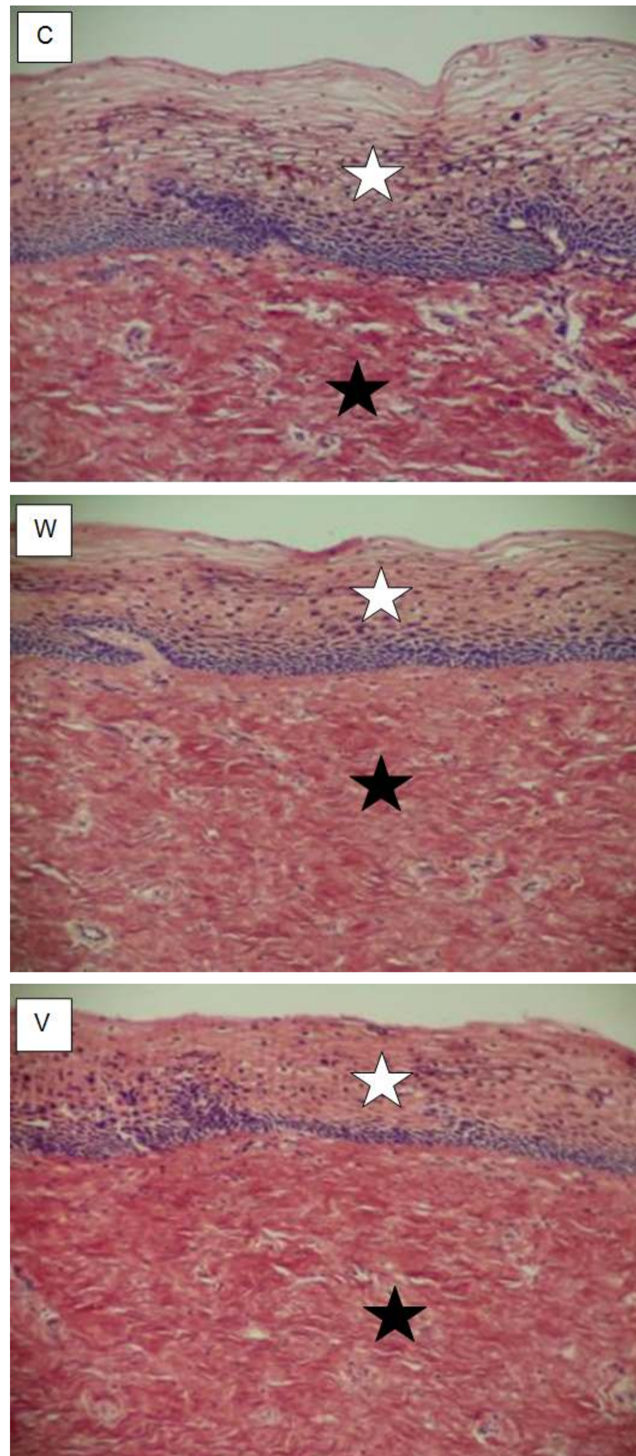

Plate 4. Human cervix demonstrating the integrity of the stroma and its relationship to the epithelium. The integrity of the tissue is better maintained in the microwave with vacuum method (Plate 4.V) than in the other two methods. Staining intensity is however comparable among the three trechniques. White stars= stratified squamous epithelium, Black stars= collagen fibre. Phosphotungstic Acid Haematoxylin stain. 100X
C=conventional method, W= microwave without vacuum method. V= microwave with vacuum method

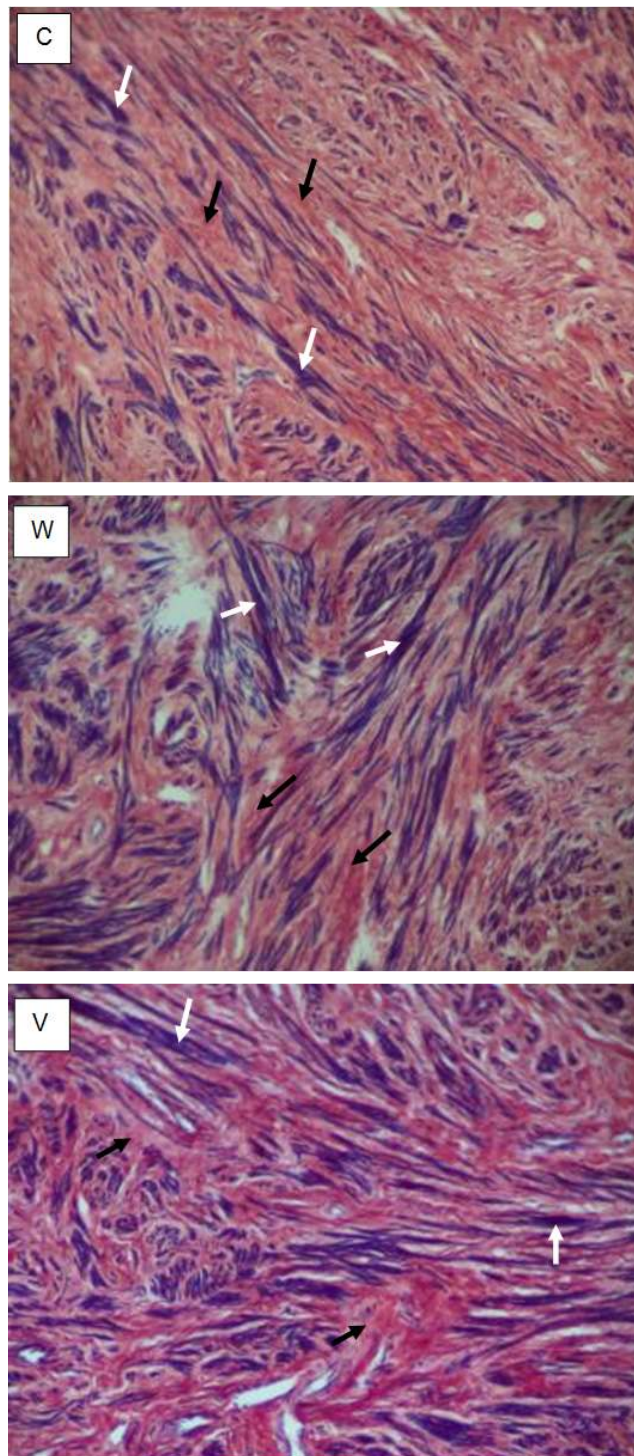

Plate 5. Human Fibroid clearly differenciating between smooth muscles and collagen fibres. The three methods support the histochemistry of the fibroid tissue where collagen fibres (black arrows) take their characteristic red-brown colouration while smooth muscles (white arrows) take their characteristic blue colouration. Phosphotungstic Acid Haematoxylin. 100X
C=conventional method, W= microwave without vacuum method. V= microwave with vacuum method

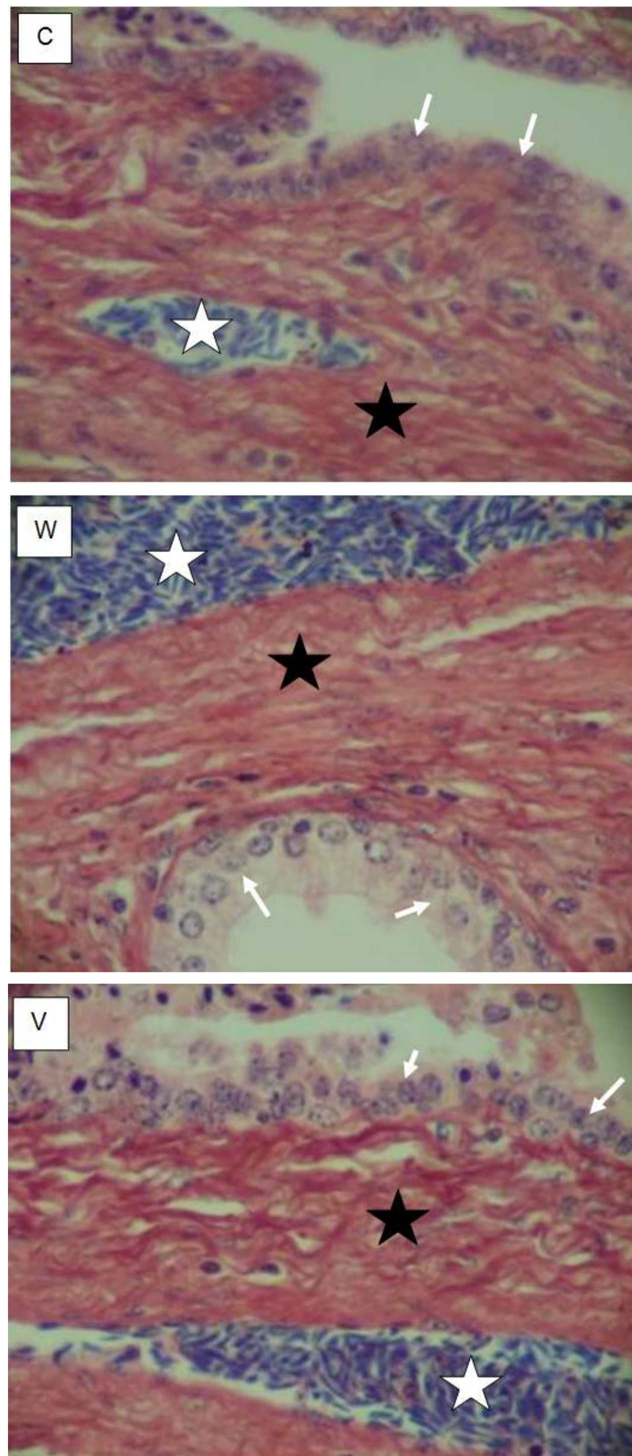

Plate 6. Human Prostate demonstrating the staining abilities of the prostate tissue components. The characteristic blue staining of red blood cells (white stars) is well demonstrated in the three processing methods. These techniques also showed how the epithelial cells (white arrows) are well preserved. Collagen fibres presented with their characteristic red brown colour. Phosphotungstic Acid Haematoxylin. 400X
C=conventional method, W= microwave without vacuum method. V= microwave with vacuum method

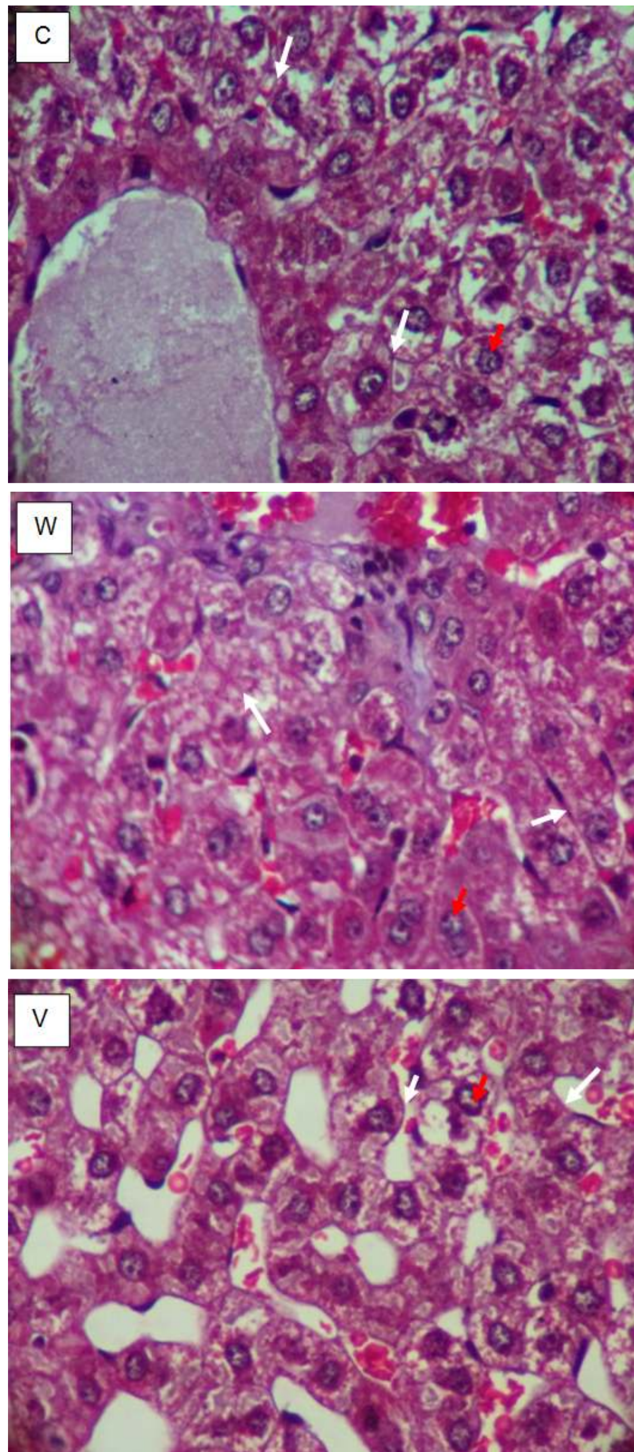

Plate 7. Liver tissue of a rabbit processed by the three techniques. The morphology of the hepatocytes is clearly demonstrated as revealed by cellular demarcations (white arrows) and intact nuclei (red arrows). Masson Trichrome stain. X400

C=conventional method, W= microwave without vacuum method. V= microwave with vacuum method

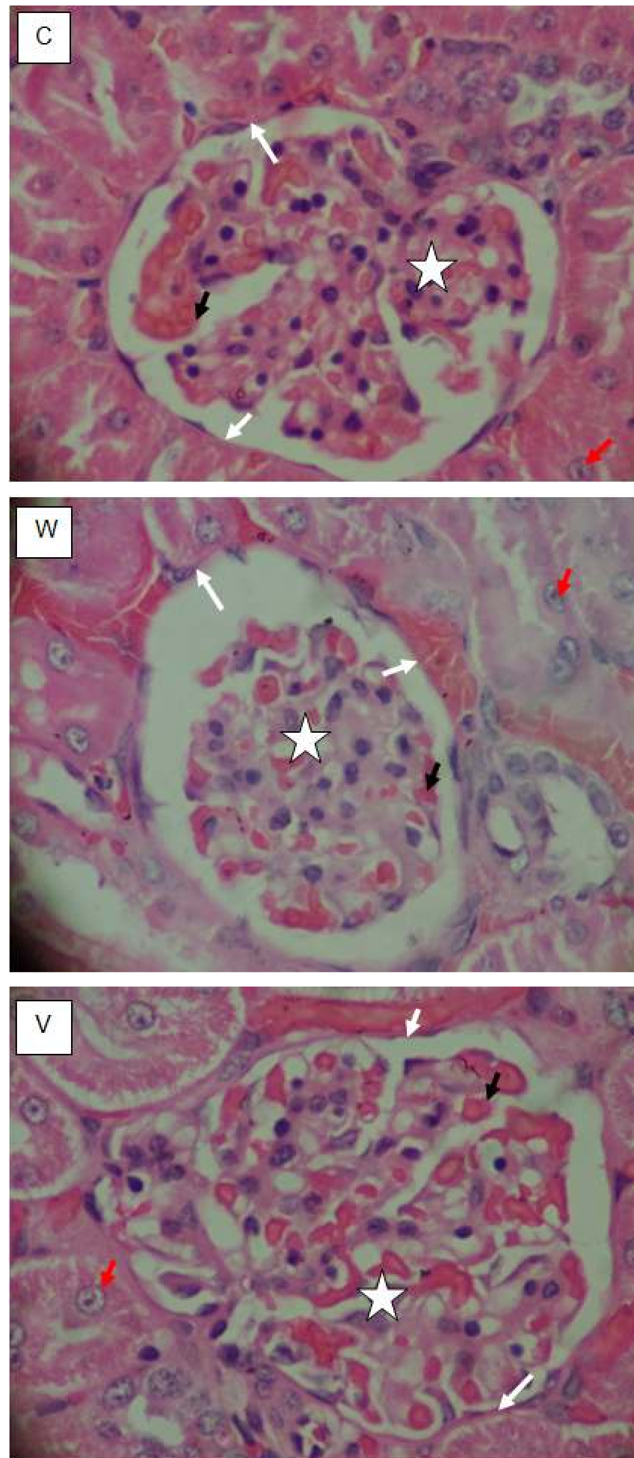

Plate 8. Cortical area of a rabbit kidney processed by the three techniques, showing the glomeruli (white stars), nuclei (red arrows), and red blood cells (black arrows). Glomerular capsules (white arrows) are clearly demonstrated. H&E X400

C=conventional method, W= microwave without vacuum method. V= microwave with vacuum method

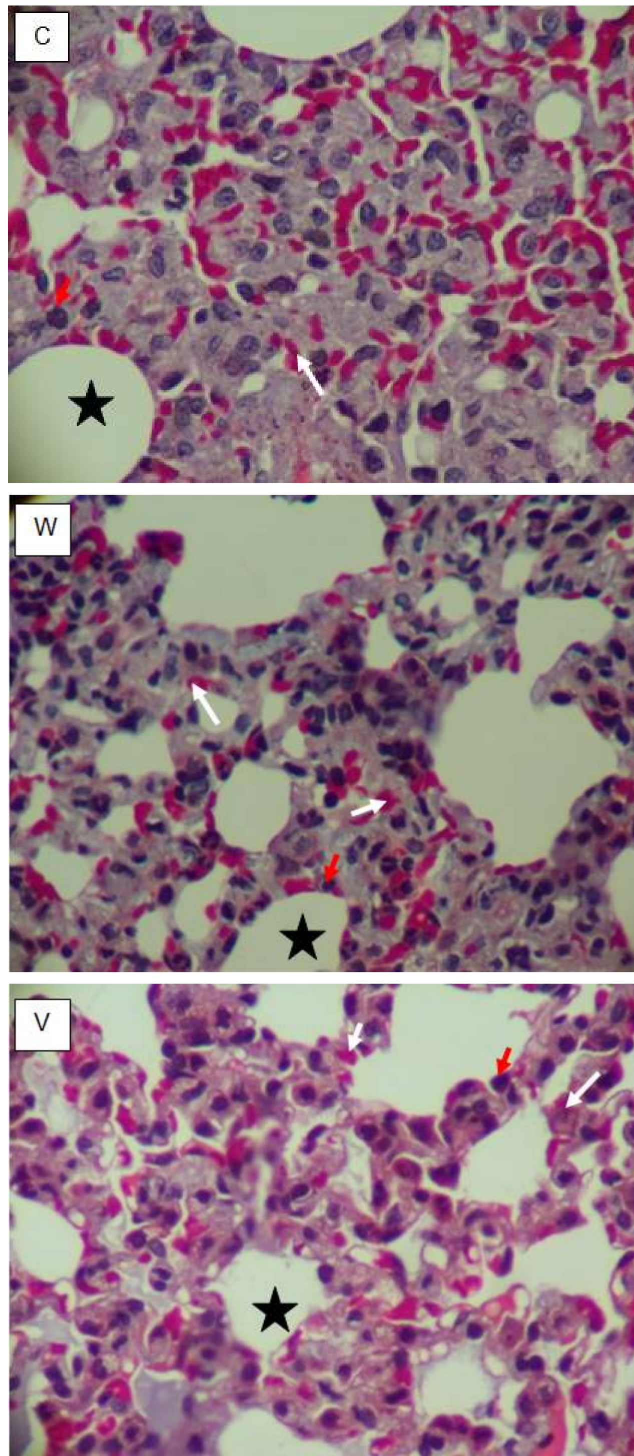

Plate 9. Lungs of a rabbit processed by the three techniques and stained by the Masson Trichrome techniques. Black stars= alveolar spaces, red arrows= cell nuclei, white arrows= red blood cells. Masson Trichrome X400

C=conventional method, W= microwave without vacuum method. V= microwave with vacuum method

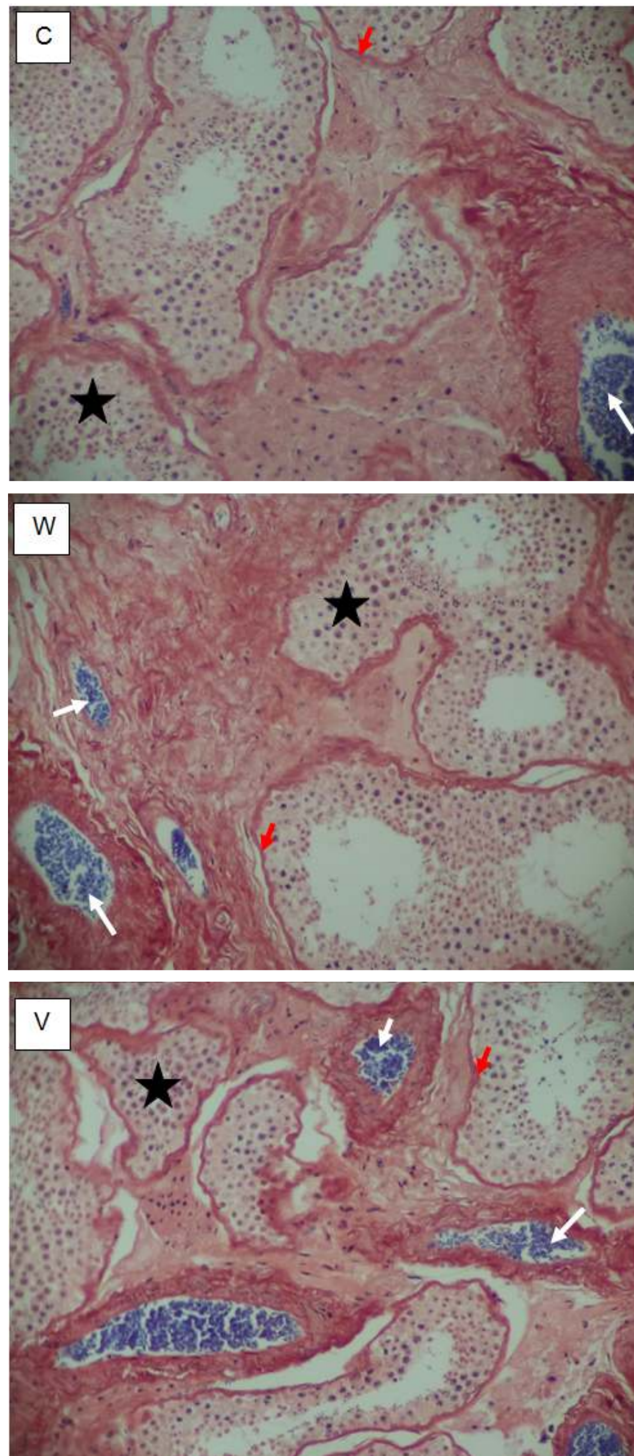

Plate 10. Human testis processed by the three techniques and stained by the Phosphotungstic Acid Haematoxylin Technique. Balck stars= seminiferous tubules, red arrows= basement membrane, white arrows= red blood cells. PTAH X100

C=conventional method, W= microwave without vacuum method. V= microwave with vacuum method

Table 1. average scores for details of section preparation mounting and block storage among the three techniques on a 0-2 scale pointer

Parameter	Group C	Group W	Group V
Dehydration & clearing	1.3	1.7	1.7
Cohesiveness	2	2	1.7
Flattening	1.7	1.7	1.7
Specimen shrinkage	2	1.7	2
Opacity	1.7	2	1.7
Drying	2	2	2

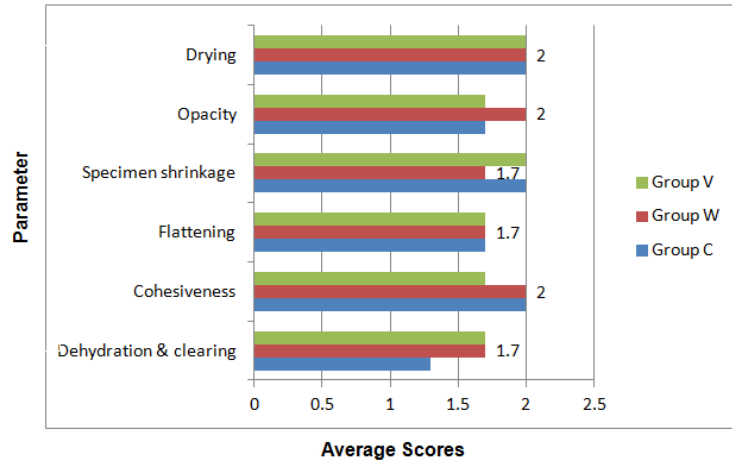

Fig. 1. Bar chart showing a comparison of average scores for details of section preparation mounting and block storage among the three techniques on a 0-2 scale pointer. The three techniques show no disparity for the evaluation of dryness and flattening while the microwave with vacuum and the conventional method show equal and a lower score than the microwave without vacuum when it comes to opacity

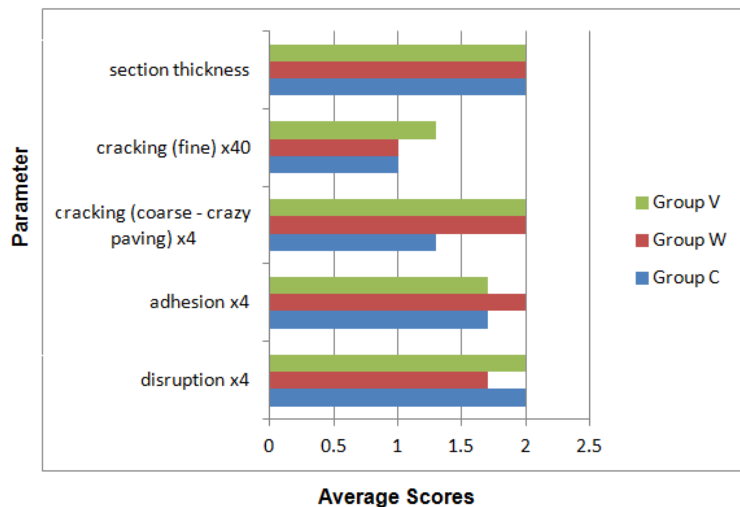

Fig. 2. Bar graph comparing the average scores for details of microscopic assessment and physical quality of section among the three techniques on a 0-2 scale pointer. The graph shows that there is no variation in terms of section thickness among the three techniques, while the degree of cracking as revealed under fine (X40) objectives shows that the microwave technique with vacuum is better than the other two techniques. In terms of section adhesion, this graph shows that the microwave without vacuum is better than the other two techniques

Table 2. Average scores for details of microscopic assessment and physical quality of section among the three techniques on a 0-2 scale pointer

Parametre	Group C	Group W	Group V
Disruption x4	2	1.7	2
Adhesion x4	1.7	2	1.7
Cracking (coarse - crazy paving) x4	1.3	2	2
Cracking (fine) x40	1	1	1.3
Section thickness	2	2	2

4. CONCLUSION

The paraffin wax method of tissue processing which is characterized by at least a 24 hour delay, coupled with the use of hazardous substances like xylene has now received a boost with a less than 100 minutes duration of processing and a drastic reduction in the quantity of reagents used. These new innovations are seen to be fast and cheap as well as compatible with the phosphotungstic acid haematoxlin technique; here, tissue histochemistry is not compromised as the reactions demonstrated in the conventional method were comparable with the rapid microwave methods. Red cells, epithelial cells, cell and nuclear components are well preserved in the three techniques hence the microwave techniques can be used for the diagnosis of diseases associated with the deposition of of collagen. Studies on the cardiac muscles can now be done at a cheaper, faster and more convenient atmosphere by utilizing the microwave innovation. And were electricity supply is interrupted, the microwave technique provides an avenue for the tissues to be removed for continues processing using alternative techniques. This work agrees with other research carried out [18-20] and should be encouraged. It is good for laboratories where electricity supply is erratic as it can be achieved within the shortest time possible even with an electricity generation plant.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Gruntz SL, English V. Expedited bone throughput using microwave decalcification. Histologic. Technical Bulletin for Histotechnology. 2011;54(1):1-3.
2. Hafajee ZAM, Leong AS-Y. Ultra-rapid microwave-stimulated tissue processing with a modified protocol incorporating microwave fixation. Pathology. 2004;36: 325–329.
3. Boon ME, Wals-Paap CH, Visinoni FA, Kok LP. The two-step vacuum-microwave method for histoprocessing. Microwave Newsletter 13. Eur J Morphol. 1995;33:349-58.
4. Falkeholm L, Grant CA, Magnusson A, Möller E. Xylene-free method for histological preparation: A multicentre evaluation. Laboratory Investigation. 2001; 81(9):1213-1221.
5. Kandyala R, Raghavendra SC, Rajasekharan ST. Xylene: An overview of its health hazards and preventive measures. Journal of Oral Maxillofacial Pathology. 2010;14:1-5.
6. Dapson R. Dye-tissue interactions: Mechanisms, quantification and bonding parameters for dyes used in biological stains. Biotechnology Histochemistry. 2005;80:49-72.
7. Meier-Ruge WA, Bruder E. Current concepts of enzyme histochemistry in modern pathology. Epub. 2008;75(4):233-243.
8. Vargas SO, Sampson BA, Schoen FJ. Pathologic detection of early myocardial infarction: A critical review of the evolution and usefulness of modern techniques. Mod. Pathol. 1999;12(6):635–645.
9. Berghes C, Parvu M, Cuca D. Anatomic studies about rabbit spermatics ways. Lucrări ştiinţifice Zootehnie şi Biotehnologii. 2009;42(1).
10. Hristov H, Kostov D, Vladova D. Topographical anatomy of some abdominal organs in rabbits. Trakia Journal of Sciences. 2006;4(3):7-10.
11. Grizzle WE. The effect of tissue processing variables other than fixation on histochemical staining and immunohistochemical detection of antigens. The Journal of Histotechnology. 2001;24;213-219.

12. Boon ME, Kok LP, Noordam EO. Microwave - Stimulated diffusion for fast processing of tissue: Reduced dehydrating, clearing and impregnating times. Histopathology. 1986;10:303-309.
13. Chaudhari K, Chattopadhyay A, Dutta SK. Microwave technique in histopathology and it's comparison with the conventional technique. Indian Journal of Pathology Microbiology. 2000;43(4):387-394.
14. Leong ASY, Daymon ME, Milios J. Microwave irradiation as a form of fixation for light and electron microscopy. Journal of Pathology. 1985;146:313-321.
15. Rolls GO, Farmer NJ, Hall JB. Artifacts in histological and cytological preparations. 1st edition. Melbourne. Leica Micro-systems; 2008.
16. Warmington AR, Wilkinson JM, Riley CB. Evaluation of ethanol-based fixatives as a substitute for formalin in diagnostic clinical laboratories. The Journal of Histotechnology. 2000;23(4):299-308.
17. Kok LP, Boon ME. Microwaves for microscopy. J Microsc. 1990;158:291-322.
18. Morales AR, Essenfeld H, Essenfeld E, Duboue MC, Vincek V, Nadji M. Continuous-specimen-flow, high throughput, 1-hour tissue processing. Archives of Pathology Laboratory Medicine. 2002;126:583-590.
19. Mathai AM, Naik R, Pai MR, Rai S, Baliga P. Microwave histoprocessing versus conventional histoprocessing. Indian Journal of Pathology and Microbiology. 2008;51:12-16.
20. Avwioro OG. Staining reactions of microwave processed tissues compared with conventional paraffin wax processed tissues. European Journal of Experimental Biology. 2011;1(1):57-62.

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