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Evaluation of the Conventional Versus Two Rapid Microwave Processing Techniques Using the Masson Trichrome Histochemical Methtod

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Authors' contributions

This work was carried out in collaboration between all authors. Author TPPC carried out the experiment, author KPJ redesigned the experiment. Authors SIO and SDL prepared the reagents used while authors AAN and TPPC read and graded the slides. Author SCC carried out the staining. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To evaluate the quality of paraffin blocks produced using the microwave and without the use of xylene. To evaluate the compatibility of the rapid microwave processing techniques on the histochemistry of collagen and muscle fibre as well as nuclear, cytoplasmic and extracellular components.
Study Design: Harvesting and fixation of tissues in 10% formalin. Grossing into triplicates, processing using three different techniques, checking for retraction of blocks, checking the quality of paraffin ribbon, Staining using the Masson Trichrome technique, grading of section quality and staining characteristics.
Place and Duration of Study: Anatomy Department, Nnamdi Azikiwe University, Nnewi Campus and National veterinary Research institute, Vom, Nigeria. The work was done between August and

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December, 2014.
Methodology: Liver, kidney and oesophagus were harvested from an apparently healthy rabbit. Human cervix, prostate, testis and prostate were obtained. All samples were fixed in 10% buffered formalin, cut into triplicates and processed using the three techniques, sectioned and stained simultaneously using the Masson Trichrome technique and evaluated.
Results: Processing cycles and reagent consumption per cycle were greatly reduced in the microwave techniques. Paraffin blocks as well as ribbons produced from the three techniques were of comparable quality. Though tissue integrity was comparable among the three techniques, the stroma of the cervix was better retained in the microwave with vacuum method. Dye uptake as well as section thickness were comparable among the three techniques. Nuclear, cytoplasmic and extracellular components presented with comparable histochemistry.
Conclusion: The rapid microwave techniques are cheap and fast with good ribonning. Reagent toxicity is greatly reduced as xylene is excluded. Muscle fibre and collagen fibre histochemistry for the rapid microwave methods are comparable to that of the conventional method. Cervical stroma is better retained in the rapid microwave methods than in the conventional method of paraffin wax processing. Microwave techniques should be encouraged since they can help to produce quality paraffin section within the shortest time possible at a cheaper rate than the conventional method without compromising section quality and tissue histochemistry.

Keywords: Tissue processing; reagent toxicity; xylene; isopropanol; microwave processing; vacuum; masson trichrome; muscle and collagen.

1. INTRODUCTION

Solid tissues need to be fixed and processed, to preserve their structures, and eventually impregnated with an appropriate hardening substance to permit making thin slices suitable for staining and microscopic evaluation. For almost 100 years, the steps followed to prepare tissues for microscopic review have remained practically unchanged. One Substantial shortcomings associated with that practice include at least a 1- day delay in providing the diagnosis [1,2] reagent toxicity, and degradation of nucleic acids. This work will try to investigate the possibility of reducing the total time required for tissue processing without compromising histochemical and/or morphological quality. Although microwave radiation seems to overcome some of the problems, microwavebased processing methods have not gained widespread acceptance. Microwave techniques generally utilize only absolute ethyl alcohol or isopropanol and paraffin. Graded alcohols are not necessary and the use of clearants is eliminated because carry-over alcohol is evaporated during the final paraffin step. In this process, the penetrative properties of the microwave and the conversion of this incident energy into heat, is made use of, the advantages include shorter processing times, eliminating noxious chemicals like xylene and lesser degree of denaturation of nucleic acids [3]. 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]. It is a histological technique used for studying chemistry of tissues and cells hence different substances can be demonstrated. A “trichrome” in today’s histopathology laboratory usually means a section with the nuclei stained with hematoxylin, and the connective tissue and cell cytoplasm stain different colors, rather than just a section with three colors. Most popular in present

times is the Masson's trichrome introduced in 1929 that stains nuclei blue–black, cytoplasm red, and connective tissue green or blue depending on the particular stain chosen. Pretreatment of the section with Bouins fluid, or picric acid solution alone, is common before trichrome staining. Many trichrome methods will demonstrate fibrin, which is useful to histopathologists. The trichrome stain is used to differentiate between collagen and smooth muscle in tumors, and the increase of collagen in diseases such as cirrhosis. It is a routine stain for liver and kidney biopsies. It assessing the quality of a trichrome stain therefore, it is good to use tissues that are known to contain collagen fibres, smooth mucles and keratin. Such tissues include the cervix, fibroid, protrate, oesophagus. The liver, kidney and testis are also good for this assessment since diseases conditions cause the deposition of collagen in them, for example, the trichrome stain is performed on medical liver biopsies to assess the degree of fibrosis in the liver [8].

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 oesopagus, liver and kidney were harvested and fixed in 10% buffered formalin for three days. Each of the seven 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°C 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°C for 15 minutes [12-14]. 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 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 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 Thermo Scientific) 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 ųm).
- 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: Masson's Trichrome Stain Staining Technique

- Sections were deparaffinized and rehydrated
- Sections were washed in distilled water.
- Sections were re-fixed in Bouin's solution for 1 hour at 56°C.
- Sections were rinsed in running tap water for 5-10 minutes to remove the yellow color.
- Sections were stained in Weigert's iron hematoxylin working solution for 10 minutes.
- Sections were rinsed in running warm tap water for 10 minutes.
- Sections were washed in distilled water.
- Sections were stained in Biebrich scarlet-acid fuchsin solution for 10-15 minutes.
- Sections were washed in distilled water.
- Sections were differentiated in phosphomolybdic-phosphotungstic acid solution for 10-15 minutes.
- Sections were transfered directly (without rinse) to aniline blue solution and stained for 10 minutes.
- Sections were rinsed briefly in distilled water and differentiated in 1% acetic acid solution for 2-5 minutes.
- Sections were washed in distilled water.
- Sections were dehydrated very quickly through 95% ethyl alcohol, absolute ethyl alcohol and cleared in xylene.
- Sections were mounted in DPX and coverslipped.

2.6 General Criteria for Evaluation of Quality of Sections

The following criteria were used [5,14] 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 [15].
ii. **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), uniformity of preservation (includes zonal fixation) [16].
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 [17,18].

The following grading was done:

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

3. RESULTS AND DISCUSSION

Good ribbons were obtained from the three techniques without any difficulty. The conventional method takes one thousand, four hundred and forty minutes per cycle while the microwave techniques take an average of ninety five minutes to complete (Fig. 1). For the number of reagents used, the three techniques used an average of three reagents. However, in terms of volume of reagents used, the conventional method used over five times, the volume of ethanol used per cycle for each of the microwave methods (Fig. 2). While the conventional method uses 3200 mL of xylene per cycle, the microwave methods do not use xylene at all. However, the microwave methods, each utilizes 2000 mL of isopropanol per cycle which is not used in the conventional method. The three techniques, each, makes used of molten paraffin wax, and while the conventional method consumes 3200 mL of molten paraffin wax, the microwave methods, each consumes 2000 mL of molten paraffin wax per cycle. Blocks obtained in the three techniques after embedding showed no retraction as observed after one week, two weeks, three weeks, four weeks, eight weeks and sixteen weeks. Their texture and cohesiveness was similar too and there was no colour change after this period of observation. Fine ribbons were obtained in the three techniques with good elasticity and ability to flatten easily on 20% ethanol. There was no fragmentation of ribbons or sections when placed on warm water in the water bath during floating-out. No case of section detachment was recorded in the three techniques as evidence after passing through different staining fluids and mounted.

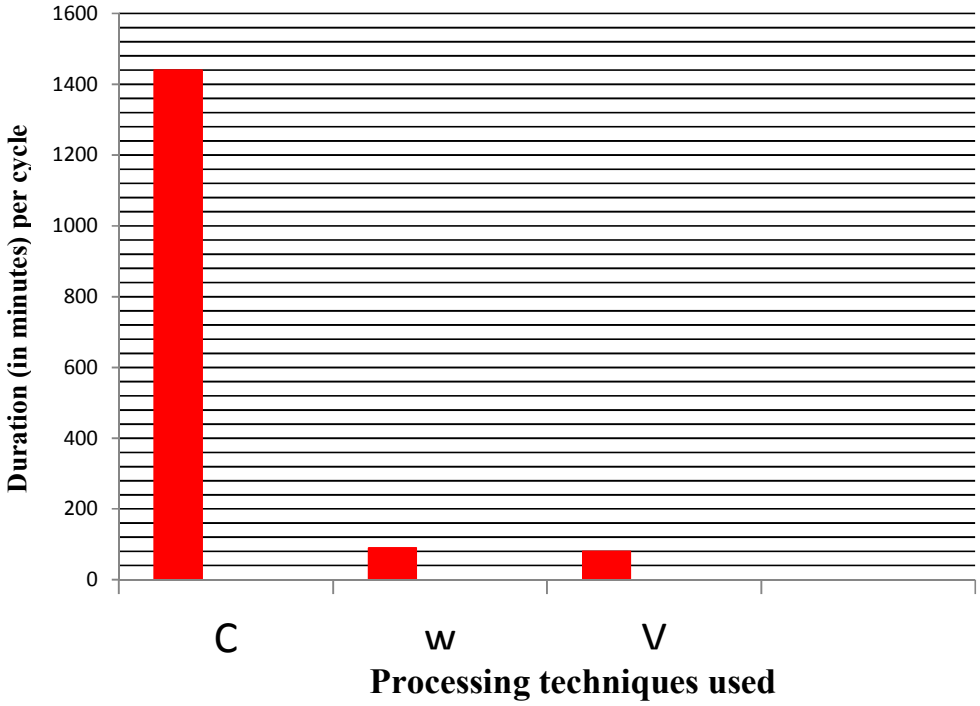

Fig. 1. Duration per cycle for each of the three processing Techniques. The figure shows that the conventional method (C) takes one thousand, four hundred and forty (1440) minutes for a complete cycle, the microwave without vacuum (W) takes ninety (90) minutes while the microwave with vacuum (V) takes eighty (80) minutes

Section thickness was uniform in the three techniques as shown with even stain intensity per section (there were no differences in staining intensity within an individual section) (Plates 1 and 2). In terms of section disruption and cracking, there is variability in terms of organs;

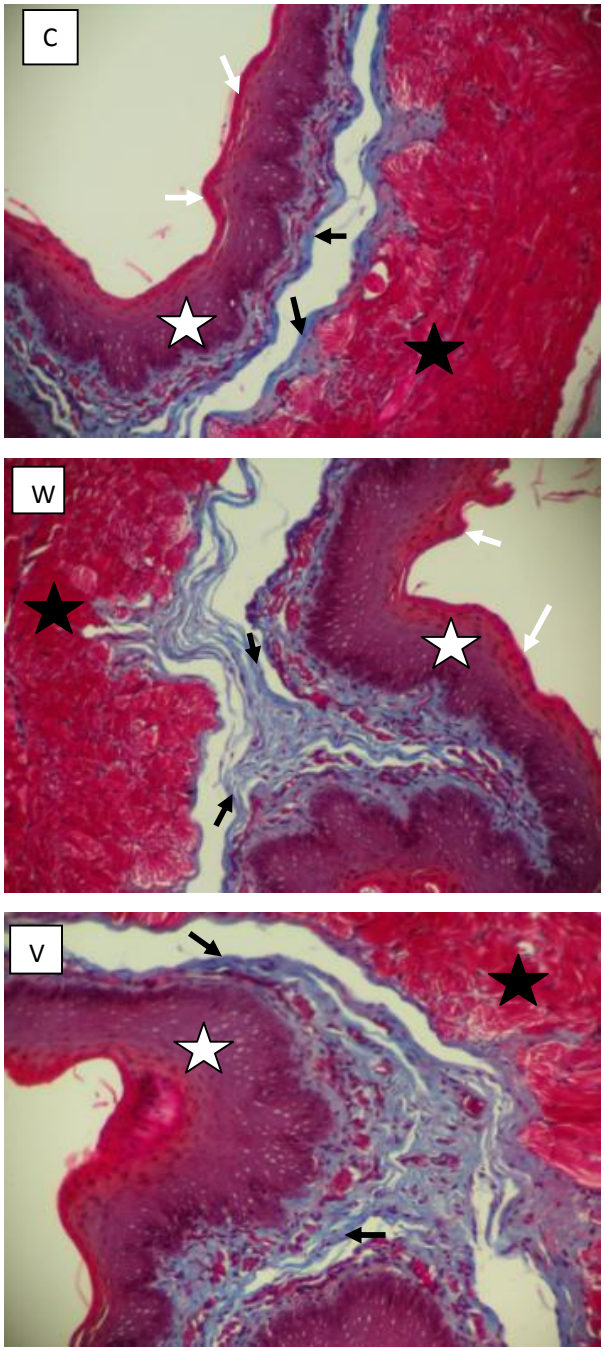

Plate 1. Rabbit oesophagus (Transverse section); control slide for Mason trichrome staining technique. A very sharp contrast for the different tissue components is maintained among the three techniques Muscle fibre (black stars) demonstrate their distinct reddish colour against the collagen fibres (black arrows) picking the characteristic blueish colour. White stars= stratified squamous epithelium. White arrows=keratin. Mason Trichrome Stain. 400X

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

while there are no appreciable differences in most of the organs used, the human cervix (plate 6) showed a greater degree of cracking in the conventional method as compared with the microwave techniques. Nuclear and cytoplasmic details, special features like kidney-basement membrane definition, liver-sinusoidal endothelium definition (Plates 3, 4 and 8) are clearly demonstrated in the three techniques. Red blood cells are well preserved in the three techniques. Tissue components are clearly differentiated based on their different affinities to the dyes used (Plates 5 and 7).

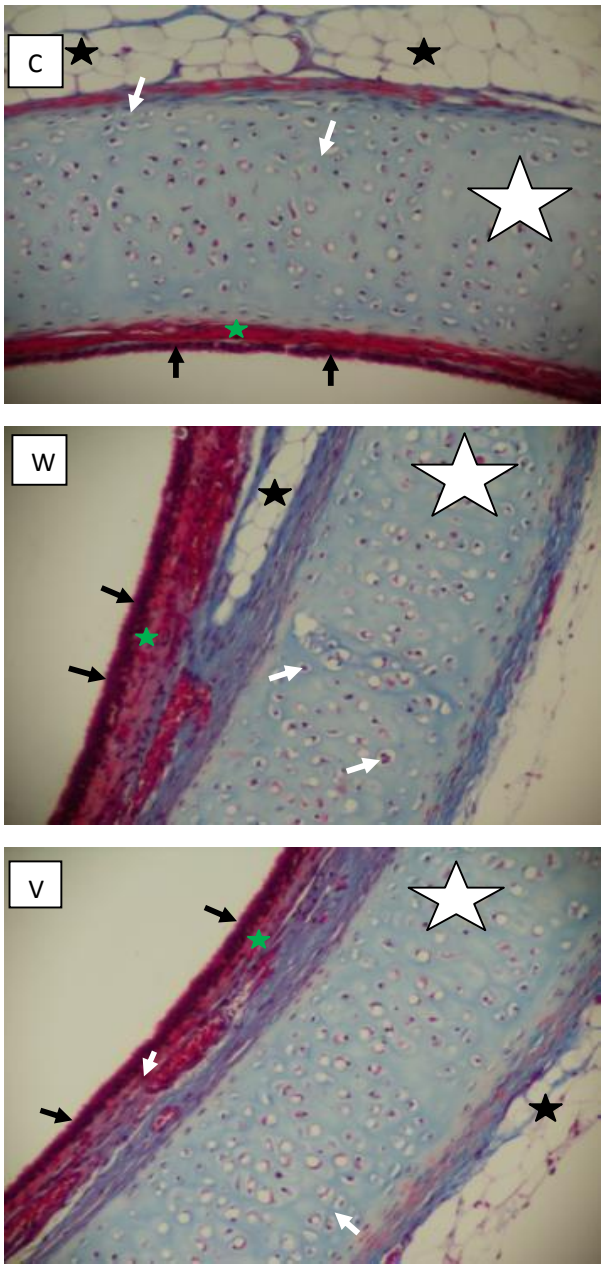

Plate 2. Tranverse section of a rabbit Trachea. Respiratory epithelium (Black arrows) is adhered well to the lamina propria (green stars) in the three techniques. Spaces previously occupied by lipid (black stars) are clearly demonstrated. White stars= hyaline cartilage. Masson Trichrome stain. 400X

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

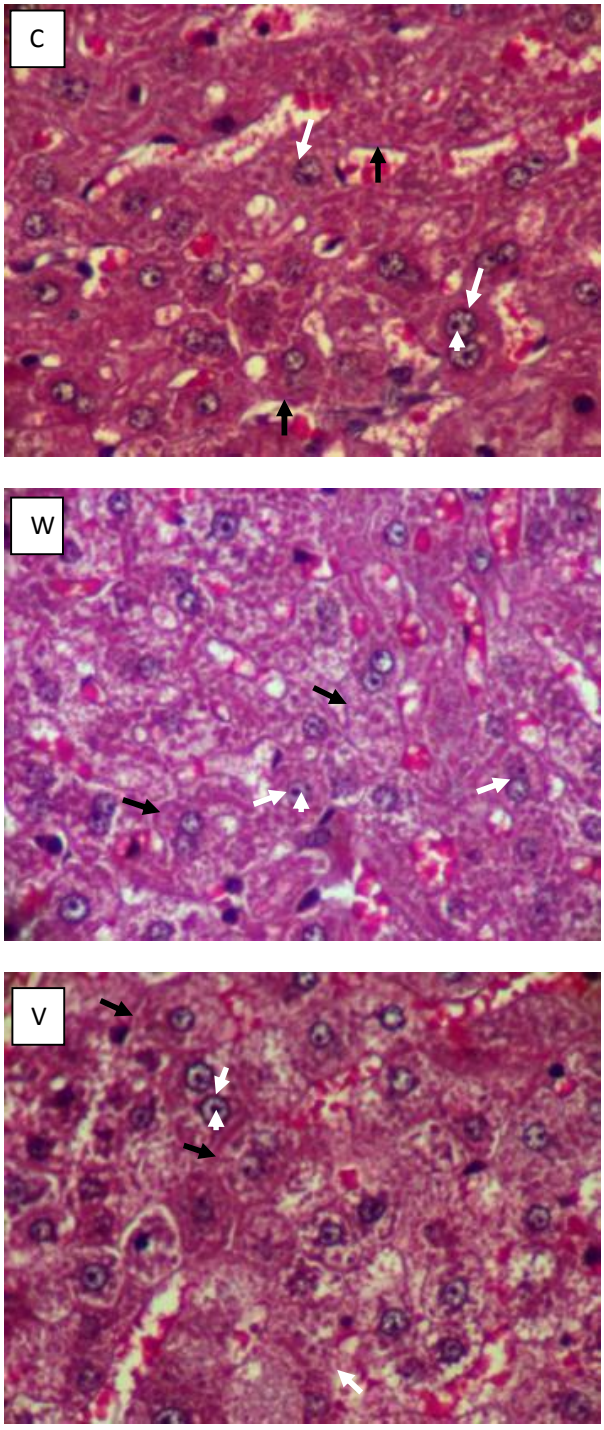

Plate 3. Rabbit liver processed by the three technique. Cellular demarcations (black arrows) are clearly demonstrated. Nuclei (white arrows) as well as nucleoli (white arrowhead) are clearly demonstrated in the three techniques. Because this is a normal liver, there is no deposition of collagen hence the characteristic blue staining of collagen is absent here, showing that the trichrome stain is specific to the substances of target (collagen and muscle fibre). Masson Trichrome stain. 400X

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

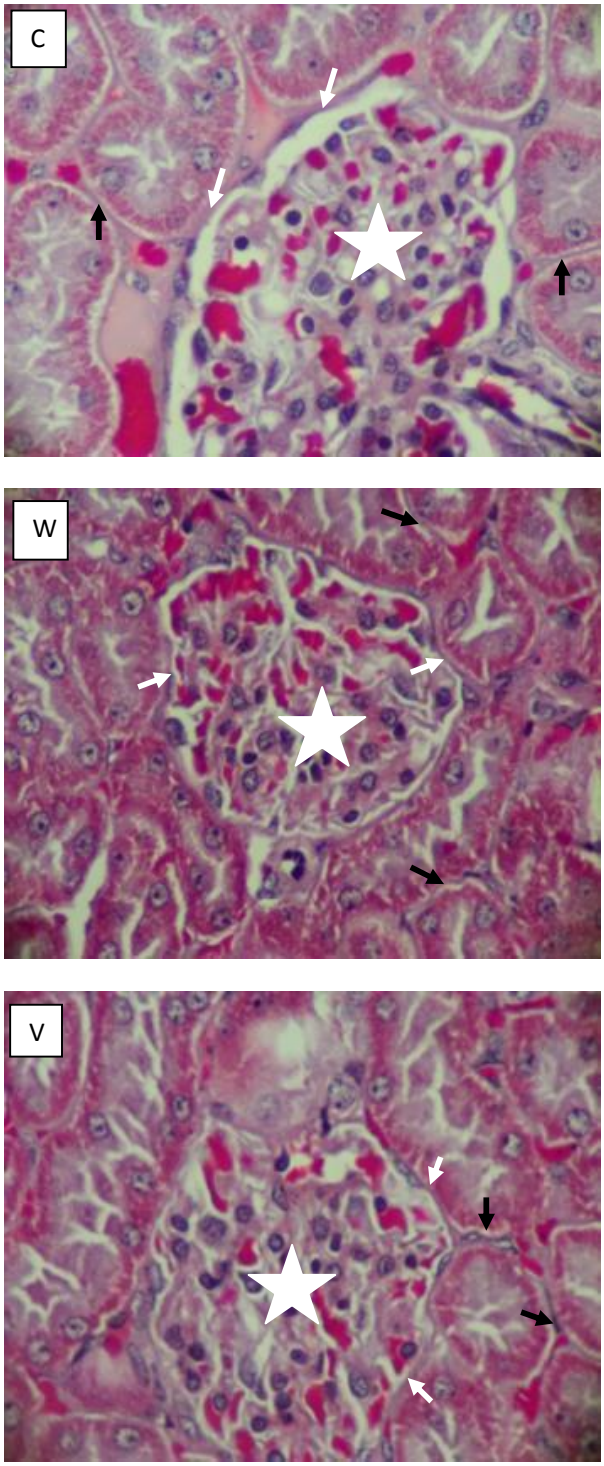

Plate 4. A normal rabbit kidney showing the glomerulus (white stars). The glomerular capsules (white arrows) as well as cellular demarcations are clearly defined in the three techniques. The characteristic blueish staining of collagen and reddish staining of muscle fibre, known with this technique is absent since these substances are absent in a normal kidney like this. Masson Tri‹.hrome stain. 400X

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

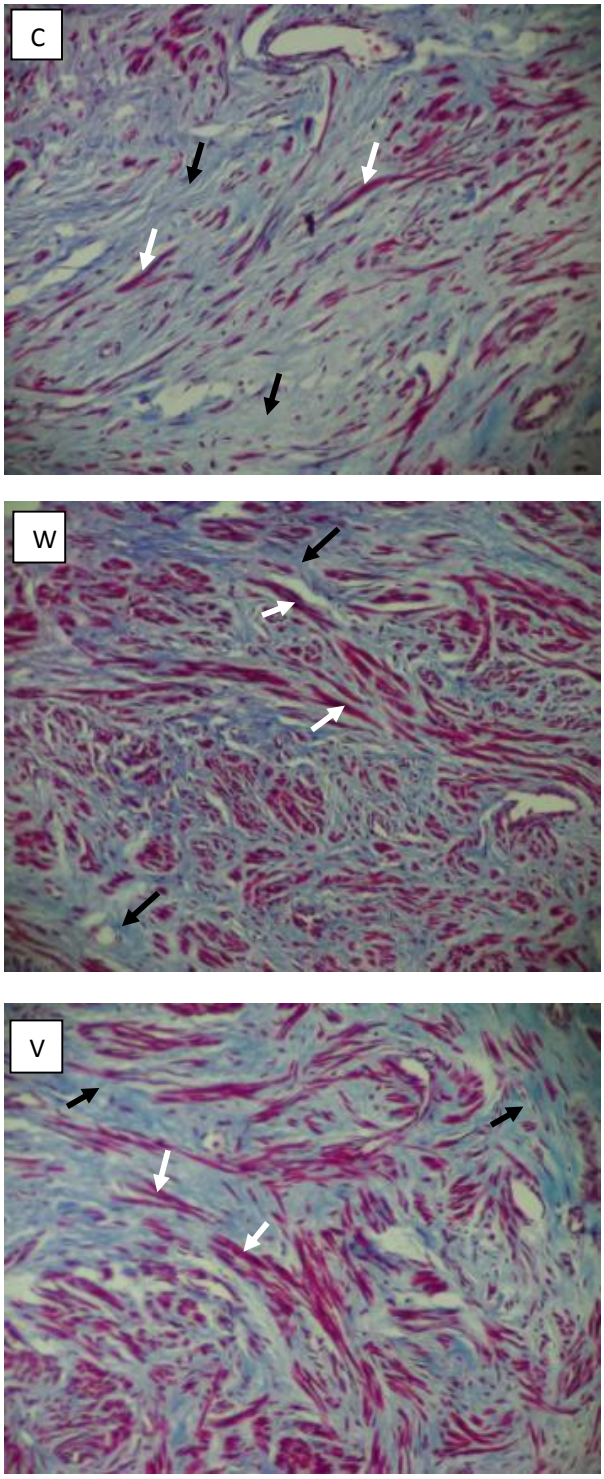

Plate 5. Human Fibroid. This histochemical technique clearly differenciates between the two major components of fibroid, which is composed of smooth muscles (white arrows) and collagen fibres (Black arrows). Mason Trichrome Stain. 100X

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

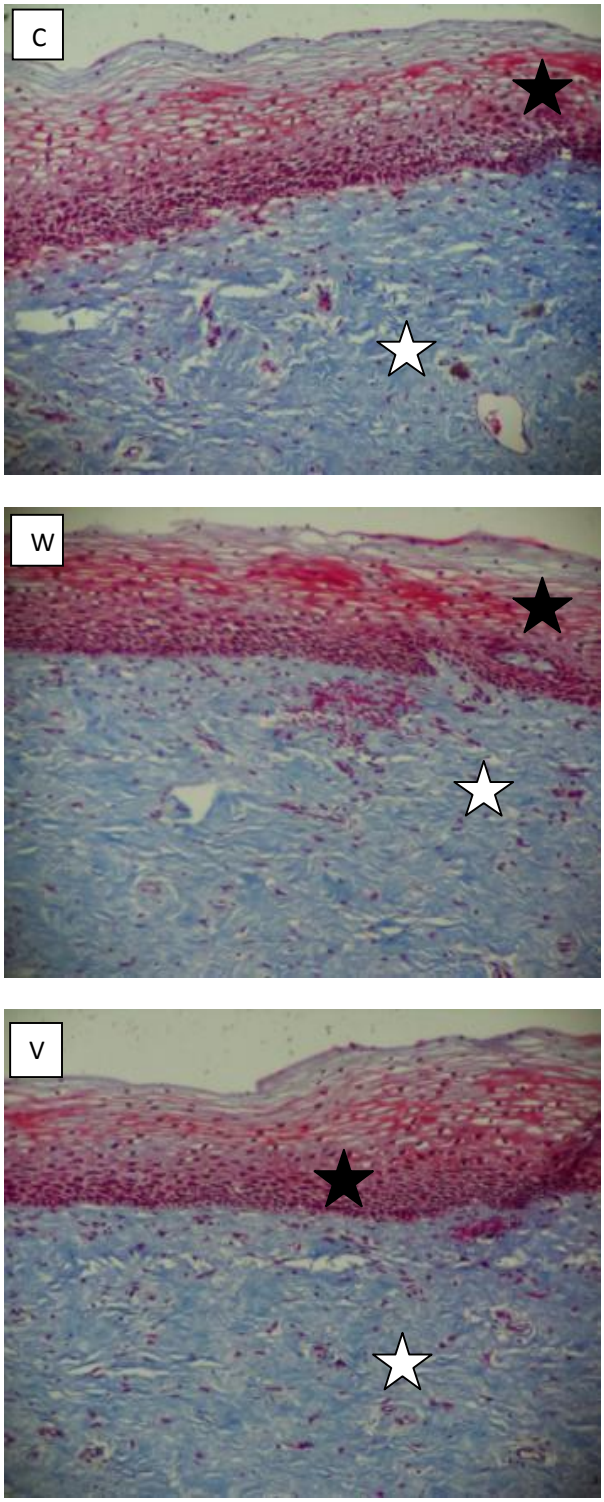

Plate 6. Human cervix. The stain demonstrates the characteristic red staining of epithelial cells (black stars) and blue staining of collagen fibre (white stars). This technique will give a detailed extend of invasion in terms of cancer of the cervix. Mason Trichrome. 100X

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

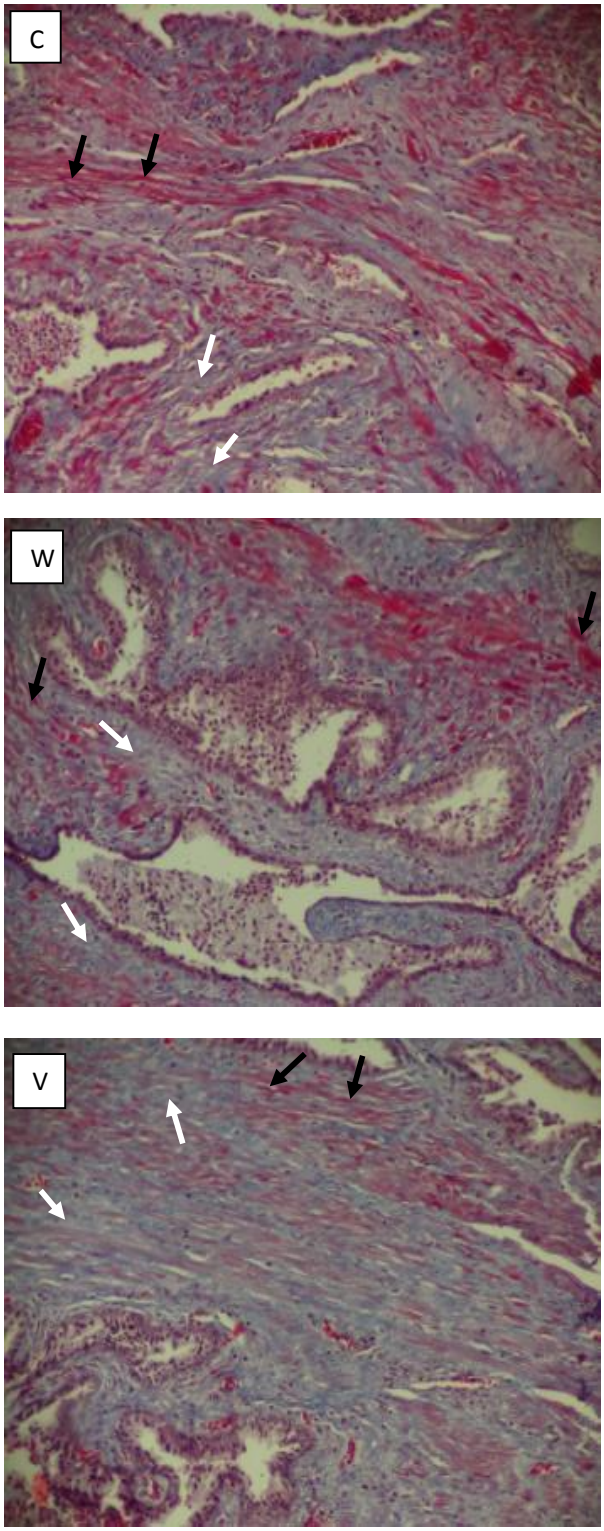

Plate 7. Human prostate. White arrows= collagen fibres, black arrows= smooth muscles. Mason Trichrome. 400X

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

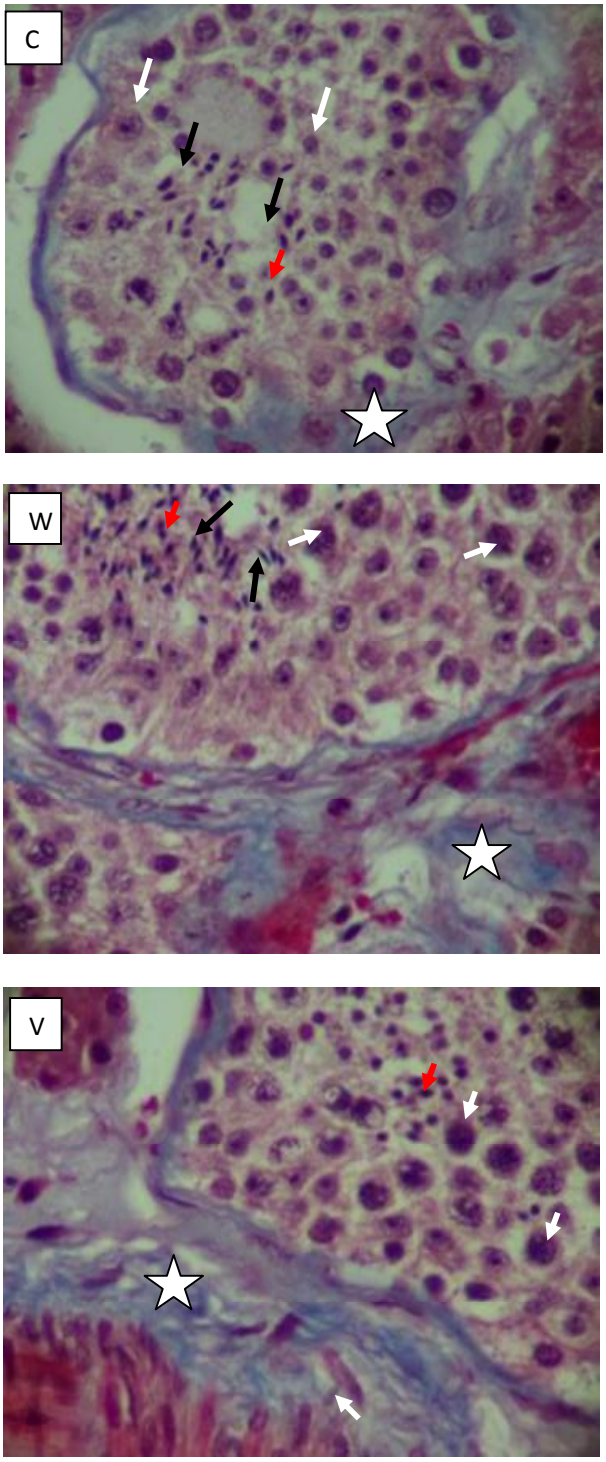

Plate 8. Human testis seminiferous tubule. Spermatogonia (white arrows) are clearly demonstrated alongside spermatozoa (black arrows) and spermatids (red arrows). All these are comparable in the three techniques. White stars= collagen fibres. This shows that the three techniques can be used for the study of the spermatogenic series. Mason Trichrome. 400X

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

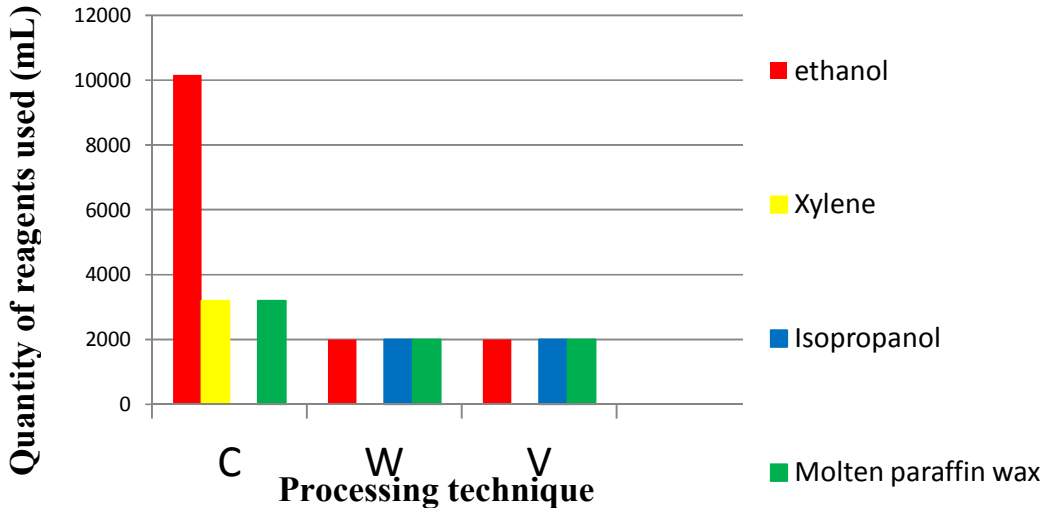

Fig. 2. An evaluation of the quantity of reagents used among the three processing techniques
C=conventional method, W= microwave without vacuum method. V= microwave with vacuum method

4. CONCLUSION

This work shows that same day turn-around is possible in histology without a negative effect on the histochemistry of both smooth muscles as well as collagen fibres. This agrees with the work carried out by other researchers [19,20]. The integrity and staining intensity is seen to be comparable among the three techniques though integrity is better retained in the microwave with oven technique. Studies on liver and kidney, which require the trichrome stains can now be carried out within the shortest time possible. Xylene can now be eliminated from the paraffin wax tissue processing schedule due to its toxicity and negative effect on the tissue. Tissues processed in this way can be subjected to imunohistochemistry as well as molecular techniques to give a wider scope.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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