A COMPARATIVE STUDY OF MICROWAVE ASSISTED AND CONVENTIONAL TISSUE PROCESSING FOR PREPARING RAT (Rattus norvegicus) TISSUES FOR MICROSCOPY.

BY

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A dissertation submitted in partial fulfilment of the requirements for the Bachelor of Science (Hons) Degree in Applied Biosciences and Biotechnology

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APPROVAL FORM

This is to certify that the dissertation entitled "A comparative study of microwave assisted and conventional tissue processing for preparing rat (*Rattus norvegicus*) tissues for microscopy.", submitted in partial fulfilment of the requirements for Bachelor of Science Honors Degree in Applied Biosciences and Biotechnology at Midlands State University, is a record of the original research carried out by YEUKAI GILMORE MAGARA under my supervision and no part of the dissertation has been submitted for any other degree or diploma.

Name of supervisor……

Signature…….

Chairperson's signature…….

ABSTRACT

The discovery of microwaves is believed to have brought a revolutionary improvement in many scientific fields with microscopy and histopathology being among them. Microwave assisted tissue processing of pathologic material is becoming increasingly desirable to fulfil the needs of clinicians treating acutely ill animals and patients. This technique shortens the time for tissue processing from days to hours and execute the needs of the patients and animals in need of diagnosis as well as those of the physician by improving the use of reagents while reducing or eliminating their toxicity and places the laboratory in a better position to meet the demands of the pathologists. Few data exist comparing quality of microwave-processed tissues with that processed by conventional means on rat (*Rattus norvegicus*) for microscopic analysis. This study was conducted to determine the efficacy of the microwave tissue processing method for rat specimens by comparing microwave assisted tissue processing with the conventional method, based on the clarity of nuclei, cytoplasm, staining intensity and cellular detail of tissues processed by each method. A total of eight specimens were cut into two equal parts and each part was processed by the two processing techniques. Haematoxylin and eosin staining was performed at the same time and grading was done by two pathologists. An independent samples t-test using SPSS 21 was used to test for significant differences. The parameters (staining intensity, nuclear, cytoplasmic and cellular details) were scored higher in conventionally processed sections as opposed to the domestic microwave method. The P-values for the mean scores showed a significant difference for all the parameters on sections processed by CTP (conventional tissue processing) and DMTP (domestic microwave tissue processing), nuclear detail , $t\ (14) = 2.170$, ($p= 0.048$, $p < 0.05$), cytoplasmic detail $t\ (14) = 4.194$, ($p= 0.001$, $p < 0.05$) ,staining intensity $t\ (14) = 2.297$ ($p= 0.038$, $p < 0.05$), and cellular detail $t\ (14) = 2.668$ ($p= 0.018$, $p < 0.05$). Domestic microwaves can thus be used for tissue processing which shortens the time for tissue processing but however cannot produce quality slides comparable to those made from conventional processing.

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DEDICATION

This dissertation is dedicated to the memory of my mother Tendai Mtandavari. Although she was my inspiration to pursue my undergraduate degree, she was unable to see my graduation. This is for her. I also dedicate it to my father who helped me in all things great and small.

CONTENTS **PAGE**

LIST OF FIGURES **PAGE**

LIST OF TABLES **PAGE**

LIST OF APPENDICES **PAGE**

CHAPTER 1. INTRODUCTION

1.1 Background

Tissue processing is a biological procedure carried out in pathology departments of health institutions, for the purpose of studying the microscopic structure of animal tissues through identification of morphological changes, to observe characteristics of certain diseases under a microscope. Bancroft and Gamble (1995) proposed tissue processing as a method in which animal tissues are preserved by manual or automatic means to retain their life like state and cellular components for further analysis by pathologists. The whole course of tissue processing for the basis of tissue diagnostics is referred to as histopathology and as a means of tissue visualization on a cellular level, it is a fundamental tool in the study of diseases that infect plants, animals and humans. An example is cancer in humans and liver diseases in rats that can be diagnosed through analyzing the tissue structure in terms of cell development, cytoplasmic details, nuclear details, tissue orientation and other related details, hence there is continuous need for timely delivery of quality histological materials (plant and animal specimens) for pathological evaluation in plant pathology, veterinary, hospital and clinical laboratories (Leong, 1989).

In most cases, the tissue must undergo preparatory treatment before being sectioned, which involves impregnation with a suitable embedding medium to provide support and a suitable constituency for microtomy. According to various protocols by Hopwood *et al*. (1984) Mathai *et al.* (2004) and Kango and Deshmukh (2011), stages of tissue processing include fixation, dehydration, clearing, impregnation and embedding, each of a designated duration to ensure completion of the procedure. The whole process is driven by diffusion of various substances into and out of the stabilized porous tissues and the diffusion process results from the thermodynamic tendency of processing reagents to balance concentrations inside and outside the tissue block. The aim of tissue processing is to prevent tissue changes that occur within the cells soon after tissue samples have been cut.

Biomedical research has led to the development and application of biological techniques in animal epidemiology through microscopic analysis of cells and tissues because the efficacy of detection and control of human and animal diseases is reliant on a solid understanding of their nature and implementation of scientifically sound methods by people who are well trained. The study of

tissues under a microscope has brought a revolution within the field of biology through the introduction and requirements of very thin quality sections mounted on a glass slide and appropriately stained to demonstrate normal and abnormal tissue structures (Cullings *et al.*, 1985). For more than 100 years there has been one main method of processing tissues to obtain thin sections for microscopic examination that is referred to as conventional tissue processing, which is the oldest and first method that remains the gold standard against which all other new technologies such as microwave assisted tissue processing have been assessed and still being assessed (Valle, 1986). Bancroft *et al.* (1984) postulated that each of these different approaches is unique with their own advantages and disadvantages. The traditional (Conventional tissue processing) steps that were used to prepare tissues for microscopic review have remained practically unchanged. However, with the need to support the specimen firmness to timely produce strong tissues for better sectioning, tissue processing underwent a drastic transformation from the latter years of the eighteenth to early twentieth century (Giberson and Demaree, 1999). In the last 30 years microwave tissue processing has become increasingly useful in histological preparations during treatment of acutely ill patients in hospitals and animals in the veterinary clinics (Kango and Deshmukh, 2011). Although conventional tissue processing is the most followed method, it is a laborious and tedious procedure, consumes a lot of time, has a high cost of operation and also uses toxic chemicals such as xylene and formalin (Cullings, *et al*., 1985).

The turnaround time became increasingly important in this age of managed care and commitments to overall reduction of time and costs for healthcare services. Turnaround time refers to the time needed for the fulfilment of a request, therefore, a faster and cost-effective method which is the microwave assisted method has achieved acceptance in the last decades after the American Society of Clinical Pathologists reviewed microwave techniques (Mayers, 1970). They further produced a report in 1993 acknowledging the use of microwave techniques in disease diagnostics laboratories and since that report, the increased popularity of microwave assisted tissue processing has led to the production of commercial laboratory microwave ovens that are designed to ensure uniform rapid tissue processing under precisely controlled specimen temperatures (Boon and Ouwerkerk-Noordam, 1986). However, a significant number of studies (Hopwood et al, 1984; Mathai et al, 2004; Kango and Deshmukh, 2011) have proved that, kitchen and ordinary laboratory drying microwaves can be used successfully in tissue processing since the physicochemical basis of tissue

processing lies behind the diffusion of reagents in and out of the cells, hence microwave has electromagnetic radiation properties that can be used to provide heat to the regents to facilitate faster diffusion of regents instantaneously.

Boom *et al.* (1986) postulated that the quality of structure and preservation of tissue components is determined by the choice of reagents and exposure time to the reagents during processing and the diffusion of reagents to the tissue to be processed which can be accelerated with the application of heat. Conventional tissue processing depends on relatively slow infiltration of solutions from the outer surface hence taking long hours for completion of whole process. Since heat is the key factor in tissue fixation, if it is applied it must work its way into the interior of the specimen by thermal conduction exposing these sections of the specimen to microwave energy affecting the entire specimen instantaneously and simultaneously facilitating the exchange of solutions and accelerating the rate of diffusion of solution into and out of the cells. Thus, microwaves quickly and uniformly heat materials without the use of convectional heat (transfer of heat from one place to another by movement of fluids within the automated chamber). This is because a microwave is a form of non-ionizing radiation that produces alternative electromagnetic radiation that releases alternating electromagnetic field which results in the generation of instantaneous heat, thereby helping in the faster cooking of biological materials (Kango and Deshmukh, 2011).

We are unaware of any previous research study comparing the quality of microwave processed and conventional processed slides produced form using matched specimens of rat tissues procured from an ordinary histopathology laboratory. In this study, we will examine the usefulness of domestic microwave assisted tissue processing and staining so as to determine if it can produce results as good as the conventional method in medical biotechnology and pathology fields. The study aims to determine whether microwave assisted tissue processing can replace standard formalin and paraffin embedment overnight processing (conventional processing) as well as the validation of histologic quality, reliability of histological sections and the positive impacts on the turnaround times in biomedical research laboratories using rat tissue samples.

1.2 PROBLEM STATEMENT

Scores of animals and people die due to lack of inadequate medical and veterinary facilities. In countries with poverty and attendant health and hygiene issues such as Zimbabwe, diseases tend to spread fast and hence the need to stay ahead in medical biotechnology. Tissue processing has enabled scientists to be able to study and analyze characteristic changes of tissues for the basis of disease diagnosis and treatment (Boon *et al.*, 1986). For more than 100 years, conventional methods remained the gold-standard; however, they are slow and costly since they use highly automated machines. The process is laborious, tedious and time-consuming and also involves the use of potentially carcinogenic chemicals when inspired, such as xylene and formalin. The turnaround time has also been an issue for many years and has become increasingly important in this age of managed care and commitment to overall reduction of costs for healthcare service. Leong (1989) proposed that efforts are directed toward reducing specimen processing time when a rapid diagnosis and initiation of emergency therapy based on the histopathology findings is needed. Therefore, biomedical technology aims to prevent the spread of animal and human disease, diagnose them early and also find solutions that can manage the medical problems. Hence, the adaptation of microwave assisted tissue processing due to its ability to reduce turnaround time and use of noxious chemicals and same day reporting is increasingly becoming acceptable as the method produces histological samples with the same quality as those produced by conventional means.

1.3 JUSTIFICATION OF THE STUDY

While a considerable number of studies done by Leong (1989)., Ruehl-Fehlet (2000) and Boon *et al.* (1989) have reviewed the technique and reported satisfactory results of microwave fixation of surgical and autopsy specimens processing, to the best of our knowledge, there are no previous research studies comparing microwave processing and conventional processing from matched specimens of rat tissues for microscopy using a domestic microwave.

The microwave has brought a marked improvement as it shortens the time for tissue processing from days to minutes, hence, fulfilling the needs of patients and technicians by improving the processing time and eliminating the use of toxic substances, Cullings *et al*. (1999). The study is being carried out to support and understand the efficiency of microwave tissue processing in microscopy by comparing the two different techniques which are microwave assisted tissue

processing and conventional tissue processing basing on the clarity of staining, nuclear detail, cytoplasmic detail, and colour intensity and also assessing the turnaround time between the two methods using randomly picked tissue samples from two dissected rats of species *Rattus norvegicus*. The study will document the experience and usefulness of domestic microwave assisted processing and compare it with the conventional method so as to determine the extent to which it can replace standard formalin and paraffin embedded overnight processing, validate histological quality and reliability of histological sections and test the reduction of turnaround time in biomedical laboratories

1.4 Objectives

- **Main objective**

- To assess the usefulness of microwave assisted tissue processing using rat tissue samples so as to determine the extent to which it can replace conventional tissue processing in microscopy.

- **Specific objectives**

    a) To compare conventional and microwave assisted tissue processing in terms of quality and reliability of histological sections focusing on cellular, nuclear and cytoplasmic details and staining colour intensity.
    b) To evaluate the turnaround times for microwave tissue processing as against conventional tissue processing**.**

CHAPTER TWO: LITERATURE REVIEW

2.1 Microscopy

Microscopy refers to any technique used to attain images of nearby objects at resolutions that greatly exceed the resolving ability of the unassisted (naked) human eye. It is the art and science of creating, recording, and interpreting magnified images for the basis of tissue observation which offers help in fields such as human and animal pathology (histopathology) where a microscope is an essential tissue diagnostic tool (Sakurai and Watanabe, 2000). The world at large is concerned with the diagnosis of disease in humans and animals due to high mortality rates as a result of the inability to diagnose diseases in some areas especially the developing countries that are still thriving to meet first world technology standards. Disease diagnostics is based on the gross examination, microscopic, and molecular examination of organs, tissues, and whole bodies (necropsy). Therefore, microscopy has made a huge impact in academic, government research institutions, public and private diagnostic laboratories (including those at zoos), government regulatory agencies, industry (pharmaceutical and biotechnological companies, chemical companies, contract research organizations) (Locard and Edmond, (2008). Microscopy specialists have a diverse knowledge base in materials and have specialized skills of visual literacy (Patels *et al*, 2003). Object visualization may be facilitated by light or electron beams using optical or magnetic lenses respectively, or using a physical scanning probe that measures one of a wide range of different sample characteristics. In this document, one of the fields in medical and biological sciences that involves the use of microscopes to visualise thin sections of tissues produced from microwave assistance and conventional tissue processing for basic tissue examination will be discussed. We shall be using microscopic techniques to compare the outcomes of tissues processed from both methods. This knowledge can be applied successfully in biomedical fields such as pathology which involves the microscopic examination of tissue in order to study the architecture and manifestations of disease.

2.2 History of histology in biological and medical studies

Histology is a branch of biomedical pathology that deals with the study of diseases in tissue sections. It refers to the study of cells and animal tissues using simple to sophisticated microscopes that use either light or electrons for the visualization of minute biological entities within a cell (Baker, 1962). The visualization and analysis of tissues is done by biological technicians usually

referred to as histologists that have the skills necessary to process and stain various tissue samples and also to interpret the histological slides. Early researchers have used micro anatomy (microscopic study of tissue architecture) to draw a relationship around differences in cells as well as differentiating a normal plant cell structure from that of the animal (Bancroft and Layton, 2013). Biological researchers have advanced in the microscopic study of diseased tissue diagnosis since accurate diagnosis of diseases such as cancer and other diseases usually require histopathological examination of tissue samples (Binchat, (2009). Microscopical disciplines such as histology are used in forensic investigations, autopsy diagnosis (a dissection performed on a cadaver to find possible causes of death), plant, animal pathology and education.

The specialty of histopathology technique dates back to 1838, when Johannes Miller published his book, On the Nature and Structure Characteristics of Cancer, the first book on histopathology (Baker, 1962). However, many authors argued that there is not a single event that demarcates the beginning of pathology as a defined interest for early biomedical practitioners but in fact the history has roots in common with all other medical specialties arising in antiquity when men reasoned about the physiological elements that affected them (Ruehl-fehlet, 2000). For probable reasons those gross features of disease that were directly visible either in life or after death in funeral preparations came first to notice and over the last century archaeological discoveries increasingly have been linked with paleontological investigations focusing a wealth of knowledge and observation of gross external features of diseases from pre-historic people to present time (Bancroft and Layton, 2013). Currently we know from recent investigations of mummies that bone tumour and tuberculosis of the spine occurred in ancient Egypt as well as atherosclerosis, gallstones and abscesses, yet there is little evidence that Egyptians developed any systematic knowledge of those phenomena (Morales *et al*, 2002). The rise of pathology studies using a microscope came into light when the first compound microscope was constructed earlier in 1591 although it suffered from severe optical problems (Giberson and Demaree, 1999). In 1673 Anton van Leeuwenhoek started the development of simple microscopes with single lenses that gave improved magnification and resolution (Baker, 1962). As time progressed different laboratory chemicals were investigated for use as fixatives. A fixative was a necessity since visualisation of tissues under a microscope required fixed tissues mounted on a transparent glass. Formalin, widely used today, was the first fixative to be used in 1893 although many other fixatives were later

discovered to date (Leong and Gilham, 1989). The invention of a microscope by an Italian Marcello Malpighi in the 17th century for the basis of studying tiny biological entities made an introduction to an academic discipline in the field of biomedicine hence the introduction of the concept of tissue processing in anatomical studies by a French anatomist named Binchat (Morales *et al*, 2002).

This proves that the development of the microscope totally changed the scope of disease from whole organ to focus upon cell. It enabled the practice of histology and spawned numerous attendant advances in techniques necessary for modern practices. During the 19th century many fixation techniques designed to preserve the tissues under study were established by many scientists (Adolph Hannover-solutions of chromates and chronic acid; Franz Schulze and Max Schultze-osmic acids; Alexander Bullerov-formaldehydes; Bundikt Stillings-freezing) (Kok and Bon, 1990). In the beginning slices of fresh tissues where cut fresh by hand, examined unfixed and unstained, this led to a lot of bias on the analysis of the tissues as many changes would occur on the samples as a result of necrosis (Minot, 1989).

By contrast in the last decades of the century this crude approach had given way to fixed tissues, embedding techniques, microtomes, a plethora of biological stains and greatly improved microscopes. The first microtome suitable for sectioning animal tissues was constructed in 1848 and a sledge microtome manufactured later in 1910 (Kok and Bon, 1990). Paraffin wax for infiltration and support during sectioning was introduced during the mid1800s, hence all of this led to the emergency of a process referred to as tissue processing that was designed to fix, process and make tissue blocks for sectioning on a microscope to permit staining so as to analyze the features of the cell via a microscope (Ruehl-fehlet, 2000). Morales *et al*. (2002) stated that automated tissue processors replaced hand processing starting in 1945, and they further highlighted early studies in support of the use of automated machines in processing tissues which were done by Ludowing Aschoff in 1964 who described the histopathology of the heart in rheumatic fever and also the findings of the role of cholesterol in atherosclerosis. Furthermore, in support of this process is another study by Lanchsteiner (1966) who came up with a new understanding of kidney diseases through thin processed tissue samples viewed on a microscope that were processed using designed automated machines.

Over a span of 4,000 years, concepts of medicine and disease have changed, driven at times by

remarkable men and women, and more recently also by the relentless progress of technology (Bancroft and Layton, 2013). In looking for the cause of disease, the earliest physicians embraced the entire body, and often also the gods and goddesses, the stars and the heavenly bodies in their orbits (Bancroft and Layton, 2013). Then came other theories, holding physicians in thrall for almost 2,000 years, yielding only in the last few hundred years to the notion of organ-based disease and the rise of anatomical pathology (Valle, 1986). Next came the advent of the microscope as a scientific tool, concepts were re-focused from organ, to tissue, to cell, ever smaller, effecting the birth of histopathology that has held sway in pathology for just a century and a half (Bancroft and Layton, 2013). Morales *et al* (2002) postulated that, as the second millennium drew closer, powerful new technologies began to force yet another revision of the ideas, from cell-based disease, to gene-based disease, to individual molecules and their interplay.

From study of the past experience, we may catch a glimpse of the changes that occurred within the field of pathology be it animal or human up to current date were different protocols have been developed together with the production of more machines required for the production of small thin sections of tissues for the basis of diagnosis, for example the introduction of conventional tissue processing and subsequently the introduction of microwave assisted tissue processing which was reported by many authors (Chaudhari *et al.* 2000, Ruehl-fehlet 2000, Boon and Kok 1985) to produce histological slides same as the ones produced by conventional means.

The examination of tissues with a microscope usually requires a slice of tissue, which is thin enough to transmit light and the preparation of such thin slices is called section cutting or microtomy. In most cases, the tissues must undergo preparatory treatment before being sectioned, which involves impregnation in a suitable embedding medium to provide support and a suitable consistency for microtomy. This preparatory treatment is known as tissue processing. The stages of tissue processing include dehydration, clearing, impregnation and embedding, each of a designated duration to ensure completion of the procedure. For decades, instrumentation used in tissue processing remained relatively unchanged and three most commonly employed means of tissue processing are routine manual method, rapid manual method and the microwave method (Leong *et al*, 1987). Routine manual tissue processing (conventional processing) has been the most commonly employed method for the past 100 years (Leong *et al*, 1987). It includes fixation, dehydration, clearing and impregnation which is usually completed in 21-24h or even more hours

as according to a study by Hopwood (1984). The advantage of this method is its reliability and it can produce slides with less degree of shrinkage or tissue distortion. As according to a study by Leong *et al* (1987), the disadvantages is that it is time consuming and the need to work with noxious chemicals like xylene and formalin which have a potential carcinogenic effect if inhaled.

Rapid manual tissue processing is of a shorter duration than the above method, requiring 3-4h. It includes the same steps as in routine method, but for shorter durations. The advantage is that it consumes only 20% of time as compared to routine method and the disadvantages are that noxious chemicals like xylene and formalin need to be used and there is a greater degree of tissue distortion and shrinkage (Hopwood 1984). Microwave method is a recent tissue processing technique, first used by Boon and Kok in 1985 and it seems to reveal the new face of pathology as it continues to yield better and more precise diagnoses with a reduced turnaround time (Kennedy and Foulis 1989). In this process, the penetrative properties of the microwave and the conversion of incident energy into heat utilized. The other advantages include shorter processing times, eliminating noxious chemicals like xylene and lesser degree of denaturation of nucleic acids. The disadvantage is the high costs involved as a result of the use of highly specialized microwave machines.

Considering these facts, two of the three different methods of tissue processing in the study of microscopy were compared in terms of the effect on the outcomes of the histologic samples processed from a laboratory rat (*Rattus norvegicus*) and also a comparison was made on the amount of time elapsed for each method to finish processing. The two compared methods were microwave assisted tissue processing using a domestic microwave (Ecco Microwave Oven, model MT 2817) and conventional tissue processing using automated tissue processor (Dip and Dunk Automatic tissue processor, ZHCHEING, CHINA), and an automated stainer (Autostainer, ZHCHEING, CHINA).

2.3 Fixation

Fixation stands as the first and most significant stage before processing is employed to tissues. It involves the submerging of the sampled tissues in a chemical substance (fixatives) in order to prevent tissue digestion by enzymes or bacteria and preserve most of its morphologic and chemical characteristics. Fixed tissues have an advantage that they can be more easily stored and reproducibility of sections at a later date is easier. Fixatives provide crosslinking between proteins and form a gel-like substance that maintains the in vivo relationship of tissue components of each

other (Kango and Deshmukh, 2011). They are various reagents that can be used for fixation and each of which has differing penetration rates. The most commonly used solution to date is 10% formaldehyde (HCHO) which was advocated by Isaac Blum (1833-1903) and his son Ferdinard Blum (1865-1959) (Boon *et al*,1994). The crosslinking of proteins produces a denaturing or coagulation of proteins in the tissue sample so that the semifluid state is converted into semisolid state and this semisolid state facilitates easy manipulation of tissues. Advancements have been made in the development of machines that aid in fixation, for example, the commonly employed conventional method which involves the use of automated tissue processors that have chambers for the completion of the whole tissue processing stages from fixation up until embedding of the tissue with molten wax ready for sectioning on a microtome (Kango and Deshmukh, 2011). However, there are still advancements made now that are seen to overtake the use of automated convectional machines as they are signified as slow and expensive processes that also use noxious chemicals with carcinogenic effects (Leong *et al*, 1987).

2.4 Tissue processing

The study of the morphological structure of cells, tissues and organs requires small sections of tissues that are preserved, sectioned, stained and mounted on a transparent glass slide. Morphology's main research tool is the microscope, hence, for tissues to be observed under a microscope, they have to be transparent for light and electrons to pass through. To make the tissues transparent they are cut in thin slices and mounted on a glass slide, therefore, for thin sections to be cut, tissues have to be hard and preserved from decay through fixation and embedding with paraffin wax. The process ensures the treatment of a tissue necessary to impregnate it into a solid media so that the tissue is rendered sufficiently firm yet elastic for the tissue sections of desired thickness to be sectioned on a microtome. It is not only employed for tissue sections as sections can also be produced by means of cryostat of freezing microtome on frozen tissues. However, in this study we are mainly focused on the treatment of tissues impregnated with paraffin wax.

The whole process that involves the production of a thin tissue mounted on a glass slide for diagnostic purposes involves five key stages which are fixation, dehydration, embedding sectioning, staining and lastly mounting. Before proceeding on tissue processing, as soon as the tissue is received it is very important that the tissues are labelled so as to avoid any confusion regarding duplication of the same name or giving wrong diagnosis to the patient. The labels should

remain throughout the entire process and later as permanent record keeping. Fixed tissues are embedded in a solid medium by first removing water which is then replaced by any solid medium such as paraffin wax so that the tissue is rendered firm enough to enable thin sections to be cut while at the same time the tissue is soft enough to enable microtome knife to cut sections. The embedding media has to thoroughly penetrate the tissue in fluids form so that it solidifies with no damage to the tissue. The man who introduced paraffin embedding in 1869 was Edwin Klebs. To improve the embedding process, hardening and dehydration were necessary. The most satisfactory embedding medium used in tissue processing is paraffin wax.

Tissue processing can be performed manually (hand processing), but where multiple specimens should be dealt with it is more convenient and much more efficient to use an automated tissue processing machine (a tissue processor) in a process called conventional tissue processing. These devices have been available since the 1940's (Leong and Gilham, 1989) and have slowly evolved to be safer in use, handle larger specimen numbers, process more quickly and to produce better quality outcomes. Since the development of these machines up to the current date, conventional tissue processing has been considered as the gold standard upon which other techniques emerge from. There are two main types of processors, the tissue-transfer (dip and dunk) machines where specimens are transferred from container to container to be processed, or the fluid-transfer (enclosed) type where specimens are held in a single process chamber or retort and fluids are pumped in and out as required. Most modern fluid-transfer processors employ raised temperatures, effective fluid circulation and incorporate vacuum/pressure cycles to enhance processing and reduce processing times.

However, in this study the dip and dunk machines were used where specimens are transferred from container to container with varying reagent concentration through automated rotation of a steel basket with tissue samples. The need for a reduction in tissue processing time has led to the realization of alternative methods that can produce histological slides within a short period of time. Time is of the essence in any laboratory, but even more so when sample turn-around time and patient care is critical. Striking a balance between the time required to optimally process tissue and the turn-around time preferred by pathologists or clinicians is an ongoing effort in any histology laboratory. Studies by Kennedy and Foulis (1989) proved that the rate of fixation can be improved by using a microwave for assisting in the faster fixation of tissues as they managed to fix their

tissues for less than 3 hours as compared to conventional fixation which can take 21-24 hours (overnight processing). This made them to draw a conclusion that 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.

2.4.1 The general importance of tissue processing

Patients rely on quality tissue processing and most laboratory supervisors would emphasize to their staff the importance of tissue processing. It is worthwhile to stress that use of an inappropriate processing schedule or the making of a fundamental mistake (perhaps in replenishing or sequencing of processing reagents) can result in the production of tissue specimens that cannot be sectioned and therefore will not provide any useful microscopic information. This can be disastrous if you are dealing with diagnostic human or animal tissue where the whole of the specimen has been processed (Ruehl-fehlet, 2000). There will be no spare tissue more so there will be no diagnosis but there is a patient or the owner of an animal to whom an explanation must be provided. McArdle (2014) proposed that, although mechanical or electrical faults occasionally occur in tissue processors, processing mishaps where tissues are compromised, mainly occur because of human error. It is important to emphasize the value of proper education and training for those carrying out tissue processing and the need to apply particular care when setting up a processor for any processing run (Minot, 1989).

2.5 Overview of the steps in tissue processing for microscopy

2.5.1 Obtaining a Fresh Specimen for Processing

Fresh tissue specimens will come from various sources. It should be noted that they can easily be damaged during removal from patient or an animal. It is important that they are handled carefully and appropriately fixed in a proper fixative as soon as possible after dissection. Ideally fixation should take place at the site of removal, perhaps in the operating theatre, or, if this is not possible, immediately following transport to the laboratory. Materials obtained for tissue processing come from the surgery and autopsy room, therefore in the surgery room they are attained as biopsy tissue which is a small piece of lesion or tumour which is sent for diagnosis or a full removal of the lesion or the tumour. They are also obtained from the autopsy room for the study of disease as sections of tissues dissected from specific tissues of an animal or a human carcass.

2.5.1.1Types of Tissue Specimens

Specimens can be prepared as whole mount, sections and smear:

a) Whole mount

These are preparations of the entire animal for example fungus, parasite. These preparations are not more than 0.2 - 0.5 mm in thickness so as to permit light electrons to pass through under microscope so as to facilitate visualization.

b) Sections

Sections are used in the majority of tissue preparation in biomedical and veterinary departments (Bancroft and Gamble, 1995). The tissue section is cut about 3 - 5mm thick in piece and processed so that 4 - 5 microns are cut on a microtome. These are stained and permanently mounted on a glass slide. To cut sections on the microtome, the tissue must be hard enough to not get crushed and they are two methods that have since been designed to harden the tissue. One is freezing the tissues in cryostat and the other is by embedding them in a hard material such as paraffin wax.

c) Smear

Smears are made from blood, bone marrow or any other fluid for example pleural acid and semen. These are immediately fixed in alcohol to preserve the cellular structure and then stained and mounted.

2.5.2 Tissue Preservation

Once tissues have been obtained, they should be placed in a liquid fixing agent (fixative) such as formaldehyde solution (formalin). This will slowly penetrate the tissue causing chemical and physical changes that will harden and preserve the tissue and protect it against subsequent degradation by enzyme by a process known as autolysis (the breakdown of plant or animal tissue by the action of enzymes contained in the tissue affected; self-digestion). There are a limited number of reagents that can be used for fixation as they must possess properties that make them suitable for tissue preservation. For example, tissue components must retain some chemical reactivity so that specific staining techniques can be applied subsequently. Formalin, usually as a phosphate-buffered solution, is the most popular fixative for preserving tissues that will be processed to prepare paraffin sections. Ideally specimens should remain in fixative for long enough for the fixative to penetrate into every part of the tissue and then for an additional period to allow

the chemical reactions of fixation to reach equilibrium (Bhushan and Bharat, 2010). Most laboratories will use a fixation step as the first station on their processor and following fixation, the specimens may require further dissection to select appropriate areas for examination. Specimens that are to be processed are usually placed in suitable labelled cassettes (small perforated baskets) to segregate them from one another. The duration of the processing schedule used to process the specimens depends on the type and dimensions of the largest and smallest specimens, the processor employed, the solvents chosen, the solvent temperatures and many other factors (Carson, 2006).

2.5.3 Dehydration

The process of dehydration involves the removal of water from tissues, this is because melted paraffin wax is hydrophobic (immiscible with water), hence most of the water in a specimen must be removed before it can be infiltrated with wax (Minot, 1886). This process is commonly carried out by immersing specimens in a series of ethanol (alcohol usually 60% to 100%) solutions of increasing concentration until pure, water free alcohol is reached. Ethanol is miscible with water in all proportions hence all the water in the specimen is progressively replaced by the alcohol during dehydration. A series of increasing concentrations is used to avoid excessive distortion of the tissue as a result of using a single concentration. This process is also affected by a number of factors and one of them is temperature. Leong and Gilham (1989) proposed that the basis of tissue processing lies behind the diffusion of chemicals in and out of the cells, therefore alteration of temperature and agitation procedure will have both negative and a positive impact on the processing of tissues.

2.5.4 Clearing

Unfortunately, although the tissue is will essentially be water free after the dehydration step, tissues still cannot be infiltrated with wax since wax and ethanol are largely immiscible (they totally do not mix). Hence an intermediate solvent that is fully miscible with both ethanol and paraffin wax must be employed. This solvent should have properties that will enable it to displace the ethanol in the tissue, which in turn will be displaced by molten paraffin wax (Ludo wing and Aschoff, 1964). This stage in the processing of tissues is called clearing and the reagent used is called a clearing agent. The term clearing was chosen because many (but not all) clearing agents impart an optical clarity or transparency to the tissue due to their relatively high refractive index (Giberson and Demaree, 1999). Another important role of the clearing agent is to remove a substantial amount

of fat from the tissue which otherwise presents a barrier to wax infiltration. A popular clearing agent is xylene and multiple changes are required to completely displace ethanol (Kango and Deshmukh, 2011).

2.5.5 Wax Infiltration

Once tissues have been cleared, they can now be infiltrated with a suitable histological wax. Although many different reagents have been evaluated and used for this purpose over many years, the paraffin wax based histological waxes are the most popular. A typical wax is liquid at 60°C - 75°C and can be infiltrated into tissue at this temperature then allowed to cool to 20°C where it solidifies to a consistency that allows sections to be consistently cut. These waxes are mixtures of purified paraffin wax and various additives that may include resins such as styrene or polyethylene (Ruehl-fehlet, 2000). (Dye, 1991) proposed that, it should be appreciated that these wax formulations have very particular physical properties which allow tissues infiltrated with the wax to be sectioned at a thickness down to at least 2 µm, to form ribbons as the sections are cut on the microtome, and to retain sufficient elasticity to flatten fully during flotation on a warm water bath. Histological waxes such as paraplast are popular and multiple changes are required to completely displace the clearing agent (Hopwood *et al*, 1984).

2.5.6 Embedding or Blocking Out

Now that the specimen is thoroughly infiltrated with wax it must be formed into a block which can be clamped into a microtome for section cutting. This step is carried out using an embedding center where a mold is filled with molten wax and the specimen placed into it. The specimen is very carefully orientated in the mold because its placement will determine the plane of section, an important consideration in both diagnostic and research histology and pathology (Boon and Ouwerkerk-Noordam, 1986). A cassette is placed on top of the mold, topped up with more wax and the whole thing is placed on a cold plate to solidify. When this is completed the block with its attached cassette can be removed from the mold and is ready for microtomy. If tissue processing is properly carried out, the wax blocks containing the tissue specimens are very stable and represent an important source of archival material (Hopwood *et al*, 1984). After tissues have been blocked, they can then be sectioned using a rotary or fully automated microtome, usually with a thickness of 4 - 5µ. Before tissue can be stained and viewed, it must be prepared so that a very thin section, only one cell thick, can be cut and placed onto a microscope slide.

2.5.7 Staining

Staining is a secondary technique used in microscopy to enhance contrast in the microscopic visualization. Stains and dyes are used in biology and medicine to highlight structures in biological tissues for viewing, often with the aid of different microscopes. Stains may be used to define and examine bulk tissues (highlighting, for example, muscle fibers or connective tissue), cell populations (classifying different blood cells, for instance), or organelles within individual cells (Dye, 1991). Staining tissues with special stains play a critical role in tissue-based diagnosis or research. By coloring otherwise transparent tissue sections, these stains allow highly trained biologists, pathologists and researchers to view, under a microscope, tissue morphology (structure) or to look for the presence or prevalence of cell types, structures or even microorganisms such as bacteria. In the pathology laboratories, staining is usually done with haematoxylins and eosin stain (H&E) to reveal the underlying tissue structures and conditions (Boon and Ouwerkerk-Noordam, 1986). However, there are other stains that are referred to as special stains and have long been used as alternative staining techniques when the H&E does not provide all the information the pathologist or researcher needs. Haematoxylin and Eosin is commonly employed because of its ability to stain properly and it achieves this by clearly staining cell structures including the cytoplasm, nucleus, and organelles and extra-cellular components (Minot, 1989). This information is often sufficient to allow a disease diagnosis based on the organization (or disorganization) of the cells and also shows any abnormalities or particular indicators in the actual cells (such as nuclear changes typically seen in cancer). Bhushan (2010) proposed that even when advanced staining methods are used, the H&E stain still forms a critical part of the diagnostic picture as it displays the underlying tissue morphology which allows the researcher to correctly interpret the advanced stain. In a clinical histology laboratory, all specimens are initially stained with H&E and special or advanced stains are only ordered if additional information is needed to provide a more detailed analysis, for example to differentiate between two morphologically similar cancer types. Because of the volume of H&E staining needed, most clinical laboratories use fully automated systems and manual staining is now rare, however, it is currently seen being replaced by the employment of highly sophisticated microwave ovens designed for fast and more proper staining. To some researchers (Bendayan, 1987), microwave assisted staining produced better results than conventional staining (use of automated stainers) but however Pennycook *et al*. (2011) proposed that microwave staining can have some limitations such as poor respondent tissues to stains as a

result of absence of standardized temperature, power control and non-availability of vacuum processing. In this study microwave assisted processing and staining was conducted to try and determine if tissues will respond well to stains by using a domestic microwave during staining and then compare the outcomes with those stained by automated means, more so to determine if microwave staining can shorten the turnaround time.

2.6 Important Factors Affecting Tissue Processing

Before tissues can be utilized for important biomedical and drug discovery research, they must first be properly prepared. Tissue processing protocols are intended to remove water from the tissues, exchanging the water with supporting mediums that increase tissue rigidity and ease of preparation by sectioning. Better tissue sections reduce damage to sensitive structures within the tissue, and so improve their use for in-vitro research (Culling's *et al* 1985). Boon and Ouwerkerk-Noordam (1986) asserted that samples processing protocols should follow a series of well-established steps. When tissue is immersed in fluid reagents, interchange occurs between the tissue fluids and the fluid medium hence there are key factors that impact the rate of interchange between the fluids which may in turn reduce the turnaround time of processing tissues and also affect the morphological outcomes of the samples prepared leading to an impaired diagnosis (Ruehl-fehlet, 2000).

2.6.1 Agitation

To increase the rate of fluid exchange of infiltrating agents, there is need to maximize the tissue surface area. Agitation using manual or automated processors increases the flow of fresh fluids in and around the tissues therefore snowballing reagent exchange (Kango and Deshmukh, 2011). Most tissue processing protocols such as the one utilized in this study use automated processors with rotary oscillation mechanisms to speed fluid exchange. Without agitation, tissues tend to settle to the bottom of the processing device or become too tightly packed, therefore reducing surface area available for fluid exchange (Giberson and Demaree, 1999). According to histology experts, efficient agitation can reduce overall processing time by 30 percent (Winsor, 1994)

2.6.2 Heat

Elevated tissue processing temperatures can increase the rate of fluid penetration and exchange. However, heat must be carefully applied. Heat increases molecular kinetic energy and diffusion rates, which decreases solution viscosity (Bancroft and Gamble, 1995). Too much heat causes

tissue shrinkage, hardening and brittleness, and negatively affects the research. Conversely, at low temperatures, tissue structures are stabilized against solvent effects, such low temperatures also increase the viscosity of reagents used in tissue processing protocols, reducing the rate of diffusion and therefore increasing processing time (Leong and Gilham, 1989). Giberson and Demaree (1999) proposed that by applying moderate temperatures, in the range of 37° - 65°C, for a limited time can speed up tissue processing protocols although careful attention must be paid to this step to limit tissue shrinkage and viability.

2.6.3. Vacuum and Pressure

Reduced pressure can increase the infiltration rate and decrease time needed to complete steps in tissue processing. Studies show that vacuum will extract reagents from tissue only if these fluids are more volatile than the reagent being replaced (Clayden, 1997). Vacuum application during tissue infiltration improves processing quality. It can aid in removal of trapped air from, for example, lung tissue, or other porous tissue. Using vacuum during tissue processing protocols can reduce the infiltration time when dealing with dense and fatty tissue specimens (Dye, 1991). Hopwood *et al.*, (1984) proposed that top quality, carefully sourced and properly prepared tissue specimens will accelerate tissue architecture examination, biomedicine and drug discovery research programs therefore there is still a need for scientists to continue trying to develop protocols that produce quality specimens at a reduced turnaround time.

2.7 Conventional Tissue Processing

Conventional tissue processing is as old as 100 years and still remains the gold standard against which all new technologies and methods are being assessed to current date (Bhushan, 2010). The first technique known as routine manual tissue processing is a laborious and tedious manual sequence that is completed in 21 to 24 hours. Although developments in this modern day have been done to come up with automated machines that at least have an excellent performance, however, the technique consumes a lot of time for processing hence the name overnight processing. A waiting period of 1 day or longer for a pathologic diagnosis following any surgical procedure is customary (Shruthi, 2013). Anxiety from patients or animal owners at veterinary or pathology labs, 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 (Pennycook *et al.*, 2011). In particular, the tissue processing stage of

diagnostic tissue preparation takes 8 hours or longer. The second technique known as rapid manual tissue processing is similar to the routine manual tissue processing equivalent in terms of steps but for shorter durations, requiring 3 – 4 hours. Its advantages are its reliability and its inexpensive nature. A major concern associated with this conventional processing method is the toxicity of reagents used, especially formaldehyde and xylene. Formaldehyde, for instance, is known to irritate conjunctiva and the respiratory mucosa. Locard, (2000) argued that formaldehyde can also cause allergic dermatitis and asthma and has been implicated in the etiology of cancer of the oropharynx and the respiratory tract. There is also a greater degree of tissue distortion and shrinkage. Changing the standard technique for tissue fixation and preparation from the currently used overnight processing to same day microwave tissue preparation could substantially reduce turnaround times, permitting same day diagnosis that would facilitate patient diagnosis and management on a one-day basis (Dye, 1991). This improvement in turnaround time could reduce costs associated with diagnosis and patient dissatisfaction.

2.8 Microwave

Microwaves are non-ionizing radiations with electromagnetic properties. Their frequencies range from 300 MHz to 300 GHz and wavelengths from 1 mm to 1 m. All domestic microwaves operate at 2.45 GHz, corresponding to a wavelength in vacuum of 12.2 cm (Kok and Bon, 1990). Microwave excitation of molecules is a process in which applied energy penetrates the tissues to a greater depth as compared to the other methods of tissue processing. Dipolar molecules, which are present in the field are forced to oscillate and this leads to an increase in thermal agitation (Leong, 1993). The kinetic energy thus generated is converted into heat energy. As in other forms of tissue processing, here too, diffusion is the key factor. The basic effect of microwave irradiation is stimulation of diffusion and enhancement of reaction rates with internal heating, which makes them suitable for use in processing tissues. These properties make it relevant to investigate domestic microwave assisted tissue fixation, processing and staining to examine if it can produce results comparable to conventional processing by automated means and also to determine if it can also reduce the turnaround time.

2.8.1 Application of microwave in tissue processing.

The physicochemical basis of fixation and tissue processing lies in the diffusion of reagents into the substance of the tissue to be processed hence it is suggested by a number of authors Boon et

al. (1989); Giberson and Demaree (1999) and Leong and Gilham (1989) that a microwave can be ideal whenever fast processing of tissues is needed. This is because it has a reduced turnaround time as it has non-ionizing radiations that have electromagnetic properties which lead to faster heating of reagents thereby permitting fast diffusion to occur. A study conducted by Carson (2007) proposed a reduced turnaround time from days to 72 minutes and same day reporting when he processed tissue samples from a variety of organs using a specific laboratory microwave system specifically designed by Microwave Materials Technologies, Knoxville (2001). This suggests that a microwave has brought revolutionary improvement in biomedicine, pathology and drug development as it has proved to shorten the time of tissue processing from days to minutes. Hence, this fulfils the need of patients as well as the physicians by improving the use of reagents while reducing or eliminating their toxicity to meet the demands of surgeons. Microwave irradiation reduces time required for incubation in fixation of tissues and the minimization of incubation time in fixatives reduce disruption of tissues morphology.

Unlike conventional heating, this irradiation effect occurs simultaneously throughout the whole material being microwaved. The application of microwave energy in histotechnology was first recognized by Mayers in 1970, who successfully fixed human and mouse tissue with a microwave generator used in physiotherapy (Valle, 1986). At the same time, Stavinoha employed microwaves to stop enzymatic degradation by acetylcholinesterase in rodent brain. The most detailed early studies were published by Bernard in 1974, who successfully produced fixation of a wide variety of organs by microwave irradiation (Hopwood *et al*, 1984). This was supported by the findings of Boon *et al.* (1986), Chaudhari *et al.* (2000), and Morales *et al*. (2002) who found that the tissue architecture, stroma, secretory products, cell and nuclear morphology were same between conventionally processed and microwave processed tissue, except that the microwave processed tissue showed a reduced turnaround time as compared to the manually processed sections. This study tried to document the effects of using domestic microwave on fixation of randomly selected rat tissues and to compare the outcomes of tissues with paired samples processed by conventional means.

Leong and Gilham (1989) studied an ethanol-based fixative with microwave stimulated processing in post mortem material. Their results indicated good preservation of morphological details, histochemical and antigenic properties of the tissue which were comparable with formalin fixed

material. The rapid stabilization of the tissues by microwave irradiation was very useful in biomedicine and forensic pathology. The microwave irradiation prevented autolysis and putrefaction and reduced the artefacts on the subject tissues. Ethanol was found to be a good preserving agent of nucleic acid and protein material. Leong and Gilham (1989) further reported that the use of an ethanol-based fixative provided a safer working environment. Various investigators analyzed the use of domestic microwave for urgent tissue processing and the concurrent use of ethanol and isopropyl alcohol and found that ethanol was a good dehydrant and isopropyl alcohol was effective as both dehydrant and clearing agent, the slow rate of diffusion being overcome by microwave heating. In this study alcohol and isopropyl alcohol were used since they eliminated the use of formalin and xylene that have potential carcinogenic effects. The former is a good fixative and the latter a good dehydrant and a clearing agent.

2.8.2 Application of microwave on staining

Obtaining good histological images for successful interpretation is largely governed by good sample preparation and staining. Staining of tissue sections and cell preparation is based on diffusion of dye into the tissue and its binding to the substrate (Bhushan, 2010). Microwave irradiation has been beneficial for both. Microwave irradiation can be applied for accelerating routine, special, metallic, as well as immunofluorescent stains (Clyden, 1971). Staining methods that normally take minutes can be done in a microwave oven in seconds; those that take hours and minutes or days and weeks can be completed in a matter of hours using microwave techniques. The optimum temperature for most non-metallic stains such as Congo Red and Masson's Trichrome is between 55ºC and 60ºC and for metallic stains between 60ºC and 75ºC such as Weigert's van Gieson iron haematoxylin, Verhoeff's van Gieson iron haematoxylin and Haematoxylin eosin (H and E) (Erko, 2008). Microwave-accelerated processing is as effective as slower traditional staining, has the potential of reducing the time up to 70% and sections stain identically with several methods such as Periodic acid – Schiff's, Van Gieson, Congo red, Masson's trichrome, Alcian blue, Mayer's mucicarmine, and silver methods (Hopwood *et al*, 1984).

2.9 Basis of the study

During the last two decades, as a consequence of the introduction of microwave energy into biology laboratories, progress has been made in the development of rapid processing methods and

the elimination of toxic reagents (Cullings, 1985). Methods reported so far, however, are neither sufficiently practical nor shorten tissue-processing time to the extent required for their widespread acceptance (Bhushan, 2010). Time is important in any laboratory, but even more so when sample turn-around time and patient care is critical. Striking a balance between the time required to optimally process tissue and the turn-around time preferred by pathologists or clinicians is an ongoing effort in any pathology laboratory. If any step of tissue processing is rushed, subsequent processing steps may fail, and tissue will be under-processed and even undiagnosable. The present study was conducted to document the usefulness of domestic microwave assisted in rat tissue specimens over conventional processing and staining comparing their histologic quality and the total turnaround time. Morphometry was done on both conventional and microwave processed sections to assess the cellular details, cytoplasmic detail, colour intensity and nuclear detail to potentially improve reproducibility of microscopic diagnosis of tissues.

CHAPTER 3. MATERIALS AND METHODS

The present study was aimed at assessing the microwave-assisted tissue processing and staining method so as to determine if it can replace the standard formalin-fixed and paraffin-embedded processing and staining technique which is referred to as conventional tissue processing.

Sources of materials

Specimens were selected from two laboratory rats (*Rattus norvegicus*) that were bought from the Central Veterinary Laboratory in Harare. The rats were subjected to euthanasia treatment using chloroform inhalation. They were then dissected using surgical kits borrowed from the Anatomy department of the Midlands State University and the dissected samples were collected and immediately preserved in 10% formalin (HCHO) (aproximatly4% formaldehyde) solution as shown in Figure 3.2, a process referred to as pre-fixing to prevent autolysis and decomposition of tissues. The samples were then photographed before being cut into equivalent halves to be processed by both conventional and microwave processing methods and subsequently stained with Hematoxylin and Eosin (H and E).

Figure.3.1. Pre-fixed tissues in 10% formalin solution.

3.1. Experimental design

The study involved eight randomly collected rat organs that were dissected to produce tissue samples with equivalent dimensions i.e. 5 mm × 5 mm ×4 mm thickness, in a process referred to as grossing (trimming of tissues into required dimensions).

Inclusion criteria

Only soft-tissues without bony component were processed.

The tissues processed were;

(i) Kidney, (ii) liver, (iii) small intestines, (iv) large intestines, (v) lungs, (vi) heart, (vii) spleen and (vii) muscle.

Exclusion criteria

Small and bony specimens e.g. blood vessels, oral and skin biopsies were excluded because shrinkage occurs in the smooth muscles especially the endothelium cells. Bancroft and Gamble (1995) proposed that bony cells require decalcification which requires a very long time, that is, 5–6 days with 10% formic acid; hence, this would be time consuming. The eight selected tissues from different organs, each with dimensions measuring 5mm × 5mm ×4mm, were divided into two groups. One group was labeled as the Control group (Group A) which was subjected to conventional processing as according to the Cullings (1974) method and the other as the Experimental group (Group B) which was subjected to processing by a domestic microwave (Ecco, Microwave Oven, MT2817, India). Both the experimental and the control groups consisted of eight different tissue samples producing a total of 16 slides. These paired samples, eight slides for each method, were put in labeled plastic cassettes and subjected to both microwave-assisted and conventional tissue processing.

Steps for both methods of tissue processing included:

1) Dehydration - the removal of water from tissue cells with alcohol,
2) Clearing - the use of clearing agents to remove alcohol from tissue cells because alcohol is not immiscible with paraffin wax,
3) Impregnation with paraffin wax to maintain the cell structural integrity.
4) Embedding with paraffin wax to make blocks so as to permit sectioning of tissues.

5) Microtomy - sectioning of tissue blocks to 4µm with a full automatic microtome.
6) Deparaffinization - to remove paraffin on tissues so as to allow for penetration of staining agents.
7) Haematoxylin and eosin staining on tissues to enhance tissue visualization under microscope.

3.2. Conventional and Microwave-assisted Tissue processing

3.2.1. Conventional tissue processing

The steps that were followed in the conventional tissue processing were in accordance with those outlined by Cullings (1974). This was followed by processing in a Dip and Dunk automatic tissue processor (ZHCHEING, CHINA) (Figure. 3.3). Formalin 10%, graded alcohol concentrations, xylene and paraffin wax were used according to the schedule in Table 3.1 below.

Table 3.1. Conventional tissue processing protocol

Step	reagent used	time taken (mins)
dehydration	70% alcohol	20 min
	85% alcohol	30 min
	95% alcohol	30 min
	95% alcohol	30 min
	100% alcohol	30 min
	100% alcohol	30 min
	100% alcohol	30 min
clearing	Xylene	40 min
	Xylene	30 min
impregnation	Paraffin wax	10 min
	Paraffin wax	20 min
	Paraffin wax	10 min
Total time taken		**310mins**

Plastic cassettes with tissues were inserted into a metal basket on one chamber of the processor to be fixed overnight in 10% formalin for 24 hours at room temperature from 10 am to 10 am the next day. The following morning, they were transferred to running tap water for 5 minutes to remove excess formalin and they were put back in the basket and processed by automatic means during the day. The automated processor had 12 chambers, one for a fixative (formalin), six for dehydrating agent (graded alcohol concentrations), two for a clearing agent (xylene) and three chambers for paraffin embedding. Tissues were processed through a dip and dunk mechanism at room temperature as a result of timely automatic rotation of the metal basket with tissues from 1 hour 30 minutes in three changes of absolute alcohol (100%) and 30 minutes in graded absolute alcohol i.e. 70%, 80% and90% for dehydration, two changes of 30 minutes in xylene as a clearing agent and 1 hour in paraffin wax by three changes for impregnation. Finally, the tissues were embedded to make tissue blocks on the Embedding center (KD-BMIV, ZHCHEING, CHINA) (Figure. 3.4) with paraffin at 75ºC ready for sectioning by a microtome.

Figure 3.2. A Dip and Dunk Automatic Tissue Processor (ZHCHEING, CHINA)

Figure 3.3. Tissue block on Embedding Centre (KD-BMIV, ZHCHEING, CHINA)

3.2.2 Conventional sectioning and staining

The conventional tissue staining procedure followed that in Culling (1985). Embedded tissues in block forms were sectioned to 4 µm by means of a Full Automatic Microtome (ZHCHEING, CHINA) (Figure 3.4) and ribbon like structures from the eight selected and sectioned tissues processed by conventional means were inserted on transparent glass slides which were put on the slide rack to be stained by automatic means using an Autostainer (ZHCHEING, CHINA) (Figure.3.5). This was followed by automatic dewaxing in xylene in two changes of 10 minutes each. The tissue sections were hydrated in water for five minutes. Slides were then transferred to hematoxylin stain solution for seven minutes and then placed in running tap water for three minutes for bluing. Sections were differentiated in absolute alcohol (two dips). They were water washed for five minutes and then transferred to a jar containing eosin stain solution for 30 seconds. They were then water washed for five minutes to remove excess stains and were later dehydrated in absolute alcohol (two changes) one dip each and were air dried and mounted with a coverslip using DPX mountant for microscopy and lastly, they were labeled.

Figure 3.4. Full Automatic Microtome (ZHCHEING, CHINA) cutting a tissue block

Figure 3.5. Autostainer (ZHCHEING, CHINA)

3.2.3 Microwave assisted tissue processing (Group B)

This procedure required the following equipment; an Ecco Microwave Oven (model MT 2817) (Figure 3.6) with a maximum output of 700 W, two microwave-resistant glass beakers of 200 ml each, 100% alcohol as dehydrating agent (Qualigens, Product no. 32407), 100% isopropyl alcohol as intermedium (Qualigens, Mumbai, India Product no. 26897), paraffin wax (Qualigens, Product no. 19215), thermometer, plastic tissue cassettes and a hand towel.

Figure3.6. Ecco Microwave Oven (model MT 2817) Maximum output 700W.

3.2.2.1 Standardization

A pilot study using 4 tissues (lung, large intestine, kidney and trachea) was conducted in accordance with the protocol by Klump *et al.* (2010) although they employed a specific type of laboratory microwave (Micro MED Mega Time Temperature microwave oven, Milestone, Bergamo, Italy). This was done to develop my own protocol to be used in processing tissues with a domestic microwave. Isopropanol (100%) was used as a clearing agent for microwave tissue processing instead of xylene. Xylene has a high boiling point and low microwavability since it takes double the time for the same amount of reagent to get heated. Hence, the four pre-fixed tissues were washed under tap water to remove excess formalin and these were transferred to a beaker containing 200 ml of 100% alcohol. The tissues were dehydrated in 100% alcohol for 15 minutes at 65^{o} C in two changes in a microwave with agitation at every three minutes to allow for rapid diffusion of molecules in and out of the cells. After 15 minutes tissues were removed and cleared in 100% isopropanol for 15 minutes at 75^{o} C in two changes with agitation followed by infiltration in liquid paraffin for 5 minutes at 65^{o} C. All the reagents were directly heated in the microwave except paraffin. Paraffin was melted to 75^{o} C on a hot plate separately and then placed in the microwave. The temperature of the reagents was recorded at intervals manually using a mercury thermometer. Tissue blocks were then made using the tissue embedment center, later sectioned, stained with hematoxylin and eosin and mounted on a glass slide to observe if tissues could be analyzed on a stereo microscope. The tissues showed shrinkage and the staining intensity was low, hence, the amount of time when tissues were subjected to dehydration was adjusted. Sectioning of tissues was also difficult suggesting that the time for impregnation with paraffin wax

was inadequate. Therefore, the time allocation for each reagent and process were adjusted accordingly to come up with a standard procedure as shown in Table 3.2 below.

3.2.2.2 Standardized procedure

A total of eight prefixed tissue samples were water washed for five minutes to remove excess formalin from the tissue to reduce alcohol and formalin interference. The tissues were transferred to a beaker containing 200 ml of 100% absolute ethanol for dehydration and the microwave was set at 300 Watts. The tissues were dehydrated in 100% alcohol for 15 minutes at 65^{o} C in two changes in a microwave with agitation at every three minutes to allow for rapid diffusion of molecules in and out of the cells. The tissues were then transferred to a beaker containing 100% isopropyl alcohol which acted as a clearing agent to remove alcohol at 75^{o} C for two changes each for 10 minutes with the same microwave settings. Clearing was essential because alcohol is not immersible with paraffin wax. The specimens were then transferred to a beaker containing 200 ml of molten paraffin wax for impregnation. The paraffin wax was heated separately on a hot plate before being inserted into a microwave because wax has a lower boiling point. The tissues were then embedded in molten paraffin wax immediately to form tissue blocks that permit sectioning of tissues.

Table 3.2. Protocol for domestic microwave tissue processing

Step	**Reagent used**	**Oven temperature (℃)**	**Duration (mins)**
Dehydration	100% ethanol	65	10
	100% ethanol	65	10
Clearing	100% isopropyl alcohol	75	10
	100% isopropyl alcohol	75	10
Impregnation	Molten paraffin wax	65	10
	Molten paraffin wax	65	10
Total time taken			60

3.2.4 Microwave hematoxylin and eosin staining

The ribbon sections obtained from sectioning eight tissues from both groups were inserted on glass slides and dewaxed in an oven at 80 °C for 10 minutes. This allowed the wax to melt from the tissues leaving the tissues exposed on the slide for staining to take place. A hundred milliliters of hematoxylin stain were put in a heat resistant beaker and the slides containing tissue sections were placed in the beaker then placed in the microwave oven at 300 W for 30 seconds. The slides were immediately transferred to running tap water for bluing. The sections were then differentiated in acid alcohol (two dips) and then water washed in running tap water for five minutes. A hundred milliliters of eosin stain were taken into a beaker and the sections were transferred to it. The Microwave was then set at 300 W for 30 seconds. The sections were water washed in running tap water for three minutes to remove excess stain. They were then dehydrated in absolute alcohol (two changes) for 60 seconds. Finally, the slides were air dried, mounted and then labeled.

3.2.5 Technical Grading

The stained slides from each group were randomly numbered for a blind study and issued to two observers. The observers were tasked with judging four parameters namely; staining colour intensity and cellular, cytoplasmic and nuclear details. Each observer was to grade each parameter based on four categories i.e. Excellent/Good/Fair/Poor in a data sheet. Grading involved observing slides on a stereo microscope (XSZ-107BN Series) that uses an ISC Capture software which is linked to a computer (Hewlett-Packard (HP) ProBook, 4510s) (Figure 3.7).

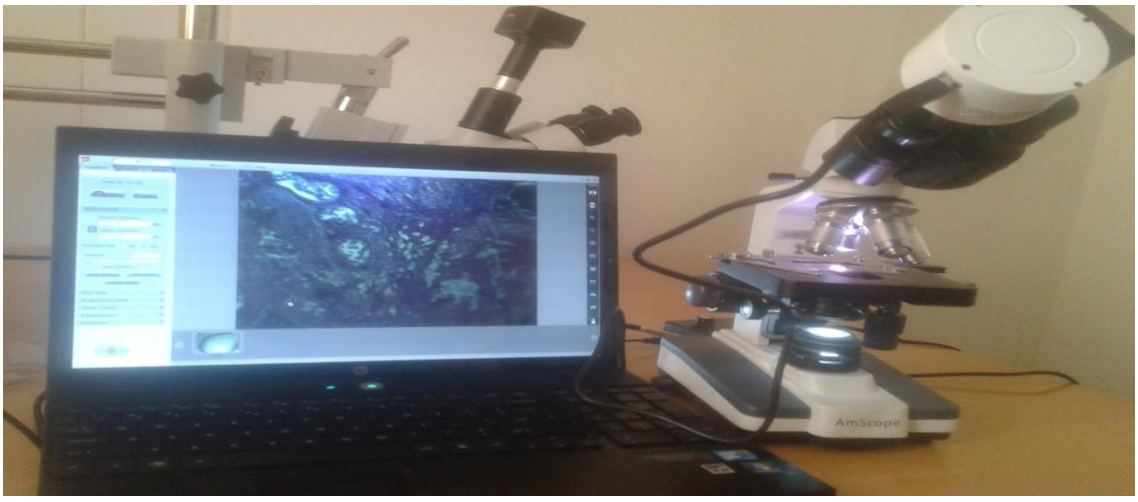

Figure 3.7. Stereo microscope (XSZ-107BN Series) linked to an HP-ProBook 4510s with ISC Capture software.

The ISC capture software was used to capture tissue images that were used in the evaluation of each slide from each particular group (experimental A and control B group). Gradings were given a score of 4, 3, 2 or 1 depending on the observer's judgment and the observers were referred to as O1 and O2 respectively. Histological experts scored the slides for each criterion i.e. Mr. F. Chibhabha (Anatomy Lecturer and Department Chairperson) and Mr. T. Burukai (Anatomy Senior Technician) who are both members of the Department of Anatomy at Midlands State University Histology laboratories by applying the scale of 1 to 4. The scoring criterion was constructed as follows:

Score (1) was given for below average (Poor or indistinct) – if none of the parameters as explained below are satisfactory

Score (2) was given for average (Fair) – if one or two parameters are satisfactory

Score (3) was given for above average (Good)– if three parameters were satisfied

Score (4) was given for excellent (distinct) - if three or more parameters were satisfied.

Criteria

a) For cellular detail and cytoplasmic evaluation, the following were evaluated; 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 inflammatory cells. If most features were present, then it was called distinct but if there was granularity of cytoplasm, focal condensation of stroma, cellular outline blurred, mucin not seen, red blood cells lysed (focal or generalized) then it was called indistinct.

b) Slides were evaluated for nuclear detail on the basis of chromatin condensation, prominent nuclear membrane, crisp staining of the nucleus and mitotic activity if appreciable. It was distinct if all features were appreciated and indistinct if smudging and pyknosis of nuclei were seen.

c) Staining of tissues was evaluated as poor if the tissue failed to take up stain adequately, stained unevenly or had artifacts in processing or staining. Fair 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.

3.3 Turnaround times for microwave tissue processing vs conventional tissue processing.

There was a suggestion in previous work by Cullings et al. (1985), Giberson and Demaree (1999) and Bancroft and Gamble (1995) that the quality of structural preservation of tissue to facilitate staining and visual observation on a microscope can be determined by the choice of reagents and exposure time of tissues to the reagents during processing time. The physico-chemical basis of tissue processing is the diffusion of reagents to the substance of the tissue to be processed and can be accelerated with application of heat. Therefore, another aim of this study was to evaluate the turnaround time for completion of processing tissues under a domestic microwave oven and automated machines.

To evaluate the turnaround times for microwave against conventional processing a stopwatch was used to time each and every step taken during processing tissues with both either of the methods. The steps included dehydration, clearing, impregnation, embedding, Deparaffinization and staining with hematoxylin and eosin (H and E). Thus, time intervals for each step were timely recorded and the overall processing time was also documented. Pre-fixing time for tissues soon after dissection of the rats was not included in the time comparisons as this was a crucial step for both methods. Pre-fixation time of tissues was not under evaluation also because tissues must be quickly preserved soon after dissection to halt the metabolic activities of proteins within tissues, that is, to stop autolysis as a result of enzyme activities. Post fixation time was taken from the onset of actual fixation in microwave and on automated microtomes and this was included in the overall processing time comparisons.

Statistical Analyses

Data obtained from the 16 specimens after evaluation by all two observers was subjected to statistical analysis to determine whether there was any significant difference in the staining color intensity, cellular, cytoplasmic and nuclear details among the two techniques. Observed data was analyzed by SPSS 21 and scores obtained for both the techniques were presented as scores with mean and standard error. Measure of agreement was calculated using Kendall's Coefficient of Concordance to check the extent to which observations tallied to factor out observer bias. Independent samples t-test was carried out on the four parameters test for the quality of DMTP

and CTP for the independent samples. A type 1 error level (α) of 5% was used as the level of significance.

CHAPTER 4: RESULTS

4.1 Staining intensity

Mean scores were calculated for staining intensity. Values of 1.9 ± 1.1 for DMTP and 3.1 ± 0.8 for CTP were obtained. An independent samples t test showed that there was a significant difference between the two means, t (14) = 2.297 (p= 0.038, p < 0.05) (appendix 1d).

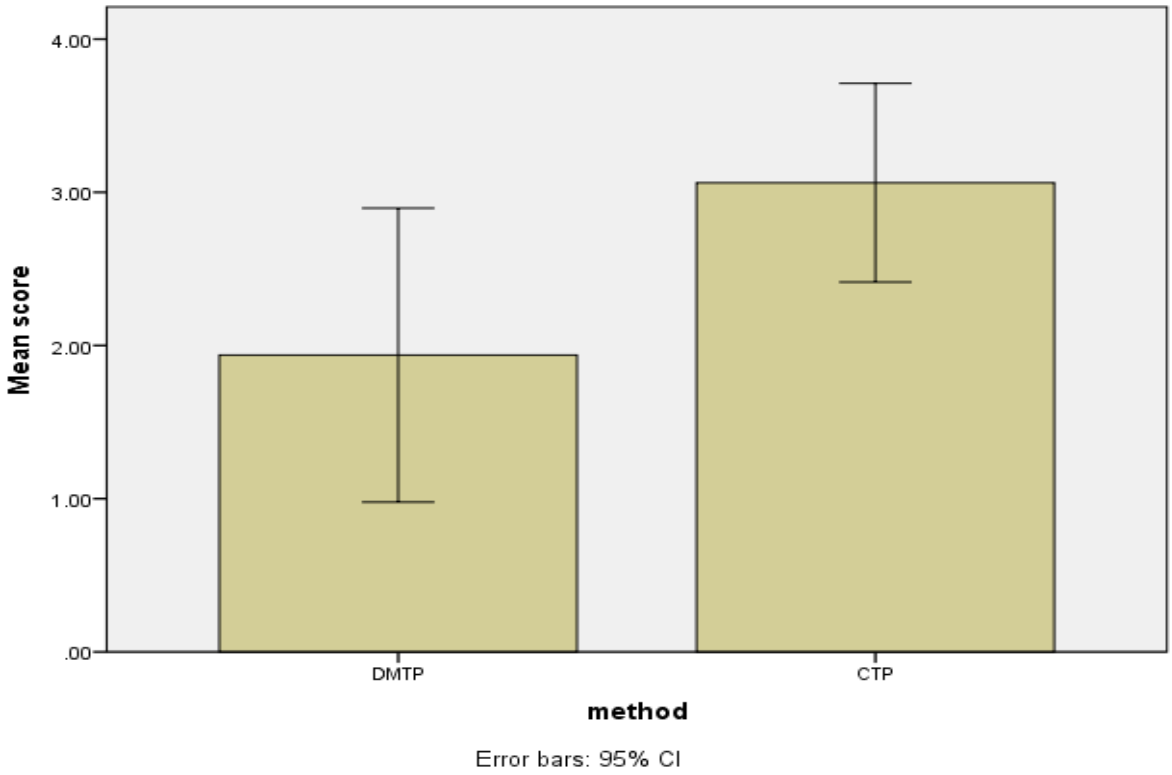

Figure 4.1: A comparison of staining intensity mean scores for domestic microwave method (DMTP) and conventional method (CTP).

There was therefore a higher mean score on tissues processed by conventional means as compared to the domestic microwave. This indicated that the domestic microwave method was less efficient in regard to staining tissues because the slides produced from the stained tissues were of poorer quality than those produced from the conventional processing method

4.2 Cellular detail

Values of 1.5 ± 0.9 for DMTP and 2.9 ± 1.2 for CTP were obtained. An independent samples t-test showed a significant difference between them with values t (14) = 2.668 (p= 0.018, p < 0.05) (appendix 1a).

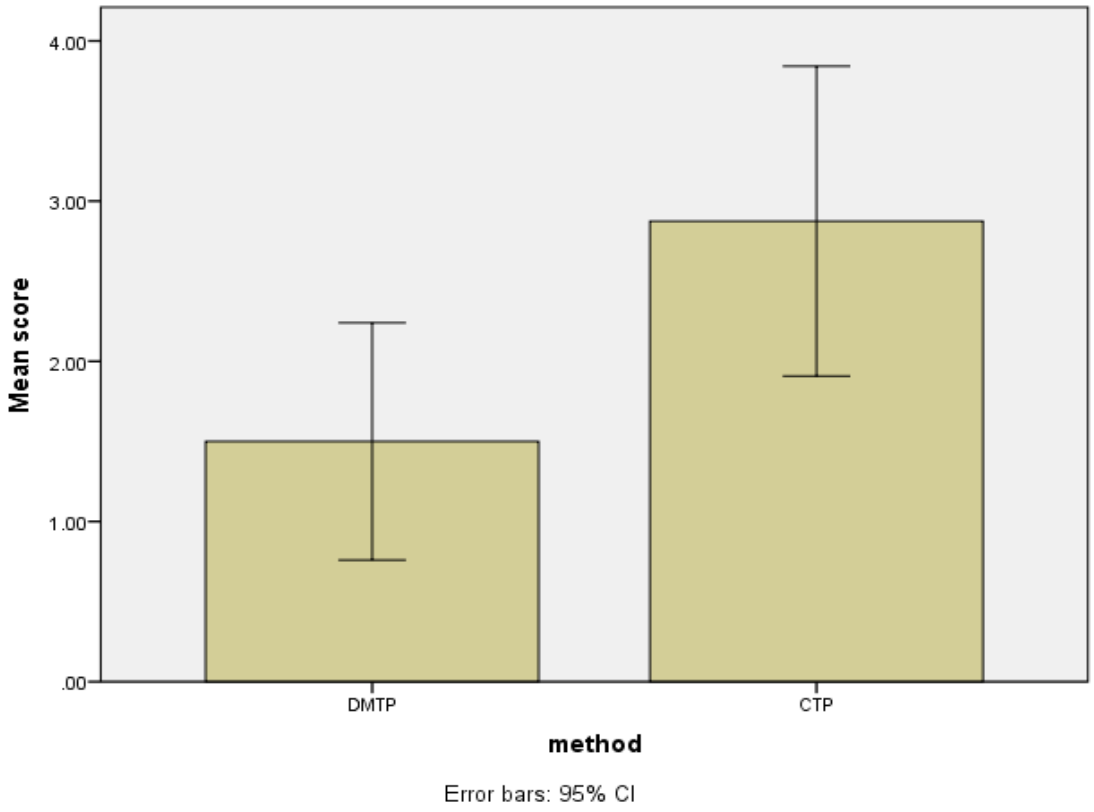

Figure 4.2. Cellular detail mean scores for the domestic microwave method (DMTP) and the conventional method (CTP).

There was, therefore, a higher mean score (2.9 ± 1.2) on tissues processed by conventional means as compared to the domestic microwave. This indicated that the domestic microwave was also inefficient on producing enough cellular detail in tissue slides. The technique did not produce slides of good quality as those produced by the conventional processing method.

4.3 Nuclear detail

Mean scores were obtained on nuclear detail with values of 1.1 ± 0.17 for DMTP and 1.8 ± 0.96 for CTP. There was a significant difference between these two means according to an independent samples t-test, t (14) = 2.170, (p= 0.048, p < 0.05) (appendix 1b). Therefore, this pointed to the fact that there was a significant difference between the two processing methods on staining intensity.

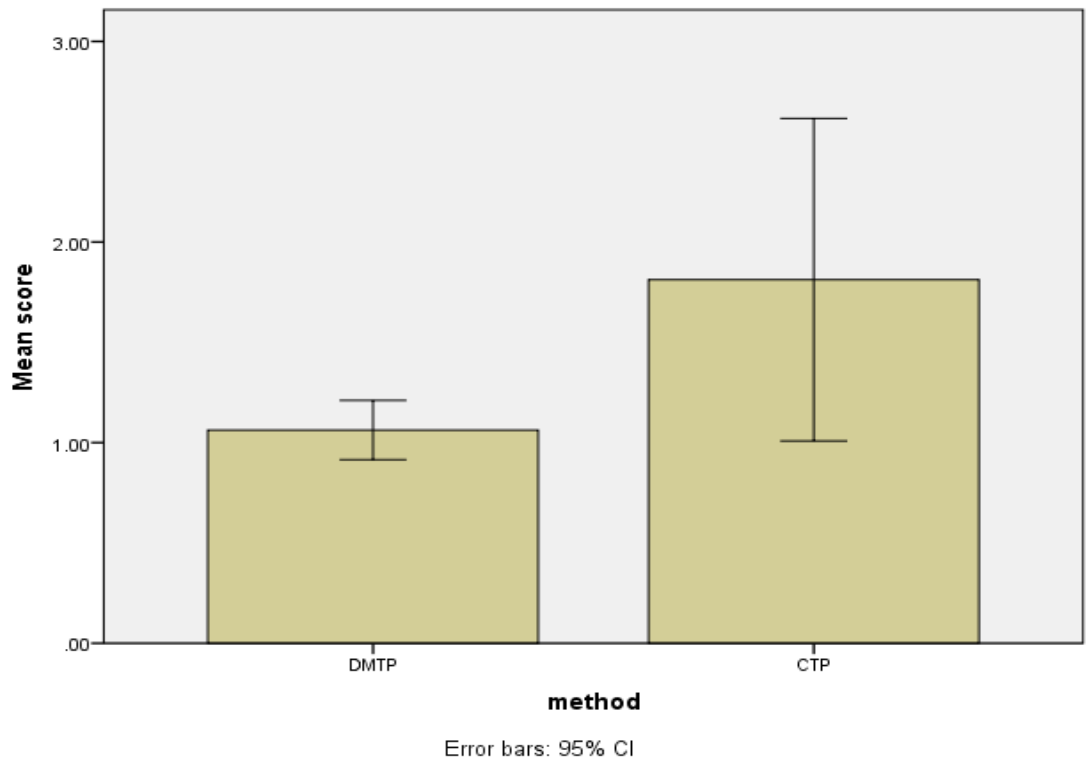

Figure 4.3. A comparison of nuclear detail mean scores for the domestic microwave method (DMTP) and conventional method (CTP).

A higher mean score (1.8 ± 0.96) on tissues processed by conventional means was evident. This showed that the domestic microwave produced slides of poorer quality in terms of nuclear detail as compared to the conventional processing method. However, the fact that the mean for the conventional method was just 1.8, this indicated that even this method was not able to produce slides of high quality.

4.4 Cytoplasmic detail

Mean scores for cytoplasmic detail were obtained for both DMTP and CTP with values of 1.8 ± 0.25 and 2.7 ± 0.53 respectively. There was a significant difference between the means as an independent samples t-test showed, t (14) = 4.194, (p= 0.001, p < 0.05) (appendix 1d). This suggested a significant difference in quality of the slides produced by the two processing methods on cytoplasmic detail.

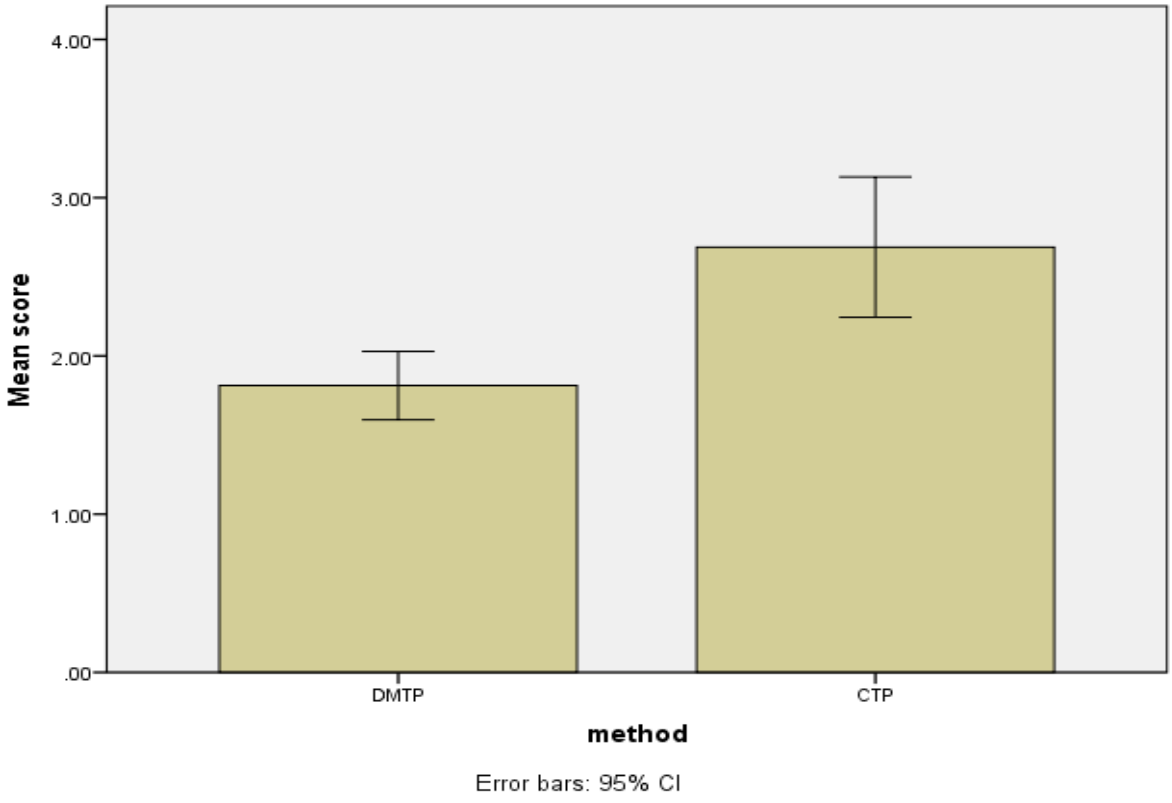

Figure 4.4. A comparison of nuclear detail mean scores for the domestic microwave method (DMTP) and conventional method (CTP).

It, therefore, follows that tissues processed by conventional means had a higher mean score (2.7 ± 0.53). Hence, according to observations, the domestic microwave once again fared poorly against the conventional method for producing slides with cytoplasmic detail.

Summary table

Table 4.1. Mean scores of the 2 observers on 4 parameters for samples processed by DMWTP and CTP and independent samples t-test

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Parameter	**Processing method**	**Observer 1**				**Observer 2**				**Mean±SD**	**T-value (p value)**
		scores				**scores**					
		1	2	3	4	1	2	3	4		
Staining intensity	DMTP (i)	2	4	1	1	0	1	5	2	1.9±1.1	2.297 (0.038)
	CTP (ii)	1	1	3	3	0	1	5	2	3.1±0.8	
Cellular detail	DMTP (i)	5	2	0	1	6	1	1	0	1.8±0.25	4.194 (0.001)
	CTP (ii)	0	3	1	4	3	0	2	2	2.7±0.53	
Cytoplasmic detail	DMTP (i)	5	2	0	1	2	6	0	0	1.1±0.17	2.170 (0.048)
	CTP (ii)	0	2	6	0	1	3	4	0	1.8±0.96	
Nuclei detail	DMTP (i)	8	0	0	0	1	7	0	0	1.5±0.9	2.668 (0.018)
	CTP (ii)	4	2	2	0	4	2	1	1	2.9±1.2	

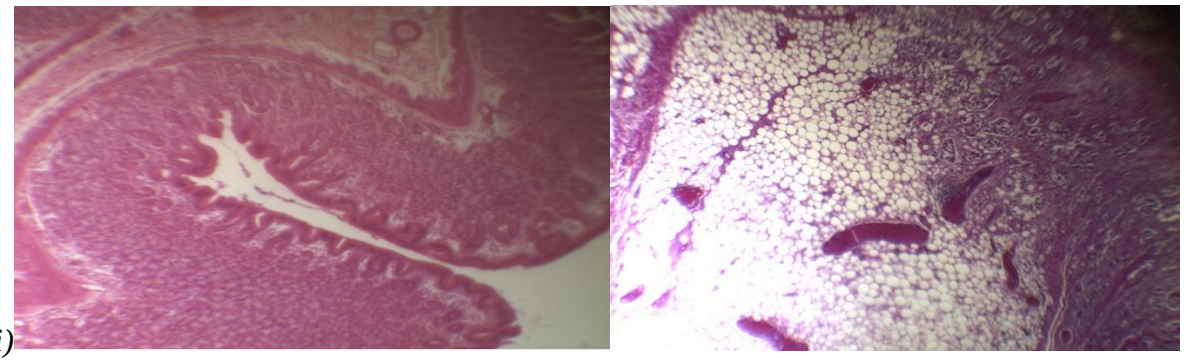
Microscopic photographs (mag x10)

Microscopic pictures of the sections which were processed with both CTP and DMWTP have been included below (Fig4.5-4.8), shows the difference in staining intensity, cytoplasmic detail, cellular detail and nuclei detail

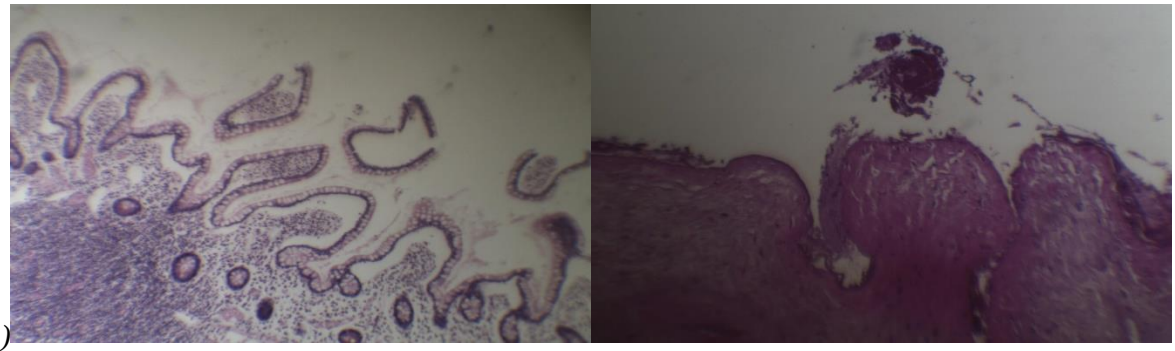
CTP (conventional tissue processing) DMTP (domestic microwave assisted tissue processing

A **B**

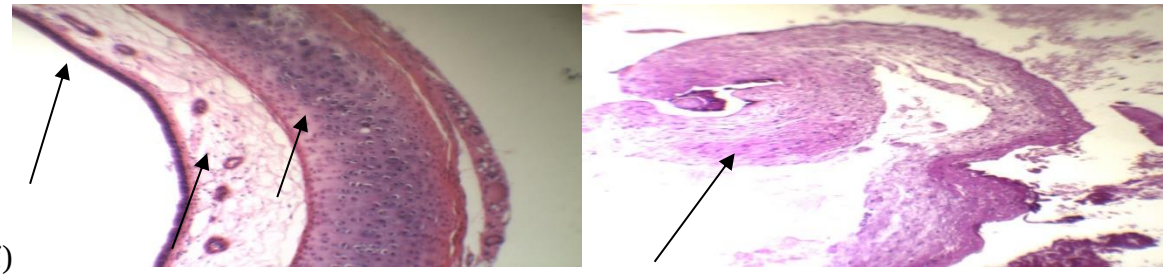
(i)

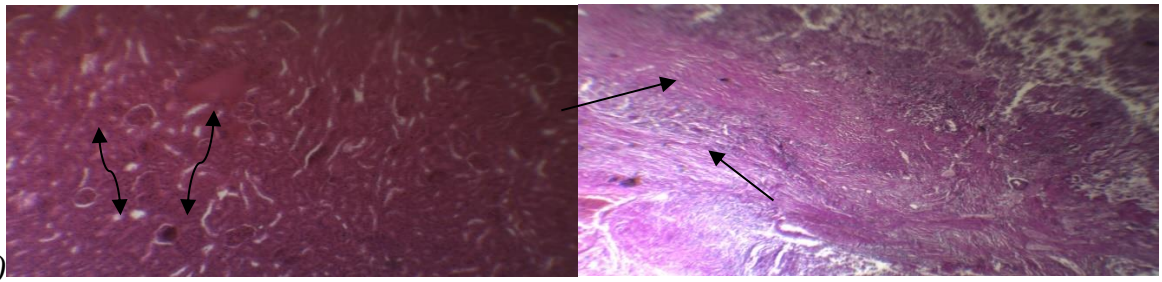


(ii)



(iii)



(iv) 

(i) **Fig 4.5.** Stomach showing staining intensity (a) conventional tissue processing (H and E, x10), (b) Microwave assisted tissue processing (H and E, x 10)

(ii) **Fig 4.6.** Small intestines showing cytoplasmic detail (a) conventional tissue processing (H and E, x10), (b) Microwave assisted tissue processing (H and E, x 10)

(iii) **Fig 4.7.** Spleen showing cellular detail (a) conventional tissue processing (H and E, x10), (b) Microwave assisted tissue processing (H and E, x 10)

(iv) **Fig 4.7.** Kidney showing nuclei detail (a) conventional tissue processing (H and E, x10) (b) Microwave assisted tissue processing (H and E, x 10)

4.5 Measures of agreement

Measures of agreement on the scores produced by the two independent observers were calculated to check the extent to which their observations tallied. This was necessitated by the need to factor out observer bias. Agreement was measured on the tissues processed by both DMTP and CMTP for the four parameters under analysis i.e. staining intensity, nuclear detail, cellular detail and cytoplasmic detail (Table 4.3). The extent of agreement was expressed as a single value, the Kendall's Coefficient of Concordance, W. Kendall's W ranges between 0 and 1 with perfect agreement equal to 1 and perfect disagreement equal to 0.

4.5.1 Staining intensity

There was reasonable agreement (Kendall's W =0.897) on scores between the two observers for tissues processed by DMTP. The observers also agreed to a large extent on tissues stained via the conventional method (Kendall's W=0.750).

4.5.2 Cellular detail

A complete concordance of opinion was observed (Kendall's W =1.000) on scores between the two observers regarding cellular detail for tissues processed by a domestic microwave. A high level of agreement on cellular detail was also achieved (Kendall's W =0.839) for tissues processed by conventional means.

4.5.3 Cytoplasmic detail

Agreement between the two independent observers was low (Kendall's W=0.212) on scoring slides processed by domestic microwave for their cytoplasmic detail. However, there was a high concordance of opinions (Kendall's W= 0.839) between the judges on tissues processed by conventional means.

4.5.4 Nuclear detail

There seemed to be high level of agreement (Kendall's W =0.839) on scores between the two judges for tissues processed by DMTP in their nuclear detail. A strong measure of agreement was also exhibited between the two observers (Kendall's W=0.955) on tissues processed by conventional means.

Table 4.2. Summary of Kendall's Coefficient of Concordance (W) values (0 = perfect disagreement, 1 = perfect agreement)

Parameter	**Processing method**	**Kendall's W**
STAINING INTENSITY	DMTP	0.897
	CTP	0.750
CELLULAR DETAIL	DMTP	1.000
	CTP	0.879
CYTOPLASMIC DETAIL	DMTP	0.212
	CTP	0.839
NUCLEI DETAIL	DMTP	0.839
	CTP	0.995

4.6. Time taken in processing tissues by microwave and conventional method

The time taken during the processing of tissues by both methods was recorded and tabulated. The conventional method uses automated machines that were timed according to the protocol by Cullings (1974) for overall processing using the dip and dunk processor and staining using automated stainer. The overall processing time by conventional microwave was 391 minutes 3 seconds whereas the overall processing time by domestic microwave was only 80 minutes 10 seconds. Though it has its limitations, the domestic microwave method has a significantly shorter processing time. Tables 4.4 to 4.6 give summaries of the time taken for the steps in each method.

Table 4.4. Time taken for tissue processing with the microwave and conventional methods**.**

Stages	**Microwave method** (Time/ minutes)	**Conventional method** (Time/minutes)
Dehydration	20	200
Clearing	20	70
Impregnation	20	40
Tissue processing time required	60	310

Table 4.5. Staining time for the microwave and conventional methods.

Stages	**Microwave method** (Time/ minutes)	**Conventional method** (Time/ minutes)
Deparaffinization	10	20
Hematoxylin staining	0.30	7
Bluing and differentiation	1	3
Tap water wash	5	5
Eosin staining	0.30	0.30
Tap water wash	3	5
Alcohol dehydration	0.10	0.10
Total staining time	20.16 min	40.67 min

Table 4.6. Overall working time for the two methods.

Microwave method total time	**Conventional method total time**
80 min 10 sec	**391 min 3 sec**

CHAPTER 5. DISCUSSION

Processing techniques that give rise to adequate tissue quality were established for most animal tissues many years ago. Many of these protocols take days to complete, for numerous applications and the lengthy duration required for processing tissues in microscopy are an impediment (Login and Dvorak, 1994). This is true, for example, in clinical samples in which microscopic examination or diagnosis are based on - at least in part - on microscopic structural findings and for research studies in which procedures or protocol modification must await microscopic structural results (Ragazzini *etal,* 2005). The reluctance by many laboratories, usually in developing countries, to switch to methods that can give better results is understandable since alternative technologies like microwave assisted procedures require an initial investment in equipment and time to establish.in the present study to establish an overview of whether a significant difference in the quality of tissues processed by domestic microwave and the conventional means (automated machines) exists. From our study we observed that there exists a significant difference in the quality of tissues processed by domestic microwave and conventional tissue processing. We observed that sections produced by the domestic microwave were not of the same standard as those from the conventional processing technique.

Domestic microwave, however, has the potential to improve diagnosis of tissues since it can speed up the rate at which staining of tissues occurs. In this experiment, the staining on microwave processed tissues was not as good as compared to conventional staining. Kango and Deshmukh (2011) in their study found that microwave processing showed good colour contrast. In this study we observed that good staining intensity was in favor of tissues processed by conventional means because sections produced by CTP showed good stain colour retention therefore enabling visualization of cells, but those produced by DMTP did not retain stains as efficient (table 4.2 (i) Fig 4.5). This might be due to the inability to appropriately control pressure, temperature and the presence of hot and cold spots within the oven therefore suggesting that the heat might not be uniformly distributed throughout the tissue as expected.

According to this study the domestic microwave did not produce slides with cellular detail as good as the conventional processing method. A perfect agreement was observed between our observers on their scoring system on cellular detail meaning they had similar views on the slides produced.

There was an inability to differentiate between the cytoplasm and the intracellular structures (table 4.2 (iii) Fig 4.5). This could have arisen as a result of poor fixation and less time allowed for dehydration and clearing since completion of each stage was marked by complete evaporation of reagents diffusing in and out of the cells from the reaction beaker.

Cytoplasm contrast was seen to be better in tissues processed by conventional means than those processed by microwave assisted tissue processing (table 4.2 (ii) Fig 4.5). Condensation of connective tissue was occasionally seen, but this did not affect interpretations for results. There was no granulation observed on tissues processed by the microwave method although it achieved differentiation between the cells and the cytoplasm.

A significant difference between the two processing methods was observed microscopically on nuclear detail of tissues. According to Morales *et al. (*2004), in microwave-processed tissues nuclear detail was of better quality than the conventionally processed one, however, in this study nuclear detail was noted to be of good quality on tissues processed by conventional means although the tissues showed some focal condensation (table 4.2 (iv) Fig 4.5). Morales *et al. (*2004) got good nuclei detail of tissues processed by DMTP than those processed by CTP and proposed that an increase in time for dehydration has an effect on the protein and hydrogen bonds within the nuclei hence microwave reduces the time for dehydration therefore avoidance of tissue shrinkage enabling good nuclei retention.

We have observed that a domestic microwave reproducibly can be used in microscopy for the basis of tissue diagnosis as it yielded histological material comparable in quality to conventional tissue processing. Unfortunately, it did not produce results as good as the conventional method. Moreover, the use of domestic microwave tissue processing has several advantages over routine methods from the perspective of laboratory personnel, microwaves are readily available, affordable, they also enhance safety by eliminating formalin and xylene from the procedure. Formaldehyde poses a significant danger to human health. In 2011, the United States National Toxicology Program described formaldehyde as known to be a human carcinogen (Panja *et al.* 2007).

Regarding the final product, slides, microwave irradiation substantially shortens the time for processing which can be an advantage in clinical laboratories from specimen reception to diagnosis. In the present study, the slides were produced in 80 minutes 10 sec as compared to 391

min 3 sec in routine processing. This allowed same-day rapid tissue processing although sections did not produce high quality tissue section. This is partly in agreement with Login and Dvorak (1994) where microscopic slides were produced in 2-3 hours using microwave irradiation.

5.1 CONCLUSIONS

Microwave assisted tissue processing yields paraffin sections that can be compared with conventional method of processing but with no superior quality to the ones that are produced by conventional tissue processing. By this innovative method, technicians in in the field of microscopy e.g. pathologists can be able to offer an early final diagnosis by using a microwave. Moreover, the application of domestic microwave in clinical and veterinary can eventually result in more efficient and better management of patients. Since the only equipment required for this method in microscopy is a microwave oven, the technique if improved and done well has a potential to be suitable for hospital and veterinary laboratories as well as research laboratories where microscopic materials are routinely processed. However, for now, conventional tissue processing can be still regarded as producing results that are more reliable and usable in microscopy.

5.2 RECOMMENDATIONS

It is recommended that where possible protocols be modified by including use of the domestic microwave to increase rapidity and suit local practices and working environments. Domestic microwave tissue processing can be implemented in diagnostic pathology laboratories handling small number of specimens daily, provided that a way is found to evenly control temperature distribution and pressure which are the major draw backs on its application.

6.0 REFERENCES

Bancroft , J. D. & Gamble , M., (1995). **Bancroft's Theory and practice of Histological Techniques, 7th ed**. Chicago: Elsevier Health Sciences.

Bendayan M, N. A. (1987). **Sectioning. Journal of Histochemistry and Cytochemistry**. India.

Bhushan, B. (2010). **Springer handbook of nanotechnology (3rd rev. & extended ed.)**. Berlin: Springer.

Boon , M. E., Ouwerkerk-Noordam, E. (1986). **Microwave-stimulated diffusion for fast processing of tissue: reduced dehydrating, clearing and impragnation times**., India: Pathol.

Boon, M. E. et al. (1989). **Microwave-stimulated staining in plastic embedded bone marrow sections with the romanwsky-giemsa stain: improved staining patterns. journal of histotechnology**, vol. **12**(no. 4), pp. **295**-297.

Clyden E.C. (1971). **Practical section cutting and staining**. Churchill Livingstone: Edinburgh.

Culling's, C. F., Allison, T. R. & Barr, W. T. (1985). **Chap 4. Processing**. London: Butterworths and Co-publishers.

Erko, A. (2008). **Modern developments in X-ray and neutron optics**. Berlin: Springer.

Giberson, R. T. & Demaree, R. S. (1999). **Microwave processing: Technique for electron microscope**. Meth-Molec, Bio, pp. 117, 145-158.New York.

Hopwood, D. et al. (1984). **Microwave Fixation**. **In: Its Potential for routine techniques, histochemistry, immunocytochemistry and electron microscopy**. Jersey: J Histochemical.

Hopwood D. (1996). **Fixation and Fixatives. In S. A. Bancroft J., Theory and practice of histological techniques**. (pp. 201-4). New York: Churchhill Livingstone.

Kango, P. G. & Deshmukh, R. (2011). **Microwave Processing**. *A boon for oral pathogist*, voll15(**7**), pp. 6-13.

Kennedy A, F. A. (1989). **Use of microwave oven improves morphology and staining of cryostat sections**. J Clin Pathol.

Leong, S. Y. A. & Gilham, P. (1989). **"A new, rapid microwave-stimulated method of staining melanocytic lessions"**. Staining technology, vol. **64**.pp. 81-85.

Leong AS. (1993). **Microwave. Techniques for diagnostic laboratories**, Scanning, 88-98. New Jersey.

Locad., W. (2000). **Tissue processing. In W. A. eds., Laboratory histopathology** (pp. 4.2-1-4.2-39.). New York: Churchill Livingstone.

Locard, E. (2000). **"The Analysis of Dust Traces. Part II". The American Journal of Police Science**, Vol. **1,** No. **4,** pp. 401-418.

Login, GR, Dvorak, AM. (1994) **The microwave tool book. A Practical Guide for Microscopists**. Boston, p145-6.: Beth Israel Hospital

Mathai, A. M., Naik, R., Rai, S. & Baliga, P. (2008). **Microwave histoprocessing versus conventional histoprocessing**. India, J Pathol Microbiol.

Mayers , C. P. (1970). **Histological fixation by microwave heating**, India: J Clin Pthol.

McArdle, P. (2007). **Histotechnology.2nd ed**. Chicago: ASCP Press.

Morales A., R, Nassiri M, Kanhoush R, Vincek V, Nadji M. (2004). **Validation of histologic quality and impact on the timeliness of diagnostic surgical pathology**. Am J Clin Pathol.

Panja P, Sriram G, Saraswathi TR, Sivapathasundharam B. (2007) **Comparison of three different methods of tissue processing**. J Oral Maxillofac Pathol.

Pennycook, S., Varela, M., Hetherington, C., & Kirkland, A. ((2011). **"Materials Advances through Aberration-Corrected Electron Microscopy**. MRS Bulletin: New Jersey.

Ragazzini T, Magrini E, Cucchi MC, Foschini MP, Eusebi V. (2005) **The fast-track biopsy (FTB)**: Int J Surg Pathol

Ruehl-fehlet, C. (2000). **Revised guides for organ sampling and trimming in rats and mice**, Pathol India: Tixol.

Shashidhara R, Sridhara SU. (2011) **Kitchen microwave - Assisted accelerated method for fixation and processing of oral mucosal biopsies: A pilot study**. World Journal of Dentistry.

Shruthi, E. A. (2013). **Microwave Histotechnology, 354 Journal of Cancer Research on Therapeutics**. Volume **9** - Issue **3**, 50-63

Valle, S. (1986). **"Special stains in the microwave oven". Journal of histoprocessing**, vol. **9**(no **4**), 237-239.

7.0 APPENDICES

SPSS OUTPUT FOR independent samples t-test

Appendix 1a**: Staining intensity**

Group Statistics					
	method	**N**	**Mean**	**Std. Deviation**	**Std. Error Mean**
score	**DMTP**	**8**	**1.5000**	**.88641**	**.31339**
	CTP	**8**	**2.8750**	**1.15728**	**.40916**

Independent Samples Test					
		Levene's Test for Equality of Variances		**t-test for Equality of Means**	
		F	**Sig.**	**t**	**df**
score	**Equal variances assumed**	**2.851**	**.113**	**-2.668**	**14**
	Equal variances not assumed			**-2.668**	**13.110**

Independent Samples Test					
		t-test for Equality of Means			
		Sig. (2-tailed)	**Mean Difference**	**Std. Error Difference**	**95% Confidence Interval of the Difference**
					Lower
score	**Equal variances assumed**	**.018**	**-1.37500**	**.51539**	**-2.48040**
	Equal variances not assumed	**.019**	**-1.37500**	**.51539**	**-2.48748**

Independent Samples Test		
		t-test for Equality of Means
		95% Confidence Interval of the Difference
		Upper
score	**Equal variances assumed**	**-.26960**
	Equal variances not assumed	**-.26252**

Appendix b: Cellular detail

T-Test

Group Statistics					
	method	N	Mean	Std. Deviation	Std. Error Mean
score	DMTP	8	1.0625	.17678	.06250
	CTP	8	1.8125	.96130	.33987

Independent Samples Test					
		Levene's Test for Equality of Variances		t-test for Equality of Means	
		F	Sig.	t	df
score	Equal variances assumed	21.125	.000	-2.170	14
	Equal variances not assumed			-2.170	7.473

Independent Samples Test						
		t-test for Equality of Means				
		Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
					Lower	
score	Equal variances assumed	.048	-.75000	.34557	-1.49118	
	Equal variances not assumed	.064	-.75000	.34557	-1.55678	

Independent Samples Test		
		t-test for Equality of Means
		95% Confidence Interval of the Difference
		Upper
score	Equal variances assumed	-.00882
	Equal variances not assumed	.05678

Appendix c: Nuclei detail

T-Test

Group Statistics					
	method	N	Mean	Std. Deviation	Std. Error Mean
score	DMTP	8	1.8125	.25877	.09149
	CTP	8	2.6875	.53033	.18750

Independent Samples Test					
		Levene's Test for Equality of Variances		t-test for Equality of Means	
		F	Sig.	t	df
score	Equal variances assumed	1.759	.206	-4.194	14
	Equal variances not assumed			-4.194	10.155

Independent Samples Test					
		t-test for Equality of Means			
		Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference
					Lower
score	Equal variances assumed	.001	-.87500	.20863	-1.32247
	Equal variances not assumed	.002	-.87500	.20863	-1.33890

Independent Samples Test	
	t-test for Equality of Means

		95% Confidence Interval of the Difference
		Upper
score	Equal variances assumed	-.42753
	Equal variances not assumed	-.41110

Appendix d: Cytoplasmic detail

T-Test

Group Statistics					
	method	N	Mean	Std. Deviation	Std. Error Mean
score	DMTP	8	1.9375	1.14759	.40573
	CTP	8	3.0625	.77632	.27447

Independent Samples Test					
		Levene's Test for Equality of Variances		t-test for Equality of Means	
		F	Sig.	t	df
score	Equal variances assumed	1.526	.237	-2.297	14
	Equal variances not assumed			-2.297	12.297

Independent Samples Test					
		t-test for Equality of Means			
		Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference
					Lower
score	Equal variances assumed	.038	-1.12500	.48985	-2.17563
	Equal variances not assumed	.040	-1.12500	.48985	-2.18944

Independent Samples Test		
		t-test for Equality of Means
		95% Confidence Interval of the Difference
		Upper
score	Equal variances assumed	-.07437
	Equal variances not assumed	-.06056