REVIEW OF LITERATURE

Microscopy:

- Microscopy is a procedure used to obtain images at resolutions that exceed the resolving capacity of the unassisted (naked) human eye.

- It is an art and science of creating, recording, and interpreting enlarged images on the basis of tissue observations that provide assistance in areas like human pathology (histopathology). Microscope is a tool for diagnosing vital tissues. The international community is concerned with high prevalence of disease as well as high rate of deaths in humans due to the inability to diagnose diseases especially in developing countries that are still not able to meet technology standards.

- Disease diagnosis depends on the gross and microscopic examination as well as ancillary test examination of tissues. Therefore, microscopy has a profound impact on educational & research institutions, diagnostic laboratories & industries

- In present study, thin tissue sections produced by two different methods that were microwave and routine (conventional) method and comparison of results were done by the use of microscope.

Histopathology and medical sciences:

- Histopathology is a branch of Pathology that executes microscopic study of the tissue affected by disease. Thus, it is one of the most effective means of diagnosing diseases.

- The development of simple microscopes with single lense had been done by Antonie van Leeuwenhoek in 1673 that gave advanced magnification & resolution[16].

- The speciality of histopathology technique is since 1838, when Johannes Muller published the book, 'Nature and Structure Characteristics of Cancer'. It was the foremost book on basics of histopathology and was the first person to believe that microscopy can aid the medical work.[17]

- This determines that by manufacturing the microscope, it has completely changed the spectrum of disease from whole organ, to tissue, to cell and even smaller. It enhanced the practice of histology and led to development of many auxiliary techniques required for modern practice.

- As time progressed, many laboratory chemicals were examined for its application as fixatives. A fixative is a necessity as examination of tissues under a microscope requires fixed tissues covered by coverslip on a glass slide. In 1893, the first fixative, formalin was the used, which is widely used today. Although various other fixatives were also discovered till date.[16]

- The first acceptable microtome for cutting animal tissues was built in 1848 and a sledge microtome built afterwards in 1910.Automated tissue processors replaced hand processing in 1945.[16]

- From a study of the past experience, we may able to look the differences that have been occurred in the field of pathology to the point where various protocols have been prepared along with the manufacturing of additional instruments needed to produce small thin sections of tissues for rapid diagnosis, like a Microwave oven.

- In 1970, the initial introduction of microwave technique was for fixation of tissues in histopathology section by Mayers[1] whereas processing of tissues were done by Kok and Boon in 1985.[7]

- The microscopic examination of tissues commonly needs a slice of tissue, that should be thin enough for transmission of light. Preparation of the thin slices of tissues is known as microtomy or section cutting. Before being sectioned, the tissues should undergo preliminary treatment, that includes impregnation within a proper embedding medium to give adequate support and an appropriate firmness for section cutting. This treatment given for preparation is called as tissue processing.

- In the past 100 years, routine (conventional) tissue processing has been widely used method. The steps of tissue processing comprise of dehydration, clearing, impregnation and embedding. These steps are assigned a fixed time

interval that ensure successful procedure completion. For past 10 years, instruments used for tissue processing is unchanged and three frequently used methods of tissue processing include routine manual method, rapid manual method and the microwave method.[19]

- The advantage of the routine method is its consistency of results and it can prepare slides with lesser degree of shrinkage or tissue distortion. But drawbacks is that it takes more time and require to work with harmful reagents like xylene and formalin that can have carcinogenic effect if inhaled[20].

- Microwave method is a latest method of tissue processing, and it appears to produce the new look of pathology as it gives good and more accurate diagnosis with decreased turnaround time. Here, the infiltrating property of microwaves and the change of electromagnetic energy into heat is utilized[21].

- The increase in usage of microwave-assisted tissue processing has inspired to manufacture commercially available laboratory microwave ovens. They were specially manufactured to ensure even and faster tissue processing along with accurate control of temperature of specimen. These devices also automatically and accurately control the on-off cycle of the heating. [21] Thus, rapid processing and diagnosis of specimen can be done without negotiating the overall quality of histopathology section. Such commercial units have favoured achievement of tissue processing on the same day but they are costly. However, domestic microwave is cheaper and readily available in comparison to laboratory microwave and provides equivalent results as that of later.[22].

- In contrast to conventional heating, the heating of material in microwave is from inside (heating of material from inner side) and hence whole material shows the effect of irradiation uniformly.

Mechanism of action of Microwave oven

- Microwaves are nonionizing electromagnetic waves with 300MHz to 300 GHz frequency range. It corresponds to the wavelength of 12.2 cm[2,3]. Microwaves are shorter than radio waves but longer than infrared waves.

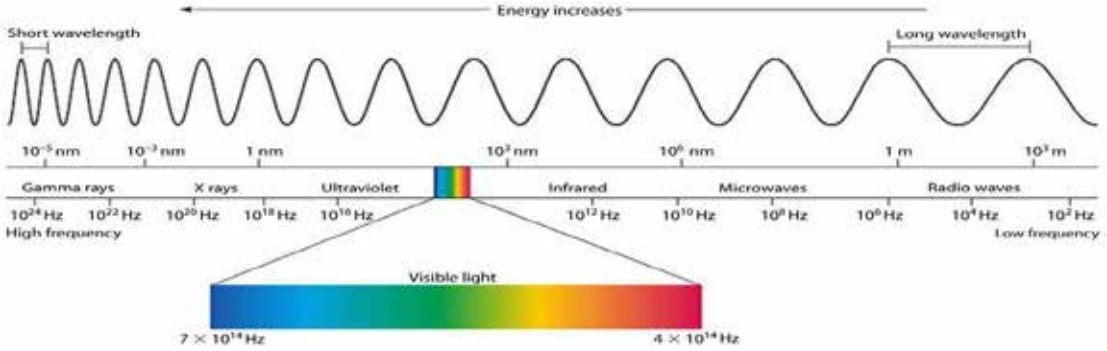

Figure 3.1 Electromagnetic spectrum

- Various research articles and books of Physics and Chemistry demonstrates that the viscosity of fluid reduces as the temperature increases and thereby increasing the diffusion.

- Microwave work on same mechanism that is 'rotation of water molecules' to generate heat. A water molecule consists of a single large atom of oxygen and two small atoms of hydrogen. The part of the molecule that contains two hydrogen atoms is positively charged, and the part that contains oxygen is negatively charged. Thus, polarity is created in water molecules as there is asymmetric distribution of positive and negative charges.

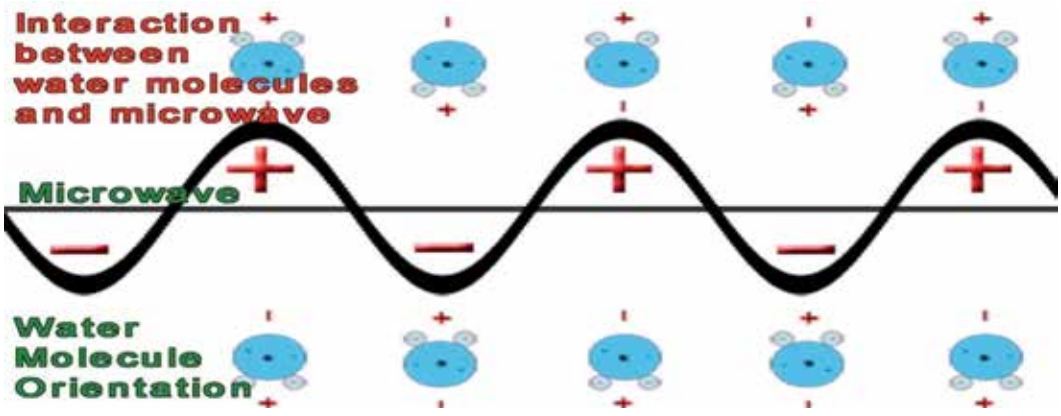

Figure 3.2 shows polar water molecule. It shows interaction between the oscillating electromagnetic microwaves and water molecules.

- When microwave energy is applied to water molecules, the electric charge of bipolar molecules oscillates them. The dipoles of molecule flip backward and forward in three-dimensional orientation causes rapid spinning of water molecules in an effort to bring into alignment with opposite polarity electromagnetic charges. By this mechanism, microwaves can cause spinning of water molecules and can change orientation. To align with negative charges in electromagnetic field, repulsion of molecules occurs because of their similar charges. This causes rotation of molecules because they are not symmetrical.

- The electric field charges force the molecules of substance in same direction or at 180^0 within electric field that depends whether it has positive or negative charge. This is also applied for bipolar molecules of water. Polar molecules exhibits torque in its electromagnetic field by the energy produced by the electric field. Thus generated energy by rotation of molecules causes random movement of molecules by Collision with other molecules. Oscillation of dipoles causes frictional forces, which produces heat energy.

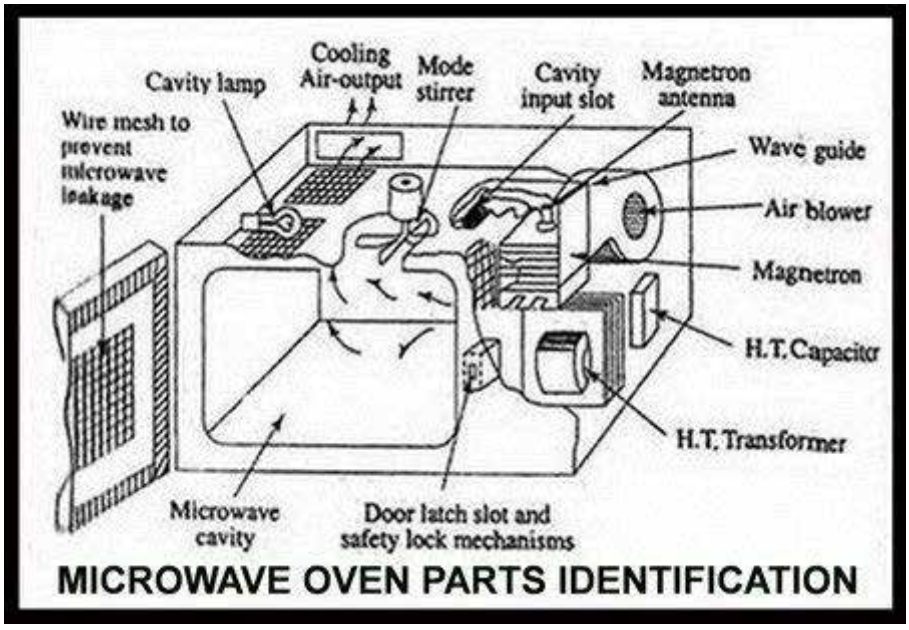

Figure 3.3 Parts of Microwave oven

- **Magnetron** produces microwaves from electricity of the microwave oven.

Thus, filament generates electrons when the electric voltage heats the centre of the magnetron.

- **Transformer** is used to stimulate the magnetron, which transforms the normal household electricity from 120 volts to approximately 4,000 volts or higher.
- **Antenna** It transmits microwaves in cooking chamber.
- **Wave guide** directs the microwave into cooking chamber. The microwaves then bounce off the inner walls of the oven and pass through material kept on turntable.
- **Metal mesh** present in microwave door with holes that are small enough so that microwave can't escape but visible light can be seen from outside. So content of chamber can be visualized.
- **Turntable** revolving glass tray rotates the food/cooking material in a circular path within microwave so it can heat material evenly.

Types of substances based on microwave absorption:

1. Microwave transparent, e.g., Plastic, crystals, glass, alumina-based ceramics, paraffin wax
2. Microwave reflectant: e.g. Metals like steel, silver, copper
3. Microwave absorbent e.g. water, tissues, food

Obtaining a Fresh Specimen for Fixation and Processing

- It is important that fresh specimen should be handled carefully and properly. It should be immediately fixed in a appropriate fixative as soon as possible after removal from body. Appropriate measures must be taken in dissection area which is the operation theatre. If it is not manageable, transport the specimen immediately to the histopathology laboratory[6].

Three Major protocols followed for tissue preparation in Histopathology laboratory

- **Fixation**
- **Processing**
- **Staining**

1. Fixation:

- Fixation stands as the first, most important and basic step before tissue processing. Any faults in fixation cannot be corrected at any further stage. It includes immersing sample tissues in a fixatives that is known chemical substance to avoid tissue destruction by enzymes or bacteria. Therefore, the tissue retains its maximum morphologic and chemical properties and preventing autolytic and putrefactive changes.

- There are only few of the chemicals that can be used for fixation as they should have peculiarities that make them appropriate for tissue morphology preservation. So that, tissue components retain its chemical reactivity in order to apply specific staining techniques subsequently.

- Fixed tissues have a benefit that they can be preserved, stored and maintained very easily. Thus, availability of quality of sections is excellent in later time also. Fixatives form crosslinking among proteins of tissues and form a semisolid gel-like substance. In this way, the tissue components retains architecture same as in vivo.[8] The fixatives are different reagents which can be used for fixation and each fixative has different penetration capacity. The widely used solution till date is 10% formaldehyde (HCHO), which was promoted by Ferdinard Blum (1865-1959)[23].

- Formalin, commonly used as a neutral phosphate buffer solution. It is the most accepted fixative solution used for fixation of tissues which will be later processed to make paraffin blocks. Preferably specimens must be kept in fixative solution for recommended duration to penetrate fixative throughout the tissue sample and later for an extra time to permit the chemical reactions of fixation to gain equilibrium[14].

- The procedure of fixation causes crosslinking of proteins molecules. It causes coagulation of tissue that leads to semifluid structure to be converted into semisolid state. Thus, easy manipulation of tissues is possible due to its semisolid state. [24]

- Progress has been made in the manufacturing of assistive devices which can help in fixation e.g. the routinely used conventional method that includes the usage of automated tissue processors with reagent containers to complete whole tissue processing steps commencing from fixation to embedding by molten paraffin wax[7].

1.1 Properties of ideal Fixatives[25]:

- Prevent autolysis and putrefaction.

- Preserve cells of tissue and its constituents as nearly as possible in life like manner i.e. preserve gross and microscopic anatomy of tissue.

- Make the cell components insoluble in the reagent used in tissue processing and prevent the loss of certain soluble molecule from their original locations.

- The fixative should penetrate the tissue quickly and uniformly. It should prevent short-term and subsequent long term destruction of the tissue micro-architecture. Thus, the molecular components of tissue is not altered by catabolic enzymes and/or by microorganisms during long term storage.

- Mildly hardening of tissues.

- Devitalize and inactivate infectious agents.

- Solidification of colloid material.

- Increases optical differentiation of cells.

- It should support high quality and consistent stain procedure.

- Preserves tissue volume, maintain shape, should be isotonic and cause minimal alteration of tissue and its components.

- Should be safe to handle, non toxic, non inflammable and non allergic for user.
- Should be economical(cheap), stable, readily disposable and recyclable.
- Useful for wide variety of tissues.

1.2 Factors affecting fixation[25]:

- **Size and thickness of tissues**: Fixative can't penetrate a piece of tissue of more than 1cm thickness. The best thickness is 3 to 5 mm.
- **pH of fixative**: pH range of 6 to 8 is satisfactory for fixation. Neutral pH is preferable. High acidity or alkalinity interferes fixation.
- **Osmolarity**: Isotonic solutions give best results. Hypotonic solutions cause swelling while hypertonic solutions cause shrinkage of cells.
- **Duration for fixation:** Most fixative fix the tissue at rate of 1mm per hour. So an adequate fixation requires 12 to 24 hours depending on size of tissue. The presence of blood in tissue may interfere the infiltration of the fixative. Thus, It is recommended to clean the tissue specimen appropriately before immerging it in a fixative reagent. To section the tissue of 3-5 mm thickness, the tissue should be optimally fixed. Fixation of tissue by formalin occurs within 24 hours. Fixation for longer duration may result in loss of lipid, protein and marked decrease in the cell enzymatic activity. This may also lead to hardening of tissue.
- **Temperature**: Increase in temperature accelerates the fixation process.
- **Penetration capacity of fixative**: It depends on diffusion and rate at which fixative reacts with tissue.
- **Agitation**: Improves the speed of penetration of fixative and gives better infiltration and impregnation.
- **Concentration of fixative** : Optimum concentration is required. Neutral pH with mild low concentration is recommended. Too low concentration increases

the fixation time and at higher concentration, faster fixation of tissue occurs but with undesirable effect in it.

- **Type of tissue**: Tissue covered with mucus or blood, organ containing large amount of fatty tissue and blood fix slowly. Hence tissue sections should be thin and requires more time than average.

- **Additives**: By adding electrolytes and non electrolytes, morphology of the fixed tissue can be improved.

- **Fixative agents:**

1. Formaldehyde
2. Glutaraldehyde
3. Osmium Tetroxide
4. Methyl and Ethyl Alcohol
5. Acetone for enzyme study and immunocytochemistry.
6. Bouin's Fixative
7. Mercury Salt-Containing Fixatives

   - Zenker's Fluid
   - Helly's Fluid
   - B5 Fixatives

1.3 Fixative used in present study:

- Formalin is most commonly used fixative in histopathology.

- Formalin (commercially available saturated solution) consists of formaldehyde ($H.CHO/CH_2O$) gas available as 40% (by weight) solution in water. i.e.

- Formalin =40% formaldehyde and

- 10% formalin=formalin (40% formaldehyde) 10 ml and water 90 ml.[25]

- Formalin consists of polymerized form of formaldehyde, whereas 10% formalin consists predominantly monohydrate methelene glycol along with monomeric form of formaldehyde. The rate of depolymerization of formaldehyde is pH dependant. Hence, it requires some time for depolymerization unless the pH of solution is made neutral or alkaline. Thus fixative of choice is 10% neutral buffered formalin.

10% Neutral buffered formalin (pH 7.2 to 7.4) consists of[26]	
Formaldehyde; 40%	100.0 ml
Distilled water	900.0 ml
Sodium dihydrogen phosphate	4.0 g
Disodium hydrogen phosphate	6.5 g

1.4 Mechanism of action:

- Formaldehyde reacts with proteins and forms cross-links between the protein molecules giving rise eventually to an insoluble end product. Formaldehyde reacts easily with the amino groups and when the pH is 8 to 9, almost all amino groups become involved.

1.5 Microwave fixation:

- Heat generated by microwaves accelerates the diffusion and thereby fixation. It can reduce the required time for fixation from more than 12 hours (16-24 hours; overnight fixation) to less than 20 minutes. Hence rapid fixation reduces time for technical processing of tissue. This lead to the conclusion that the irradiation of microwaves is effective to accelerate distribution of solutions and to augment reaction rates. Heating of material occurs from inner side which is a significant factor in the process.[6,9,10, 27]

- However, microwaving tissue in formalin produces large amounts of harmful vapours. So if there is no facility to handle this dangerous vapour, it is not safe to use it. However microwave fixation preserves tissue antigen better than routine methods for fixation.[27]

2. Tissue processing

- Research on the morphological structure of tissues requires tissue slices or biopsy fragments which should be 3-4 mm thick and not more than 4 mm thick[25].
- To observe under a microscope, the tissue should be transparent so that light and electrons can enter in it and pass from it. The tissues should be cut in thin slices and mounted on a glass slides to make them transparent. Hence, in order to section thin slices, tissues must be hardened and conserved from decomposition by fixation and embedding in paraffin block.
- When the tissue is received in laboratory, it is mandatory that the tissues should be labelled before processing. So that any confusion about duplication or giving incorrect diagnosis to the patient can be avoided. The labels must present in the whole process and afterwards as a permanent record.
- Tissue bits along with its designated number is wrapped well in square piece of filter paper and placed in embedding perforated cassette.
- The tissues which are going to be processed are placed in appropriately labelled cassettes (small baskets with holes) to separate them from each other. The specimen processing time schedule depends on the type and size of the specimens, the processor used, the reagents selected, the temperature of solvent and many other factors[28].
- The whole process consists of five important steps which are fixation, dehydration, embedding, sectioning and staining.
- Fixed tissues are first dehydrated and then embedded in a solid medium like paraffin wax. Therefore the tissue will become enough firm that can be cut into thin sections. In addition, the tissue also becomes soft and elastic which allow microtome knife to cut sections.
- The embedding media should be infiltrated well in the tissue in fluid state so that it prevents deleterious effect on the tissue. For improvement of embedding technique, hardening and dehydration are mandatory. Paraffin wax is the most satisfying embedding medium that is used in tissue processing. The paraffin embedding was introduced by Edwin Klebs in 1869 [29].

- Tissue processing can be done manually (hand processing), but when many specimens to be processed simultaneously, it is much easier and more effective to use an automated tissue processing machine (an automated tissue processor) through a technique called conventional tissue processing. These automated machines are available since 1945 and have evolved gradually such that it becomes safer in use, handle large number of specimens, process rapidly and yield results of higher quality[17].

- As the manufacturing of automated machines, till current date, conventional tissue processing has been regarded as the gold standard from which other methods are derived. Recently, two types of tissue processors are used. First is tissue- transfer machines (dip and dunk) in which specimens are transferred from one container to another container for processing. And second is fluid-transfer machines (enclosed) type in which specimens are kept in a processing chamber and fluids are moved inside and outside the chamber as needed[26]. Many newer fluid-transfer processors use raised up temperatures, effective fluid flow and vacuum/pressure cycles to accelerate processing and lessen processing times.

- In present study, for routine processing method, the dip and dunk automated tissue processor machine was used in which specimens were transferred from one container to another container at different concentration of reagents by automated rotation of a metal bucket with tissue samples. Here, the tissue processor kept in enclosed glass wooden box to prevent inhalation of fumes of noxious chemical like xylene.

- The need to reduce time of tissue processing, forced the scientists for development of alternate techniques which prepares histological slides in short duration. Reduction of turn around time is of great significance for the laboratory, but it will become more important when patient care and treatment depend on laboratory report. Facilitating a balance between the time needed by the laboratory for tissue processing and analysis and turn-around time preferred by physicians is an ongoing process in any histopathology laboratory.

2.1 Factors influencing rate of processing[25]

- **Specimen size**: Ideally 3-4 mm thick tissue slices or biopsy fragments is required for optimal tissue processing as fluids used in processing should permeate thoroughly in tissue.

- **Agitation:** To increase the fluid exchange rate of penetrating reagents, there is need to increase surface area of the tissue. Agitation of reagents by manual or automated processors intensify the flow of fluids in the tissues[10]. Slow and gentle agitation is recommended as rapid agitation can damage the tissue. As per histology specialists, adequate agitation can decrease time of total processing by 30 percent[30]**.**

- **Heat:** Elevated tissue processing temperatures can increase the rate of entry and exchange of fluids. Heat increases molecular kinetic energy and diffusion capacity and reduces solution viscosity[6]. Giberson and Demaree (1999) suggested that by using moderate temperatures for a limited duration could accelerate tissue processing protocols, although these steps should be carefully monitored to limit heat induced tissue shrinkage and viability[31].

- **Viscosity**: Viscosity is inversely proportionate to the flow of the fluid. When molecular size of particles in the solution is small, the rate of fluid penetration is more. Most of the solutions used in tissue processing, dehydration and clearing have same viscosity. However viscosity of embedding medium varies. Paraffin has low viscosity when in the liquid state(melted) and thereby increases the rate of impregnation[25].

- **Vacuum and Pressure:** Decreased pressure can accelerate the penetration rate and reduces time required to end the tissue processing steps. Studies have shown that solutions can be removed from tissue only when the fluids are more volatile than the solution going to be exchanged. Application of vacuum improves processing quality in the process of tissue infiltration. It can help to clear trapped air, such as , of lung tissue, or other porous tissues. Tissue processing procedures by using a vacuum can decrease the penetration time when working with thick and fatty tissues[6].

- **Ultrasonics**: It reduces the tissue processing time. However it is not used because heat generated by ultrasonic technique may be the source of artefact[32].

2.2 Overview of tissue processing steps for microscopy

2.2.1 Dehydration

- Dehydration is a process of elimination of water and fixative from the tissue. As melted paraffin wax is hydrophobic (it does not mix with water), maximum water from the specimen should be extracted before penetrated with wax[25]. This process is usually done by submerging specimens in increasing concentration of alcohol solution (usually 60% to 100%) till purity of alcohol is nearly without water. Alcohol mixes with water at all concentrations that is why the water present within the specimen is gradually substituted by the alcohol in dehydration step. In dehydration step, increasing concentrations of alcohol are used so that tissue distortion can be avoided. There are many factors that affect the dehydration step and heat is one of them.

- **Dehydrating agents:**

1. Ethanol
2. Methanol
3. Isopropyl alcohol
4. Butanol
5. Tetrahydrofuran (THF)
6. Acetone

2.2.2 Clearing

- Although the tissues are water free at the end of dehydration stage, still the tissues can't be penetrated with wax as wax and alcohol are non-miscible. Therefore a transitional solution which can be miscible with both alcohol and paraffin wax should be used. This solution must have property that will allow it to remove alcohol from the tissue, and then be removed by molten paraffin wax[33]. This step of tissue processing is known as clearing and the solution used is called a clearing agent.

- The ‘clearing’ term was selected as most of the (but not all) clearing agents cause an optical clarity or transparency to the tissue because of their higher refractive index.[31] Other significant effect of clearing agent is removal of excess of fat from the tissue which creates a barrier for entry of wax. The most widely used clearing solution is xylene and numerous changes are needed for complete removal of alcohol. [10]

Properties of good clearing agent:

1. Quick removal of dehydrating agent
2. Rapid penetration of tissues
3. Clear the tissue quickly without hardening or tissue damage
4. Easily removed by molten paraffin wax
5. Low flammability, toxicity and cost
6. Not dissolved out dyes used for inking
7. Not evaporate too quickly in the wax baths

Clearing agents are:

1. Xylene(xylol)
2. Toluene(toluol)
3. Chloroform(carcinogenic)
4. Carbon tetrachloride
5. Benzene (toxic, not used)
6. Cedar wood oil
7. Citrus fruit oils
8. Paraffin wax
9. Histoclear (recently introduced non-toxic)
10. CNP 30 and inhibisol

- Xylene is a widely used clearing agent in the histopathology laboratory and it is recyclable. Refractive index of xylene is 1.50.

2.2.3 Wax impregnation and embedding:

- Impregnation is the technique of the complete elimination of clearing agent through embedding media. Once tissues have been cleared, they requires infiltration by support media. Microscopic examination of tissue requires sectioning by microtomes. This requires infiltration and embedding of the tissues in a medium that permit thin sections to be cut easily.[34]

2.3 Embedding (casting or Blocking)

- As the tissue is completely penetrated by wax, it should be embedded into a block of paraffin wax that can be clamped into a microtome to cut the tissue sections. Tissue transferred in the final wax bath (in tissue processor) and later block preparation is carried out with pairs of Leuckhart's L mould in which molten paraffin wax is poured and the tissue is kept within it. The orientation of specimen should be done very carefully in the mould as its position will decide the plane of tissue cutting, which is an important reflection in both diagnostic and research histopathology.[18]

- On cooling, it forms a solid paraffin block and later mould can be detached. Now the tissue is ready for section cutting. When tissue processing is done optimally, the paraffin blocks having the tissues can be preserved for longer duration and can be stored. The tissue blocks are an important archival material for further reference.

- After tissues have been embedded in supporting media, the tissues can be cut by a rotary or fully automatic microtome machine of 4-5µ thickness. Before tissue staining procedure, it should be ready such that a thin section, having only one cell layer is possible to cut. Later it is kept on a glass slide for microscopy.

- Although various solutions have been tested and they are in use since decades, the most popular is paraffin wax based histological waxes. Most laboratories use wax with melting point of 58-60^{0}C for infiltration in tissue and then allow to cool to 20^{0}C. It solidifies the tissue such that it allows sections to cut by microtome machine[34].

- It should be noted that the paraffin wax media for tissue embedding have special physical properties that allow embedding as well as sectioning of tissues. There is a ribbon formation as the sections cut on the microtome. In addition, it also retains enough elasticity to flatten fully in tissue flotation bath after microtomy.

2.3.1 Different embedding reagents:

- Parffin wax
- Paraplast
- Paraplast plus
- Water soluble wax
- Ester wax
- Polyeaster wax
- Micro-crystalline wax
- Resins-Acrylic, epoxy, urea-formaldehyde
- Celloidin
- Agar
- Gelatin
- Carbowax

2.3.2 Paraffin wax additives: Various additives are added to paraffin wax to[25]-

- Modify its consistency and melting point and to improve the efficiency during microscopy.
- Cut thinner sections.
- Increase hardness.
- Get good ribbon sections
- Alter crystalline structure of wax.

- Commonly used additives are bees wax, rubber, ceresin, plastic polymers,

microcrystalline wax, Bayberry wax and diethelene glycol distearate. In present study, paraffin wax had been used as embedding medium and Beeswax added as an additive.

2.4 Methods and techniques for tissue processing

2.4.1 Routine manual method:

- It was the commonest method used in past decades. Its advantages include the reliability and affordable cost. But it is time consuming (about 16-24 hours) and use large amounts of harmful chemicals such as xylene and formalin.

2.4.2 Automatic tissue processor

- Automatic histoprocessor was first time designed before half a century. It has modified, latest and updated histoprocessing by decreasing the duration of processing of biopsy specimens from few days to 16-18 hrs. Better results of tissue quality is achieved in this machine because of constant agitation of tissues.

- Automatic tissue processor has two benefits. First is automatic movement of tissues from one container to another during day and night and another benefit is continuous agitation of tissues within the reagent containers decreases the time needed in the reagent. This automated technique excludes the possibility of human error and forgetfulness. The tissues can be processed in a night, such that it becomes ready in the morning for embedding.

- The conventional tissue processing is reliable and cost-effective, but it has also major disadvantages like delay in histopathological diagnosis as consumption of time and also toxicity of reagents.

- These automatic tissue processor are rarely used in organizations and laboratories where smaller quantity of tissues are processed. Processing schedule is also affected by power failure. Larger slices of tissues can't be processed properly in automatic tissue processor.[6]

- ***Tissue processing Methods for early diagnosis:***

F It is known fact that the reproducible and relatively low expense methods are commonly employed and continued to implement as a valuable tool for diagnostic purposes in laboratories. However, with the need for immediate or early reporting, new processing methods are being established. Each of them has its own advantages and disadvantages.[6,14,23]

F Often there is a need of urgent reports because early diagnosis and implementation of urgent treatment is largely dependent on histopathological findings. Numerous methods and techniques have been developed and still being established and implemented in this matter.

F Methods for early diagnosis are:

- Frozen sections
- Rapid tissue processing method,
- Microwave assisted tissue processing method.

2.4.3. Frozen section method

F By frozen section method, sections from fresh tissues can be produced by excluding various steps of tissue processing like fixation, dehydration, clearing and embedding. The main benefit of frozen sections is rapid preparation of histopathology slides for reporting.

F Frozen section method has been developed for histopathology specimen, however later it was used to demonstrate soluble substances like lipid. Lipid can be exhibited in tissues by using special stains like Sudan III, Oil Red O. Frozen section method has been acquired for urgent histopathological specimen reporting in emergency situations, for example, intraoperative biopsy specimen examination and consultation. But the drawback of frozen section is fairly decline in quality of tissue sections. [7,22,35]

2.4.4 Rapid tissue processing method

- The rapid tissue processing method was derived on basis of the common finding that tissues extracted under vacuum from step of dehydration to impregnation in wax were prepared with excellent results and also in short period of time. By this method, tissue impregnation occurs in 5-15min. In addition, there was observed that the quality of tissue sections gained by the method was of better quality than the conventional method and therefore it is appropriate for faster biopsy processing. This method allows tissue processing and preparation of H & E slides in few hours. Thus, by modification of routine processing technique, rapid reporting can be made possible. But the drawback is that this method is limited only for small biopsy specimen.[36]

- The rapid manual tissue processing method has major drawbacks such as usage of harmful chemicals, more tissue distortion and shrinkage of tissue. These caused researcher to study for newer short duration processing schedules[9].

- It is also observed that acidified dimethoxypropane can be used for dehydration and clearing both. This allows tissues to be impregnated by paraffin within an hour after the step of fixation. By the use of Dimethoxypropane, the tissues become totally water free because it reacts chemically with water and thereby ensure dehydration. Thus, after the dimethoxypropane procedure histology and histochemistry of tissues is similar to that after conventional dehydration and clearing.[37]

2.4.5 Microwave assisted tissue processing

- Recently newer microwave method is added in the list of methods for rapid processing of tissues, that has revolutionized histotechniques[38]. In 1945, the microwave oven was first introduced and later US patent award was given in 1950.[39] Microwave technique was initially used in the histopathology laboratory for tissue fixation in 1970 by Mayers[1] and for tissue processing in 1985 by Kok & Boon[7].

- Microwaves are non-ionizing electromagnetic waves having frequencies of 300 MHz to 300 GHz that correspond to 1m to 1mm wavelength respectively. All

domestic microwave oven work at 2.45GHz which correspond to 12.2 cm of wavelength. [39, 40]

- Research papers of Physics and chemistry illustrate that the viscosity of liquid reduces as the temperature of liquid increases, thus increasing its diffusion. Thus heat is known to increase diffusion.[6,14] Thus, initially conventional heating was used in histoprocessing to attain increased diffusion of reagents and thus decreasing the processing time. But this caused uneven distribution of thermal power, that caused firmness of outer layer while inner part persisted as unprocessed, and as a result softened. [36,39]

- The mechanism of action of microwaves is based on 'rotation of water molecules' where a single molecule of water has one large oxygen atom and two small hydrogen atoms which are attached with one another. Water molecules have a positive charge and a negative charge. When negative charges are taken in the electromagnetic field, repulsion occurs because of their same charges. This lead to rotation of molecules as they are not symmetrical. The charges in the electric field force the molecules to rotate in same direction or at 180^0 at the frequency of 2.45 billion cycles/second in the electric field depending on positive or negative charges The rotational movement of molecules produces heat[40].

- In contrast to conventional heating, the heating by microwaves is from inner side of the material (heating starts from inner core) and thus effect of irradiation of microwaves occurs throughout the material. [39,40]

- The benefit of this method include short processing time, elimination of harmful chemicals and less tissue distortion. The disadvantage of this method is the high cost of microwave oven.[40]

3. Staining

- Successful interpretation of the histopathological specimen is basically dependent on good sample preparation and staining. Staining of tissue specimens is depends on two key factors: 1. A physical process of distribution of dye in cells of tissue. 2. Physical and chemical process of combining dye to the substrate. It is known that heat application decreases the viscosity of the liquids

and causes increase in diffusion of liquid. Heat speeds up the staining process and the microwave provides uniform heating and helps in fasten the staining procedure along with providing better results[38].

- Staining is a procedure used to enhance contrast and highlight structures for microscopic visualization and hence it is used to examine tissues. For histopathology tissue sections, haematoxylins and eosin stain (H&E) stain is usually used as it stains the tissues optimally. It is achieved by staining cell structures like the nucleus, cytoplasm, cell membrane and extra-cellular components to identify the tissue structures and various pathological conditions.

- When required details can not be provided from H&E sections, special stains comes for rescue. It is important for tissue-based diagnosis or research as these stains allow pathologists, biologists and researchers to examine tissue morphology (structure), identification of cell types, differentiation of morphologically similar pathological conditions or even microorganisms such as bacteria.[41]

- In present study processing and staining by microwave method were performed to determine whether the tissues could give better results or not. By usage of a domestic microwave, staining results were compared with slides stained by routine method. It was determined that turnaround time was reduced in addition to comparable or better quality of stain by microwave staining method.

3.1 Factors affecting staining[25]:

1. pH of Solutions: it determines whether or not a dye will bound by certain tissue elements.

2. Temperature: increases diffusion rate of dye molecules and increase rate of staining.

3. Concentration of dye: Increase in concentration of dye molecules increases dye binding.

4. Presence of other salts in dye: can increase or decrease staining intensity.

5. Fixatives used: Formaldehyde, mercuric chloride and osmium tetroxide increase tissue basophilia. Picric acid increases acidophilia while ethyl alcohol is intermediate between these groups.

3.2 Microwave assisted staining

- Stimulation of molecules by microwave is a technique in which energy is absorbed within the tissues to a higher depth. In the electromagnetic field, dipolar molecules are forced to rotate which causes an increase in thermal agitation by effect of absorbed energy. Thus the generated kinetic energy is transformed into heat energy. If dipole movement of a molecule is higher, then the influence of the alternating electrical fields is greater , and th e heating process occurs faster. The dipolar value of water is 6.17, ethanol-5.64, and 2-propanol-5.54. In contrast, dipolar movement of paraffin wax is 0, that means its molecular structure is not affected by microwaves[42].
- As in different methods of tissue processing, in microwave assisted staining also diffusion is an important factor.[8,43] The primary effect of microwave energy is to promote the diffusion and augmentation of reaction rates by internal heating. Tissue processing prepares tissues appropriate for staining. Thus tissues processed by domestic microwave was stained by microwave staining method to determine whether they are compatible or not. At the end, the results of staining were compared for both the methods along with difference in turnaround time.
- Microwaves travel through material without any effect or little effect, or the waves can be reflected or absorbed. Material can be penetrated by microwaves. The infiltration power of microwaves depend on the electric conductivity of the material. Some materials, like glass, plastics and paraffin wax are considered "microwave transparent" as on exposure of microwave energy, the material is not affected . Some materials, like metal, causes reflection of microwaves as the penetration depth is very small. When substances are exposed to microwave irradiation, the stimulated material become excited and produce internal heat. In addition, it is also known fact that when microwave energy is absorbed in tissues, it is transformed into kinetic and chemical energy.[44]

4. Temperature control of microwave in tissue processing technique:

- Accurate control of temperature is crucial for microwave based methods, specially when the method is designed for histoprocessing.[43]

- The factors that affect the escalation in temperature of microwave-irradiated homogenous media are the level of radiation, the dielectric and thermal characteristics of the material, and other physical properties like evaporation, melting, dimensions and position of the material in the microwave in relation to radiation, added extra loads etc. To accelerate molecule diffusion and to speed up the reaction rates, heating by microwave is evidenced as very effective.[43]

- The recommended temperature range should be 70ºC to 85ºC.[6] The temperature rise should be monitored by thermometer.

Comparison between domestic microwave and laboratory microwave [42]

- Domestic and laboratory microwave ovens both can be used for performing number of procedures within a histology laboratory. However safe environment, reproducible cost, and quality of sample are of prime importance when choosing the best equipment for the procedure. The 2.45 GHz frequency is chosen for domestic microwave ovens since at this frequency polar molecules, mainly water molecules, react powerfully and the microwaves can generate enough decent energy. This property is important for cooking of food and for histology laboratory work also.

- Domestic microwave oven is more economical than the laboratory microwave however both provide nearly equivalent results.

- In house determination of volume of reagents and number of tissues, temperature monitoring, period of cycle time and different levels of power at various settings should be established before usage of domestic microwave oven for optimal tissue processing, whereas the laboratory microwaves are preprogramed for different procedures.[44]

- In contrast to domestic microwave ovens, the laboratory microwave oven

produce even heating in tissues without hotspots because of presence of magnetic stirrer which provides an equal field of radiation.

- In domestic ovens, harmful and inflammable solvent produces fumes which cannot vented adequately. In contrast, laboratory microwave is designed in such a way that suitable ventilation is present so that fumes generated can be removed from working area.

5. Uses of Microwaves in Histopathology

5.1 Application of microwave in fixation

- Fixation is the process that prevents autolysis and keeps the tissue morphology nearly similar to the living state of their life. This can be attained by tissue protein cross-linking, that makes proteins semisolid and insoluble.[14,45] Microwave energy was initially used for histological fixation of fresh specimen in the field of histopathology. The observed shrinkage was very less and customary artifacts were slight or non-existent. There was preservation of microscopic findings and uniform staining of tissues. Cellular and nuclear details were maintained well but there was vanishing of erythrocytes and collagen fibres to a small scale which was unexplained.[17] Subsequent studies were performed for histological tissue fixation by microwave energy as a source of heat.[35]

5.2 Use of microwave in tissue processing.

- The physiological basics of tissue processing mainly depends on the diffusion of solutions within the tissue to be processed. Therefore many authors have proved that a microwave is very useful and perfect device whenever faster processing of tissues is required. Because the microwave tissue processing shortens turnaround time as it emits non-ionizing radiations with electromagnetic properties that lead to quick heating of solutions and therefore allowing faster diffusion of solutions in tissue.[12,31,43]

- Unlike conventional heating, the effect of radiation occurs simultaneously throughout the whole tissue being microwaved.[46]

- In histoprocessing, diffusion is an important factor. The rate of diffusion defined by the formula is $<X^2> = 2Dt$, where the "X" represents total distance traversed by a particle in reagent in a particular path; " t" is the time period of diffusion; "D" is the diffusion constant of the material; < > represents for the mean value. As per the formula, the average squared distance which is passed through by a particle in reagent is equal to the time of diffusion. This shows that the biopsies should be thin, however tissue length and breadth do not alter results.[47,48]

- In present study, methanol was used followed by Isopropyl alcohol. In processing step, the residual alcohol of the tissue would be vaporized with the help of microwave power at a step of impregnation and thus abolishing the requirement for a separate procedure of clearing. Hence it reduces the price and materials with harmful effects like xylene. This is consistent with the research of Raju[49] and Pritam et al.[12]

- The method reported here produces histologic material of equivalent or higher quality than those produced by time-consuming conventional processing. The method has many benefits that include speed, safe laboratory environment, molecular integrity preservation of specimens and improvement in the laboratory workflow, which allows the preparation of diagnostic material in a reduced turn around time.[50]

- A study by Carson suggested that a decreased turnaround time from few days to 72 minutes. Variety of tissue samples were processed by a special laboratory microwave oven, where the reporting could be done on the same day.[28]

- Thus it proves that a microwave has carried about dynamic development in biomedicine, histopathology and development of drug because of reduced tissue processing time from days to minutes. Thus, this satisfies the requirement of patients and physicians.

- Current study was performed to determine the usage of domestic microwave for processing of histopathology tissues and for comparison of results of tissues with twin sample that was processed by routine method. Here, Methanol, isopropyl

alcohol and melted paraffin wax were used for processing of tissue in domestic microwave. Here use of xylene is eliminated that have potential carcinogenic effects.

5.3 **Use of microwave in staining**

- Histopathological tissue sample preparation and staining is of immense importance for effective interpretation and diagnosis. Tissue sections staining and slide preparation is largely depend on diffusion of dye in the tissue and availability of its binding site. Therfore, microwave irradiation is useful for staining procedures also.[51]

- Brinn introduced rapid metallic staining by Microwave oven.[52] by incorporating microwave radiation to the periodic acid oxidation step, he could decrease time of the methenamine silver staining method. It ususally takes 90-180 min that was reduced to below 20 min. Microwaves have been used to hasten a number of histochemical stains in smears, tissue blocks and plastic sections. Brinn also augmented various procedures by microwave exposure like Pascual's modification of Grimelius', Masson-Fontana's, and Perls'. In these cases, time of staining as well as background precipitation were considerably decreases.[52]

- When dye solution is exposed to microwave irradiation, it causes stimulation of polar molecules (such as water or alcohol) and ions (e.g. silver ions) . Hence diffusion of dye molecules into tissue sections is speeded up and binding of dye and substrate is increased. Heating from inner part of tissue is an important factor for these processes. Microwave assisted staining has the advantage of rapidity and brighter staining with a clear background as compared to conventional staining.

- Microwave radiation may be used for enhancing various stains like routine, special, metallic, and immunofluorescent stains.[43] Thus various staining procedures that usually take minutes can be performed in a microwave oven within seconds; and the procedures that takes hours, minutes or days can be finished in a considerable short duration by microwave techniques.

- By usage of microwave irradiation, fastest and very thin sections can be prepared for electron microscopy. The stained slides had better contrast, a little artifact as precipitate and better even staining in overall tissue. Rapidly stained sections by microwave were of improved quality as compared to routine stained sections. The process decreases the turn around time per specimen, without deteriorating the quality and thus increases the efficiency of laboratory along with cost reduction.[31]
- For rapid staining of acid and alcohol-fast organisms, microwave method abolishes the laborious stage of dewaxing and also the dangers of naked open flame in the laboratory. In addition, mycobacteria are killed by microwave irradiation for 30-60 seconds at the microwave power of 640 W. Faster staining of the microorganisms occur by enhancing permeability, that leads to fast penetration of bacterial enzymes and staining reagents.[15]
- The maturation and proportion of the various cell lineage in normal and pathological bone marrow can be easily detected. Staining of tissues substances (like melanin, epithelial basement membrane, argyrophilic granules, , fungi and acid-fast bacteria) with reduced background stain can be attained by silver staining procedures in microwave[52] and also the microwave assisted Ziehl-Neelsen method.[15]
- Microwave-accelerated special staining is also comparable with conventional special staining. Sections stained by microwave for special stain methods such as Periodic acid – Schiff's, Van Gieson, and toluene blue are similar to routinely stained special stain with added benefit of having the ability of reducing the time.[51]

5.4 Microwave in immunohistochemistry [54,55]

- The interaction of Avidin-biotin complex (the ABC method) is usually used for identification of specific protein types by use of primary antibodies.
- Though ABC method is established procedure for immunohistochemical investigations, the conventional protocols takes 2-3 hours. The duration of procedure and background noise is decreased by Microwave irradiation. Microwave irradiation is applied after deparaffinization in this procedure.

5.5 Antigen Retrieval in Microwave: [56,57]

- Samples embedded in Paraffin fixed with neutral buffered formalin can not be used for immunohisto-chemical staining or in situ hybridization because of masking of antigenic sites by protein-protein cross- linking by formaldehyde.
- Therefore, samples fixed in formalin need antigen retrieval by use of microwave generated heat induced antigen retrieval. It is performed to retrieve the epitopes by unmasking of antigens.

5.6 Microwave stimulated decalcification: [58,59]

- Decalcification is important step after formalin fixation of bone so that better quality of tissue sections can be obtained. Decalcification of bone for histopathological examination requires longer duration i.e. 2–4 days by use of 10% formic acid or 1-2 weeks by 10% EDTA.
- By usage of microwave irradiation, the processing time can be decreased to 1/5–1/10 of the initial preparation time.
- The duration of procedure must be determined by the researcher and required duration must be adjusted as per the bone size and hardness.

5.7 Microwave assisted cryosectioning:[60]

- Histopathological diagnosis, detection of enzymes and detection of antibodies by immunofluorescent microscopy, frozen section procedure is used.
- When tissue is frozen for cryosectioning, water crystallization occurs. It causes damage of tissues. Water crystal formation in tissues can be reduced by microwave irradiation which maintains good architecture of tissue.
- Microwave irradiation blends well with embedding medium, i.e. TissueTek OCT compound. So it can be cut very effortlessly. In comparison to nonirradiated specimens, microwave-irradiated samples have less bubbles and artifacts within the tissues and morphology is also well maintained.

5.8 Microwave processing of Cell block prepared from FNAC:[61,62]

- Cell block refers to collecting sediment, blood clot or grossly visible tissue bits from cytologic specimen which are processed as paraffin block.
- Routine cell processing varies from 12 to 24 hours by manual method which would require at least 5-6 hours. In contrast microwave processing takes less than 10 minutes for processing [62].
- The advantage of Cell block processing in microwave is that FNA smears and cell block were available on same day and almost at same time of cytology smear examination.
- In addition, ancillary tests like IHC can be done in indicated cases as well as Cell block can be compared with respective histopathology sections whenever available.

5.9 Application of microwave for Processing Fatty Tissues[63]

- Whenever the tissue is poorly fixed, it causes tissue damage and bubble formation in paraffin embedded sections.
- Fatty tissue fixation is difficult due to low penetration of paraformaldehyde solutions. To overcome problem of fixation, fatty tissues need to be treated by xylene and methanol mixture after fixation.
- Generally, for fixation, fatty tissues needs treatment of approximately 10–30 hours. However by microwave irradiation, the time of fixation can be markedly decreased to approximately 1/20–1/30 with proper fixation.
- Radiation time must be adjusted as per the amount of the fatty tissues. However, the exact duration of radiation exposure should be established by the researcher.

5.10 Microwave assisted Immunofluorescence Microscopy[64,65]

- For immunofluorescence microscopy, microwave irradiation cuts the duration of incubation period to approximately 1/5–1/10 of the original time.

5.11 Microwave assisted Electron Microscopy.[66,67]

- The tissues that is going to be analysed by electron microscopy are embedded within the resin for ultra thin section cutting procedure.
- As per the chemical composition, the duration of embedding procedures vary however they usually require approximately 1 week for processing. For electron microscopy sample preparation, the use of microwave irradiation decreases the procedure duration to about 2 days without depriving fine tissue structure in the samples.
- For fixation, the technique of microwave irradiation is effective for transmission and scanning electron microscopy.

5.12 Slide drying in Microwave[68]

- Slide drying in conventional oven is done at temperature between 60^0C and 80^0C, for 20 to 60 minutes. In contrast, slide drying in microwave oven takes 2 to 5 minutes.

Basis of the study:

- Microwaves are the utmost flexible type of energy that work in variety of deciplines like radar, communication, chemistry, rubber vulcanisation, drying, food processing, medical treatment and diagnosis and variety of materials processing fields.[69] For more than past two decades, thanks for introducing energy of microwaves in biological laboratories. Because of their introduction, faster processing methods has been developed and the elimination of toxic substances also occurred.[14]
- Turn around time is very important for better reputation of the laboratory. But this becomes more significant and critical when patient treatment is dependent on the laboratory report. It is of utmost important to balance turn around time of laboratory by pathologist and expected reporting time recommended by clinician. It remains always as an progressively improving ongoing effort by the laboratory. During tissue processing, if any step is

performed inadequately or rapidly, the following processing steps may be unsuccessful, and tissue will be under processed and also nondiagnosable.

- The current study has been conducted to compare the benefits of domestic microwave assisted tissue processing and staining with conventional processing and staining. The results were evaluated on basis of comparing the histologic quality and the total turnaround time. Evaluation by expert and experienced pathologist was done on both conventional and microwave processed sections to evaluate cellular details, cytoplasmic details, nuclear details and staining characteristics. Thus, the results were evaluated to determine whether any potential improvement in reproducibility of microscopic morphology of tissues.