MATERIAL AND METHOD

- The aim of present study is to compare the microwave-assisted tissue processing and staining method with routine method. By comparison of results of two methods, present study can determine whether microwave assisted method gives results comparable or better than conventional tissue processing and staining method. Thus, one method is compared with the other method.

Sources of materials:

- Present study is a cross sectional analytical prospective study.

- Here, 350 paired tissues (total 700) were selected from Histopathology Laboratory of Pathology Department, S.S.G. Hospital and Medical College, Baroda. The selected specimens were fixed for at least 24 hours in 10% neutral buffered formalin (HCHO) (approximately 4% formaldehyde) solution, a process referred to as fixation to prevent autolysis and decomposition of tissues.

Method of data collection:

- The sections taken from the specimen range from 0.8 x 0.8 x 0.6 cm to 1.5 x1.5 x0.8 cm in size.(Some tissues like appendix, wall of gall bladder, TURP chips, cyst wall, umbilical cord shows variation in size). The section were cut into two equal halves of 3mm to 4mm thickness. One part was processed and stained by conventional (routine) method and the other by microwave method.

- Before and after both the procedures, specimen type and gross features, namely colour, consistency and dimensions were recorded. The tissue processed by routine method was stained by routine staining method and microwave processed tissue by microwave staining method. The stained slides in each group were coded and labelled as described below and evaluated by expert and experienced pathologist blindly (without knowing the method performed). The evaluation proforma is attached as annexure 1 and list of specimen is annexure 2.

- Whenever applicable, special stains and immunohistochemistry was performed on tissue processed by both methods.

Coding and labelling of slides:

- Two slides were prepared from a specimen by both methods. For coding, both the slides were labelled as A or B randomly with specimen number. In the same manner, further samples had been labelled progressively as number progresses. i.e. specimen number 25 is labelled as 25A and 25 B where evaluator doesn't know by which method the tissue had been processed and slides stained.

Inclusion Criteria:

- Medium to large sized tissue fixed in 10 % neutral buffer formalin for atleast 24 hours.

Exclusion Criteria:

- Small biopsy (<1x1x0.6)
- Bony hard tissues (bone tissue need decalcification before tissue processing that requires a longer duration, which is nearly 5 to 6 days; hence, this would be time consuming)

Period of study:

- Three years. The samples received in Pathology department from September 2018 to July 2020 were included for the study.

Temperature control of microwave oven:

- In present study, Samsung microwave oven model no. MS23K3513 with maximum output of 800W was used (Figure 6.1).

- Tissue processing was carried out with a power output of 300 W (38% power). In present study, total six cycles each of 10 minutes setting was adopted, as this did not show any detrimental effects on final microscopic results of the specimen. The reagents were changed at each cycle to prevent overheating of tissue. The temperature was maintained between 70^0 to 85^0C which was monitored by laboratory thermometer.

- Temperature of methanol was recorded by thermometer. Methanol was kept inside microwave oven and at the end of cycle 1, the thermometer was kept in methanol for 15 to 30 seconds and results were recorded. In the same manner, for the 4^{th} cycle, temperature of Isopropyl alcohol was recorded at the end of cycle by thermometer. The cycles were performed consecutively without any interruption. For each cycle, reagents taken were of room temperature.

- For Hematoxylin and Eosin staining, the microwave oven was used at output of 180 W (23% power). Tap water was kept in petridish beside glass slide and was changed periodically to avoid evaporation and drying of stain.

Basic steps for tissue processing and staining included:

1. **Dehydration**: Alcohol removes water from the tissue.
2. **Clearing:** Clearing agent removes alcohol from tissue because alcohol is not miscible with paraffin wax.
3. **Impregnation:** By molten paraffin wax to preserve the cell structural integrity.
4. **Embedding:** Keep tissue in molten paraffin wax kept in L mould and allowed it to cool. This makes solid blocks of tissue. So that sectioning of tissues can be possible.
5. **Microtomy**: Cutting of paraffin blocks at 4µm thickness by a rotary microtome.
6. **Deparaffinization:** To remove paraffin from tissues to allow penetration of water based staining reagents.
7. **Haematoxylin and eosin staining-**To examine tissue under microscope.

Routine (Conventional) tissue processing: Equipment required:

- Automatic tissue processor
- Metal basket
- Glass beaker 2 liter (total 11)
- Paraffin wax
- Metal/plastic tissue cassettes

Reagents required:

- Isopropyl alcohol in graded concentrations
- Xylene
- Paraffin wax

Protocol for routine(conventional) tissue processing:

- Plastic/metal cassettes with labelled tissues were kept overnight to fix tissue in 10% neutral buffered formalin for 24 hours at room temperature from 5 pm to 5 pm of the next day (Figure 6.2).

- Routine tissue processing had been performed by automatic tissue processor (Yorko) (Figure 6.3) having uninterrupted power supply by 8 KV UPS attached to it. Graded concentrations of isopropyl alcohol, xylene and paraffin wax were used as per the schedule in Table 4.1.

- The cassettes were kept in two metal bucket of the automatic tissue processor and processed from 5.30 pm to 9.30 am of the next day (total 16 hours). The automatic processor had 11 chambers, six for dehydrating agent (graded alcohol concentrations), three for a clearing agent (xylene) and two chambers for paraffin impregnation. Tissues were processed in it at room temperature in alcohol and xylene while temperature of paraffin wax was kept at 58^0C-60^0C. Bucket containing tissue capsule rotates in beaker to allow agitation of fluids and better penetration in tissues. In built timer of tissue processor move bucket from one beaker to next beaker.

- Finally, the tissues were embedded in paraffin wax using L moulds (Figure 6.4).Thus tissue blocks became ready for sectioning by a rotary microtome. Leica 2125 RT was used for section cutting (Figure 6.5).

Table 4.1 Protocols for automatic tissue processing by routine method

Reagent	Duration	Temperature
Dehydration		
70% Isopropyl alcohol	3 hours	Room temperature
80% Isopropyl alcohol	1 hours	Room temperature
90% Isopropyl alcohol	1.5 hours	Room temperature
99% Isopropyl alcohol	1.5 hours	Room temperature
99% Isopropyl alcohol	1.5 hours	Room temperature
99% Isopropyl alcohol	1.5 hours	Room temperature
Clearing		
Xylene	1 hour	Room temperature
Xylene	1 hour	Room temperature
Xylene	1 hour	Room temperature
Impregnation		
Molten Paraffin wax	1.5 hour	60^0C
Molten Paraffin wax	1.5 hour	60^0C
Total time	**16 hours**	

Tissue processing by microwave method:

Equipment required:

- Samsung Domestic Microwave oven (model MS23K3513) with a maximum output of 800W
- Microwave-resistant glass beakers of 500 ml (total 6),
- Beaker no. 1 and 2 for methanol(Dehydrating agent)
- Beaker no. 3 and 4 for isopropyl alcohol (Intermedium)
- Beaker no. 5 and 6 for paraffin impregnation
- Laboratory thermometer
- Plastic tissue cassettes
- Hand towel.

Reagents required:

- Methanol
- Isopropyl alcohol
- Paraffin wax

Table 4.2. Protocol for tissue processing by microwave method

Reagent	**Microwave method**	**Temperature/Power**
Running water	5 minutes	Room temperature
Dehydration:		
Methanol	10 minutes	300 W
Measurement of temp.1	1 minute	--
Methanol	10 minutes	300 W
Isopropyl alcohol	10 minutes	300W
Isopropyl alcohol	10 minutes	300 W
Measurement of temp 2	1 minute	--
Impregnation		
Molten Paraffin wax	10 minutes	300W
Molten Paraffin wax	10 minutes	300W
Total time	**67 minutes**	

- Present study was conducted in accordance with the protocol established for tissue processing in domestic microwave oven by T Mahesh Babu *et al* (Table 4.2) [4]

- The procedure was performed in microwave proof vessel kept at marked constant position on rotating plate to achieve most consistent results.

- Plastic cassettes must be used instead of metal cassettes during tissue processing. Because metals and metallic utensils can not be used in microwave because of total internal reflection of microwaves, which can cause spark inside the chamber.[20] Plastic cassettes are relatively cheap and can be reused.

- Four pre-fixed tissues were washed under running tap water for 5 minutes to remove additional formalin from the tissue to decrease alcohol and formalin interference.

- Samsung domestic microwave oven (model MS23K3513) was used for present study. It shows six power levels as per table 4.3.

Table 4.3 Power levels of microwave oven Samsung (model MS23K3513)

Power level	Percentage	Output	Power Used in present study
High	100%	800W	-
Medium high	75%	600W	-
Medium	56%	450W	-
Medium low	38%	300W	Tissue processing
Defrost	23%	180W	Slide staining by H&E Method
Low	13%	100W	-

- Two tissues were transferred in beaker no. 1 and remaining two in beaker no. 2, each containing 300 ml of methanol (Figure 6.6).

- Methanol was used as a dehydrating agent for microwave tissue processing. The microwave oven was set at 300 Watts (38% power). The tissues were dehydrated in two cycles, each by methanol 300 ml at 38% power for 10 minutes (total 20 minutes). Temperature was recorded at the end of first cycle (Figure 6.7).

- The methanol was kept in storage container and respective tissues were transferred to beaker no. 3 and 4 each containing 300 ml of isopropyl alcohol. Two cycles were done each for 10 minutes (total 20 minutes) at 300 W (38% power). Temperature was recorded at the end of 4^{th} cycle (Figure 6.8).

- Clearing agent is not required in microwave assisted method because the temperature facilitates evaporation of alcohol from tissue before paraffin infiltration. In addition, the boiling point of xylene is higher and microwavability is lower. Thus it takes double time for heating the same quantity of reagents.

- Then, subsequent four tissues were transferred in two beakers each of 300 ml molten paraffin wax. The tissues were impregnated by molten paraffin wax by two cycles of 10 minutes each (total 20 minutes) at 300W (38 % power). Except paraffin wax, all other reagents were heated directly inside the microwave.

Paraffin wax was melted at 60° C on a hot plate separately and later kept in the two microwave safe glass beaker (Figure 6.9).

- After each cycle, the remaining reagents were kept in labelled containers for reuse. (Before processing for next cycle of tissue processing, microwave oven has to be allowed to cool)

- Tissue blocks were then made using Leuckhard's 'L' moulds, later sectioned, stained with haematoxylin and eosin stain and mounted on a glass slide to analyse tissue under a microscope.

Troubleshooting:

- If tissues show shrinkage with low staining intensity, the dehydration time can be adjusted.

- If sectioning of tissues is difficult suggesting inadequate impregnation with paraffin wax, time for impregnation can be adjusted.

Microtomy and Section cutting: Equipment required:.

- Rotary microtome
- Low profile microtome blade
- Tissue floatation bath
- Egg albumin coated glass slides
- Diamond marker pen

Protocols for Microtomy and Section cutting:

- Tissue block surface smoothen for cutting the tissue by applying it on hot plate.

- Embedded tissues in paraffin block were sectioned at 4 µm thickness by means of a Rotary Microtome (Leica RM 2125 RT). SLEE low profile microtome blade was used for cutting the tissue sections. (Figure 6.10)

- Ribbon like structures of sectioned tissues were kept in tissue floatation bath that is set at temperature of 56^0C.

- The tissue sections were taken on slides coated with egg albumin to adhere tissue during staining. The unique ID number is given on slide for further reference. The sections were kept on hot plate at 60^0 C for 10 minutes. This was followed by dewaxing in three changes of xylene for 10 minutes each. Later keep in isopropyl alcohol for 2 minutes and then tissue sections were hydrated in running tap water for 2 minutes.

Conventional hematoxylin and eosin staining : Equipment required:

- Container for reagents
- Slide holding rack
- Container to hold slide in running tap water
- Glass slides
- Coverslips

Reagents required:

- Xylene
- Isopropyl alcohol
- Hematoxylin stain
- 1 % acid alcohol
- Eosin stain
- DPX mountant

Protocols for Conventional hematoxylin and eosin staining (Table 4.4):

- For conventional tissue staining procedure, slides were kept in hematoxylin stain solution for three minutes and then placed in running tap water for three minutes.
- Sections were dipped in 1% acid alcohol (two dips) and then slides were washed for five minutes in running tap water.
- Then slides were dipped in a jar containing eosin stain for 30 seconds. They were

washed in three changes of isopropyl alcohol as one to two dip to remove excess eosin stain.

- The slides were air dried and cleaned in warm xylene for 10 minutes and later mounted with a coverslip using DPX mountant for microscopy and lastly, they were labelled.

Method of H&E Staining by Microwave oven[8]: Equipment required:

- Two petridish
- Dropper bottle of 200 ml each containing hematoxylin, acid alcohol, eosin, isopropyl alcohol and tap water.

Protocols for microwave assisted hematoxylin and eosin staining (Table 4.4):

- For deparaffinization, slide were heated on hot plate at 60^0C for 10 minutes and then kept immediately in three changes of xylene for 10 minutes each.
- After air drying, slides were kept in isopropyl alcohol for 2 minutes and then kept in running water for two minutes to hydrate tissue. Now the slides are ready for hematoxylin and eosin stain.
- The petridish was kept at constant marked position during the procedure to get consistent results.
- Two petridishes were taken each containing a single slide with few drops of tap water. The tissue was covered by 1 to 2 drops of hematoxylin and microwave at 180W for 30 seconds (Figure 6.11).
- Keep slide in running tap water for 30 seconds to remove excess hematoxylin.
- One drop of 1% acid alcohol was kept on tissue for 2 to 3 seconds for differentiation.
- The slide was kept in running tap water for 30 seconds. Later slide was dipped in petridish containing tap water and microwave at 180 W.
- Then, one drop of eosin was poured on tissue for 2 to 3 seconds followed by few

drops of Isopropyl alcohol to remove excess eosin stain.

- Slides were air dried and cleaned in warm xylene for 10 minutes and later mounted with a coverslip using DPX mountant for microscopy and lastly, labelled.

Table 4.4 Protocols for tissue staining by microwave and routine methods.

Reagent	Microwave	Routine
Running tap Water	2 minutes	2 minutes
Hematoxylin in microwave at 180 W	30 seconds	3 minutes
Running tap water	30 seconds	3 minutes
Differentiation by 1 % acid alcohol	1 drop for 2-3 seconds	Dip for 2-3 seconds
Running tap water	30 seconds	5 minutes
Tissue dipped in petridish containing tap water in microwave at 180 W.	30 seconds	-
Eosin	1 drop for 2-3 seconds	Dip
Isopropyl alcohol	Few drops for 2-3 seconds	Dip
Xylene	10 minutes	10 minutes
Total time	14 minutes	23 minutes

Embedding, section cutting and staining had been done simultaneously for both tissue blocks of same tissue. Slides were numbered and coded before reporting.

Scoring and Grading:

The stained slides of each method were randomly numbered as unique ID number with A or B for a blind study and evaluated by expert and senior pathologist. The observer was tasked with judging four parameters and ten subparameters (Table 4.5)

Table 4.5 Parameters and subparameters for stained slide assessment.

	Parameters		**Sub parameters**
1	Cellular details	1.	Cellular outline
		2	Clarity
		3.	Integrity of tissue
2	Cytoplasmic details	4.	Nuclear cytoplasmic contrast
		5.	Eosinophilia /granularity
3	Nuclear details	6.	Clarity of nucleus and nucleoli
		7.	Clarity of nuclear membrane
		8.	Clarity of chromatin
4	Staining characteristics	9.	Colour intensity
		10.	Uniformity

Parameters evaluation by microscopic examination: A. Cellular Details:

1. Cellular outline: It was evaluated by examination of cell boundary. It should be appreciated at cell- cell or cell- stroma interface.

2. Clarity: Clarity of cellular details like intracytoplasmic or extracytoplasmic secretary products, red blood cells, inflammatory cells, muscle tissue, fibrous tissue etc. should be well appreciated.

3. Integrity of tissue: Tissue should be intact, without fragmentation and serrations. Stroma and epithelial cell interface should be well preserved.

B. Cytoplasmic details:

1. Nuclear cytoplasmic contrast: basophilic nucleus and greater eosinophilia of cytoplasm produces enhancement of the nuclear cytoplasmic contrast. Greater resolution of slides can be done by better nuclear cytoplasmic contrast.

2. Eosinophilia/granularity: Intracytoplasmic details should be clearly identifiable i.e. normal/tumor/hurthle cells with eosinophilic/granular cytoplasm should be well appreciated.

C. Nuclear details

1. Clarity of nucleus and nucleoli: crisp staining of nucleus is mandatory for evaluation of various nuclear details like nucleoli, mitosis etc.

2. Clarity of Nuclear membrane: it should be stained sharp and crisp to distinguish nuclear and cytoplasmic details.

3. Clarity of chromatin: Chromatin details like vesicular, hyperchromatic, powdery, clumped, coarse should be well appreciated. Changes of nuclear smudging and pyknosis of cells should be identified.

D. Staining characteristics:

1. Colour intensity: Intensity of stain should be adequate for interpretation of microscopic examination.

2. Uniformity: the tissue should be evenly stained throughout without any variation of staining in different areas.

Microscopic evaluation of tissue:

· Parameters were evaluated on basis of subparameters.

· Marks of each subparameters-4

· Total marks were given out of 40.

· Grade was given as per marks obtained.

i.e.

Grade I(Poor)- Less than 10 marks

Grade II(Average)- Between 11 to 20 marks

Grade III(Good)-Between 21 to 30 marks

Grade IV(Excellent)- Between 31 to 40 marks.

Turnaround times

- Turnaround time was evaluated in conjuction with the quality of structural preservation of tissue to facilitate staining and evaluation under microscope.
- For tissue processing, 16 hours of conventional tissue processing vs 67 minutes of microwave assisted tissue processing suggests major advantage of microwave method without any detrimental effects on outcome.
- Staining of tissue also shows difference of 14 minutes by microwave method and 20 minutes by routine method. It is due to heat that enhances diffusion of reagents and allows better penetration in tissue and reduction in turn around time.

Statistical Analysis::

Data obtained after evaluation of histopathology specimens were subjected to statistical analysis to determine whether there is any significant difference among the two techniques. Observed data was statistically analysed and scores obtained for both techniques were presented as mean, standard deviation and standard error of difference.

Statistical Analysis:

1. Descriptive statistics (Frequency, Mean, Standard Deviation, Confidence interval).
2. Independent t test to compare quantitative data.

Table 4.6 Result evaluation proforma:

Parameter	Processing method	Mean	Standard Deviation	Standard error of difference	T value	p value	Confidence interval at 95% level	
							Lower limit	Upper limit
Cellular details								
Cellular outline	Routine							
	Microwave							
Clarity	Routine							
	Microwave							
Integrity of tissue	Routine							
	Microwave							
Cytoplasmic details								
Nuclear cytoplasmic contrast	Routine							
	Microwave							
Eosinophilia/ Granularity	Routine							
	Microwave							
Nuclear details								
Clarity of nucleus & nucleoli	Routine							
	Microwave							
Clarity of nuclear membrane	Routine							
	Microwave							
Clarity of chromatin	Routine							
	Microwave							
Staining characteristics								
Colour intensity	Routine							
	Microwave							
Uniformity	Routine							
	Microwave							