1. INTRODUCTION

1.1 Purpose of study

Rapid processing of histopathologic tissue is becoming increasingly desirable to fulfill the needs of clinicians treating acutely ill patients. Turn-around time is an important issue for many years and has become increasingly important in this age of managed care, commitments and health care services.

Histopathological diagnosis of specimens is greatly dependent on good sample preparation and staining. Both of these processes are governed by diffusion of fluids and dyes in and out of tissue, which is the key in tissue processing and staining.

It is known fact that application of heat decreases the viscosity of fluids thereby increasing diffusion of fluids that reduces the time of processing and staining from hours to minutes. Domestic microwave is used for the generation of heat to produce better results[1]. From the perspective of the final product, my purpose is to establish microwave irradiation tissue processing and staining method that shortens the time from specimen reception to diagnosis without compromising the overall quality as well as antigenic property of histological section.

1.2 **Aims and Objectives:**

- To modify routine processing and staining techniques of biopsy specimen by domestic microwave oven[2].
- To compare mean shrinkage of tissue, quality of histopathologic slides, time duration and antigen preservation for routine and microwave method.

2. REVIEW OF LITERATURE

Microwave tissue processing technique was introduced by Boon and Kok in 1985[2], but the potential application of microwave energy was first recognized by Mayers in 1970, who successfully fixed tissue with a microwave generator[3].Boon *et al*reported that it was possible to produce a significant acceleration of tissue processing using microwave radiation[2].

Examination of tissues under a microscope requires a slice of tissue that is thin enough to transmit light, and the preparation of such thin slices is called section cutting or microtomy. The soft tissues must undergo preparatory treatment before being sectioned. This requires fixation, processing and impregnation in a suitable embedding medium to provide support and a suitable consistency for microtomy.

2.1 Mechanism of action of Microwave oven:

Microwave oven is an integral part of our daily lives and its applications in the pathology laboratory has an inevitable outcome. Microwave method is a recent tissue processing technique, and it seems to reveal the new face of pathology as it continues to yield better and more precise diagnosis with a reduced turnaround time. In this process, the penetrative properties of the microwave and the conversion of electromagnetic energy into heat is utilized[4].

Microwaves emits nonionising radiation. Microwave causes 'rotation of water molecules' to generate heat. One molecule of water has one big atom of oxygen to which, two little hydrogen atoms are attached. The portion of the molecule containing two hydrogen molecules is positively charged, and the oxygen-containing portion is negatively charged. This asymmetric distribution of charge subsequently creates polar water molecules.

The excited molecules cause oscillation with adjacent molecules producing friction and cause production of heat within the material itself until the radiation ceases. The heat produced enhances the rate of diffusion of fluids to permeate into the tissues. The rise in temperature decreases the viscosity of processing fluids that facilitates diffusion. Therefore, it is possible to fasten the tissue processing and staining. This results in a substantial reduction in turnaround time and providing earlier diagnosis[5].

2.2 Tissue fixation:

It is important that fresh specimen should be 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, that is in the operation theatre, or, if this is not possible, immediately transport specimen to the laboratory[6]. Fixative used in present study is 10% neutral buffered formalin.

2.3 Tissue processing by Routine method:

Tissue processing is routinely performed in automatic tissue processor. Specimens that are to be processed are usually placed in suitable labelled cassettes (small perforated baskets) to segregate them from one another. Fixed tissues are first dehydrated and then embedded in a solid medium such as paraffin wax so that the tissue is rendered firm enough yet to enable thin sections to be cut while at the same time the tissue is soft and elastic enough to enable microtome knife to cut sections.

The routine tissue processing takes hours to complete procedure. The need for a reduction in tissue processing time has led to the realization of alternative methods that can producecomparable histological slides within a short period of time. Reduction of turn around time is of the essence in any laboratory, but even more so when patient care is critical. The stages of tissue processing include dehydration, clearing, impregnation and embedding. Each stage of tissue processing has a designated duration to ensure completion of the procedure.

2.3.1 Dehydration:

The process of removal of water and fixative from the tissue is termed as dehydration. As melted paraffin wax is hydrophobic (immiscible with water), most of the water in a specimen must be removed before so it can be infiltrated with wax[7]. This process is commonly carried out by immersing specimens in solutions of increasing concentration of alcohol (usually 60% to 100%) until pure, water free alcohol is reached. Alcohol 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.

2.3.2 Clearing:

Although the tissue is water free after the dehydration step, tissues still cannot be infiltrated with wax since wax and alcohol are immiscible. Hence an intermediate solvent that is miscible with both alcohol and paraffin wax must be employed. This solvent should have properties that will enable it to displace alcohol from the tissue, which in turn would be displaced by molten paraffin wax[8]. 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[9]. 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 alcohol [10].

2.3.3 Wax Impregnation:

Impregnation is the process of the complete removal of clearing agent by embedding media. Once tissues were 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[11].

2.3.4 Embedding (casting or Blocking):

After the tissue is infiltrated by paraffin wax in automated tissue processor, it becomes ready for embedding. Tissue is embedded into paraffin block. The tissue can be sectioned using a microtome, usually with a thickness of 4 - 5µ. When tissue block cut on microtome, it forms ribbon. In addition it also retains sufficient elasticity to flatten fully during flotation on a warm water bath.

2.4 Tissue processing by Microwave method:

Literature from Physics and chemistry suggest that the viscosity of liquid decreases as the temperature of fluid increases, thereby increasing diffusion. Thus heat is a known factor to increase diffusion[6,12]Microwaves emits electromagnetic waves that causes 'rotation of water molecules'. This rotational movement produces heat.[13]

Initially conventional heating was employed into histoprocessing in order to achieve increased diffusion thereby reducing the processing time. But this led to uneven distribution of heat energy, which resulted in hardening of outer layer whereas the central part remained unprocessed, and therefore soft[13].Unlike conventional heating, heating in microwave is from within (internal heating) and its effect occurs throughout the material being irradiated.[14]

In this study microwave assisted processing was conducted by modification of steps of routine method. Thus adequacy of tissue processing determined by quality of final product that is stained slides and advantages related to turn around time.

2.5 Staining:

Successful interpretation of the histopathological specimen is basically dependent on good sample preparation and staining. Staining is a technique used to enhance contrast and highlight structures for microscopic visualization. Hence is used to examine tissues. In the pathology laboratories, staining is usually done with haematoxylins and eosin stain (H&E) because of its ability to stain tissues properly and it achieves this by clearly staining cell structures including the cytoplasm, nucleus, cell membrane and extra-cellular components to reveal the underlying tissue structures and conditions.

Microwave causes rotation of polar and charged molecule of water. The excited molecule causes friction of adjacent molecule and produces friction that leads to generation of heat. Heat produced by microwave facilitate faster diffusion of staining dye molecules that causes reduction in staining time and better quality of tissue stain.

In this study microwave assisted staining was conducted on microwave processed tissue to determinethe quality of tissue section. In addition, comparison was also done for two methods regarding turn around time and antigen preservation by histochemistry and immunohistochemistry.

2.6 Advantages and unique benefits of microwave assisted technique:

- The microwave assisted procedure eliminates the need of xylene and formalin which are toxic chemical agents and carcinogens (environmental advantage)[6] It also gives us cost benefit.
- The use of microwave for tissue processing will be boon for the patients whose rapid histopathological diagnosis is required to initiate life-saving antimicrobial, immunosuppressant or chemotherapeutic intervention as it reduces turn around time.
- Preservation of antigens on certain tissues assessed by histochemistry (like PAS, Reticulin, Alcian blue, Massion Trichrome) and immunohistochemistry[7]which shows comparable results. Thus microwave technique processed tissue is equally indicated for special investigations[15,16].
- Graded concentration of solutions do not require so protocols for tissue processing can be established and followed with minimal training of individual[1].
- The cost of domestic microwave is 10 to 15 times lesser than the commercially available laboratory microwave. However there is uniform radiation in commercially available laboratory microwave as compared to nonuniform emission of radiation in domestic microwave. This pitfall can be minimized by performing procedure at constant marked position on rotating plate as it provides most consistent results during each procedure[17].

3. RESEARCH METHODOLOGY:

3.1 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.

3.1.1 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.

3.1.2 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 as described below and evaluated by expert and experienced pathologist blindly (without knowing the method performed).
- Whenever applicable, special stains and immunohistochemistry was performed on tissue processed by both methods.

3.1.3 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 were labeled 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 was processed and slides stained.

3.1.4

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 (bony cells require decalcification which requires a very long time, that is, 5–6 days; hence, this would be time consuming)

3.1.5 Period of study:

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

3.1.6 Temperature control of microwave oven:

- In present study, Samsung microwave oven model no. MS23K3513with maximum output of 800W was used.
- 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[1]. 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 operated 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.

3.1.7 Basic steps for tissue processing and staining included:

1. **Dehydration**: The removal of water from tissue cells by alcohol.
2. **Clearing:** To remove alcohol from tissue cells because alcohol is immiscible with paraffin wax.
3. **Impregnation:** By molten paraffin wax to maintain the cell structural integrity.
4. **Embedding:** Keep tissue in molten paraffin wax (kept in L mould) and allowed it to cool. This makes solid block of paraffin wax containing tissue. Now, sectioning of tissues can be possible.
5. **Microtomy**: Tissue is sectioned by rotary microtomeof 4µm thickness.
6. **Deparaffinization:** There is remove paraffin wax from tissues o that f water based staining reagents can be penetrated.
7. **Haematoxylin and eosin staining-**The procedure is performed to stain cytoplasmic and nuclear details.Then tissue becomes ready for microscopic examination.

3.1.8 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.
- ☞ Routine tissue processing was performed by automatic tissue processor (Yorko) having uninterrupted power supply by 8 KV UPS attached to it. Graded isopropyl alcohol concentrations, xylene and paraffin wax were used according to the schedule in Table 3.1.
- ☞ The cassettes were inserted into two metal basket 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 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 allow to move bucket from one beaker to next beaker.
- ☞ Finally, the tissues were embedded in paraffin wax using L moulds. Thus tissue blocks became ready for sectioning by a rotary microtome. Leica 2125 RT was used for section cutting.

Table 3.1 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**	

3.1.9 Microwave assisted tissue processing:

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 3.2. 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 3.2)[1]
- ☞ 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 are contraindicated for use in microwave due to total internal reflection of microwaves, leading to sparkling.Plastic cassettes are relatively cheap and can be reused.
- ☞ Four pre-fixed tissues were washed under running tap water for 5 minutes to remove excess formalin from the tissue to reduce alcohol and formalin interference.
- ☞ Samsung domestic microwave oven(model MS23K3513) was used for present study. It shows six power levels as per table 3.3.

Table 3.3 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.
- ☞ 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.
- ☞ 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.
- ☞ Clearing agent is not required in microwave assisted method because the temperature facilitates evaporation of alcohol from tissue before paraffin infiltration.. In addition, Xylene has a high boiling point and low microwavability since it takes double the time for the same amount of reagent to get heated.
- ☞ 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). All the reagents were directly heated in the microwave except paraffin wax. Paraffin wax was melted at 60^{o} C on a hot plate separately and then placed in the two microwave safe glass beaker.
- ☞ 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' molds, 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.

3.1.10 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.
- ☞ 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.

3.1.11 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 3.4):

- ☞ 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 for bluing.
- ☞ Sections were dipped in 1% acid alcohol (two dips) and then slides were washed for five minutes in running tap water.
- ☞ Then slides were transferred to 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.

3.1.12 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 3.4):

- ☞ 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.
- ☞ 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 and later slide was dipped in petridish containing tap water and microwave at 180 W for 30 seconds.
- ☞ 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 3.4 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	
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	20 minutes

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

3.1.13 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 3.5).

Table 3.5 Parameters and subparameters for stained slide assessment.

	Parameters		Subparameters
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:

i. 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.

ii. 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/hurthlecells with eosinophilic/granular cytoplasm should be well appreciated.

iii. 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.

iv. 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 subparameter-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. .

3.1.14 Turnaround time

- ☞ 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 6 minutes i.e.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.

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3.1.15 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 analyzed and scores obtained for both techniques were presented as mean, standard deviation and standard error of difference.

Statistical Analysis:

1. Descriptive statistics (Frequency, Mean, SD).
2. Independent t test to compare quantitative data.

3.2 RESULTS:

3.2.1 Gross findings:

- ☞ The colour of tissues was examined and predominant primary colour of tissue was noted. Maximum tissues were of grey colour followed by brown colour.
- ☞ Consistency of tissues was recorded while grossing of specimen and subsequent sections were taken. Maximum tissues were firm in consistency.
- ☞ Tissue section was taken from specimen and cut into two equal halves. The volume of each section was assessed before and after processing for length, breadth and width.
  Percentage of shrinkage was calculated. Shrinkage of tissue by routine method was 36.7% and by microwave method was 42.5%. Thus, difference of shrinkage between two methods was 42.5%-36.7%=5.8% which showed that shrinkage of tissue was slightly more in microwave method than routine tissue processing method.

3.2.2 Microscopic findings:

The evaluation of microscopic slides processed and stained by both methods were based on four parameters and ten subparameters by expert and senior pathologist. Evaluation result of all tissues was assessed as Grade IV (Excellent)-Marks between 31 to 40.
Analysis of data was done which showed following results.

- ☞ Cellular outline, nuclear cytoplasmic contrast, clarity of nucleus and nucleoli, clarity of chromatin and colour intensity were better in microwave method as compared to routine method and the difference was statistically significant.
- ☞ Clarity of cellular details, integrity of tissue and clarity of nuclear membrane were equally preserved in both the methods.
- ☞ There was slight difference in Eosinophilia/granularity and uniformity in staining by both the methods. However the difference was not statistically significant.

Special stain:

- ☞ In Histopathology, the major part of the routine work consists of examination of slides stained by hematoxylin and eosin stain. But some of the tissue components can not be discriminated by routine hematoxylin and eosin stain.
- ☞ Various special stains like Periodic acid Schiff, Alcian blue, Massion trichrome, Fontana Massion were performed on tissues processed by both the methods and the results were comparable. Thus, tissue components retain their specific chemical characteristics not only by routine processing but also by microwave processing.

Immunohistochemistry:

- ☞ Immunohistochemistry procedure is a technique for identifying cellular or tissue constituents (antigens) by means of antigen-antibody interaction.
- ☞ The immunohistochemistry was performed on tissues processed by both the methods and the results were comparable. Thus, antigens of tissues were preserved in microwave processing.

Time taken for the processing and staining of tissues by both the methods were recorded. Routine tissue processing was performed by dip and dunk tissue processor and staining was done by manual method. Overall processing time by Routine method is 16 hours whereas by microwavemethod it was only 67 minutes. Time taken for staining by manual method was 20 minutes while for microwave method it was 14 minutes.

The reagents utilised in routine method consists of 11 buckets of 2000 ml (2 litre) capacity which can process approximately 20 tissues at a time optimally. While microwave method utilises 6 buckets reagents of 300 ml each that can process 4 tissues at a time optimally. As routine method is performed on room temperature, evaporation of reagents are minimal. In contrast, in each 10 minutes cycle of microwave oven approximately 30-50 ml reagents are evaporated. Thus, Evaporation of reagent is comparatively more in microwave method.

Overall time taken for processing and staining by routine method was 16 hours and 20 minutes respectively while by microwave assisted tissue processing and staining method was 67minutes and 14 minutes respectively.Though it has its limitations, the domestic microwave method has a significantly shorter processing and staining time.

3.3 DISCUSSION

Rapid processing of histopathologic material is becoming increasingly desirable to fulfill the needs of clinicians treating acutely ill patients. Traditional techniques for routine processing of tissues require 16 to 24 hours, delaying treatment for some critically ill patients. Microwave processing shortens this time, allowing rapid histopathologic tissue processing and staining.

Various data exist comparing quality of routine processed tissue with that of microwave processed tissue. In present study, 350 paired tissues (total 700) were randomly selected. One part was processed and stained by routine method, while the other was processed and stained by the microwave method. Then, both the tissues were compared for quality of histologic preparation in a blinded fashion.

On analysis of results, microwave processing and staining considerably shortens the preparation time for permanent histologic sections without demonstrable decrease in section quality or “readability.”

Turnaround time is an important issue for many years and has become increasingly important in this age of managed care and commitments to overall reduction of costs for health care services.

Changing the standard technique for tissue preparation from the currently used overnight processing to microwave assisted tissue preparation could substantially reduce turnaround times, permitting early diagnosis that would facilitate patient diagnosis and management. Thus, reduction in turn around time reduces patient and clinician dissatisfaction and increase patient compliance as patients with critical illness or neoplastic diseases can be diagnosed early and therapy can be initiated.

Comparison of Turn around time of various studies:

Name of study	Processing time (Routine method)	Processing time (Microwave method)	Staining time (Routine method)	Processing time (Microwave method)
N Amrutha et al[18]	7 hour	2 hour	31 minutes 20 seconds	16 minutes 45 seconds
ShankarGouda Patil et al[19]	7 hours	2 hours	31 min 20 sec	16 min 45 sec
Mahesh Babu[1]	310 minutes	75 minutes	40 minutes	33 minutes
Anamika Sinha et al[20]	17 hours	35 min 25 sec	-	-
Anita Choudahury[21]	11-12 hours	15 minutes	-	-
Kartesh Singla et al[17]	7 hour	1 hour	-	-
Suyog Tupsakhare[22]	29 hour	1 hour 5 minutes	-	-
Harshkumar et al[19]	18 hour 30 minutes	55 minutes	-	-
Anil Pandey[20]	28 hour	2 hour	-	-
Rajat Nangia[23]	48 hours	1 hour	-	-
B.S. Shruthi et al[24]	60 minutes	15 minutes	-	-
Promil Jain[25]	960 minutes	8.5 minutes	-	-
Ralph Rohr[26]	8 hour (small biopsy)	15 minutes	-	-
	12 hour (large biopsy)	60 minutes	-	-
Bhuvanamha Devi et al[27]	7 hour	1 hour 22 minutes to 2 hour 22 minutes	-	-
Archana Mukunda[15]	-	-	1 hour 15 minutes	20 minutes
Present study	16 hour	67 minutes	20 minutes	14 minutes

Number of authors have studied the techniques and results of microwave-facilitated tissue processing and staining. The purpose of the present study is to determine whether microwave assisted technique is comparable or better than standard overnight routine processing technique for tissue preparation.

Ralph Rohr[26] studied randomly selected 158 paired samples. One member of each pair was processed routinely overnight according to 1 of 2 schedules, that is, a small or a large biopsy specimen schedule using a vacuum tissue processor. The other member of each pair was processed according to two different microwave schedules, a short schedule for small biopsy specimens less than 2 mm thick and less than 10 mm in diameter, and a long schedule for biopsy specimens more than 2 mm thick or containing abundant blood, mucus, or both. The microwave schedule was performed in temperaturecontrolled microwave processor. Total microwave processing times were 15 minutes for small biopsy specimens and 60 minutes for larger biopsy specimens. These times compare favorably with traditional processing times of 8 hours for small biopsy specimens and 12 hours for large and fatty specimens. The study concluded that there is positive impact on turnaround time in microwave method and the quality of microscopic tissues from the traditional processing and the microwave-processing methods were extremely similar. It was not possible to distinguish between the 2 techniques by studying the tissue sections. In present study, there is considerable time saving in microwave method which takes 67 minutes for processing in contrast with the overnight procedures that is routinely used which takes 16 hours. Hence, its use can be given priority for whom a rapid histopathologic diagnosis is required to initiate life-saving antimicrobial, immunosuppressant or chemotherapeutic intervention.

The tissues processed by both methods were examined and assessed for change in colour, consistency and shrinkage. The colour and consistency (at 58^0C) were consistent as before processing while minimal shrinkage was identified in microwave processed tissue. The study by Mahesh Babu et al[1] used reagents identical to present study. The study examined dimensions of tissue in terms of volume and considered reduction in volumes as shrinkage. A 4% excess shrinkage as compared to routine processing was experienced. Here it is explained that it could be due to the heat generated by microwave oven. Present study also shows excess shrinkage of 5.8% by microwave method. However, tissue shrinkage was not observed by Kango and Deshmukh [10]and Kartesh Singla et al[17] et al. It could be due to different protocols and reagents used in these studies.

Rajat Nangia et al[23] examined 20 oral mucosal biopsy specimens. They were cut into three equal parts and each processed by routine, domestic microwave and commercially available laboratory microwave techniques. Parameters studied were colour intensity, uniformity of staining and nuclear and cytoplasmic details. The results were slightly better in labotatory microwave method followed by domestic microwave method and routine method. *P* values were statistically insignificant for all the parameters except colour intensity. Present study also evaluated for former two methods of 350 specimens for various parameters.

Kango and Deshmukh et al[10] processed 50 specimen (total 100 tissues) and compared histopathological evaluation of slides processed by microwave and conventional technique. The sections were evaluated for cellular morphology, nuclear morphology and staining characteristics. The slides were examined by four experienced pathologists. The alpha values in percentage, where in all observer were 75% in concordance for cellular morphology, 60% for nuclear morphology and 56% for staining quality. The study results showed that microscopic quality of the sections of microwave processed tissues were comparable to, or slightly better than, conventionally processed tissue having the same formalin fixation time. Thus, rapid microwave-assisted tissue processing is the optimal method for producing quality sections. Also, excellent microscopic sections obtained by this technique revealed no differences in the cellular and nuclear morphology in several types of tissues.

Raju Shashidara et al[28] studied kitchen microwave assisted accelerated method for fixation and processing of oral mucosal biopsies. The parameters included for evaluation were diagnostic evaluation of tissues, section quality, cellular outline, nuclear details, staining quality and occurrence of artifacts (section folds, heat artifacts). On comparison, larger numbers of samples were graded as good/optimal in the microwave fixed and processed tissue as compared to routine fixation in all parameters evaluated other than in the occurrence of artifacts. However, no statistically significant differences were observed in any of the parameters assessed.

T Mahesh Babu et al[1] studied 15 oral mucosal biopsies and processed by conventional and microwave methods and then subsequently stained H & E by conventional and microwave methods. They were evaluated blindly by six observers. The parameters included in the data sheet were cellular clarity, cytoplasmic details, nuclear details, color intensity, interface of epithelium and connective tissue and fibrous tissue. The overall quality of microwave-processed and microwave stained slides appeared slightly better than routinely processed and routinely stained slides. The total processing time involved in microwave was 42 minutes and in conventional method was270 minutes. H and E staining in microwave took 33 minutes and 40 minutes for conventional method. The study concluded that microwave technique of tissue processing can be adopted in the regular histopathology laboratory on a regular basis, and considering the considerable shortening of time period, microwave technique can replace the well-established routine tissue processing.

Promil Jain et al[25]conducted the study to compare the time taken and quality of sections in processing of prostatic tissue by rapid microwave and conventional techniques using morphometry. The study included paired fifty prostatectomy specimens of four to five mm thickness. One tissue piece of the pair was processed routinely overnight by conventional tissue processing and the other by microwave processing method. Time taken were compared for processing by both techniques which showed that time taken for steps of dehydration, clearing and impregnation in microwave technique was significantly less as compared to histoprocessing done by conventional technique. Morphometric study was done on slides of prostatic tissue processed by both conventional and microwave techniques. Morphology, staining patterns of prostatic tissue processed within minutes by microwave technique, were comparable to those sections which were processed in days using standard technique. The study concluded that domestic microwave can be used for histoprocessing to accelerate the processing with preservation of morphology and is cheaper than commercially available laboratory microwave and processing time was considerably reduced from days to minutes.

Archana Mukunda et al [15] compared the reliability of modified kitchen microwave staining technique against technique of routine staining. Sixty different tissue blocks were used to prepare 20 pairs of slides for 4 different stains namely hematoxylin and eosin, Van Gieson's, 0.1% toluidine blue and periodic acid-Schiff. One slide from each tissue was stained routinely, and the other stained inside a microwave. Microwave staining considerably cut down the staining time from hours to seconds. In addition, the cellular details, nuclear details and staining characteristics of microwave stained tissues were better than or equal to the routine stained tissue. The overall quality of microwave-stained sections was also found better than the routine stained tissue in majority of cases.

Similarly a new approach to the Ziehl-Neelsen stain using microwave oven was done by S. Hafiz et al [29] for the stage of heating. the smears were flooded with carbolic magenta which were exposed to microwave irradiation for 30 seconds at full power of 640 Watts. The staining of sections of tissue by microwave method with carbolic magenta was comparable with the results obtained by the conventional Ziehl-Neelsen method. In addition, the advantages for microwave irradiation method were that the slides were cleaner; there were no crystalline deposits on the smears; processing of smears was much quicker as the time was reduced by 15 minutes for each batch; no flames were required, and thus there was no risk of the slides cracking as a result of direct heat or of fires starting due to naked flames in the laboratory; and the smears were safe because mycobacteria were killed by microwave irradiation in 30-60 seconds. The characteristics of decolourisation remained the same, and a particular advantage for staining histological sections was that the tedious stages of dewaxing were eliminated.

Godwin O. Avwioro[14] compared staining quality of microwave processed tissues with the staining quality of the conventional paraffin wax processed tissues. Normal lung, kidney, liver, intestine and heart tissues obtained from an adult Wistar albino rat were fixed in 10% formol saline and processed by the conventional paraffin wax method. The twin specimens were also processed by the microwave method. Sections were cut with the rotary microtome, paired and stained by heamatoxylin and eosin method for general tissue structure, Weigert's van Gieson iron haematoxylin for collagen fibres, Verhoeff's van Gieson iron haematoxylin for elastic fibres,periodic acid Schiff reaction for neutral mucopolysaccharides, Gordon and Sweet's method forreticular fibres, Alcian blue (pH2.5) for carboxylated and sulphated mucopolysaccharides,Congo red method for amyloid, Masson's trichrome for collagen and muscle fibres and Gomori'saldehyde fuchsin method for elastic fibres. Microscopically, there were no significant differencesin the staining reactions of all the techniques when they were compared with the microwaveprocessed tissues. Nuclear, cytoplasmic, extracellular and intracellular materials appeared the same with the conventionally processed tissues by the paraffin wax method. Muscles, collagenfibres, elastic fibres, reticular fibres, neutral carbohydrates and amyloid were also stained the same way as the conventionally processed tissues.

Hendrik E. Moorlag et al[30]performed study by modification of staining technique for Perl's, Alcian blue, Fontana-Masson and Romanowsky-Giemsa stain using microwave stimulation. It was found that this stain modification proved to be highly successful in daily practice for individual cases in which fast diagnosis is required. With this method the total time for staining is a matter of few minutes at the most. Results obtained were excellent and consistent and the method was economically advantageous as only small quantities of staining solutions were used.

Lyska L. Emerson et al[16] studied whether microwave-assisted rapid tissue processing adversely affects the quality of immunohistochemical staining. There were 30 specimens (20 neoplastic and 10 nonneoplastic) from routine surgical pathology workload were selected. Paired large tissue blocks were made from each specimen type, one for microwave-assisted rapid processing and the other for conventional tissue processing. Two microarrays of 60 punches each were made from the donor blocks. The microarray blocks were examined for intensity and extent of staining by 44 commonly used antibodies. Slides were reviewed independently by 2 pathologists blinded to the type of processing used. In 5,280 tissue punches examined, it was found a high degree of concordance in quality, as measured by intensity and extent of immunohistochemical staining, between microwave and routinely processed tissues. Thus, the study demonstrates that quality of immunohistochemical staining is similar between rapid microwave and conventional tissue processing method. Thus, immunohistochemical analysis is not a contraindication in tissues processed by microwave-assisted rapid tissue processing.

Nitin Gangane et al[31] determined if microwave processed cell blocks prepared from residual material on sampling devices could add to the diagnostic accuracy of cervico-vaginal smears. The material remaining on the spatula after making cervical smears was rinsed in ethanol and used to prepare microwave-processed cell blocks in 260 patients. Both Papanicolaou (Pap) smears and cell block sections were examined independently. The sensitivity and specificity of both techniques for detection of malignancy and intraepithelial lesions were calculated. Sensitivity and specificity for cytology smear for detection of malignancy were 79.16% and 100%, respectively. The sensitivity and specificity of cell block were 86.3% and 100%, respectively. The quality of the section and staining after microwave processing was excellent. In addition, modified technique using microwave fixation and processing substantially reduced the turn around time. Microwave processed cell blocks prepared from residual cervico-vaginal specimens on sampling devices can add to the diagnostic accuracy of Pap smear without patients needing to undergo an additional procedure.

Thus microwaves can be used for tissue processing and staining without affecting architecture of cells and considerable shortening of turn around time. It is boon for pathologist as rapid diagnosis is desirable to fulfil the needs of clinicians treating acutely ill patients. It is encouraging to see the growth of this beneficial technology in our discipline.

4. SUMMARY AND CONCLUSION

- In gross examination, colour and consistency were not altered. Mean tissue shrinkage examined after processing by both methods was slightly more in microwave method as compared to routine conventional method. However, no any adverse outcomes were noted in microscopic examination.
- In microscopic examination, cellular outline, nuclear cytoplasmic contrast, clarity of nucleus and nucleoli, clarity of chromatin and colour intensity were better in microwave method as compared to routine method and the difference was statistically significant.Clarity of cellular details, integrity of tissue and clarity of nuclear membrane were equally preserved in both the methods.There was slight difference in Eosinophilia/granularity and uniformity in staining by both the methods, however the difference was not statistically significant.
- Thus, microwave assisted tissue processing yielded morphology of cells and architecture of histologic material similar or superior quality to that provided by time-honored conventional processing method.
- Turn around time was assessed for both routine and microwave assisted processing and staining methods The overall processing time by routine method is 16 hours whereas the overall processing time by domestic microwave was only 67 minutes. Time taken during staining by manual method takes 20 minutes while domestic microwave method utilised 14 minutes for staining. Thus, overall processing and staining time by routine method was 16 hours and 20 minutes while time taken by microwave assisted tissue processing and staining method was 81minutes.
- Microwave method has many advantages, including expediency, safety, potential for preservation of molecular integrity of specimens that might be used in subsequent studies, and improvement in the workflow of the laboratory, permitting the preparation of diagnostic material during the day at family-friendly hours.

Limitations of present study:

- ☞ The limited throughput owing to the small size of the microwave allows limited samples to process at a time. Thus, it make it difficult and time consuming for processing routine biopsies in large sample sized laboratory on a regular basis.
- ☞ When microwave exposure occurs on reagent, release of noxious fumes occurs due to heating of reagents. Thus, adequately ventilated room is recommended to process tissue in domestic microwave.
- ☞ The procedure is labour intensive as manual processing and staining is done in microwave which requires constant supervision and caution.
- ☞ The technician should be trained, attentive and dedicated for the procedure so that optimum results can be obtained. Temperature inside domestic microwave should be monitored.
- ☞ Thus, further exploration in the field is required for the development of cost effective microwave histoprocessor that provides comparable results as conventional methods with added advantages.

Impact and future scope

Routine use of formalin fixation, overnight dehydration, paraffin infiltration, manual embedding and sectioning has served well in producing relatively uniform, good quality tissue sections, but it is the major bottleneck in the workflow of histopathology laboratories.

As we moved into the 21st century, the standard practice is now increasingly challenged because of its inability to meet the support required by current clinical demands. Because the routine manual histoprocessing remains time-consuming and requires toxic chemicals, alternative methods such as microwave tissue processing are the "future ray of hope."

The microwave-assisted tissue processing is believed to have brought a revolutionary improvement in histopathology. The technique shortens the tissue processing time from hours to minutes. The technique is responsive to the patient and physician needs, improves the use of reagents while reducing or eliminating their toxicity, creates a personnel-friendly workflow and places the laboratory in a better position to meet the demands of the rapidly expanding field of molecular medicine**.**

5. CHAPTERWISE PLANNING OF THESIS

Chapter 1: Introduction

The introduction chapter will introduce the present research topic in context with background information and answering question that why it is necessary to study this topic. There will bean overview on invention of microwaves and its application in histopathology laboratory for tissue processing and staining.This chapter will also highlight uniqueness and advantages of boththe methods.

Chapter 2:Aims and Objectives

The chapter will describe aims regarding research project and objectives focus on how the aim will be achieved. Routine tissue processing and staining techniques of biopsy specimen will be compared by domestic microwave assisted processing and staining technique. Both the methods will be assessedfor mean shrinkage of tissue, quality of histopathologic slides, time duration and antigen preservation.

Chapter 3: Review of literature

The chapter will be framed on basis of research literaturelike most relevant books and journal articles. It will stateexisting research and literature on the topic in relation with present study. It will describe general problem regarding conventional (routine) technique and modification in technique can improve result outcome of the diagnostic laboratories. The chapter will also includebackground informationwhich is necessary to understand the problem(s) related to the topic and also provides the justification for the study as itwill describe the gaps and weaknesses in the existing research.

Chapter 4:Materials and methods

The chapter explains the research methods and design that will be used to conduct the study. The critical part of writing present chapter is to describe the procedures that will be used to conduct the study. Thus, it describes how the studywill be conducted. Here, itwill include detailed descriptions about every aspect of present study like sources of specimen collection, period of study, inclusion and exclusion criteria, detailed steps of procedure and statistical data analysis approach in brief.

Chapter 5: Results

The chapterwill describe the findings of the data collected and final interpretation. This chapter will include a narrative, graphical, numerical and finally compiled data as tabular form to know the outcomesof the study. The reported statistics were analyzed as mean, standard deviation, standard error of difference, T value, p value and confidence interval at 95% level.Thus interpretations of statistics indicate whether differences observed in results of both the methods were significant or not.

Chapter 6 Photographs

Photographs of instruments, procedures and results related to present study will be included in this chapter.

Chapter 7: Discussion

The chapter willdraw the conclusions about the present study's findings. Discussion will include the relationship of results of present study findings to the finding of previous research conducted in the literature.

Chapter 8: Limitations of study

The chapter acknowledges the limitations or weaknesses of the new techniquesintroduced in context to utilization of domestic microwave oven.

Chapter 9: Summary and conclusion

In this chapter, the important conclusions about the results and their implications will be summarized.

Chapter 10:The impact and future scope

The chapter will include recommendations for future areas of research that can be conducted related to microwave assisted procedures so that better and committed services can be provided to the community by the laboratory.

Chapter 11: References:

The chapter will include existing literature that reviewed and applied as per need in present study.

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