Scholars Academic Journal of Biosciences (SAJB) **ISSN 2321-6883 (Online)**
Sch. Acad. J. Biosci., 2016; 4(10A):796-804 **ISSN 2347-9515 (Print)**

(An International Publisher for Academic and Scientific Resources)
www.saspublishers.com DOI : 10.36347/sajb.2016.v04i10.003

Original Research Article

A comparative study of tissue processing using microwave without xylene and conventional method

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Abstract: Tissue processing is the method in which tissues are prepared by paraffin embedding for sectioning. Conventional tissue processing is the gold standard, it is more than hundred years old procedure and hence against which all the new techniques and methods need to be assessed. This study consist of randomly selected twenty paired specimens, first member of the pair was processed by conventional tissue processing method while the second member of the pair was processed by the microwave technique. The slides were examined for accuracy of histologic preparation in a blinded fashion by six oral pathologists for nuclear staining, cytoplasmic staining, uniformity of staining, clarity of staining and intensity of staining. Adequate nuclear staining was noted in 99.16% of conventional processed tissue slides and 94.16% of microwave processed tissue slides, Adequate cytoplasmic staining was noted in 100% of conventional processed tissue slides and 96.6% of microwave processed tissue slides, Adequate uniformity of staining was noted in 100% of both conventional processed tissue slides and microwave processed tissue slides, Adequate clarity of staining was noted in 96.6% of conventional processed tissue slides and 100% of microwave processed tissue slides, Adequate intensity of staining was noted in 97.5% of conventional processed tissue slides and 92.5% of microwave processed tissue slides. From the present study it can be concluded that microwave processed tissues were more superior for the diagnosis as compared to conventional method.
Keywords: Conventional tissue processing, Microwave technique, Xylene

INTRODUCTION

In the recent years to complete the needs of clinicians those who are treating the acutely ill patients, rapid processing of tissues for the histopathological diagnosis becoming increasingly desirable [1].

Since from the past hundred years conventional manual tissue processing has been the most commonly employed method which is completed in twenty one to twenty four hours. It is reliable and inexpensive method but consumes more time and required noxious chemicals like Xylene [2].

Conventional tissue processing still remains the gold standard, hence against which all the new techniques and methods need to be assessed [3].

Microwave method is a new tissue processing technique as Kok and Boon in 1985 first time used the microwave method of tissue processing [4].

In this method, the heat generated by incident energy from the penetrative properties of the microwaves is used for the tissue processing. The advantages are shorter processing time, avoiding noxious chemical like Xylene and poor degree of denaturation of nucleic acids [2].

Xylene is an aromatic hydrocarbon. According to the National Institute for Occupational Safety and Health, the exposure limits for Xylene at 100 ppm as a time weighted average (TWA) for upto a ten hours work shift and fourty hours work week and 200 ppm for ten minutes as a short term limit. Inhalation and eye or skin contact is the most common route of exposure to Xylene. Xylene is metabolised in the liver by oxidation of methyl group and conjugated with glycine to form a methyl hippuric acid and is excreted in urine. It has its deleterious effects on central nervous system, lungs, reproductive system, liver, kidney, blood and also has carcinogenic effect. Xylene in the laboratory is used during tissue processing, deparaffinization of tissue sections, cover slipping, cleaning tissue processors and

recycling [5]. Various substitutes to clearing of tissues without xylene include vegetable oils and terpenes. These alternative materials have different success level to replace the xylene. Some vegetable oils like Olive and Coconut oils have some disadvantages like they are difficult to displace during infiltration and also they are more expensive than xylene. Terpenes are isoprene polymers found in essential oils from plants. It includes AmeriClear, Histoclear and Histosolve but their personnel safety concerns and cost limit their use. Instead; Paraffin itself can act as a clearing agent [6].

The basic aim in any field of life sciences is to utilize eco-friendly materials which are nontoxic, less bio hazardous and are economical [5]. Hence by knowing these facts, we tried to compare the two different methods of tissue processing in terms of nuclear staining, cytoplasmic staining, uniformity of staining, clarity of staining and intensity of staining.

MATERIALS AND METHODS

Twenty unmatched formalin fixed tissue samples were collected from the archives of Department of oral pathology and microbiology, from our college. Every sample was cut into two parts, first part was sent for conventional tissue processing and the second for microwave tissue processing. The steps followed in the conventional tissue processing are outlined in table 1. After the initial trial and errors the microwave tissue processing method was standardized. The steps followed in the microwave tissue processing are outlined in table 2.

Domestic microwave oven (SAMSUNG GW73BD) was used for microwave tissue processing method. All the tissues were processed in microwave oven safe glass containers and the microwave oven was operated at 100 watt mode. The solutions were not covered with the lid because we had two containers, the first container contained 200 ml alcohol along with the tissue inside and the second one contained a water load of 200 ml and placed next to the first container, in this way the excess heat was controlled, which was absorbed by the water [7]. Embedding and Microtomy were done similarly for both the processing methods.

Harris's Hematoxyline and Eosin Y (H & E) staining protocol for slides from conventional tissue processing and from microwave tissue processing were similar except for the microwave processed tissues the time of both the Harris's Hematoxyline and Eosin Y were increased by one minute and two dips respectively.

A total of forty slides (twenty pairs) were obtained, one each from microwave tissue processing and conventional tissue processing. The slides were evaluated independently by six oral pathologists with no information of the type of processing used. Each pathologist evaluated the slides according to the criteria used by Sravya T. *et al*. [5] according to the following scheme.

- Nuclear staining (Adequate = score 1, Inadequate = score 0)
- Cytoplasmic staining (Adequate = score 1, Inadequate = score 0)
- Uniformity of staining (Adequate = score 1, Inadequate = score 0)
- Clarity of staining (Adequate = score 1, Inadequate = score 0)
- Intensity of staining (Adequate = score 1, Inadequate = score 0)

As the scoring was done by six examiners, to find out the central tendency mode value was recorded instead of mean/average. The score of each slide was scored and mode value obtained from each parameter was totalled. A score of ≤ 2 was graded as inadequate for diagnosis while score 3-5 was graded adequate for diagnosis. After the completion of evaluation, the processing code was broken and the results were analysed.Inter examiner reliability was tested for both conventional processed tissue (Table -3) and the microwave processed tissue (Table -4) by Cohen's Kappa test and inter group comparison were done by Chi- square test (Table -5,6,7,8 and 9).

RESULT

All the forty slides were found to be adequate for the diagnosis and scored five. All staining scores were summarized in table-10 and Fig. 1a to 9 shows the staining pattern. Graph-1 shows the percentage of adequate scores for the different parameters for diagnosis of H & E stained slides in both conventional processed and microwave processed tissue slides.

- Adequate nuclear staining was noted in 99.16% of conventional processed tissue slides and 94.16% of microwave processed tissue slides.
- Adequate cytoplasmic staining was noted in 100% of conventional processed tissue slides and 96.6% of microwave processed tissue slides.
- Adequate uniformity of staining was noted in 100% of both conventional processed tissue slides and microwave processed tissue slides.
- Adequate clarity of staining was noted in 96.6% of conventional processed tissue slides and 100% of microwave processed tissue slides.
- Adequate intensity of staining was noted in 97.5% of conventional processed tissue slides and 92.5% of microwave processed tissue slides.

Table-1: Conventional tissue processing protocol

Washing, 2 hour 30 min.
70% alcohol, 3 hour 30 min.
90% alcohol, 1 hour 30 min.
Absolute alcohol – 1, 16 hours
Absolute alcohol – 2, 2 hours
Xylene – 1, 15 min.
Xylene – 2, 15 min.
Wax bath for 3 hours

Table-2: Microwave tissue processing protocol

Absolute alcohol – 1, 15 minutes
Absolute alcohol – 2, 20 minutes
Molten paraffin wax -1, 15 minutes
Molten paraffin wax -2, 15 minutes

Table-3: Inter-examiner reliability of 6 examiners for Conventional processed tissue slides

Parameters	Cohen's kappa
Nuclear Staining	0.96
Cytoplasmic staining	1
Uniformity of staining	1
Clarity of staining	0.92
Intensity of staining	0.94

Table-4: Inter-examiner reliability of 6 examiners for microwave processed tissue slides

Parameters	Cohen's kappa
Nuclear Staining	0.86
Cytoplasmic staining	0.90
Uniformity of staining	1
Clarity of staining	1
Intensity of staining	0.82

Table-5: Inter group comparison of adequacy percentage of nuclear staining between Conventional processed group and Microwave processed group († Pearson Chi-Square test)

Nuclear Staining	Conventional Processed (n=120)	Microwave Processed (n=120)	Pearson Chi-Square	p value †
Adequate Inadequate	119(99.16%) 1(0.84%)	113(94.16%) 7(5.84%)	38.67	0.062

Table-6: Inter group comparison of adequacy percentage of cytoplasmic staining between Conventional processed group and Microwave processed group († Pearson Chi-Square test)

Cytoplasmic Staining	Conventional Processed (n=120)	Microwave Processed (n=120)	Pearson Chi-Square	p value †
Adequate Inadequate	120(100%) 0(0%)	116(96.6%) 4(3.4%)	21.71	0.13

Table -7: Inter group comparison of adequacy percentage of staining uniformity between Conventional processed group and Microwave processed group († Pearson Chi-Square test)

Uniformity of staining	Conventional Processed (n=120)	Microwave Processed (n=120)	Pearson Chi-Square	p value †
Adequate Inadequate	120(100%) 0(0%)	120(100%) 0(0%)	--	--

Table-8: Inter group comparison of adequacy percentage of staining clarity between Conventional processed group and Microwave processed group († Pearson Chi-Square test)

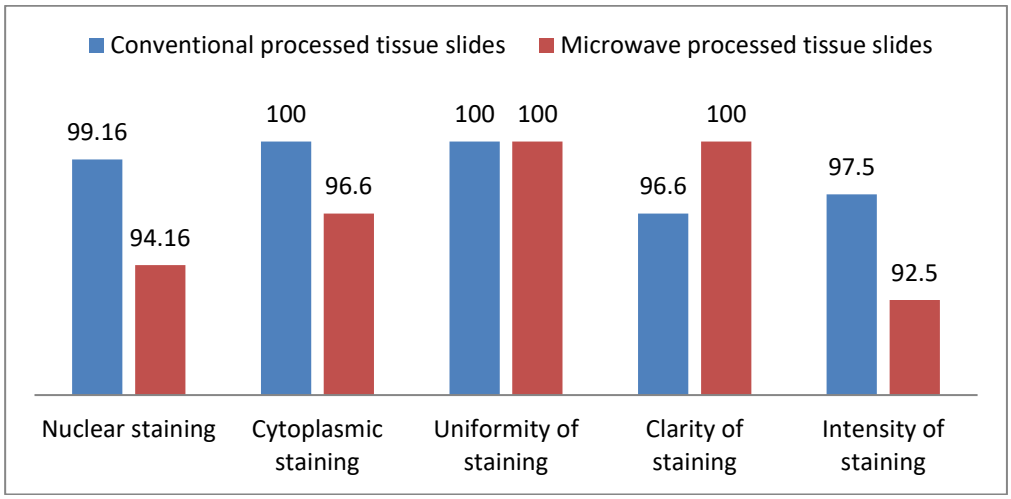
Clarity of staining	Conventional Processed (n=120)	Microwave Processed (n=120)	Pearson Chi-Square	p value †
Adequate Inadequate	116(96.6%) 4(3.4%)	120(100%) 0(0%)	21.71	0.13

Table-9: Inter group comparison of adequacy percentage of staining intensity between Conventional processed group and Microwave processed group († Pearson Chi-Square test)

Intensity of staining	Conventional Processed (n=120)	Microwave Processed (n=120)	Pearson Chi-Square	p value †
Adequate Inadequate	117(97.5%) 3(2.5%)	111 (92.5%) 9 (7.5%)	29.83	0.087

Table-10: Staining scores of each slide by six examiners.

Slide No.	Nuclear staining						Cytoplasmic staining						Uniformity of staining						Clarity of staining						Intensity of staining						Conventional Processed tissue slides from 1 to 20
Observer No. →	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	
1)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	
2)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
3)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
4)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	
5)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
6)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	
7)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
8)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
9)	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
10)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	
11)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
12)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	
13)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
14)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	
15)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
16)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
17)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
18)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
19)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
20)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
21)	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	Microwave processed tissue slides from 21 to 40
22)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
23)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
24)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
25)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
26)	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
27)	1	1	1	1	0	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	
28)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
29)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
30)	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
31)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	
32)	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
33)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
34)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	
35)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
36)	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
37)	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
38)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	
39)	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	
40)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	



Graph -1, showing percentage of adequate scores in both conventional processed and microwave processed tissue slides

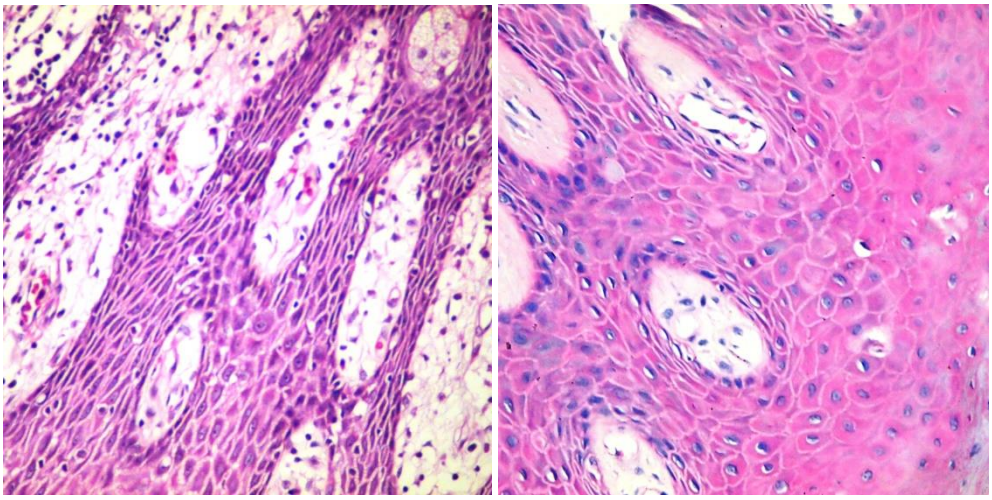


Fig-1a and 1b: shows oral epithelium and connective tissue (High power)

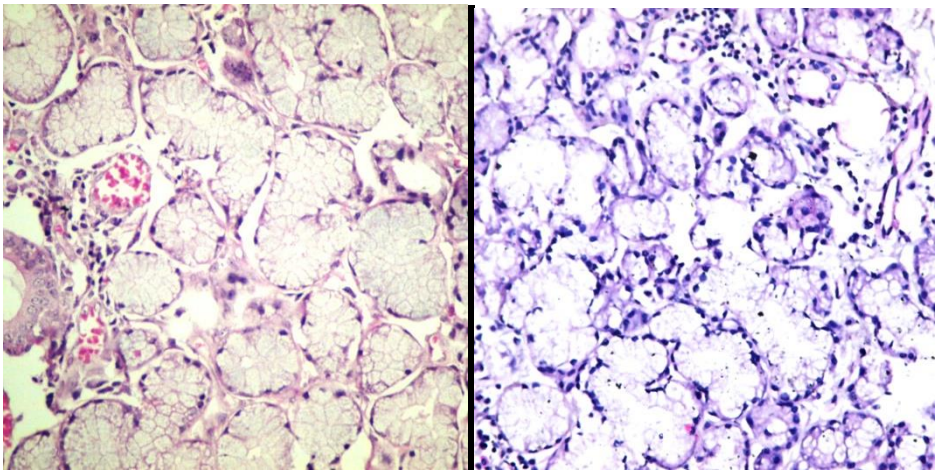


Fig-2a and 2b: shows salivary mucous acini and ducts (High power)

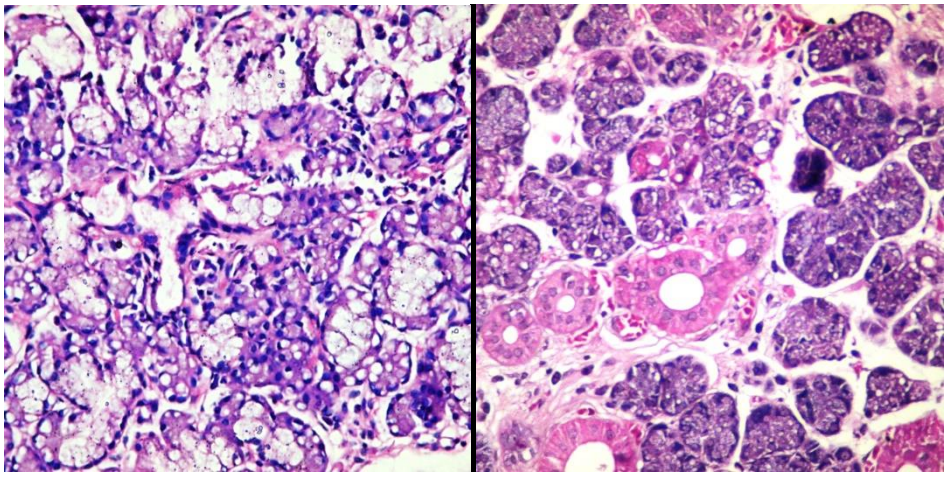


Fig-3a and 3b: shows salivary serous acini and ducts (High power)

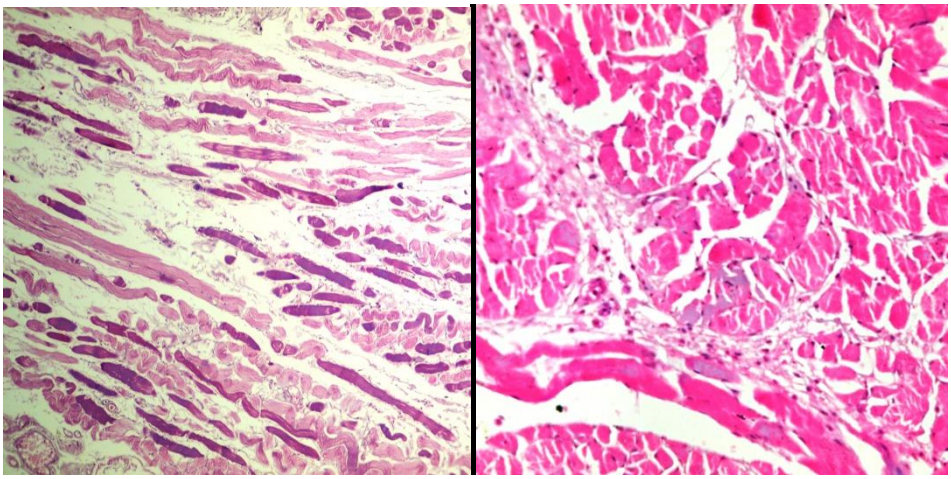


Fig-4a and 4b: shows muscle fibres (High power)

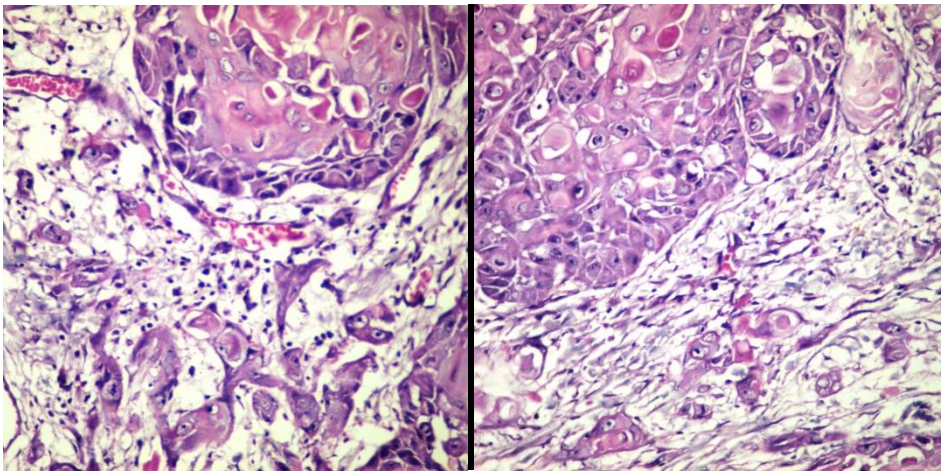


Fig-5a and 5b: shows oral squamous cell carcinoma (High power)

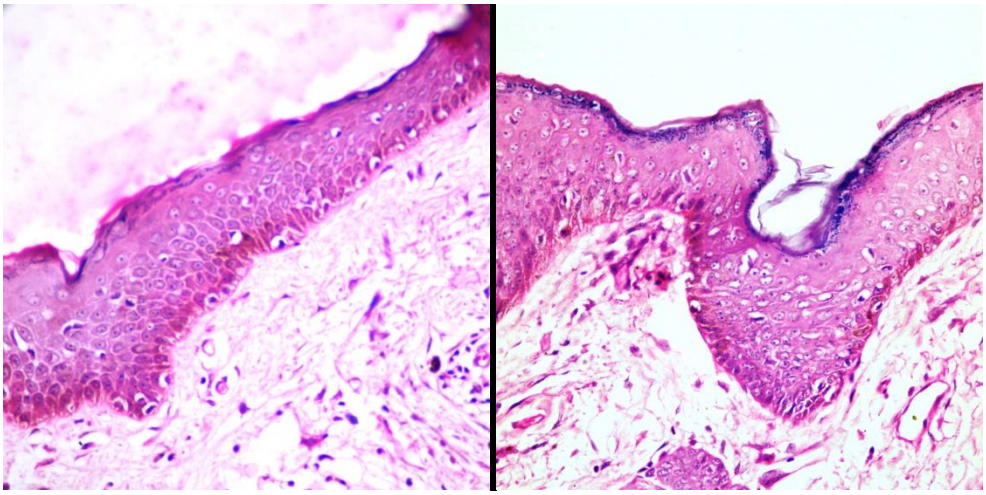


Fig-6a and 6b: shows facial skin (High power)

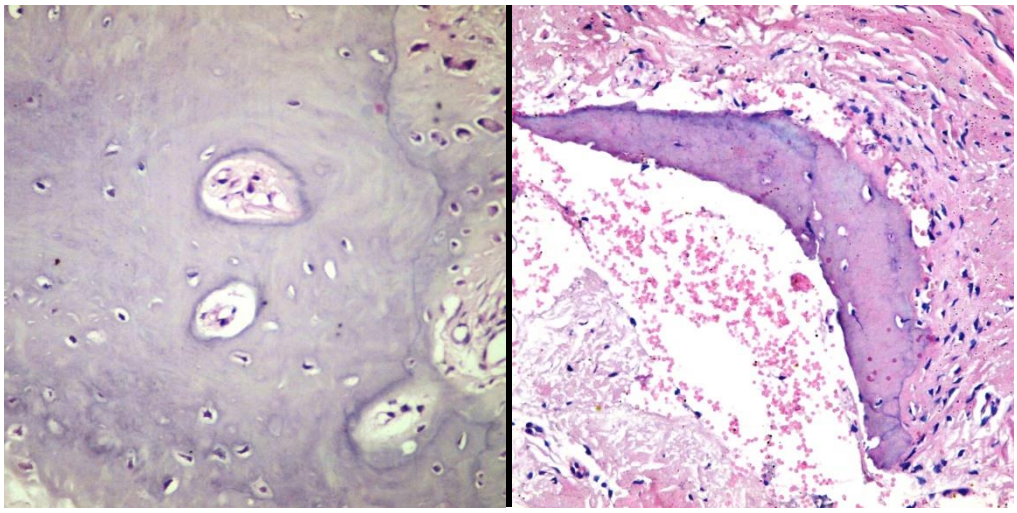


Fig-7a and 7b: shows hard tissue (High power)

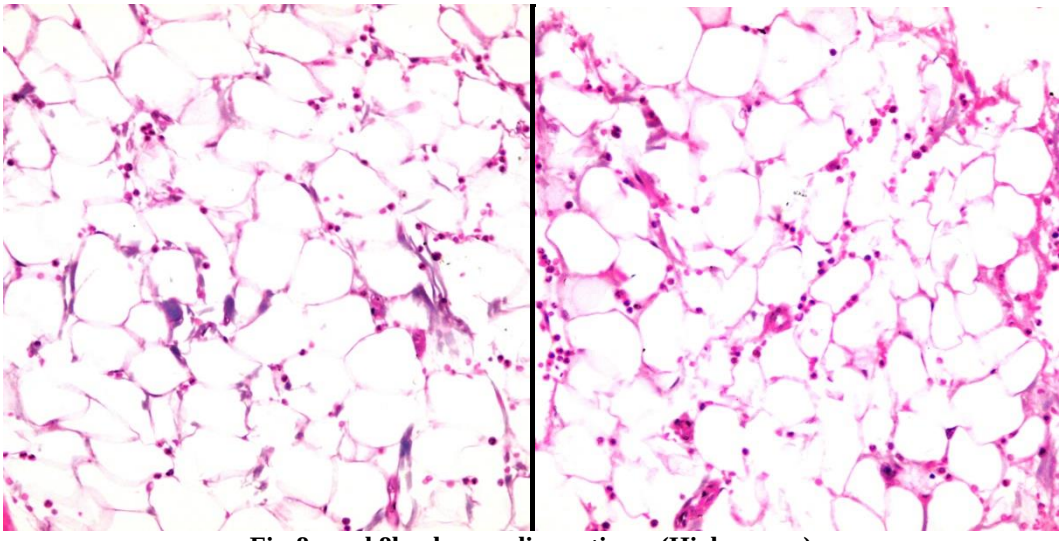


Fig-8a and 8b: shows adipose tissue (High power)

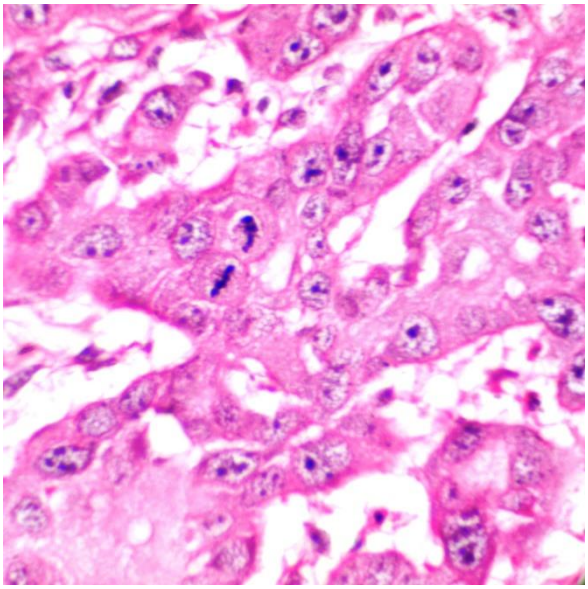


Fig-9: Shows the nuclear details with mitosis from H & E stained and microwave processed tissue of oral squamous cell carcinoma. (Oil Immersion)

Note - In the figures (a) indicate microwave processed and H & E stained tissue while (b) indicate conventional processed and H & E stained tissue.

DISCUSSION

During the last five decades the practice of surgical pathology has been enriched by advances in our knowledge of the morphologic expression of the disease and by new technologies like immunohistochemistry and molecular assays. The path of tissue samples from surgical removal till the preparation of H & E stained slides, however, has remained impervious to scientific advances. In particular, formalin fixation followed by currently used conventional tissue processing methods has been the standard for almost hundred years [8].

An essential part of all histological techniques is preservation of cells and tissues as they naturally occur. In the study of histology, preparation of tissue for microscopic viewing is an important consideration. This is because cell and tissue cannot be studied unless they are well prepared for microscopic examination on which diagnosis and treatment is depends [9].

Microwave energy is the most versatile form of energy applicable in various different fields. It was first used for radar in WWII (World War II) but, it is now applied in communication, chemistry, rubber vulcanisation, drying, food processing, medical treatment and diagnosis and variety of materials processing fields, etc [10].

Due to the presence of water molecule the embedding media can not enter the tissue. Hence the process of dehydration is required to replace the water in the tissue by alcohol or a substitute, clearing causes the replacement of alcohol by a chemical miscible with paraffin or its substitute and impregnation is the step in which the clearing agent is replaced by paraffin or its substitute. Hence the physiochemical basis of tissue processing lies in the diffusion of reagents into the substance of the tissue to be processed [2].

For decades instrumentation used in tissue processing remained relatively unchanged. A recent addition in the list of techniques involved for rapid processing of tissues is the use of microwaves, which has revolutionized histotechniques [11].

Microwaves are non-ionizing radiations and have electromagnetic properties, their frequencies range from 300 MHz to 300 GHz and wavelengths from 1 mm to 1 m. All domestic microwaves operate at 2.45 GHz corresponding to a wavelength in vacuum of 12.2 cm.[2]

Microwaves are the electromagnetic waves that can penetrate various types of material. The penetration depth of microwaves is dependent on the electric conductivity of the medium. Microwaves penetrate into the tissues and the microwave energy is absorbed by the molecules. Microwave irradiation produces oscillating electric fields which forces the dipolar molecules like water to vibrate. Vibration creates rotational energy, part of this acquired rotational energy is transferred to the random motion upon collision with other molecules. This induced kinetic movement produces instantaneous heat. This heat production increases the diffusion of the reagents and thereby decreases the tissue processing time. Unlike conventional heating, the heating in the microwave is from within (internal heating) and its effect occurs throughout the material being irradiated [11].

Diffusion is a key factor in the histoprocessing. The formula for the rate of diffusion is $<X^2> = 2Dt$, here "X" indicate the net distance covered by a particle in solution in a certain direction; "t" is the time period during which diffusion occurs; "D" is the diffusion constant for the substance and "< >" stands for the average value. The formula states that the average squared distance covered by a particle in solution is proportional to the diffusion time. This indicates that the thickness of the biopsies should be less, the length and breadth of the tissue does not matter [11]. Hence, from this study, by microwave processing "t" can be reduced without compromising the dehydration and impregnation.

Alka M.M *et al*. [12] found that when dehydration, clearing and wax impregnation were combined in the microwave method, microscopically similar features were observed. Overall, the quality of microscopic examination of tissues from conventional processing and microwave processing methods were identical. It was impossible to distinguish between the two techniques by studying the tissue section.

In this study, the effect of microwaves on the different types of tissue such as epithelium, connective tissue, squamous cell carcinoma, muscle tissue, adipose tissue, skin, serous acini, mucous acini and hard tissue were studied and found to be adequate for diagnosis.

In the previous studies, it was found that the tissue architecture, stroma, secretary products, cell and nuclear morphology were similar between conventionally processed and microwave processed tissue [6], which was also seen in this study.

Cox M.L *et al*. [13] found that there was no significant effect on RNA preservation by microwave fixation and/or processing.

There was no major difference in the quality of staining between the two different methods of tissue processing studied. Microwave tissue processing substantially shortens the time from tissue reception till diagnosis with no compromise with the overall quality of histologic section.

Microwave tissue processing achieves the three aims of reduced time taken, avoiding a noxious chemical and more economical, by eliminating Xylene from the process.

CONCLUSION

The present study revealed satisfactory results with microwave tissue processing. By using this technique profitability of any diagnostic laboratory would be increased. However further long term studies with larger sample size using a microwave oven with precise temperature control are required to arise at a definitive conclusion about the advantages of microwave tissue processing over the conventional tissue processing method.

Conflicts of Interest

There is no conflict of interest of any author regarding the preparation of the manuscript and publication of paper.

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