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Original Article

Comparative evaluation of different histoprocessing methods

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ABSTRACT

Objectives: Tissue processing for years is carried out by the conventional method, which is a time-consuming technique resulting in 1-day delay in diagnosis. However, in this area of modernization and managed care, rapid diagnosis is increasingly desirable to fulfill the needs of clinicians. The objective of the present study was to compare and determine the positive impact on turnaround times of different tissue processing methods by comparing the color intensity, cytoplasmic details, and nuclear details of the tissues processed by three methods.

Methods: A total of sixty biopsied tissues were grossed and cut into three equal parts. One part was processed by conventional method, second by rapid manual, and third by microwave-assisted method. The slides obtained after processing were circulated among four observers for evaluation. Sections processed by the three techniques were subjected to statistical analysis by Kruskal–Wallis test. Cronbach's alpha reliability test was applied to assess the reliability among observers. One-way analysis of variance (ANOVA) was used for comparing mean shrinkage before and after processing.

Results: All observers were assumed to be reliable as the Cronbach's reliability test was statistically significant. The results were statistically non-significant as observed by Kruskal–Wallis test. One-way ANOVA revealed a significant value on comparison of the tissue shrinkage processed by the three techniques. The histological evaluation of the tissues revealed that the nuclear-cytoplasmic contrast was good in tissues processed by microwave, followed by conventional and rapid manual processing techniques. The color intensity of the tissues processed by microwave was crisper, and there was a good contrast between the hematoxylin and eosin-stained areas as compared to manual methods.

Conclusion: The overall quality of tissues from all the three methods was similar. It was not feasible to distinguish between the three techniques by observing the tissue sections. Microwave-assisted tissue processing has reduced the time from sample reception to diagnosis, thus enabling the same-day processing and diagnosis.

Keywords: Conventional, fixation, kitchen microwave, rapid manual, tissue processing

Introduction

Turnaround time for any pathological laboratory is very important which depends on the preparation and diagnosis of the pathological lesions. The rapidity advantages the clinician to treat acutely ill patients and influence the work practice of the pathologist.[1] With the advent of modernization, tissue processing is modified from the point of tissue removal to embedding for instant histopathological diagnosis by various techniques or methods.

After the surgical removal, the tissue undergoes preparatory protocol for the preparation of sections, which usually involves impregnation with a suitable supporting medium. The stages of tissue processing include fixation, dehydration, clearing, impregnation, and embedding for designated durations of time to ensure completion of the procedure.[2,3]

The reproducibility and relatively low expense attached to the most commonly employed methods continue to recommend it as a valuable tool after nearly 100 years of existence. However, with the demand for faster or early reporting, newer techniques such as rapid manual and microwave processing are getting introduced. Each of them is unique with their own set of advantages and disadvantages.[2-4]

The conventional tissue processing is reliable and cost-effective, but time consumption, reagent toxicity, and delay in providing diagnosis are the major disadvantages. The rapid manual tissue processing has major disadvantages such as the use of noxious chemicals and greater degree of tissue distortion and shrinkage which led to exploration for new short-processing schedules. The microwave tissue processing eliminates the use of noxious chemicals, causes lesser distortion of tissue, and has shorter processing time, but the cost involved in instrumentation is very high.[4,5]

Microwaves were invented by Spencer in 1945, which work on the principle of producing heat by oscillating or exciting polar molecules. The microwave irradiation forces dipolar molecules of proteins to rotate through 180° at the rate of 2.45 billion cycles per second.[3,6] These excited molecules due to kinetics cause collision with adjacent molecules resulting in transfer of rotational energy. This friction causes production of heat within the material itself, leading to the accelerated diffusion of processing fluids; hence, faster processing is possible.[7]

The advantages associated with microwave processing led to the production of commercially available microwaves, specifically designed for tissue processing; however, the cost involved in these is very high.[1,8] Domestic microwaves are readily available, affordable and had been used for tissue processing with good results earlier by some authors.

Thus, the aim of the present study was to compare and analyze the efficacy of three tissue processing techniques.

Methods

In the present study, sixty specimens were selected randomly from the Department of Oral Pathology and Microbiology, Genesis Institute of Dental Sciences and Research, Ferozepur, Punjab, India. The soft-tissue specimens fixed in 10% neutral-buffered formalin for 24 h were included in the current study. Hard tissues specimens such as cartilage, bone, and tooth were not included in the study. The gross features of the specimen were recorded and the tissues were cut into three pieces of same size (approximately 2 cm × 2 cm) to be processed by three methods. The size of the tissues was recorded before processing and after impregnation with the help of a standard ruler to calculate shrinkage of the tissues. The tissue sections obtained after processing were subsequently stained with hematoxylin and eosin (H and E).

The stained slides in each group processed by three techniques were randomly numbered for a blind study and circulated among four observers referred as O1-O4. The observers graded each parameter by following specific criteria as given in Tables 1 and 2.

Microwave tissue processing

The microwave oven was calibrated as the microwave energy is non-uniform within the chamber. Thus, hot and cold spots were detected in the chamber with the use of thermal paper sheet, instead of the use of extra water load as suggested by various authors. The cold spot provided the most consistent results every time.

The technique was self-standardized by trial and error method in the domestic microwave (LG Model no. MS-285SD, India, [Figure 1]). The microwave was operated at the maximum output power of 40% (approximately 360 W) with rotating tray and ring removed. The cut piece from a fixed tissue sample was placed in a plastic tissue cassette and washed with the running tap water so that tissue was free of formalin. The tissue was irradiated in 200 ml of 100% methanol (Qualikems, Vadodara, Gujarat, India) and 200 ml of 100% isopropyl alcohol (Qualikems, Vadodara, Gujarat, India) for dehydration at cold spot for 2 cycles of 10 min each, respectively, in the microwave. After dehydration, tissue was impregnated in 200 ml of molten paraffin wax (Qualikems, Vadodara, Gujarat, India) for 2 cycles at the cold spot of 10 min each and was embedded in paraffin wax (Qualikems, Vadodara, Gujarat, India).

Conventional tissue processing

The cut piece from a fixed tissue sample was placed in a metal tissue cassette and washed with the running tap water so that tissue should be free of formalin. The tissue was dehydrated in 70% isopropyl alcohol (one change), 90% isopropyl alcohol (one change), and 100% isopropyl alcohol (Qualikems, Vadodara, Gujarat, India), three changes of 1 h each, respectively. After dehydration, tissue was cleared in two changes of xylene of 1 h each. Finally, tissue was impregnated in two changes of molten paraffin wax (Qualikems, Vadodara, Gujarat, India) for 1 h each and was embedded.

Rapid manual tissue processing

The cut piece from a fixed tissue sample was washed with the running tap water so that tissue should be free of

Figure 1: Microwave used for microwave-assisted tissue processing

Table 1: Grading criteria for assessment of cytoplasmic and nuclear details

Grade/parameter	Cytoplasmic details	Nuclear details
Distinct	• Greater eosinophilia of cytoplasm producing enhancement of the nuclear-cytoplasmic contrast • Good stroma • Appreciation of secretory products • Absence of red cell lysis • Ability to differentiate between inflammatory cells	• On the basis of chromatin condensation • Prominent nuclear membrane • Crisp staining of the nucleus and mitotic activity, if appreciable
Indistinct	• Granularity of cytoplasm • Focal condensation of stroma • Cellular outline blurring • Absence of mucin • RBCs lysis (focal or generalized) • Absence of differentiation between inflammatory cells	• Smudging and pyknosis of nuclei

RBC: Red blood cell

formalin; after that, tissue was wrapped in a filter paper and dehydrated in 95% isopropyl alcohol, 100% isopropyl alcohol (Qualikems, Vadodara, Gujarat, India) for 20 min on a stir plate. The dehydrated tissue was cleared in xylene (Qualikems, Vadodara, Gujarat, India) for 20 min on a stir plate. Tissue was impregnated in two changes of molten paraffin wax (Qualikems, Vadodara, Gujarat, India) of 1 h each and was then embedded.

The processed tissues by respective techniques were stained as per the protocol (Table 3).

The values obtained from different observers after assessment of sections processed by the three techniques were subjected to statistical analysis by Kruskal–Wallis test. One-way analysis of variance (ANOVA) was used for comparing mean shrinkage in tissues processed by the three tissue processing techniques. $P < 0.05$ was considered statistically significant.

Results

All observers were assumed to be reliable as the Cronbach's reliability test was statistically significant (Table 4). Complete concordance was found among all pathologists in most of the cases. Hence, observer 1 was randomly selected for further analysis.

The histopathological evaluation of the tissues (Tables 5-7) revealed that the nuclear-cytoplasmic contrast was good and cellular outline was distinct in tissues processed by microwave-assisted technique, followed by conventional processing and rapid manual processing techniques. The stroma was good with a distinct cellular outline. The secretory products can be easily appreciated, and the red blood cells and inflammatory cells were intact (Figures 2-4). The results were statistically non-significant as observed by Kruskal–Wallis test.

The color intensity of the tissues (Tables 5-7) revealed that the microwave sections were crisper and there was a good contrast between the H and E-stained areas.

Table 2: Grading criteria for assessment of color intensity

Grade/parameter	Color Intensity
Poor	• Tissue failed to take up stain adequately • Stained unevenly
Satisfactory	• Details not visualized up to the mark but slide suitable to give diagnosis
Good	• Good contrast between the nucleus and cytoplasm • Visibility of details along with brilliance of staining

Table 3: Protocol for staining

Reagent	Time	
	Microwave assisted staining	Routine and rapid manual staining
Xylene	10 min	10 min
Xylene	10 min	10 min
Water	10 min	10 min
Hematoxylin	10-15 s	3-5 min
Water	5 min	5-7 min
Acid alcohol	2 dips	1-2 dips
Water	5 min	3-5 min
Eosin	5-10 s	30 s
Absolute alcohol	2 changes	2 changes
Xylene	10 min	10 min
Total time	50 min	60-65 min

Table 4: Reliability test was carried out among observers to evaluate the alpha value

Details	Alpha value	*P* value[#]
Cytoplasmic details	0.649	0.000
Nuclear details	0.651	0.000
Colour intensity	0.476	0.000

[#]$P<0.001$; highly significant

One-way ANOVA revealed a significant value on comparison of the tissue shrinkage processed by the three techniques (Table 8). The dimensions of the tissues were recorded before dehydration and before paraffin embedding with the ruler,

Table 5: Histopathological evaluation for cellular morphology

Cellular morphology	Grades	Technique		
		Conventional	Microwave	Rapid manual
Distinct	0	39	45	32
Indistinct	1	21	15	28
Total		60	60	60

Table 6: Histopathological evaluation for nuclear morphology

Nuclear morphology	Grades	Technique		
		Conventional	Microwave	Rapid manual
Distinct	0	40	46	34
Indistinct	1	20	14	26
Total		60	60	60

Table 7: Histopathological evaluation for staining characteristics

Staining characteristics	Grades	Technique		
		Conventional	Microwave	Rapid manual
Poor	0	3	0	1
Satisfactory	1	18	16	27
Good	2	39	44	32
Total		60	60	60

Table 8: Comparison of shrinkage of tissues among the three methods of processing

Technique	% of shrinkage (Mean±SD)	Comparison of techniques	*P* value
A	40.08±20.71	A versus B	0.436[#]
B	35.91±20.04	B versus C	<0.001**
C	57.79±14.08	A versus C	<0.001**

[#]NS *P*>0.05 - Not significant, **Highly significant *P*<0.001. A: Conventional, B: Microwave, C: Rapid manual, SD: Standard deviation

respectively. The mean percentage of shrinkage in rapid manual technique was significantly higher as compared to the other two techniques, whereas statistically non-significant value was obtained by comparing conventional and microwave method of tissue processing.

Discussion

Microwaves are electromagnetic waves[9] which cause oscillation and excitation of polar molecules which are usually dipolar molecules of proteins in tissues. The excited molecules cause collision with adjacent molecules due to kinetics producing friction and cause production of heat within the material itself. 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 theoretically possible to fasten the tissue fixation and processing. This has resulted in a substantial reduction in the basic steps of histoprocessing, thereby reducing turnaround time and providing the same day diagnosis.[6,10]

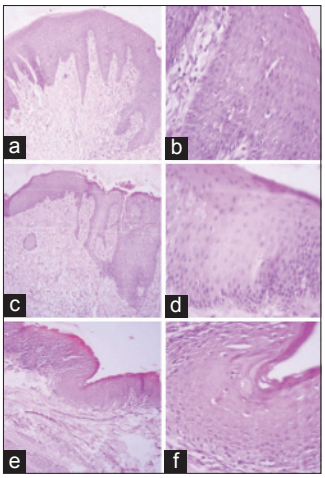

Figure 2: Squamous cell carcinoma processed by conventional method (a and b), microwave method (c and d), and rapid manual method (e and f) (H and E, ×10, ×40)

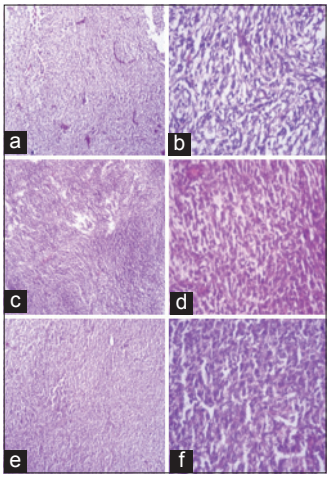

Figure 3: Fibrous histiocytoma processed by conventional method (a and b), microwave method (c and d), and rapid manual method (e and f) (H and E, ×10, ×40)

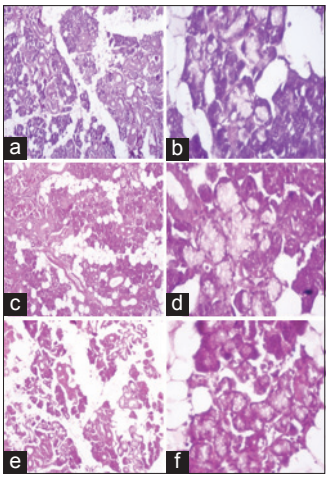

Figure 4: Submandibular salivary gland processed by conventional method (a and b), microwave method (c and d), and rapid manual method (e and f) (H and E, ×10, ×40)

The applications of microwaves are extensive which includes tissue fixation, stabilization of large specimens, tissue processing for light and electron microscopy, and histochemical and immunohistochemical staining.

Microwave tissue processing technique was introduced by Boon and Kok in 1985, but the potential application of microwave energy was first recognized by Mayers in 1970, who successfully fixed tissue with a microwave generator.[11] Boon *et al*.[12] reported that it was possible to produce a significant acceleration of tissue processing using microwave radiation. The first processor described was able to complete the processing in 30-120 min, thus reducing the processing time from 24 h to just 1-2 h providing early reporting and easier patient management.[13]

Thus, the aim of the present study was to compare the cytoplasmic and nuclear details as well as staining characteristics of tissue sections processed by conventional, rapid manual, and microwave techniques.

The noxious chemicals used in conventional tissue processing were replaced in microwave tissue processing. In the microwave processing in contrast to conventional tissue processing, isopropyl alcohol was replaced by methanol as dehydrating agent and xylene by isopropyl alcohol as intermediate agent. Molten paraffin wax remained the impregnating and embedding medium for both the techniques. The reagent selection agreed with Babu *et al*.,[14] who also used methanol, isopropyl alcohol, and molten paraffin wax for microwave tissue processing.

Microwave radiation when enters the chamber, gets reflected by chamber walls until these get absorbed by the material placed inside the chamber. However, the spreading is not even throughout the chamber, leading to formation of hot and cold spot zones.[16-18]

Hence, hot and cold spots should be detected to achieve consistent results. Various authors have described methods for detection of hot and cold spots.

Microwave processing was self-standardized by trial and error method, in which the hot and cold spots were detected using a damp thermal paper.[16] All the procedures in the microwave were carried out in the cold spot zones as suggested by Sharp and Paperiello,[19] Bernard,[20] Rangell and Keller[21] in their respective studies.

Microwavable plastic tissue cassettes were used for microwave tissue processing which are cheap and reusable as metallic utensils are contraindicated in the microwave because the electric fields of the waves produced by microwave magnetron are completely reflected at the same frequency by metals which can lead to sparking.[22]

In the present study, the staining protocol for microwave was followed as given by Babu *et al*.,[14] which included the stains used to be accelerated in the microwave. Domestic microwave oven has been used for acceleration of the various stains without the production of any deleterious effects on staining which also included H and E stain.[23,24]

In our study, the three pieces of tissue processed by three techniques sectioned by a soft-tissue microtome and stained as per their respective protocols were evaluated (Figures 5-7). We adopted the criterion for evaluation of tissue sections given by Kango and Deshmukh.[25]

The overall quality of the tissue sections processed by microwave and manual methods was comparable. The microwave processed sections had the same or similar cytoplasmic and nuclear details (Figures 2-4) with good erythrocyte integrity and lymphocytic appearance than the manual methods. We also observed that the stroma in some cases was slightly more condensed focally in microwave processed tissue sections, which is similar to the findings reported by Boon *et al*.[12] This leads to the erroneous categorization of these cases as indistinct in studies by Kango

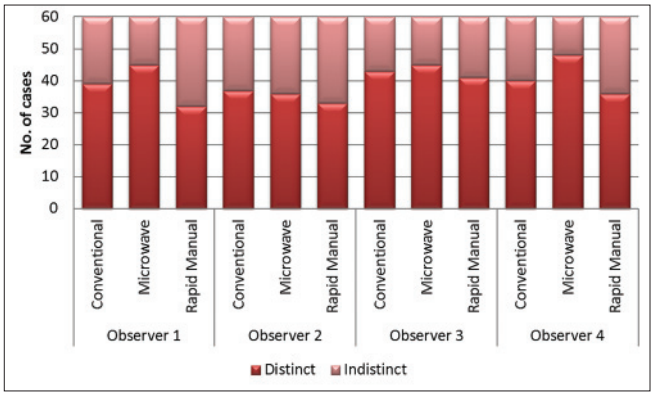

Figure 5: Comparison between conventional, microwave, and rapid manual tissue processing for cytoplasmic details

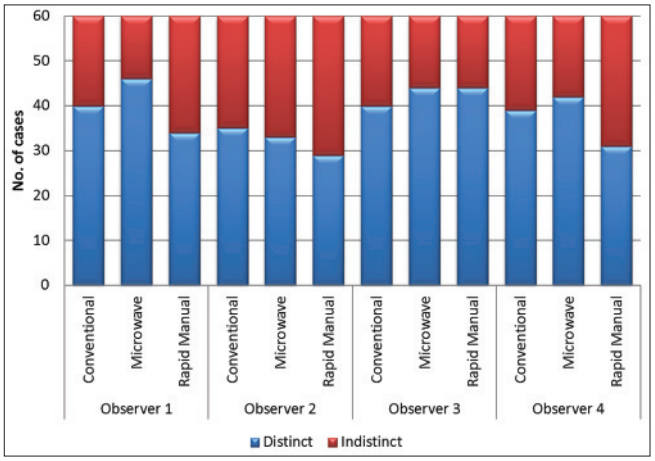

Figure 6: Comparison between conventional, microwave, and rapid manual tissue processing for nuclear details

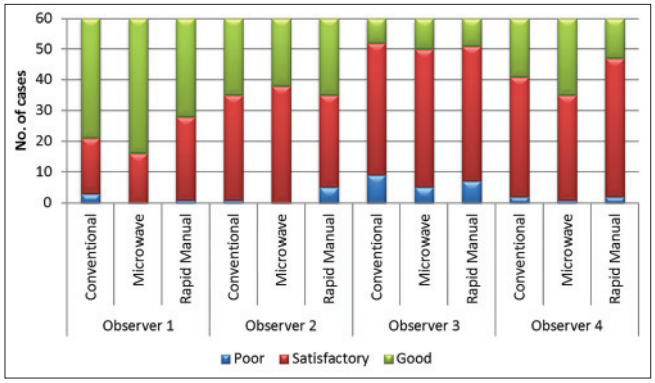

Figure 7: Staining characteristics of conventional, microwave, and rapid manual tissue processing

and Deshmukh.[25] Since our criterion was adopted from the above-mentioned study, we also placed focal condensation of stroma as indistinct. In contrast, Kok *et al*.[11] refuted the importance of focal condensation of stroma in diagnostic pathology.

The color intensity of the tissues graded by four observers revealed that the microwave sections were crisper and there was a good contrast between the hematoxyphilic and eosinophilic areas (Figures 2-4). The microwave processed tissues showed an increased reaction to H and E. The sections stained were slightly more eosinophilic as compared to the manual techniques. Similar findings were reported by Hopwood *et al*.,[26] Boon *et al*.,[12] Leong and Price,[27] and Panja *et al*.[4] Hopwood *et al*.[26] suggested that this eosinophilia could be easily corrected by altering the stain composition or staining time in eosin. In contrast, Leong and Price[27] observed that eosinophilia of the cytoplasm was advantageous as it produced good nuclear-cytoplasmic contrast and enhancement of the cellular features.

The dysplastic features, i.e. hyperchromatism, pleomorphism of tumor cells and mitotic figures, were easily appreciable in the microwave processed tissue sections of malignancy. There was also an easy appreciation of the giant cells in the tissues of central giant cell granuloma and tubercular lymphadenitis processed by microwave processing technique.

Rapid processing of histopathologic material is becoming increasingly desirable for intraoperative consultations and timely diagnosis. We found positive impact on turnaround time in microwave method as the time taken for block preparation from fixed tissue was 1h as compared to conventional method (9 h) and rapid manual method (3 h).

As assessed in our study, the effects of the three methods of histoprocessing on cytoplasmic and nuclear details of epithelial, fibrous, and glandular tissue showed no statistically significant variation. The microwave technique was comparable or slightly better than the manual methods.

Conclusion

The applications and versatility of microwave processing methods are unattainable with conventional procedures. The microwave-assisted tissue processing method reproducibly yields similar histologic quality to that provided by conventional processing. It has many advantages including feasibility, safety, and elimination of noxious chemicals that might be used for improvement in the practice of the histopathology laboratory, permitting the preparation of diagnostic material within a day. Domestic microwaves are easily available and cost-effective but have certain notable disadvantages such as uneven heating and inability to record and maintain temperature within the chamber. Further exploration in the field is required for the development of cost-effective microwave histoprocessors for histopathology, which provides similar histologic material for rapid processing and same-day reporting.

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