Original Article

Hegazy' Simplified Method of Tissue Processing (Consuming Time and Chemicals)

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ABSTRACT

Background: Processing is the next step in the histological process after tissue fixation. There are three methods commonly used for such tissue processing. They are the routine manual, rapid manual and the microwave methods. This study aimed to proceed a simple new manual method in a trial to take the advantages of rapid manual and microwave methods and avoid their disadvantages. **Methods**: One hundred samples of different tissues and cell blocks were included in this study. They were divided into two equal halves. One half is processed by the routine manual method and the other managed by new suggested technique. **Results:** The time consuming in the new method was about 7 hours vs. 20 hours in the routine processing. Also, the histologic quality was better in the new method as compared to the routine manual technique. **Conclusions**: The current simplified method of tissue and cell block processing using mild temperature and moderate agitation possess the advantages of reduction of time of processing, as well as the economic benefit of the utilization of fewer fluids.

Key Words: *Tissue processing, Cell blocks, Manual method, Histological process*

INTRODUCTION

Processing is the next step in the histological process after tissue fixation. Tissue processing constitutes several steps that cause structural formats of the tissues allowing them to be sectioned and hence stained for diagnostic purposes.[1] All tissues must be adequately supported before such sectioning. The support comes from impregnation of the tissues in paraffin. This gives a stable solid medium necessary to facilitate sectioning by microtome.[2] Many methods have evolved for a range of embedding media and applications. The most commonly used means of tissue processing are routine manual method, rapid manual method and the microwave method. Routine manual method represents the commonest one used in the past decades. Its advantages include the reliability and inexpensive nature. The disadvantages are that it is time consuming (about 21-24 hours) as well as consuming large amount of noxious chemicals such as xylene and formalin. Rapid manual tissue processing includes the same steps as in routine method but for shorter duration, requiring only 3-4 hours. The advantages are that it consumes shorter time as compared to routine method; but its disadvantages include greater degree of tissue distortion and shrinkage.[3]

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Microwave method is a recent technique, in which the microwaves possess penetrative properties with conversion of energy into heat.[4] These properties give the advantages of microwave processing including shorter processing time. The disadvantages include the high cost of the machine involved and tissue shrinkage.[5] Also, the size of tissues used must be not more than one cubic cm, otherwise complete and even penetration of microwaves will not occur.[6] In this study, we aimed to proceed a new method of manual tissue processing in a trial to take the advantages of rapid manual and microwave methods and avoid their disadvantages.

MATERIALS AND METHODS

Materials included plastic cassettes, magnetic stirrer with heat, conical-shaped glass jars, rubber stupor, descending grades of alcohol (absolute, 95%, 80% and 70%), distilled water, xylene, paraplast wax, scissors, and metallic ruler.

One hundred samples of different tissues and cell blocks were included in this study. Sections were cut at 3mm thickness, then placed in cassettes, then immersed in 10% neutral buffered formalin, for 12 hours. We divided the specimens into two equal parts. Half of the specimens were processed by the routine manual method mentioned by Kiernan[7] with modifications and the other by new method.

The new tissue processing method was proceeded as follows:

The cassettes were placed in:

70% ethanol; (the volume of alcohol to tissues was adjusted to a range of 20 alcohol: 1 tissue) at a temperature range of 30-45 ˚C with agitation of

tissues and fluids. This method of processing used a magnetic stirrer for 45 minutes (cursor of stirring at the medium and that of heating at 37 ˚C);
80% ethanol, in temperature of 30-45 ˚C, with stirring, for 45 minutes;
95% ethanol, in temperature: 30-45 ˚C, with stirring, for 45 minutes;
Absolute ethanol, in temperature of 30-45 ˚C, with stirring, for 45 minutes;
Xylene (I), in temperature of 30-45 ˚C, with stirring, for one hour;
Xylene (II), in temperature of 30-45 ˚C, with stirring, for one hour;
Paraffin (I), in temperature of 60-65 ˚C , without agitation, for 30 minutes;
Paraffin (II), in temperature of 60-65 ˚C, without agitation, for one hour;
Paraffin (III), in temperature of 60-65 ˚C, without agitation, for one hour [Table 1].

Table 1: A summary of our method in comparison to the routine method of tissue processing

Steps	Placed in	Item of technique	Routine method	Our method
Dehydration	Alcohol 70%	Duration Temperature Stirring	2 hours Room None	45 minutes 30º-45ºC Medium
	Alcohol 80%	Duration Temperature Stirring	2 hours Room None	45 minutes 30º-45ºC Medium
	Alcohol 95%	Duration Temperature Stirring	2 hours Room None	45 minutes 30º-45ºC Medium
	Absolute Alcohol	Duration Temperature Stirring	2 hours Room None	45 minutes 30º-45ºC Medium
Clearing	Xylene I.	Duration Temperature Stirring	2 hours Room None	45 minutes 30º-45ºC Medium
	Xylene II.	Duration Temperature Stirring	2 hours Room None	1 hour 30º-45ºC Medium
Embedding	Paraffin I.	Duration Temperature Stirring	2 hours 48º-68ºC None	30 minutes 60-65ºC None
	Paraffin II.	Duration Temperature Stirring	2 hours 48º-68ºC None	1 hour 60-65ºC None
	Paraffin III.	Duration Temperature Stirring	4 hours 48º-68ºC None	1 hour 60-65ºC None
Time consumed	-	-	20 hours	7 hours,15 minutes

After paraffin embedding, serial sections at 2-5 um were cut, deparafinized, hydrated, stained with Harris hematoxylin, and eosin (H&E) and some special stains such as periodic acid-Schiff (PAS), Masson's trichrome and Papanicolaou's (PAP) stain.
There were some modifications done according to type of the tissues [Table 2], such as:
For cell block, temperature was adjusted at 30ºC or slightly higher (30-32ºC).
For uterine specimens: lowering the duration to 30 minutes was done, to avoid hardening of the tissue, and so, difficult cutting in the microtome.
For bone specimens, decalcification was achieved by mixture of formic acid and hydrochloric acid, according to other literatures[1].
An evaluation of times required for each step of tissue processing by the routine manual method was performed and compared with that of our new technique [Table 1]. Then, the slides were examined independently by two examiners for tissue shrinkage, quality of staining, and clarity of nucleo-cytoplasmic differentiation in various cells and the presence of artifacts. We demonstrated the causes of artifacts and methods involved to mange or avoid them[8] [Table 3].

Table 2: Modifications in the procedure.

Specimens	Modifications
Cell block	30ºC or slightly higher
Uterine specimens	Duration: 30 minutes
Fatty, lymphoid, thyroid, GIT endoscopy	Ideal for processing
Bone specimens	Decalcification first

RESULTS

In the specimens examined in our method, only 10 specimens showed artifacts due to fixation; 5 specimens with shrinkage, the other 5 specimens showed tears and holes in the tissues due to hard paraffin. To manage this, we checked the concentrations of alcohols, and used mixtures of

hard paraffin and paraplast wax. The time consuming in the new method was about 7 hours vs. 20 hours in the routine processing [Table 1]. Overall histologic quality was better in the new method as compared to the routine manual technique. We demonstrated some tissue specimens processed by this new method [Figure 1-4]. For fatty tissues, lymphoid tissues, thyroid tissues, GIT and endoscopic specimens, the new technique was an ideal for tissue processing. However, there were some modifications done for other tissues [Table 2].

Figure 1: Juvenile retention polyp, H&E stain. 400 x

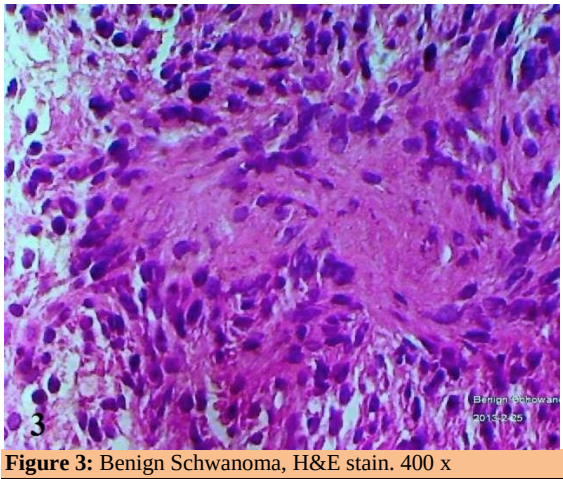

Figure 3: Benign Schwanoma, H&E stain. 400 x

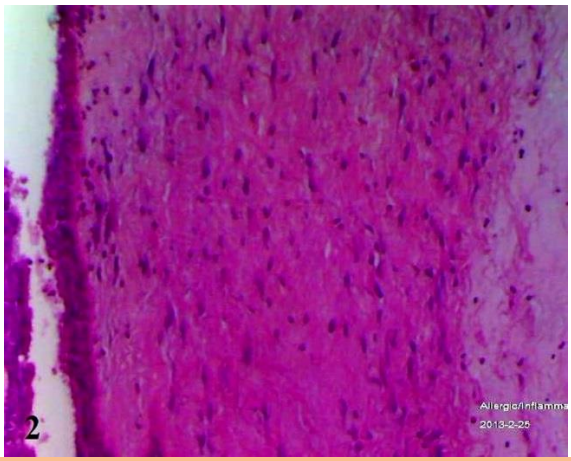

Figure 2: Allergic nasal polyp, H&E stain. 200 x

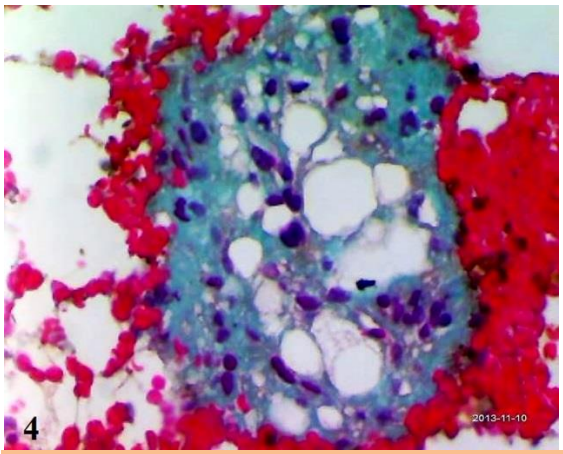

Figure 4: Atypical lipoma, cell block, PAP stain. 400 x

DISCUSSION

In this new manual method, we cut the sections into 3mm thickness to ensure good penetration of formalin into the tissues (1mm penetration/ hour).[9] Tissue processing is a process involved in the diffusion of various substances into and out of fixed tissues. This diffusion process results from the thermodynamic tendency of the reagents to take equal concentrations inside and outside the tissue, according to Fick's Law. This law states that the rate of solution diffusion through tissues is proportional to the concentration gradient which is temperature-dependent constant for each specific substance.[1] Accordingly, we can operate on the significant variables in tissue processing including the temperature, characteristics and concentrations of the reagents and properties of the tissue.[10,11] For efficient and effective processing, there should be a specimen volume to processing fluid volume ratio of at least 1:20.[10] So, we depended on this percent in starting tissue processing.

The most commonly used method of dehydration is the dilution dehydration. In such method, specimens are transferred through ascending grades of hydrophilic or water miscible fluids which dilute and then, replace free water in the tissues. Ethanol is probably the most commonly used dehydrant in histological techniques. It is a rapid, efficient and widely applicable dehydrant. Processing times in absolute ethanol should be minimal, because of the decrease in its concentration by each cycle of processing. While well fixed tissues can be transferred directly to 95% ethanol, incompletely

fixed tissues may exhibit artifacts if placed directly in higher alcohols. To minimize tissue distortion from diffusion currents, delicate tissues were dehydrated in a graded ethanol series from water through 10%-20%-50%-95%-100% ethanol. Duration of dehydration should be kept to the minimum consistent with the tissues being processed. Tissue blocks 1 mm thick should receive up to 30 minutes in each alcohol, blocks 5 mm thick require up to 90 minutes or longer in each change. In our method, we placed the 3mm-thick tissues for 45 minutes in each change. Tissues may be held and stored indefinitely in 70% ethanol without harm.[1] Therefore, we started with alcohol 70%.

Table 3: Artifacts found during tissue processing and how to manage

Type of artifact	Picture in the slide	Causes	How to manage or avoid
Squeeze artifacts	Crush, hemorrhage, split Fragmentation, pseudocyst	Due to forceps	Gentle handling of the specimens
Fulguration artifacts	-Amorphous appearance of epithelium, C.T -Appears at periphery of the lesion	Heat produced by electrosurgery	
Foreign bodies: **Cotton** **Starch**	Eosinophilic amyloid-like or black under polarized light. Retractile, glassy, PAS positive, diastase sensitive.	Due to powders on gloves.	Good wash of the specimens before fixation
Fixation artifacts:	Degenerative changes: shrinkage in cells, polarization cytoplasm, less details in nuclei, nucleoli.	-Delayed fixation	Rapid putting the specimens in fixatives, and sectioning of the large specimens before fixation.
Freezing in transport of tissues	Ice crystals, leading to vacuoles in cytoplasm, interstitium.	Due to freeze	Don`t freeze the specimens, just refrigerate.
Formalin artifacts	Black precipitates	Due to binding of heme-pigment to formaldehyde	Wash the specimens thoroughly with saline before fixation.
Processing artifacts	Shrinkage, Tissue vacuolization	Rapid removal of water	Ensure the graduation of ascending grades of alcohol.
Poor embedding	Tears, holes in the tissues	Hard paraffin or sections improper dehydrated before embedding.	Ensure the graduation of ascending grades of alcohol, check the paraffin
Microtomy	Wrinkling, curling, nicks in the tissues Alternate thick and thin tissues Tangential cut artifact	Wax is too soft or insufficient attachment of the microtome knife.	Ensure good cutting sections, temperature of water bath(45^0C) Check the paraffin, attachments of the knife.
Artifacts of the knife	Curling, separation and serrated edges.	Blunt knife	Ensure sharpening of the knife.
	Wrinkling, folding.	Very thin section	Ensure sections between 2-5um.
Floater artifacts		Improper cleaning of the water bath	Ensure cleaning of the water bath.
Staining artifacts	Patches, blotching in slides	Placing the slides in xylene before complete removal of wax.	Ensure complete removal of wax before.
	Air bubbles under cover	Thin mounting media	Ensure adequate mounting media.
	Drop of water under cover	Improper dehydration before mounting	Ensure complete dehydration before mounting
	Dark or light staining		Ensure concentration of the stains, and duration of staining.

The application of mild heat within the range 37°C to 45°C, during the dehydration and clearing steps considerably reduces processing times, but may concomitantly increase shrinkage. Tissue shrinkage during infiltration in paraffin wax results mainly from the effect of heat on collagen.[12] On this point of view, we utilized this mild heat (30°C-45°C) in each change.

Fluid interchange between processing reagents and tissues is promoted by exposure of the maximum

tissue surface area to reagents. During processing, agitated within the medium to facilitate the exchange of dilute reagent from the tissues with the more concentrated reagent replacing it. Agitation of tissues and fluids in manual processing is achieved using rotors or magnetic stirrers. Efficient agitation could reduce 30% of the time of tissue processing.[13] In our method, we utilized the magnetic stirrer at medium level.

High infiltration temperatures cause marked tissue shrinkage and hardening which can be avoided by maintaining the embedding waxes 2°C above their melting points. In the automatic processor, 10-12 reagent stations are done with temperatures adjustable between 30ºC-45°C, of which 3-4 paraffin wax stations with ranges of temperature between 60ºC-65°C.[13] In our method, we performed 3 paraffin wax stations at 60ºC-65°C, for 0.5, 1.0 and 1.0 hour respectively.

Prolonged immersion in paraffin wax at the correct temperature results in only slight tissue shrinkage, though tissues such as blood, muscle and yolk may harden and become brittle.[14] Therefore, the blocks could be left in the last paraffin station at room temperature to be used in the next day.

The extent to which tissues are affected during paraffin wax infiltration depends upon the combination of fixative, dehydrant and transition solvent used as well as the tissue type. For example, progressive removal of bound water from carbohydrates and proteins during prolonged immersion in absolute ethanol causes tissues to harden excessively and become brittle. Colloid, blood, collagen and yolky tissues are particularly affected.[5] The problem is exacerbated by heat during wax infiltration. At low temperatures structural elements of tissues are stabilized against the destructive effects of solvent changes. This is possibly because of the stiffening and strengthening effect of cold upon biopolymers resulting from diminution in thermal disruption of secondary bonds of the tissue constituents. Unfortunately at low temperatures, reagent viscosities increase and diffusion rates decrease, resulting in prolonged processing times. Isothermally processed mammalian tissues show finer detail and fewer artifacts than those processed by the more practicable, common an-isothermic techniques. Heat increases the kinetic energy of molecules and rate of diffusion, with a corresponding decrease in solution viscosity.[13] On this basis, we utilized mild heat and medium agitation.

CONCLUSION

The current simplified method of processing using mild temperature and moderate agitation possess the advantages of reduction of time of processing to 7-8 hours, as well as the economic benefit of the utilization of less fluids. We can adjust the amount of fluids to size of tissue (20:1), especially for low rate of specimen preparations. tissues should be loosely packed, suspended and

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