Original Article

Evaluation of histomorphometric changes in tissue architecture due to fixation delay

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Abstract

Introduction: All good tissue specimen preparations require complete fixation. The process of tissue handling and processing from patient to paraffin block is too frequently invisible to the pathologists. Many times due to certain emergencies or unavailability of a proper fixative, tissues are kept in different carrying media such as normal saline (NS) or local anesthetic till the availability of a proper fixative solution. This fixation delay can lead to various tissue architectural changes which can affect its diagnostic value.

Aims: The aim of this study was to assess sectioning ability, staining intensity and microscopic details of tissues kept in different carrying media at different time intervals followed by standard fixation.

Materials and Methods: Fresh tissue specimen, i.e., goat tongue was collected and its middle portion was retained and was used for study purpose. The tissue was grossed and kept in various carrying media for five different time intervals. Standard formaldehyde fixation was then carried out followed by sectioning and staining. The sections were evaluated histologically under light microscope.

Statistical Analysis: For sectioning parameter, Fisher's exact test and for staining and microscopic details, Mann–Whitney U-test was used.

Results: According to the study, NS is considered as a best carrying media followed by 10% honey and local anesthetic. Two percent hydrogen peroxide cannot be used as a carrying media.

Conclusion: It was concluded that NS should be given first preference as a carrying media till the availability of a suitable fixative. Clinicians and Pathologists should have to familiarize themselves with the advantages and disadvantages of using various carrying media and the histomorphometric changes associated with delayed fixation which may lead to incorrect diagnosis.

Keywords: Carrying media, fixation, fixation delay, incomplete fixation

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INTRODUCTION

Mummification is one of man's earliest methods of human tissue preservation. The objective of fixation is to preserve and harden cell and tissue constituents in as close as a life-like state as possible and to allow these to

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undergo further preparative procedures without change. It is the first step and the foundation in a sequence of events that culminate in the final examination of a tissue section.[1] Fixation may be described phenomenologically, that is in terms of change using living tissue as a standard. The major processes which tissue fixation must ideally prevent have been listed by Baker (1960): autolysis, attack by bacteria and change in volume and shape, especially during subsequent preparative treatment. Fixation may also be described in terms of molecular processes such as formation of macromolecular network between the various cellular constituents or in more specific terms, in reaction between the fixative and some chemical group. A complete definition of fixation is difficult or even impossible as the changes brought about are necessarily compared with living tissues which themselves are not by any mean completely characterized.[2]

Errors in fixation are permanent. Fault in fixation cannot be remedied at any later stage and the finished product can only be as good as its initial fixation.[1] Properly fixed tissue is nearly impervious to abuse during tissue processing and slide preparation.[3] Fixation arrests autolysis and bacterial decomposition and stabilizes the cellular and tissue constituents so that they withstand the subsequent stages of tissue processing.[4] There is no single fixative that is ideal.[5] Good fixation requires a compromise between rapid tissue stabilization and retention of original physicochemical properties.[6]

Ferdinard Blum has been credited as the first person to use formaldehyde as a tissue fixative.[7] Formaldehyde as 4% buffered formaldehyde is the most widely employed universal fixative, particularly for routine paraffin-embedded sections.[4]

The process of tissue handling and processing from patient to paraffin block is too frequently invisible to the pathologists. Many times due to certain emergencies or unavailability of a proper fixative, tissues are kept in different carrying media such as normal saline (NS) or local anesthetic till the availability of proper fixative solution. This fixation delay can lead to various tissue architectural changes which can affect its diagnostic value. The effect of delay in fixation on the number of mitotic figures in tissue has received little attention and it was found that the number of observable mitotic figures declined by about 30% with a delay of fixation of 2 h and by 50% with a delay of 6 h.[8] It is therefore important that the principles and practice of tissue fixation and the consequences of poor fixation are thoroughly understood by all staff involved in collecting and processing tissue specimens.

Cell preservation and staining ability of under-fixed or delayed fixed tissue show significant variability in cell structure and result in degradation of the tissue architecture which hinders the diagnosis. Oral Pathologists should have to familiarize themselves with a different set of microscopic details associated with different carrying media and their length of exposure in outcome of the end result. The present study was conducted to evaluate sectioning ability, staining intensity and microscopic details of tissues kept in various carrying media at different time intervals followed by standard formaldehyde fixation.

MATERIALS AND METHODS

This is a comparative study which was carried out over a period of 1 year. The ethical clearance for the study was obtained from the Institutional Ethical Committee.

Tissue sample used for the study

Goat tongue was used as the tissue sample [Figure 1a] which was harvested from a butcher shop. Total of eight tongue specimens were taken for each cycle and each specimen was further divided [Figure 1b] as to be used at five different time intervals.

Carrying media selected

NS (0.9%), local anesthetic (Xicaine), 10% honey (Dabur), 2% hydrogen peroxide solution (Qualigens).

Methodology

Fresh tissue specimens (goat tongue) were obtained from the slaughter house and were immediately transferred to the carrying media. No animal was harmed for the purpose of the study. Each tongue tissue was collected and its anterior and posterior-third was discarded. The middle-third portion of the tongue was retained and was used for the study purpose. The tissue was grossed into ten equal parts (1 cm each) and were kept in above-mentioned

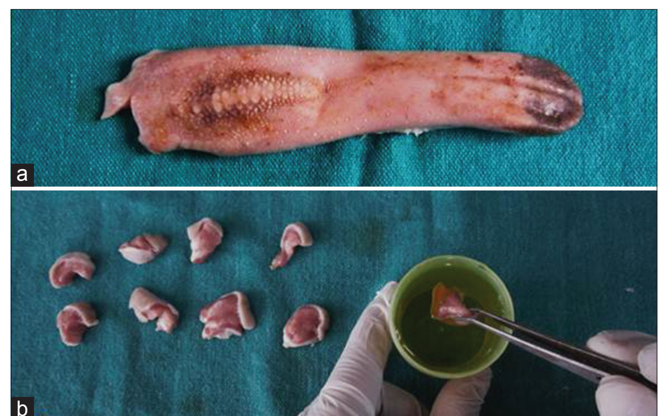

Figure 1: Goat tongue (a), immediate transfer of grossed tongue specimen (b)

carrying media for five different time intervals of 6, 12, 18, 24 and 30 h, respectively. They all were then fixed, respectively, in 10% buffered formaldehyde for 24 h. After fixation, tissue processing steps were carried out followed by sectioning and staining. These fixation and processing steps were repeated twenty times and all the data obtained were compared and statistically analyzed.

Scoring

The data hence achieved were statistically analyzed under following headings:

- Sectioning criteria: It was evaluated by the presence and absence of two factors; hard to cut and crumble
- Staining criteria: It was evaluated under light microscope at ×10 magnification by scoring the slides from 0 to 5 (score - 3 was kept as minimum score for acceptable result) by three independent observers under two parameters; nuclear staining and cytoplasmic staining
- Microscopic details: It was evaluated under light microscope at ×10 magnification by scoring the slides from 0 to 5 (score - 3 was kept as minimum score for acceptable result) by three independent observers under two parameters; nuclear and cellular shrinkage, nuclear and cellular dissolution/distortion of cellular components.

Score 0-5, indicates: 0 (very poor), 1 (poor), 2 (average), 3 (good), 4 (very good), 5 (excellent).

Tissue kept in 10% buffered formaldehyde for 24 h was taken as control [Figure 2a]. Comparison of various parameters with standard parameter was analyzed by applying appropriate statistical methods. The descriptive results were presented as median. Comparison of sectioning criteria was carried out using Fisher's exact test with $P < 0.05$ as atatistically significant. Comparison of staining criteria and microscopic details was carried out using Mann–Whitney U-test with $P < 0.05$ as statistically significant. Statistical analysis was conducted using SPSS version 20.0 (SPSS, Inc., Chicago, IL, USA).

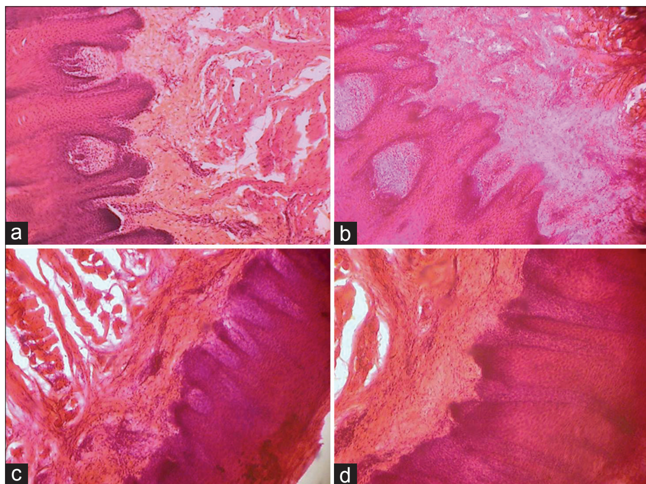

Figure 2: Photomicrographs of tissue kept in 10% buffered formaldehyde for 24 h (a), in normal saline for 18 h (b), in 10% honey for 12 h (c), in local anesthesia for 6 h (d) (H&E stain, ×10)

RESULTS

Hard to cut: Hard to cut frequency [Figure 3a] of specimens in all carrying media were statistically insignificant [Table 1] during sectioning by microtome.

Crumbling: The specimens in 2% hydrogen peroxide showed maximum crumbling frequency at 6 h ($P < 0.001$), whereas specimen in NS showed minimum crumbling frequency [Figure 3b]. At 12 h, specimens in honey and local anesthetic showed statistically significant crumbling frequency [Table 1]. From 18 h onwards, specimens in all the carrying media show statistically significant crumbling frequency ($P < 0.001$). With increase in time interval, best results were shown by specimens in NS as it can hold the tissue for longer time with less crumbling frequency and the worst results by specimens in 2% hydrogen peroxide [Figure 3b].

Nuclear and cytoplasmic staining: It was seen that as the specimens time interval in all the carrying media increase the quality of slides decreases, i.e., poor staining intensity. The best results were shown by the specimens in NS as they showed acceptable score of 3 till 18 h followed by 10% honey, local anesthetic and 2% hydrogen peroxide [Table 2].

Nuclear and cellular distortion/dissolution: Specimens kept in 2% hydrogen peroxide showed high nuclear and cellular distortion/dissolution at 6 h time interval whereas those in NS showed least [Table 2].

Specimens in NS showed the best tissue architecture and can hold the tissue specimens for longer time interval followed by 10% honey, local anesthetic, 2% hydrogen peroxide.

DISCUSSION

Carrying media are considered as holding agents rather than fixatives because they do not chemically alter tissues. They are often used to transiently prevent desiccation of tissues. They are used as a buffer system in cell culture media and aid in maintaining the optimum physiological pH and osmotic pressure providing the cells with water and inorganic ions. In our study carrying media were chosen on the basis of their easy availability (local anesthetic, NS,

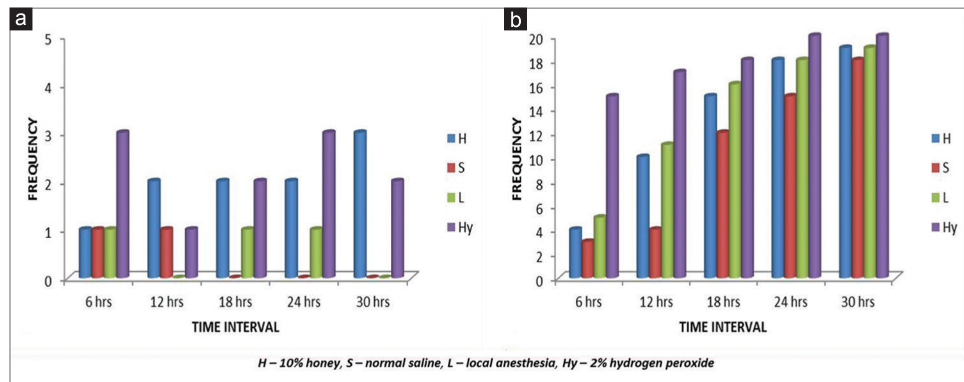

Figure 3: Graphs showing hard to cut (a) and crumbling (b) frequency during sectioning

Table 1: Sectioning criteria: *P* value using *fisher's exact test*

Time interval	6 hours		12 hours		18 hours		24 hours		30 hours	
Carrying media	Hard to cut	Crumble	Hard to cut	Crumble	Hard to cut	Crumble	Hard to cut	Crumble	Hard to cut	Crumble
Saline	1	0.605	1	0.3416	1	<0.001	1	<0.001	1	<0.001
Honey	1	0.3416	1	0.0033	1	<0.001	1	<0.001	0.605	<0.001
L.A	1	0.1818	1	0.0012	1	<0.001	1	<0.001	1	<0.001
H_2O_2	0.605	<0.001	1	<0.001	1	<0.001	0.605	<0.001	1	<0.001

Table 2: Staining and microscopic details: Overall histologic score and comparison of different carrying-medias at different time intervals

Parameters (median score)	Fixatives	6 hours		12 hours		8 hours		24 hours		30 hours	
		N	Cy	N	Cy	N	Cy	N	Cy	N	Cy
Staining	Honey	3	3	3	3	2	2	1	2	1	2
	Saline	4	4	4	4	3	3	2	2	1	2
	L.A	3	3	2	2	1	1	0	1	0	1
	H_2O_2	2	2	1	2	1	1	0	1	0	1
		N	C	N	C	N	C	N	C	N	C
Shrinkage	All Carrying-medias	Not applicable (Due to Dissolution and Distortion with increase in time interval)									
Swelling	All Carrying-medias	Not applicable (Due to Dissolution and Distortion with increase in time interval)									
Distortion and Dissolution	Honey	3	3	3	3	2	2	1	2	1	1
	Saline	4	4	3	3	3	3	2	2	2	2
	L.A	3	3	2	2	2	2	1	1	0	0
	H_2O_2	3	2	2	2	1	1	1	1	0	0

$P<0.001$ for all, Mann-Whitney U test, N: Nuclear, Cy: Cytoplasmic, C: Cellular

2% hydrogen peroxide) and easy preparation (10% honey) in dental clinics and hospitals.

NS (or N/S) is the commonly used phrase for a solution of 0.90% w/v of NaCl. The exact mechanism by which sodium chloride act on the tissue is unclear and its precise action on the tissue is unknown. Presumably, it might be a sodium specific chloride-anion and hyperosmolarity effect which leads to osmotic dehydration of cells and intercellular matrix.[9]

Honey has been known as a medicine for wound healing, tissue preservation and antibiotic properties. It is a supersaturated sugar solution, and this results in a strong interaction between the sugar molecules and water molecules. This leaves very few water molecules for growth support of micro-organisms. pH of honey is between 3.2 and 4.5, and this acidity is low enough to inhibit the growth of most microorganisms.[10] Bee honey has been shown to preserve tissue morphology.[11]

Lignocaine with methyl paraben has a more pronounced deleterious antibacterial effect as compared to lignocaine when used alone.[12] Within a pH range of 3–6, methylparaben solutions are very stable, but they will hydrolyze in an alkaline environment.[13]

Hydrogen peroxide is the simplest peroxide (a compound with an oxygen-oxygen single bond). Owing to its antibacterial property,[14] hydrogen peroxide was used in our study as a carrying media. The results obtained with its usage indicated that hydrogen peroxide has a very poor efficacy as a carrying media as it cannot retain tissue morphology for a substantial period of time, i.e., for a period of 6 h or more.

Specimens in all carrying media showed increase crumbling with increase in time interval. This could be due to the fact that, due to incomplete fixation tissue components can separate easily on the floatation bath as described by Carson.[3] Specimens in all carrying media were easy to cut which could be due to the fact that whether it is placed in water, Ringer's fluid or the aqueous fluids; some hours after excision, swelled more readily than fresh tissue. Such swollen tissue had a notably less compact character than fresh tissue or fresh soaked tissue.[15]

Specimens in NS showed the best tissue architecture as compared to other carrying media. Pure anhydric sodium chloride was used as a fixative agent at room temperature for prolonged periods of time was found to preserve the morphological and molecular structure of the studied tissues.[9] Al-Saraj also conducted a study in which the saturated sodium chloride solution was used as a fixative for tissue in pathological or histological procedures.[4] In our study, NS could only be used as a carrying media as it is neither in a saturated form nor in pure anhydrous state.

The mechanism of preservation of the molecular structure of cells by sodium chloride might be due to sodium specific, chloride anion and hyperosmolarity effect. Osmotic dehydration of cells and the intercellular matrix should also be taken into consideration.[9] It is also conceivable that sodium chloride might exert a direct effect in stabilizing reactions occurring during fixation.[16]

Our results showed that specimens kept for more than 18 h in NS showed poor/unacceptable quality of tissue architecture in form of distortion (splitting of cells). This is similar to the findings described by Culling, *et al*.[17] This finding could also be supported by the fact that, the tissue fixed in calcium carbonate solution used for buffering, exhibited prominent acantholysis of the superficial epithelium, with preservation and attachment of the basal cell layer of the underlying tissue.[18] A similar artifact was also produced by "fixing" the specimen in tap water for 48 h.[18]

Specimens in 10% honey showed acceptable results till 12 h. This finding could be strengthen by the fact that; Pine honey has been shown to preserve tissue morphology similar to that produced by formalin.[8] Also, Honey has been shown to inhibit the growth of a wide range of bacteria, fungi, protozoa and viruses. The antibacterial effect of honey depends on its osmotic effect (high sugar and low water content), acidity, hydrogen peroxide formed by enzymatic reaction and phytochemical factors (McCarthy 1995). Most unprocessed honeys, when diluted slowly, generate hydrogen peroxide owing to activation of the enzyme, glucose oxidase, which oxidizes glucose to gluconic acid and hydrogen peroxide.[10,11]

Specimens in local anesthetic showed acceptable results till 6 h. This could be due to the preservative and antibacterial action of methyl paraben which is one of its constituent. Specimens in 2% hydrogen peroxide showed unacceptable results at all the time intervals and hence its use as a carrying media was discouraged.

Tissues which were kept in these carrying media for extended time period showed dissolution and distortion of nuclear and cellular morphology in form of fading/complete disappearance, disruption of the cytoplasm and artifactual spaces around cells which is due to delayed fixation [Figure 4]. These findings were similar to the facts reported by Carson.[3]

Thus it was found that, for adequate histomorphology maximum time for the tissue which could be kept in NS was 18 h [Figure 2b], in 10% honey was 12 h [Figure 2c] and in local anesthetic was 6 h [Figure 2d]. Clinician or a dentist must be familiarized to these carrying media or holding agents which could be used in dental clinics or hospitals till the proper fixative is made available. When using a carrying media, clinician should specify the type of carrying media and the time it is kept in it, so that when it is received by the pathologist the time lapse can be calculated and thus anticipated alteration in tissue architecture would be kept in mind by the pathologist at the time of diagnostic interpretation.

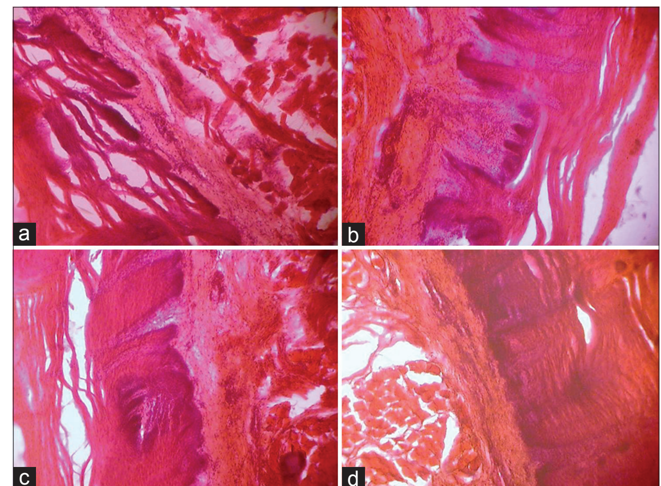

Figure 4: Photomicrographs of tissue kept in H_2O_2 for 6 h (a), in normal saline for 24 h (b), in 10% honey for 30 h (c), in local anesthesia for 12 h (d) (H&E stain, ×10)

CONCLUSION

According to the study, it was concluded that NS is considered as a best carrying media followed by 10% honey and local anesthetic. This is the first study on this aspect of certain aqueous-media to be used as holding agents. Being an animal study, further investigations are required to be carried out using same as well as other carrying media along with extensive and large sample size to support and standardize the presented facts so that it can become an integral part of the normal day-to-day routine procedure, whenever there is unavailability of a proper fixative at a given time. Furthermore, similar studies should be conducted using human pathological tissues and using different set of criteria for assessment. Delay in fixation due to transfer of tissue specimen in carrying media is a critical issue that needs to be addressed as it causes various morphological alterations in the tissue specimen.

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Conflicts of interest
There are no conflicts of interest.

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