Original Article

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Utilizing Almond Oil with Vitamin E as an Alternative Fixative to Formalin

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ABSTRACT

Introduction: The health hazards resulting from exposure to formalin have given rise to the need of developing safer alternative fixatives. Almond oil has been widely used in the aromatherapy and cosmetic industry for its good penetrating and skin-rejuvenating properties, whereas vitamin E is a widely known anti-oxidant. The present study aimed to test the fixative efficacy of almond oil with vitamin E as compared to formalin.

Material and Methods: The present study was conducted on 42 tissue specimens obtained by crown lengthening procedure or other minor surgical procedures. The specimens were divided into two groups- 21 specimens in the study group were fixed in oil fixative while others were fixed in formalin for 24 hours. Routine histopathological procedures were done and the histomorphological parameters related to the preservation of tissue architecture as well as cellular details were assessed.

Result: The preservation of tissue architecture and cellular details in tissues fixed in AO were comparable to that of tissues fixed in formalin. Although statistically non-significant, all the three investigators agreed that oil-fixed tissues exhibited superior nuclear details as compared to formalin.

Conclusion: Almond oil with vitamin E can be effectively used for the fixation of tissues, providing results that are comparable to formalin-fixed tissues. The non-toxic, non-irritant chemical nature of almond oil coupled with its ease of availability, enable it to be utilized safely even in remote areas.

Keywords: *Formaldehyde; Fixation; Histotechnique; Histopathology.*

Introduction

Fixation is the foremost fundamental process in histopathology laboratories which focuses on preventing the autolysis or degradation of the tissue. Preserving the tissue chemistry and architecture is crucial for subsequent processing and microscopic examination. At present, 10% neutral buffered formalin (NBF) is the most used fixative globally [1]. It provides impeccable results with regard to preservation of tissue architecture and nuclear-cytoplasmic details.

However, the most gruesome drawback of using formalin is the carcinogenic potential of formalin vapor emitted by these fixatives. Various studies have demonstrated that formaldehyde vapors can induce squamous cell carcinomas in the respiratory tract of histopathologists and technicians coming into frequent contact with these vapors through genotoxic and cytotoxic modes of action [2,3]. A recent meta-analysis also revealed that the risk for nasopharyngeal cancer and leukemia increased significantly with exposure to formaldehyde [4]. Therefore, the Occupational Safety and Health Administration has recommended developing safer fixatives alternative to formalin [5].

Several previous studies have utilized alternatives for fixation such as commercially available honey or jaggery syrup with satisfactory results. Almond oil (AO) or *oleum amygdalae* is used in the cosmetic industry for its penetrating, moisturizing, restructuring, and skin-rejuvenating properties [6]. On the other hand, Vitamin E is widely known for its potent antioxidant properties. Vitamin E has been subsequently proven as a radical chain-breaking antioxidant that can protect the integrity of tissues and play an important role in life processes [7].

The present study aimed to test the utility of commercially available AO with Vitamin E as an alternative fixative to formalin. The study had the objective of developing a safer, feasible, and easily available fixative that could effectively fix histopathological specimens with minimal side effects.

Materials and Methods:

The study protocol was approved by the institutional ethical review board. A sample size of 42 specimens was determined using single proportion formula. Tissue specimens that were generally to be discarded following a

crown lengthening procedure or any other minor surgical procedure were utilized in the study. It was ensured that specimens were of at least 2 mm in length, width, and depth respectively because embedding and sectioning of smaller specimens would be difficult after further shrinkage during tissue processing.

Tissues carrying diagnostic value that was intended to be submitted for histopathological diagnosis of a suspected pathology were not included in the study because the fixation potential of the novel fixative was yet unknown. Friable specimens, that were not suitable for histopathological processing, were also excluded from the study.

Upon removal from the patient, the tissues were briefly washed with normal saline to remove excessive blood. The specimens were then randomly allotted to either the 'experimental group' or 'control group', with each group comprising of 21 specimens. The tissues in study group were placed in an air-tight plastic container containing 50 ml of commercially available AO (Patanjali Ayurved Limited, India) having vitamin E (in a ratio of 99:1). The tissues in the control group were fixed in 10% NBF with similar settings.

The gross findings of specimens were then noted after fixing the tissues in respective fixatives for 24 hours. The fixed tissues then underwent routine histopathological processing comprising of dehydration in graded concentrations of alcohol, clearing in xylene, and infiltration with paraffin wax in an automated tissue processor (Yorco Scientific, India). Sections of 4µm thickness were obtained using a semi-automated microtome (Leica Biosystems, Germany). The sections were then deparaffinized and rehydrated to water followed by routine Hematoxylin and Eosin (Fisher Scientific, Waltham, MA, USA) staining, The stained sections were mounted with DPX (Merck specialties Pvt. Ltd., Mumbai) and observed under a light microscope.

Various parameters related to the preservation of overall tissue architecture and cellular details (Table-1) were scored from 1 to 4 for Poor, Satisfactory, Good, and Excellent. The grading used was based on a similar study performed earlier by Sinha et al. that compared the efficacy of jaggery syrup as a fixative to formalin [8]. To eliminate bias, three investigators independently assessed all the parameters, and they were blinded with respect to the fixative used. The mean and standard deviation (S.D) of all the five variables was calculated. The data was subsequently analyzed for statistical significance by Student's unpaired t-test using Statistical Package for the Social Sciences (SPSS v 21.0, IBM). For all the statistical tests, $P < 0.05$ was statistically significant, keeping α error at 5% and β error at 20%, yielding a power of 80%.

Results

The tissues fixed in AO displayed adequate preservation of tissue architecture (Figure-1), as well as cellular details (Figure-2). The mean, S.D, and values obtained by t-test for all the variables are listed in (Table-2). The P-value was found using a table of values from Student's t-distribution.

All the parameters exhibited a statistically non-significant difference indicating that the preservation of tissue architecture and cellular details in tissues fixed in AO was comparable to that of tissues fixed in formalin. Although statistically non-significant, all the three investigators agreed that oil-fixed tissues exhibited superior nuclear details as compared to formalin.

Table 1: Histomorphological parameters assessed and their rating scores.

Parameters	Score
Overall tissue architecture • Delineation of various tissue elements (under 4x and 10x) • Interface between the tissue elements (under 40x) **Cellular details and staining (under 10x and 40x)** • Nuclear • Cytoplasmic • Cell outline	**Poor (1)** **Satisfactory (2)** **Good (3)** **Excellent (4)**

Table 2: Comparison of fixative properties of Oil fixative with formalin by Student's t-test

	Formalin		Oil		t-test	p-value
	Mean	SD	Mean	SD		
Overall tissue architecture						
Delineation of various tissue elements	3.86	0.35	3.71	0.45	1.2058	0.235
Interface between the tissue elements	3.71	0.45	3.57	0.49	0.9643	0.3407

	Formalin		Oil		t-test	p-value
	Mean	SD	Mean	SD		
Cellular details and staining (under 10x and 40x)						
Nuclear	3.1	0.61	3.24	0.75	0.6636	0.5107
Cytoplasmic	3.23	0.53	3	0.76	1.1375	0.2621
Cell Outline	3.19	0.66	2.9	0.68	1.4024	0.1685

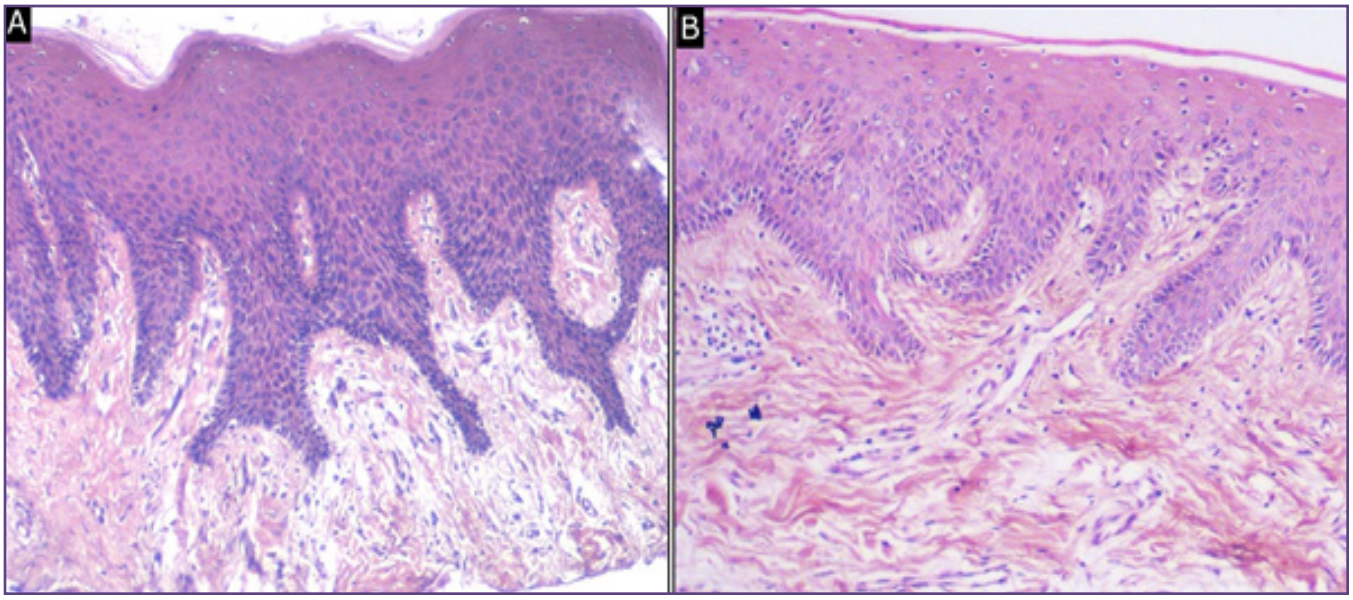

Fig. 1: H and E-stained section under 10x magnification of A) Tissues fixed in formalin; B) Tissues fixed in Almond oil with vitamin E.

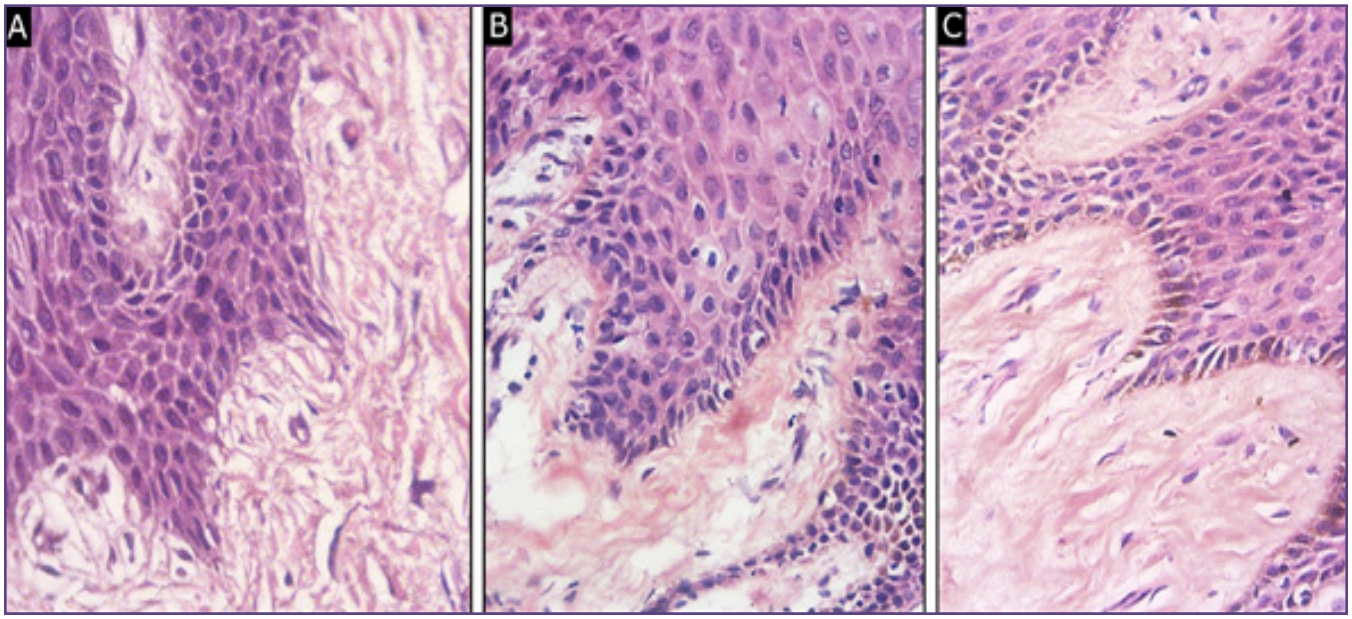

Fig. 2: H and E-stained section under 40x magnification of A) Tissues fixed in formalin; B) and C) Tissues fixed in Almond oil with vitamin E (note the melanin pigmentation in basal cells).

Discussion

AO, being a natural emollient, has been effectively used in ancient civilizations of India, China, and Greece for preservation or rejuvenation of the skin as well as for treating dryness-related skin conditions such as psoriasis and eczema [6]. It was also used as an ingredient, for fixing hairs and in the mummification of bodies in ancient Egypt [9]. Although no conclusive scientific data describes the actual process by which AO preserves the tissue, its constituents – oleic acid, linoleic acid, pantothenic acid, palmitic acid, folate, and zine may have a role in its fixative properties [6,10]. On the other hand, Vitamin E is widely known for its potent antioxidant properties that can break radical chains to protect the integrity of tissues [7].

From a histochemical perspective, fixatives consisting of acids generally have a lower pH. Such fixatives act as good nuclear fixatives although the preservation of cytoplasmic components becomes compromised [11]. Previous researchers that have attempted to use alternatives for fixative such as commercially available honey, faced issues with the preservation of cytoplasmic details owing to the low pH [11-13]. The acids present in AO are weak acids and thus, its pH is close to neutral. This was confirmed by means of a digital pH meter wherein the pH of oil used in the present study was found to be 6.8. This accounts for superior nuclear details exhibited by AO-fixed tissues without compromising the cytoplasmic preservation.

AO has been demonstrated to have good penetrating properties. The time required for the fixation of tissue depends upon the coefficient of diffusibility of the fixative and the depth of the tissue [14]. The co-efficient of diffusibility of 10% NBF is 0.79, while that of AO has not yet been scientifically determined. It has been estimated that most fixatives, including NBF, penetrate the tissue at the rate of 1 mm per hour [15]. Yet routinely, a minimum of 24-hour fixing period is recommended in histopathology laboratories, which was also followed in the present study for both the groups [8]. Even so, the exact rate and depth of penetration with time could not be determined in the present study.

Splitting a tissue into two and fixing it in either of the fixatives would have been ideal for observing and comparing the fixative properties. However, the oral tissues removed during CLP are of extremely small dimensions and cutting them would not be feasible. Therefore, a separate tissue was considered for each fixative rather than splitting a single tissue, which was also done in the study conducted by Sabarinath et al. that utilized honey as an alternative fixative [11].

Previous studies involving alternative fixatives have used them at diluted concentrations, similar to the routinely used 10% NBF [8,11]. However, AO being insoluble in water, could not be diluted and was thus, used in undiluted form. It was also difficult to wash the containers in which the specimens were stored. In the pilot study performed earlier, it was also observed that the rubber-containing caps of the vials became distorted after a few days, and thus, the use of plastic containers is highly advisable during fixing tissues with AO.

Additionally, in case of spillage, the grossing area became slippery and difficult to clean; although it did not pose any health hazards unlike those that result from formalin spillage [16]. By virtue of its chemical nature, AO is biologically non-hazardous, non-irritating, non-carcinogenic, and stable against hydrolysis. The pleasant odor of commercially available AO has earned its wide application in aromatherapy [6,17]. AO is also easily available and feasible. Therefore, it would be especially useful in dental clinics when formalin is not available or cannot be procured easily. These properties of AO overcome the drawbacks of hazardous and irritant formalin, further adding to the list of advantages of its use as a fixative.

Despite positive results, there is still a need to determine the most suitable ratio of AO to vitamin E for optimal fixation. The optimal time required for the fixation of tissue in AO also requires to be standardized by conducting further studies. Immunohistochemistry and special stains are an inseparable part of pathology, that are frequently employed in the diagnosis of cases. Further studies could also test the effects of AO fixation on these procedures.

The present study only utilized normal tissues because the actual fixative ability of AO was unknown. As a result, only a limited number of tissue elements could be studied. Future studies to check the effectiveness of AO in the fixation of specific tissue components such as mucins, lipids, and bone could provide more insight into the true potential of AO as a fixative. Consequently, AO could be employed for the fixation of tissues in various pathologies containing the variable composition of tissue elements.

Conclusion

AO+ Vitamin E can be effectively used for the fixation of tissues, providing results that are comparable to formalin-fixed tissues. The non-toxic, non-irritant chemical nature of almond oil coupled with its ease of availability, enable it to be utilized safely even in remote areas. Maintenance cost, clinician's expenditure, and in turn, patient's expenditure can be reduced by the use of such organic alternatives in histopathology. Our research could serve to

guide researchers in a direction toward the development of natural alternatives for histopathological laboratories.

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Informed Consent
A written informed consent was obtained from the patients whose tissue were used for the study.

Ethical approval
The study protocol was approved by the institutional ethical review board. Ref ID: GDCHMumbai/EthicalCommittee/4126/2020

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Competing Interests
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