International Journal of Early Childhood Special Education (INT-JECSE)
DOI: 10.9756/INT-JECSE/V14I1.394 ISSN: 1308-5581 Vol 14, Issue 01 2022 PP:3281-3286

ALCOHOL AND ANHYDROUS COPPER SULPHATE WITH ALCOHOL FOR DEHYDRATION OF ORAL TISSUE SPECIMENS - A COMPARATIVE PILOT STUDY

- **Monica. K***

Department of Oral Pathology and Microbiology,Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Science, Saveetha University,Chennai-600077.Email id:151907005.sdc@saveetha.com

ABSTRACT:

Background: The processing of human tissue for pathologic diagnosis begins with fixation, followed by dehydration with graded concentration of alcohol, clearing, infiltration and embedding. The aim of the study is to evaluate the efficacy of anhydrous copper sulphate with alcohol as a dehydrating agent for tissue processing.

Materials and methods: 25 formalin fixed tissues were used for this study. Each tissue sample was cut into two halves. One half was kept for routine tissue processing using alcohol as dehydrant and the other with alcohol and anhydrous copper sulphate. After dehydration, clearing with xylene and infiltration and embedding with paraffin wax was done. Sections were stained with H & E stain and assessed for histomorphological criteria. The scores were statistically analysed using Mann Whitney U test.

Results: Statistically significant correlation was found between alcohol and anhydrous copper sulphate with alcohol for nuclear (p value=0.002) and cytoplasmic staining (p value = 0.012). Sections folds were seen in tissue samples dehydrated with anhydrous copper sulphate.

Conclusion: Anhydrous copper sulphate reduced the dehydration time and increased the shelf life of alcohol.

Clinical significance: Anhydrous copper sulphate reduces the tissue processing time and also increases the shelf life of alcohol thereby acts as a tool for quality assurance in pathology.

KEYWORDS: anhydrous copper sulphate, dehydration, tissue processing.

INTRODUCTION:

Biopsy is a Greek word mean ing 'bios' means life and 'opsis' means vision, tissue taken from a living organism for the purpose of microscopic examination which aids in histopathological diagnosis (1, 2). The microscopic slide preparation has a proper protocol to be followed. The processing of human tissue for pathologic diagnosis begins with fixation, which requires a minimum of 12 hours (3, 4). Tissue processing is an important histotechnique after tissue fixation which involves graded concentration of various chemicals in order to make the tissue amiable for sectioning. This includes dehydration, clearing and infiltration. Dehydrating solutions are usually alcohol-based solutions which remove water and fixative from the tissue specimen and replace it with alcohol. The tissue is then placed in xylene, a clearing agent which makes the tissue receptive to wax impregnation by the removal of alcohol; followed by infiltration and embedding. This entire process takes 2–3 working days before a tissue is ready for diagnosis (3, 5).

Dehydration is an important step in tissue processing. It is the process of removing water from tissues; this can be achieved by either dilution dehydration or chemical dehydration (5, 6). It is important because paraffin is not miscible with water (7). Dehydration is usually considered complete when less than 3-4% of water remains in the tissues. Time required for this depends on permeability of tissues, continuous rotation of fluid to prevent stagnation of fluid around tissues and temperature. The most commonly used dehydrant is ethyl alcohol; others isopropyl alcohol, acetone and least used is methanol (6, 8).

Concentration of the first alcohol bath depends on the fixative and size and type of the tissue. Usually, 70% alcohol is employed as the first solution and 100% as the last solution. After tissues have passed through the first change of alcohol, it is discarded because following dehydration the alcohol gets contaminated with water. Contaminated alcohol solutions do not effectively cause dehydration which in turn affects the subsequent tissue processing steps. Hence quality check for alcohol in routine tissue processing is a mandate to maintain its efficacy. There is also distortion of tissues due to shrinkage produced by immersion in alcohols for a longer duration of time.

Anhydrous coppersulfatehas no water in it. When water is present in a sample of coppersulfate it turns blue. It is still a dry solid, because the individual water molecules are trapped within the ionic lattice surrounding the copper ions. Anhydrous $CuSO_4$ removes water from alcohol and also from tissues (3, 9). Thereby copper sulphate prolongs the shelf life of alcohol without compromising the quality of tissue processing. Hence the aim of the study is to evaluate the efficacy of anhydrous copper sulphate with alcohol as a dehydrating agent for tissue processing.

MATERIALS AND METHODS:

Sample Preparation:

The present study was carried out in the Department of Oral and Maxillofacial Pathology, Chennai. The study was approved by the Institutional Review Board, SRB/SDC/OPATH-1905/02. 25 soft tissue specimens which were divided into four categories namely i) mucosa (n=9) ii) adipose tissue (n=6) iii) skin (n=5) and iv) gland (n=5) were taken for tissue processing using alcohol (Group A) and alcohol with anhydrous copper sulphate (Group B) as dehydrating agents.

Tissue processing procedure:

Prefixed formalin tissues were retrieved for tissue processing from the department of Oral and Maxillofacial Pathology. Each tissue sample was cut into two halves. One half was kept for routine tissue processing using alcohol as dehydrant and the other with alcohol and anhydrous copper sulphate. Gross measurements of the soft tissue samples were recorded prior to tissue processing. Both the tissue processing procedures were performed at temperatures of 45 – 55 degree celsius. Steps for tissue processing technique using alcohol as dehydrantvs routine tissue processing protocol are summarized in table-1.

Table-1: Steps involved in tissue processing technique

Routine processing with alcohol as dehydrant	**Time**	**Alcohol with anhydrous copper sulphate as dehydrant**	**Time**
Isopropyl alcohol	30 minutes	Alcohol with Anhydrous copper sulphate	
Acetone (2 changes)	1 hour		
Xylene (2 changes)	1 hour	Xylene (2 changes)	1 hour
Paraffin	overnight	Paraffin	overnight

Alcohol with Anhydrous Copper sulphate dehydration:

Prior to tissue processing, copper sulphate (Aksharchem copper sulphate, hydrous blue crystals) was ground into fine powder with mortar and pestle. The hydrous copper sulphate was dehydrated at a temperature of 60 degree Celsius (colour changes from blue to almost white). A layer of anhydrous CuSO4 was placed at the bottom of a beaker and was covered with 2-3 layers of filter paper of approximate size to prevent staining of the tissue. Following which the beaker was filled with two third levels of alcohol. Then the samples are kept for tissue processing as mentioned previously. After dehydration with anhydrous copper sulphate and alcohol, the colour of the copper sulphate changed to blue in colour and alcohol appeared almost clear after 1 hour.

The dimensions of the tissues were recorded before embedding. The tissues were embedded in the L-former using molten paraffin wax and cut at 3 µm on a rotary microtome (LEICA RM - 2245) and mounted on positively charged slides. Sections were stained with haematoxylin and eosin (H & E) to assess tissue morphology. All slides were examined under light microscopy (Olympus CG20i).

Evaluation of slides:

The histomorphology of each tissue section was assessed by 2 examiners, based on intensity of nuclear and cytoplasmic staining based on a 1–4 grading system by Titford et al. 2005 (10).

1 – Poor
2 – Satisfactory
3 – Good
4 – Excellent

Score of 0-1 for section folding and tissue shrinkage were also recorded. Any disagreements between the observers were resolved by asking them to evaluate the slides again.

Statistical analysis:

The scores were tabulated and analysed using IBM SPSS (statistical package for social science), version 23. The scores of the samples processed with alcohol and anhydrous copper sulfate were compared using Mann Whitney U test. Within group comparisons were done using Pearson Chi-square tests with p value <0.05 was considered to be statistically significant.

RESULTS:

The quality check of alcohol using anhydrous copper sulphate on tissue sections was identified based on histologic examination. The percentage values were calculated based on scores obtained for each category. Routine tissue processing with alcohol and alcohol with anhydrous copper sulphate tissue processing was compared based on nuclear, cytoplasmic staining characteristics, shrinkage and section folds, summarized in table 2.

Analysis of nuclear staining showed different staining efficiency by different tissue samples. Good nuclear staining was seen in 100% of skin and gland tissue samples, whereas only 40% of skin samples had good nuclear staining with alcohol and anhydrous copper sulphate processing. 88.9% of the mucosa samples processed with alcohol and anhydrous copper sulphate had satisfactory nuclear staining (Figure 1). Routine tissue processing with alcohol had relatively better nuclear

staining compared to alcohol with anhydrous copper sulphate and the difference was statistically significant [U=168.500, p value=0.002]

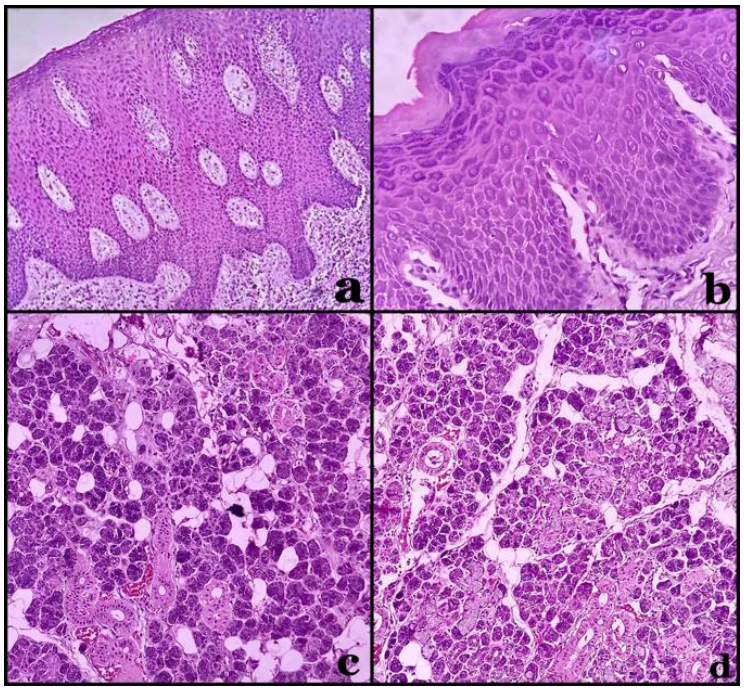

Figure 1: a) and b) Photomicrograph of oral mucosa processed with routine tissue processing using alcohol for dehydration (Magnification 40X). c) and d) Photomicrograph of gland tissue processed with anhydrous copper sulphate and alcohol for dehydration (Magnification 10X).

60% of skin tissue samples and 77.8% of mucosal samples had good cytoplasmic staining with alcohol and anhydrous copper sulphate tissue processing. Majority of the samples processed with routine tissue processing had good cytoplasmic staining. 11.1% samples processed with alcohol and anhydrous copper sulphate had poor nuclear and cytoplasmic staining. However, the difference between the two groups was statistically significant [U=206.500, p value = 0.012]. Section folding was common in samples processed with alcohol and anhydrous copper sulphate and the difference between the groups was not statistically significant [p value= 0.395], (Table 2). Shrinkage of tissues was seen in tissues processed with anhydrous copper sulphate and alcohol (16%) (Figure 2).

Nuclear staining								
	Routine tissue processing with alcohol				**Alcohol with anhydrous copper sulphate**			
Score	**Skin**	**Gland**	**Adipose tissue**	**Mucosa**	**Skin**	**Gland**	**Adipose tissue**	**Mucosa**
0	0%	0%	0%	0%	0%	0%	0%	11.1%
1	0%	0%	22.2%	0%	0%	0%	33.3%	0%
2	0%	0%	66.7%	33.3%	60%	100%	50%	88.9%
3	100%	100%	11.1%	66.7%	40%	0%	16.7%	0%
4	0%	0%	0%	0%	0%	0%	0%	0%

Cytoplasmic staining								
0	0%	0%	0%	0%	0%	0%	0%	11.1%
1	0%	0%	0%	0%	0%	0%	33.3%	11.1%
2	0%	20%	16.7%	22.2%	40%	100%	66.7%	0%
3	100%	80%	83.3%	77.8%	60%	0%	0%	77.8%
4	0%	0%	0%	0%	0%	0%	0%	0%
Section folding								
Absent	80%	80%	77.8%	66.7%	40%	80%	55.5%	66.7%
Present	20%	20%	22.2%	33.3%	60%	20%	44.4%	33.3%

Table- 2: Percentage values for nuclear staining and cytoplasmic staining between alcohol and copper sulphate with alcohol as a dehydrant.

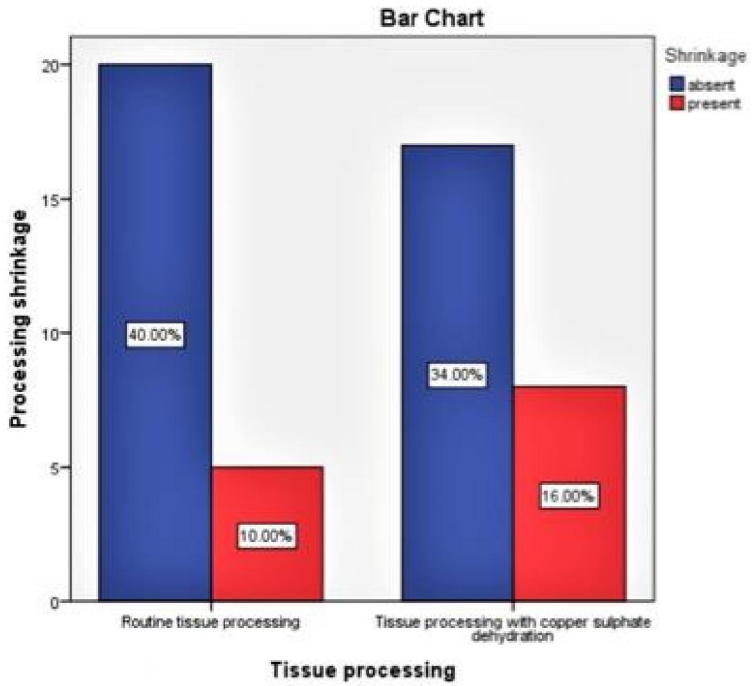

Figure 2: Percentage of tissue shrinkage and between alcohol and anhydrous copper sulphate with alcohol as dehydrant.
Comparison of nuclear staining and cytoplasmic staining and section folding between different routine tissue processing using alcohol and alcohol with anhydrous copper sulphate, summarized in table-3.

Variable	Dehydration methods	Mean Rank	Mann Whitney U	P-Value
Nuclear staining	Routine dehydration	31.26	168.500	0.002

	Anhydrous Copper sulphate dehydration	19.74		
Cytoplasmic staining	Routine dehydration	29.74	206.500	0.012
	Anhydrous Copper sulphate dehydration	21.26		
Section folding	Routine dehydration	27.00	275.500	0.395
	Anhydrous Copper sulphate dehydration	24.00		

Table- 3: Mann Whitney U test comparing parameters between alcohol and anhydrous copper sulfate with alcohol as dehydrant.

DISCUSSION:

Quality assessment in laboratory medicine is an essential requirement to ensure accuracy and precision of test results. A well processed and good quality tissue section without any artifacts is the basic requirement for making an accurate histopathological diagnosis (11). Significant number of errors occur during the pre-analytical phase, which in turn demands effective quality control and quality assurance steps during this phase of tissue processing. Current clinical demands leading to alterations in the standard techniques, results in various modifications.

Dehydration is an important technique in tissue processing, troubleshooting results in artefacts affecting the patient's histopathological diagnosis (12). Alcohol is more commonly used, easily available, hydrophilic which is miscible in water and can act as a secondary fixative. In the present study a layer of anhydrous copper sulphate is used along with alcohol to evaluate the quality check and also to increase the shelf life of alcohol (8).

The properties of tissues processed by routine tissue processing with alcohol were comparable with tissues processed with anhydrous copper sulphate and alcohol. The anhydrous copper sulphate as it is added in alcohol helps in removal of water from the alcohol and tissue samples because copper sulphate acts as a dehydrating agent by itself. Because of which the time required for tissue processing is also reduced compared to the routine tissue processing with a series of alcohols and acetone.

Section folding was present in tissues processed with anhydrous copper sulphate and alcohol compared to routine tissue processing with alcohol. This could be due to the over dehydration of tissues caused by the synergistic effect of alcohol and copper sulphate.

Limitation of the present study includes less sample size. Standardization of timings in copper sulphate with alcohol for different types of tissues, specimen thickness needs to be validated to overcome the drawbacks. Despite these limitations present study achieved reduction in time taken, cost-effectiveness, less shrinkage with comparable microscopic features. Anhydrous copper sulphate reduced the dehydration time by increasing the shelf life of alcohol.

CONCLUSION:

Technological advances in monitoring tissue processing by using copper sulphate with alcohol for dehydration increases the shelf life of alcohol and also acts as a tool for quality assurance in pathology.

CLINICAL SIGNIFICANCE:

Anhydrous coppersulfate as such acts as a dehydrant, in combination with alcohol helps us retain alcohol for next processing. Becomes more economical and reduces the tissue processing time and also increases the shelf life of alcohol thereby acts as a tool for quality assurance in pathology.

ACKNOWLEDGEMENT:

The authors would like to acknowledge the help and support rendered by the Department of Oral Pathology & Microbiology of Saveetha Dental College and Hospitals and the management for their constant assistance with the research.

CONFLICT OF INTEREST: None

FUNDING:

This research did not receive any specific grant from funding agencies in public, commercial or not-for-profit sectors.

REFERENCES:

1. Biopsy and Histopathologic Diagnosis of Oral Premalignant and Malignant Lesions. www.cda-adc.ca/jcda/vol-74/issue-3/283.html
2. Nischal U, NischalKc, Khopkar U. Techniques of skin biopsy and practical considerations. J CutanAesthet Surg. 2008 Jul;1(2):107–11.
3. Bancroft JD, Gamble M. Theory and Practice of Histological Techniques. Elsevier Health Sciences; 2008. 725 p.
4. Karnam S, Girish HC, Murgod S, Nayak VN, Varsha VK, Yanduri S. Rapid tissue processing technique: A novel method using methyl salicylate. J Oral MaxillofacPathol. 2018 Sep;22(3):443.
5. Histopathology. Vol. 24. 1994. http://dx.doi.org/10.1111/his.1994.24.issue-1

6. ReshmaPoothakulath Krishnan, PratibhaRamani, Herald J. Sherlin, GheenaSukumaran, AbhilashaRamasubramanian, GifrinaJayaraj, K. R. Don, ArchanaSanthanam. Dehydrants used in microwave tissue processing. Drug Invention Today. 2019 Feb; 14; Vol 11, Issue 10
7. Lerch ML, Bauer DR, Theiss A, Chafin D, Otter M, Baird GS. Monitoring Dehydration and Clearing in Tissue Processing for High-Quality Clinical Pathology. BiopreservBiobank. 2019 Aug;17(4):303–11.
8. Boon ME, Kok LP. Microwave Cookbook of Pathology: The Art of Microscopic Visualization. 1989. 280 p.
9. TLakshmi,InvitroAnti-ArthriticactivityofSesbaniagrandifloraEthylacetateextractResearchJournalofPharmacyandTechnology8(11),1509,2015.
10. KPavithra,TLakshmi,Awarenessofconventionalriskfactorsamong dental professionals–A survey, Journal of AdvancedPharmacyEducation&Research|Jul-Sep7(3),2017.
11. JHemashree,LThangavelu,Anti-InflammatoryactionofAcaciaCatechuseedextractJournalofAdvancedPharmacyEducation&Research|Jul-Sep8(3),93,2018.
12. Culling CFA. Handbook of Histopathological and Histochemical Techniques: Including Museum Techniques. Butterworth-Heinemann; 2013. 726 p.
13. Titford ME, Horenstein MG. Histomorphologic assessment of formalin substitute fixatives for diagnostic surgical pathology. Arch Pathol Lab Med. 2005 Apr;129(4):502–6.
14. Rao S, Masilamani S, Sundaram S, Duvuru P, Swaminathan R. Quality Measures in Pre-Analytical Phase of Tissue Processing: Understanding Its Value in Histopathology. J ClinDiagn Res. 2016 Jan;10(1):EC07–11.
15. Ralph P. A Comparative Study of some Dehydration and Clearing Agents. Vol. 13, Stain Technology. 1938. p. 9–15. http://dx.doi.org/10.3109/10520293809111361