Efficacy of Anhydrous Copper Sulphate as a Solid Dehydrant in Tissue Processing Procedure

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ABSTRACT

BACKGROUND

Tissue processing in histotechnique is an important procedure after specimen collection involving three main procedures namely, dehydration using alcohol, clearing using xylene, and infiltration by paraffin wax. Isopropyl alcohol, the widely used dehydrating agent is toxic and when exposed to heated isopropyl alcohol fumes, it leads to numerous health hazards. Anhydrous copper sulphate is less toxic than isopropyl alcohol and requires less amount of exposure to the chemicals. The aim of the study was to investigate the dehydrating potential of anhydrous CuSO₄ as an isopropyl alcohol substitute.

METHODS

A descriptive study of forty paired soft tissue specimens were obtained from the Department of Oral Pathology and Microbiology and were subjected to routine histopathological tissue processing with isopropyl alcohol and anhydrous CuSO₄ as dehydrating agents. Histomorphological criteria namely nuclear staining, cytoplasmic staining, artefacts, and background staining were evaluated and the scores were tabulated, and statistical analysis was done using SPSS version 20 by IBM.

RESULTS

Our study results showed that 40 % of alcohol dehydrated tissue samples had better nuclear staining than tissue samples dehydrated by anhydrous copper sulphate and 15 % of alcohol dehydrated tissue samples had better cytoplasmic staining than tissues dehydrated by Anhydrous CuSO₄. Background staining of more than 20 % was seen in anhydrous CuSO₄ dehydrated tissue specimens than alcohol dehydrated tissue specimens. Artefacts were seen in similar ratios in both alcohol (80 %) and anhydrous copper sulphate (75 %) dehydrated tissue specimens.

CONCLUSIONS

Anhydrous CuSO₄ has superior dehydrating properties than isopropyl alcohol which lead to over-dehydration of the tissue specimens. Further studies are required to validate the findings.

KEY WORDS

Isopropyl Alcohol, Anhydrous Copper Sulphate, Toxicity, Histopathology Alternatives

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DOI: 10.14260/jemds/2020/657

How to Cite This Article:

Sushanthi LC, Sherlin HJ, Jayaraj G, et al. Efficacy of anhydrous copper sulphate as a solid dehydrant in tissue processing procedure. J Evolution Med Dent Sci 2020;9(40):3004-3008, DOI: 10.14260/jemds/2020/657

*Submission 01-07-2020,
Peer Review 27-08-2020,
Acceptance 04-09-2020,
Published 05-10-2020.*

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BACKGROUND

Tissue processing in histotechnique is an important procedure after specimen collection. Tissue processing is a step by step procedure that exposes the biopsy specimen to various chemical agents in a sequence. It involves 3 main procedures namely, dehydration using alcohol, clearing using xylene and infiltration by paraffin wax. These procedures are mandatory in histopathological laboratories in order to prepare the tissue specimens for sectioning.¹ Dehydration is the most imperative step in diagnostic pathology. An ideal dehydrant should remove unbound water and aqueous fixatives from the tissue compartment. After dehydration, the tissue specimens are subjected to a clearing process which removes alcohol from the tissue specimens. This enables easy penetration of paraffin wax into the tissue. Infiltration by paraffin wax provides support to the tissue specimens and is embedded in paraffin wax.

Isopropyl alcohol has a chemical formula of C_3H_8O . It easily dissolves in water, ethyl alcohol and other reagents because of its low acidic nature. Isopropyl alcohol is also less toxic when compared with Xylene.² Dehydration is important because paraffin wax is not miscible with water which might interfere with sectioning of the tissue specimen and impact the diagnosis. Isopropyl alcohol is a widely used tissue dehydrant due to its ease of use and economic viability.³ Isopropyl alcohol enters the human body through fumes or vapours during handling of the chemical reagent in tissue processing method, which can be toxic in long duration. Alcohol vapours that emerge after heating of alcohol rapidly enters the human body. Its absorption is also rapid and it directly reaches the brain via arterial blood. Though not many literatures have focused on inhalation of alcohol vapours, long term exposure to alcohol vapours affects the human body and should not be neglected.⁴

Apart from isopropyl alcohol, butanol, dioxane, ethanol, dimethoxy propane, methanol, phenol polyethylene glycols and tetrahydrofuran can be used as dehydrating agents.⁵ The dry method of dehydration by substituting Isopropyl alcohol by Anhydrous copper sulphate is less toxic and also reduces the duration of exposure to the chemicals. Anhydrous Copper Sulphate are the inorganic compounds with the chemical formula $CuSO_4$ and are solid crystals in nature. Anhydrous $CuSO_4$ does not require heat to initiate the dehydration process and also frequent changes done for liquid chemical agents are also not required which marginally reduces the exposure to chemical agents. With this principle, copper sulphate crystals are used for dry dehydration method, with no pre-existing literature, to the best of our knowledge, on use of anhydrous copper sulphate as a dry dehydrant in tissue processing procedures.

Copper sulphate crystals absorb moisture from the environment when exposed to air. The reaction between Anhydrous $CuSO_4$ and water is usually used as a test for alcohol. When Anhydrous $CuSO_4$ crystals are mixed with water, they absorb water and change colour from pale blue to dark blue. Thus, one molecule of anhydrous $CuSO_4$ will contain 5 molecules of water. Quality check for alcohols used in tissue processing can be done to detect the presence of water after each cycle of tissue processing. This enables to maintain the quality of histopathological specimens and also prolongs the alcohol shelf life. Anhydrous $CuSO_4$ removes water content

from alcohol.⁶ As a result of increasing concerns about the potential carcinogenicity of the alcohols, implementing less toxic solutions in routine histopathology is necessary. The main purpose of the study was to investigate the efficacy of anhydrous copper sulphate as alcohol substitute in routine histopathological procedure.

METHODS

This is a descriptive study carried out in the Department of Oral and Maxillofacial Pathology, Saveetha Dental College and Hospitals, Chennai. The study was approved by the Institutional Review Board. Forty paired soft tissue specimens were selected based on the previous studies reported on routine tissue processing and the selected soft tissue specimens were prepared for routine histopathological evaluation using isopropyl alcohol (Group II) (20 tissue samples) and anhydrous copper sulphate (Group I) (20 tissue samples) as dehydrating agents. Each group was categorized into 4 subgroups based on the different types of tissues such as muscle, adipose tissue, salivary gland and oral mucosa with 5 tissue specimens in each subgroup.

Histopathology Processing

The department has a pre-established in-house tissue processing protocol. The reagents for histopathological processing were manufactured by MERCK. Anhydrous copper sulphate crystals by Akshar Chemicals were bought from an online commercial market. Copper sulphate crystals were finely powdered to avoid damage to the tissue. A layer of powdered $CuSO_4$ (25 grams) was placed at the bottom of a clean airtight container. The formalin fixed tissue (Group I) was washed in water, excess water was pat dried with tissue paper and was placed on a layer of $CuSO_4$ (25 grams) in an airtight container and another layer (25 grams) of powdered $CuSO_4$ was added covering the tissues completely. The container is closed tightly and placed in a dry area for a duration of 4 hours. After dry dehydration, the tissues were subjected to clearing in Xylene for 30 minutes of 2 changes respectively at 50 degree Celsius followed by impregnation in paraffin wax for one overnight at 50 degree Celsius and embedding in paraffin wax.

Simultaneous tissue processing was done using Isopropyl alcohol and Acetone as dehydrating agents (Group II). The impregnated tissues were embedded and tissue sections of 3 micron were made using semi-automated tissue rotary microtome by Leica (RM - 2245), Germany. Tissue sections were later stained with haematoxylin and eosin stain to assess tissue morphology.

Evaluation of tissue Sections: The paired tissue sections were evaluated by 2 independent Oral Pathologists blinded to the type of dehydration process. The slides were evaluated according to the histomorphological criteria's such as nuclear staining, cytoplasmic staining, background staining and artifacts. The histomorphological criteria were graded with a score of 1 - poor; 2 - fair; 3 - good; 4 - excellent for nuclear staining and cytoplasmic staining, 0 - nil; 1 - mild; 2 - moderate; 3 - severe for background staining and 0 - no; 1 - yes for artefacts.

Statistical Analysis

The scores obtained were tabulated and assessed for statistical significance using SPSS version 20 by IBM. The mean rank score of dehydration by acetone-isopropyl alcohol and Anhydrous copper sulphate for histomorphological criteria were compared using Mann-Whitney U test. Comparison of means scores between Acetone-Isopropyl alcohol and anhydrous copper sulphate within the subcategories for the histomorphological criteria were done by Kruskal Wallis Anova. P value < 0.05 was considered to be statistically significant.

RESULTS

The percentage of adequacy for the parameters of 40 tissue samples from four different tissue types, between Isopropyl alcohol (20 samples) and CuSO₄ (20 samples) were analysed. In 20 paired tissue samples, comparison of rank scores between groups demonstrated nuclear staining with superior results in Alcohol dehydrated tissues than CuSO₄ dehydrated tissues [U = 76.000; p = 0.000]. Cytoplasmic staining had superior results in alcohol dehydrated tissue samples than CuSO₄ dehydrated samples [U = 91.500; p = 0.002], which is statistically significant. Background staining was more appreciated in CuSO₄ dehydrated tissue samples compared to alcohol dehydrated tissue samples [U = 116.000; p = 0.007], which is statistically significant. Artefacts were present in similar ratios in both alcohol dehydrated and CuSO₄ dehydrated tissue samples [U = 190.000; p = 0.708] which is statistically not significant (Table 1).

Eighty percent of muscle tissue samples dehydrated in alcohol showed good nuclear staining against 40 % of tissue samples dehydrated in CuSO₄ (p = 0.045), which is statistically significant. In cytoplasmic staining, 60 % of tissue samples dehydrated by alcohol and CuSO₄ exhibited a similar fair score (p = 0.729), which is statistically not significant. Background staining was appreciated in 40 % of CuSO₄ dehydrated tissue samples against 0 % of Alcohol processed samples (p = 0.50), which is statistically not significant. Artefacts were seen in 100 % of both alcohol and CuSO₄ processed samples (p = 1.000), which is statistically not significant (Table 2).

In the tissue samples of gland, 80 % of tissue samples dehydrated by CuSO₄ demonstrated good nuclear staining against 40 % of alcohol dehydrated tissue samples (p = 0.221), which is statistically not significant. In cytoplasmic staining, 40 % of tissue samples dehydrated by alcohol and CuSO₄ showed a similar fair score (p = 0.118), which is statistically not significant. Background staining was absent in 80 % of alcohol dehydrated tissue samples against 60 % of CuSO₄ dehydrated tissue samples (p = 0.513), which is statistically not significant. Artefacts were seen in 80 % of both alcohol and CuSO₄ processed samples (p = 1.000) which is statistically not significant (Table 3).

Eighty percent of adipose tissue samples dehydrated by alcohol and CuSO₄ showed good nuclear staining (p = 0.180) which is statistically not significant. In cytoplasmic staining, 40 % of alcohol dehydrated tissue samples showed excellent staining against 0 % of CuSO₄ dehydrated tissue samples (p = 0.189) which is statistically not significant. Background staining was appreciated in 80 % of CuSO₄ dehydrated tissue

samples against 80 % of alcohol dehydrated tissue samples with no background staining (p = 0.204) which is statistically not significant. Artefacts were seen in 80 % of alcohol dehydrated tissue samples and absent in 80 % of CuSO₄ dehydrated tissue samples (p = 0.221) which is statistically not significant (Table 4).

Histopathological Criteria	Type of Dehydration	N	Mean Rank	Sum of Ranks	Median	Interquartile Range	P Value
Nuclear Staining	Alcohol Processing	20	26.70	534.00	3	1	0.000
	CuSO ₄ Processing	20	14.30	286.00	3	1	
Cytoplasmic Staining	Alcohol Processing	20	25.90	518.50	3	1	0.002*
	CuSO ₄ Processing	20	15.08	301.50	2	2	
Background Staining	Alcohol Processing	20	16.30	326.00	0	0	0.007*
	CuSO ₄ Processing	20	24.70	494.00	1	1	
Artefacts	Alcohol Processing	20	21.00	420.00	1	0	0.708
	CuSO ₄ Processing	20	20.00	400.00	1	1	

Table 1. Comparison of Mean Rank Scores of Histopathological Parameters between Routine Alcohol Dehydration and Anhydrous CuSO₄ Dehydration by Mann Whitney U Tests

*- Denotes p value is significant.

Tissue	Parameters	Types of Processing	Poor	Fair	Good	Excellent	P Value	
Muscle	Nuclear Staining	Routine Processing	0 %	0 %	80 %	20 %	0.045	
		CuSO ₄ Processing	20 %	40 %	40 %	0 %		
	Cytoplasmic Staining	Routine Processing	0 %	60 %	40 %	0 %	0.729	
		CuSO ₄ Processing	0 %	60 %	40 %	0 %		
	Background Staining	Routine Processing	Nil	Mild	Moderate	Severe	0.050	
		CuSO ₄ Processing	100 %	0 %	0 %	0 %		
	Artefacts			Present		Absent		1.000
		Routine Processing	100 %	0 %				
CuSO ₄ Processing		100 %	0 %					

Table 2. Comparison of Adequacy of Dehydration in Muscle Tissue Specimen between Alcohol Dehydrated and Anhydrous CuSO₄ Dehydrated Tissue. Specimens. Kruskal Wallis Anova, P Value < 0.05 was Considered to be Statistically Significant

*- denotes p value is significant.

Tissue	Parameters	Types of Processing	Poor	Fair	Good	Excellent	P Value	
Gland	Nuclear Staining	Routine Processing	0 %	0 %	40 %	60 %	0.221	
		CuSO ₄ Processing	0 %	0 %	80 %	20 %		
	Cytoplasmic Staining	Routine Processing	0 %	20 %	40 %	40 %	0.118	
		CuSO ₄ Processing	0 %	60 %	40 %	0 %		
	Background Staining	Routine Processing	Nil	Mild	Moderate	Severe	0.513	
		CuSO ₄ Processing	80 %	20 %	0 %	0 %		
	Artifacts			Present		Absent		1.000
				80 %		20 %		
			80 %		20 %			

Table 3. Comparison of Adequacy of Dehydration in Gland Tissue Specimen between Alcohol Dehydrated and Anhydrous CuSO₄ Dehydrated Tissue Specimens. Kruskal Wallis ANOVA P Value < 0.05 was Considered to be Statistically Significant

In mucosal tissue samples, 60 % of alcohol dehydrated tissue samples showed excellent nuclear staining against 40 % of CuSO₄ dehydrated tissue samples with good nuclear staining ($p = 0.007$, significant), good cytoplasmic staining was seen in 80 % of alcohol dehydrated tissue samples and fair cytoplasmic staining was seen in 100 % of CuSO₄ dehydrated tissue samples ($p = 0.004$ significant), which is statistically significant. Background staining was appreciated in 60 % of CuSO₄ dehydrated tissue samples against 80 % of alcohol dehydrated tissue samples with no background staining ($p = 0.221$), which is statistically not significant. Artefacts were seen in 80 % of alcohol dehydrated tissue samples and in 100 % of CuSO₄ dehydrated tissue samples with a statistically not significant value of $p = 0.317$ (Table 5).

Tissue	Parameters	Types of Processing	Poor	Fair	Good	Excellent	P Value
Adipose Tissue	Nuclear Staining	Routine Processing	0 %	0 %	80 %	20 %	0.180
		CuSO ₄ Processing	0 %	0 %	80 %	20 %	
	Cytoplasmic Staining	Routine Processing	0 %	20 %	40 %	40 %	0.189
		CuSO ₄ Processing	0 %	80 %	20 %	0 %	
	Background Staining	Routine Processing	Nil	Mild	Moderate	Severe	0.204
		CuSO ₄ Processing	80 %	20 %	0 %	0 %	
	Artifacts	Routine Processing	Present		Absent		0.221
		CuSO ₄ Processing	80 %		20 %		
			20 %		80 %		
Table 4. Comparison of Adequacy of Dehydration in Adipose Tissue Specimen between Alcohol Dehydrated and Anhydrous CuSO ₄ Dehydrated Tissue Specimens. Kruskal Wallis ANOVA, P Value < 0.05 was Considered to be Statistically Significant							

Tissue	Parameters	Types of Processing	Poor	Fair	Good	Excellent	P Value
Mucosa	Nuclear Staining	Routine Processing	0 %	0 %	40 %	60 %	0.007*
		CuSO ₄ Processing	0 %	60 %	40 %	0 %	
	Cytoplasmic Staining	Routine Processing	0 %	20 %	80 %	0 %	0.004*
		CuSO ₄ Processing	0 %	100 %	0 %	0 %	
	Background Staining	Routine Processing	Nil	Mild	Moderate	Severe	0.221
		CuSO ₄ Processing	80 %	20 %	0 %	0 %	
	Artifacts	Routine Processing	Present		Absent		0.317
		CuSO ₄ Processing	80 %		20 %		
Table 5. Comparison of Adequacy of Dehydration in Mucosal Tissue Specimen between Alcohol Dehydrated and Anhydrous CuSO ₄ Dehydrated Tissue Specimens. Kruskal Wallis Anova P Value < 0.05 was Considered to be Statistically Significant.							
*- Denotes p value is significant.							

DISCUSSION

Isopropyl alcohol is a cost effective and easily available alternative to ethyl alcohol that is being used in histopathological lab procedures from the mid-20th century. The present study is a novel study with no pre-existing literature on usage of Anhydrous copper sulphate as Alcohol substitute in tissue processing procedure. Twenty matched tissue specimens from 4 different tissue types were selected to assess Anhydrous CuSO₄ as alcohol substitute. Tissue specimens dehydrated by Alcohol showed better nuclear staining than tissue specimens dehydrated by CuSO₄. As far as

cytoplasmic staining was concerned, tissue specimens dehydrated by Alcohol showed superior results than tissue specimens dehydrated by anhydrous CuSO₄. Majority of the tissue specimens dehydrated by anhydrous CuSO₄ had background staining compared to alcohol dehydrated tissue specimens. Artefacts were equally seen in tissue specimens dehydrated by both alcohol and anhydrous CuSO₄.

The finding of our study is in accordance with the literature by Viktorov et al who stated that Isopropyl alcohol when used as a dehydrating agent in tissue processing procedure has excellent dehydrating properties.⁽²⁾ Downside of isopropyl alcohol usage includes its toxicity on the human body. Isopropyl alcohol when ingested is absorbed rapidly by 80 % within 30 minutes causes GIT irritation and individuals exposed to it might experience nausea, abdominal pain and vomiting.⁷ Histopathology technicians and oral pathologists are frequently exposed to isopropyl alcohol vapours during handling of tissue specimens in tissue processing procedure. Contemplating the health of the histopathology technicians and oral pathologists and also regarding the increasing concerns about the potential carcinogenicity of alcohols, it is necessary to implement less toxic solutions in routine histopathology.

Copper sulphate salt is created by treating cupric acid with sulphuric acid, resulting in large bright blue coloured crystals containing five water molecules. Anhydrous copper sulphate is a result of loss of water molecules when copper sulphate is exposed to heat.⁸ The alternating changing property of copper sulphate from anhydrous form to hydrous form was applied in dehydration of tissue specimens in histopathology procedures. When tissue specimens were subjected to dehydration in anhydrous copper sulphate, repeated changes of the chemical agent was not required unlike dehydration in alcohols. This reduces the consecutive exposure to alcohol fumes during tissue processing.

Anhydrous copper sulphate as a dry dehydrant is a first of its kind study in histopathology procedures. With its principle of absorbing moisture from the environment, it was used to evaluate the dehydrating accuracy in tissue specimens of histopathological biopsies. Anhydrous copper sulphate did not yield better results than alcohol. This could be due to the extensive dehydrating capacity of the Anhydrous CuSO₄ as the tissue specimens were subjected to anhydrous copper sulphate for a longer duration of 4 hours. In future, depending upon the tissue types, the duration of exposure of the tissue specimens to anhydrous copper sulphate should be altered to achieve the expected outcome.

The authors acknowledge the presence of study limitations namely lesser sample size and selection of only four different tissue types. More tissue types must be selected to evaluate the anhydrous CuSO₄ accuracy as a dehydrant at various anatomical sites. Colour deposition on the tissues were seen though it did not hinder the evaluation of histomorphologic criteria. Another limitation includes longer duration of tissue specimen exposure to anhydrous CuSO₄ which over-dehydrated the tissue specimen resulting in splitting of tissues during sectioning. Although the comparison between alcohol dehydration and Anhydrous copper sulphate dehydration showed superior results in alcohol dehydrated tissue specimens, it is proved that anhydrous CuSO₄ has extensive dehydrating properties. Duration of tissue specimens to anhydrous CuSO₄ should be addressed.

CONCLUSIONS

The present study proves that anhydrous CuSO₄ in powdered form has extensive dehydrating property which led to over-dehydration of the tissue specimens. Anhydrous CuSO₄ is a cost effective, less toxic and easily available alternative to the traditional Isopropyl alcohol. Substituting isopropyl alcohol with less toxic chemical agents will be a breakthrough in providing a favourable histopathological laboratory environment for histopathology technicians and oral pathologists.

Financial or Other Competing Interests: None.

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