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Original Article

Comparison of Honey and Alcoholic Fixatives in Tissues Using Different Types of Stains

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ABSTRACT

Background: Formalin has been used as a standard fixative for various clinical routines globally. In 1987, after formaldehyde was used as a standard, various studies have been conducted to find a substitute for formalin due to its carcinogenic effects. Since then honey has been proven to be a safe alternative to formalin. **Objective:** Comparing the tissue fixation abilities of honey, Carnoy's, Bouin's and Zenker's solution with the tissue fixation abilities of formalin using eosin, hematoxylin and Masson trichrome periodic acid Schiff is the objective of this study. **Methods:** This current study is a comparative model of the study conducted at an experimental research laboratory of the University of Health Sciences, Lahore, Pakistan. This study was designed to determine and explore more eco-friendly, more cost-effective, readily available and best substitutes for formalin fixatives, such as honey, Bouin's solution, Carnoy's solution and Zenker's solution. 30 rats were allocated into five categories, Group 1, Group 2, Group 3, Group 4, and Group 5. Group 1 was the control group which consisted of 6 rats in which the fixative employed was Formalin. The other groups were the experimental groups. They all utilized the other fixatives, Bouin's, Zenker's and Carnoy's solution and honey. This was followed by conventional dispensation and staining. The sections of the tissues were examined and assessed for nuclear details, cytoplasmic details and quality of staining under microscope. Each benchmark was rated on a scale of zero to three (where zero for poor and three for excellent). **Results:** The results of this current study demonstrated that the tissue that was fixated in honey, Carnoy's, and Bouin's solutions were comparable to the tissues fixated in formalin. The tissues that were fixated on Zenker's solution showed only minute morphological changes among some tissues. Although this didn't have an impact on accurate diagnostic supposition. **Conclusion:** Honey, Carnoy's and Bouin's solutions used for the fixation of tissues yielded good results. Hence this study concludes that they might be used efficaciously in daily routine histopathology laboratories as a substitute for formalin.

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INTRODUCTION

Fixation is defined as a histological procedure that has been used in preserving the morphology of the tissue by stabilization of the proteins preventing the autolysis and putrefaction of the tissue.[1] The purpose of doing fixation is to hinder the autolysis of the constituents of the cell, either by using a fungal or bacterial agent to preserve the fundamental structural organization of the tissue everlastingly in as much life-like condition as possible. The characteristic of a good fixative is that it should harden the specimen of the tissue and preserve its microarchitecture as much as possible for investigation.[2] The right choice of suitable fixatives, their correct concentration, duration and the storing of the fixation are all very substantial factors to be considered.[3] While formulating the sample of the tissues for histology after their treatment with formaldehyde, outstanding staining results were gathered using alanine dyes and hemotoxylin. Subsequently, since then, the formalin-fixed paraffin entrenched tissue stained with hematoxylin and eosin was used as the gold standard. The literature still lacks information on any other histopathology technique that provides so much information economically and quickly.[4]

This current study might fill this gap and provide literature and reliable evidence for another histopathology technique that can be used for this purpose. When formaldehyde was declared a precarious chemical, carcinogenic agent, the exploration for alternatives with less hazardous chemicals was encouraged by two substantial expansions, The Occupational Safety and Health Administration and the International Agency for Research on Cancer.[5] Exposure to formaldehyde for a long period is the cause of cancer in humans as declared by the Environmental Protection Agency. So, eliminating the use of formaldehyde and its toxicity in pathology laboratories is a major issue since formaldehyde has been used as a regular fixative in bulk amounts.[6] One of the alternatives for formalin this current study is considering is Zenker's solution. It is a mercury-comprising fixative. It provides outstanding preservation of the nuclear material of the cell, fibers of connective tissues and some of the characteristics of cytoplasmic portion. The mercury portion of the Zenker's solution must be removed from the sections before staining.[7] Another alternative this current study is considering is Bouin's solution.

It is a picric acid that consists of a tri-nitro aromatic compound that has been frequently used in histopathology and forensic laboratories as a fixative and staining agent. Bouin's solution has been known to preserve glycogen well. It has been recognized as an outstanding general fixative for stains of connective tissues.[8] One more alternative considered by this current study is Carnoy's solution. It is a very interesting alternative that makes the management and handling of dissection of lymph node specimens easier. [9] The Carnoy's solution has been observed as useful particularly in specimens of cancer allowing more precise and accurate staging, World Health Organization in 2010. This solution is quick in performing its action, it gives good preservation of the nuclear material and also it retains glycogen. Ribonucleic acid can also be easily extracted from a tissue fixed in Carnoy's solution.[10] Honey is considered a naturally occurring sweet substance that is obtained from the nectar of plants by honey bees. One very substantial property of honey is that it acts as a wound healer on animal tissues. This was investigated and was associated with honey which also prevents putrefaction and prevention.[11] A study conducted by Sabarinath and co-workers showed the fixation of Pericoronitis and pericoronal abscess in

formalin and honey. Their study showed that the architecture of the overall tissue and its morphological specifics were very well preserved. The tissues which were fixed in formalin and honey showed equivalent results.[3] Considering honey as an alternative fixative for oral tissues, the characteristic of tissue hardening of honey and its penetration to the inmost of the tissue inhibits autolysis and putrefaction making it comparable in action to that fixative which acts by hardening of tissues. Honey is a naturally occurring product and is readily available, it can replace the toxic and cancer-causing formalin which has been proven carcinogenic as quantified by the Occupational Safety and Health Administration and World Health Organization.[12]

Table 1: Health Hazards of Formalin

Organ System	Adverse Effects
Skin & mucous membrane	• Irritation of Mouth • Upper respiratory tract • Allergic contact dermatitis
Respiratory system	• Chronic rhinitis • Sneezing • Coughing • Degenerative diseases • Laryngospasm • Pulmonary edema • Asthma • Loss of olfactory functioning
Eye	• Irritation • Lacrimation • Conjunctivitis
Gastrointestinal tract	• Nausea • Vomiting • Gastrointestinal hemorrhage • Diarrhea • Chronic pharyngitis • Loss of appetite • Burns and ulceration • Abdominal pain
Cardiovascular system	• Tachypnea • Nodal tachycardia
Central nervous system	• Dizziness • Headaches • Sleep disorders • Memory loss and coma
Hematopoietic system	• Increase ALT • Decrease RBC, WBC, platelet and Hemoglobin
Renal system	• Renal failure
Reproductive system	• Menstrual disorder • Dysmenorrhea • Spontaneous abortion

This current study aimed to find an alternative to formalin for the fixation of the tissues. Countries like Pakistan, do need an alternative fixative for formalin, which has been proven to be carcinogenic by the World Health Organization and Occupational Safety and Health Administration. Developing countries can not afford a higher frequency of cancer-diagnosed patients and can not deal with carcinogenic agents on a higher occurrence. This current study will add more knowledge considering other options to be used as tissue fixation agents. Future researchers are recommended to conduct this study on a larger sample size and use humans as the target population to obtain more conclusive results.

METHODS

This current study is a relative and comparative model of the study at an experimental research laboratory of the University of Health Sciences, Lahore, Pakistan. This study included healthy adult male and female
rats weighing two hundred to two-hundred and fifty grams of age ranging from six to eight weeks.[14] Disease rats and rats with unfixed and necrotic tissues were excluded from this study.[14] The sample of six rats per group was considered for this study based on substantiation from previous literature.[1] Albino rats were included in this study who weighed from two hundred to two hundred and fifty grams and were selected from the animal house of the University of Health Sciences. After their selection, they were randomly allocated into five groups. A convenient sample technique was employed. Six rats were recruited in the control group in which the fixative employed was formalin. Four other groups were formed, which also recruited six rats. These four groups were experimental groups in which the specimens were fixed using different fixatives. The entire research work till the end of this study was carried out in the animal research laboratory of the University of Health Sciences.

All the rats were weighed before the beginning of the experiment. All the rats were anesthetized with chloroform and then were sacrificed in the animal house of the University of Health Sciences considering the accurate procedure. The heart, liver, lungs, kidneys and muscles of all the rats were removed and were placed in five different dishes containing, ten percent formalin, ten percent honey, Zenker's solution, Bouin's solution and Carnoy's solution for twenty-four hours of fixation to check the morphology of theses solution. Unremarkable gross examinations of all the tissue specimens were recorded. All the tissues were then washed with normal saline solution to clean blood and other contaminants and then were fixed in ten percent formalin, ten percent honey, Zenker's solution, Bouin's and Carnoy's solution for twenty hours. Grossing was conducted and then the thickness of three to five millimeters were expunged from the organ and then placed into containers after labeling them. Examinations were conducted microscopically. It was done to inaugurate the evaluation of the morphological state of each tissue. This was a subjective analysis and was carried out by at least two proficient histopathologists. The evaluation was done and the staining of the tissues was assessed as

Table 2: Fixatives Employed in Different Groups

Groups	Fixative Employed
Group I (Control Group)	10% Formaldehyde
Group II (Experimental Group)	Bouin's Solution
Group III (Experimental Group)	Zenker's Solution
Group IV (Experimental Group)	Carnoy's Solution
Group V (Experimental Group)	10% Honey

poor, satisfactory, good and excellent. Then the findings were recorded in the concerned performa.[15] Poor- Score 0: The tissue failed to take up the stain sufficiently, stained disproportionately satisfactory score 1: Pointing towards particulars but not pictured up to the mark good score 2: Regarded as a good contrast between the nucleus and cytoplasm excellent score 3: Flawless stain including both of the nuclei and cytoplasm. After the processing of the tissues, sequential sections of the blocks of the tissues were cut on a microtome with a width of three to four micrometers. Then these sections were placed on the albumenized slides made of glass. Slides were stained with hematoxylin and eosin using the method of Harris hematoxylin.[16] The Masson trichrome technique[17] and Periodic Acid Schiff Technique[18] were also employed. The data was entered, evaluated and analyzed using Statistical Package of Social Sciences version 23. The percentages and frequencies were calculated for qualitative results. The chi-square test was employed to equate and compare the percentage of the categories of fixation. Statistical tests were employed where needed to validate the significance. The p-value <0.05 was considered statistically significant.

RESULTS

The results showed us the normal histology of lung tissue. For formalin (control group) 100% good results were observed in Table 3. For Bouin's solution, 67% satisfactory and 33% good results were observed. For Zenker's solution, 50% good results 33% excellent results and 16.7% satisfactory results were observed. For Carnoy's solution 83% excellent, 17% good results were observed. Honey showed 100% good results on Periodic Acid Schiff Stain. This proves that honey might be successfully used as an alternative to formalin as a fixative. The statistical difference (p-value=0.000) was calculated

Table 3: Distribution of Percentages in Lungs (Periodic Acid Schiff Stain)

Tissue	Fixatives	Grading of Fixation				
Lung		Based on stains				
Groups		Poor	Satisfactory	Good	Excellent	p-value
	10% Formaldehyde n (%)	0 0.0%	0 0.0%	6 100.0%	0 0.0%	0.000
	Bouin's Solution n (%)	0 0.0%	4 66.7%	2 33.3%	0 0.0%	0.000
	Zenker's Solution n (%)	0 0.0%	1 16.7%	3 50.0%	2 33.3%	0.000
	Carnoy's Solution n (%)	0 0.0%	0 0.0%	1 16.7%	5 83.3%	0.000
	10% Honey n (%)	0 0.0%	0 0.0%	6 100.0%	0 0.0%	0.000
Total	n (%) within groups	0 0.0%	5 16.7%	18 60.0%	7 23.3%	0.000

Table IV: Distribution of Percentages in Muscles (Masson Trichrome Stain)

Tissue	Fixatives	Grading of Fixation				
Muscles		On the basis of stains				
Groups		Poor	Satisfactory	Good	Excellent	p-value
	10% Formaldehyde n (%)	0 0.0%	0 0.0%	4 66.7%	2 33.3%	0.035
	Bouin's Solution n (%)	0 0.0%	0 0.0%	4 66.7%	2 33.3%	0.035
	Zenker's Solution n (%)	0 0.0%	0 0.0%	4 66.7%	2 33.3%	0.035
	Carnoy's Solution n (%)	0 0.0%	0 0.0%	0 0.0%	6 100.0%	0.035
	10% Honey n (%)	0 0.0%	0 0.0%	5 83.3%	1 16.7%	0.035
Total	n (%) within groups	0 0.0%	0 0.0%	17 56.7%	13 43.3%	0.035

between comparative fixation quality among these five fixatives. Table 4 shows us the normal histology of the muscles.

DISCUSSION

Formalin has been extensively used as a fixative for histopathology and immunohistochemistry regularly but it has proven to show venomous effects on innumerable tissues. Formalin has been proven to be exceedingly toxic. The Occupational Safety and Health Administration, International Agency for Research on Cancer and World Health Organization categorizes Formalin as a cancer-causing agent in humans that can cause nasopharyngeal cancers.[19,20] Honey has been considered and used as a medication predominantly for treating wounds because it has been recognized to have antibiotic properties. Honey has been also recognized as an agent to prevent autolysis as tissues fixed with it for thirty days did not display any insignia of autolysis. It also has been recognized to contain tissue hardening properties which makes it comparable in accomplishment to fixatives which act as tissue hardeners. Honey has been recognized to contain numerous minerals, trace elements and vitamins as well as carbohydrates and acids predominantly ascorbic acid, glucose and fructose.[11] Considering this context, the objective of this current study was to compare the fixative effects of Honey and other alcoholic fixatives in tissues using various types of stains. The results of our study coincided with the results of Ozkan and coworkers. In their research, they procured seven different fresh tissues, lungs, stomach, suprarenal, omentum, uterus, endometrium, breast and placenta and fixed them using ten percent distilled water, ten percent neutral buffered formalin and alcoholic formalin. Their results also validated that tissues that were fixed in honey, alcoholic formalin and neutral buffered formalin showed similar histomorphology.[1] The results of this current study also coincided with the results of

William and his coworkers. In their study, they worked on tissues of mice, Tissues of the esophagus and gastrointestinal tract and fixed them in Neutral Buffered Formalin, Carnoy's and Bouin's solution. Their results showed that the overall morphology of tissues was comparable to those of Neutral Buffered Formalin.[21] The results of our study were also similar to the results of a study conducted by Maaini and coworkers. In their study, they worked on honey as a substitute fixative for formalin which is the same as for our study. Their study determined that tissues that were fixed using a higher concentration of honey were successful to a lesser extent and yielded a slower rate of penetration at the sites where a low concentration of honey has been proven to be encouraging and comparable to those obtained with control tissues fixed in formalin.[3,22] The results of our current study also coincided with the results of Piereira and coworkers. They worked on Carnoy's solution and Neutral Buffered Formalin used as a fixative for specimens of colorectal cancers. In their study, the performance of fixative was comparable to morphology.[23] Results of our study are also comparable to the results of Muddana and coworkers. They worked on finding naturally occurring substitutes for formalin such as honey. They took formalin as a positive control. The optimum concentration of naturally occurring fixatives was determined to be ten percent honey. They then evaluated the histomorphological features of the tissues. They observed that the preservation of tissues when using honey as a fixative was comparable to those of formalin.[13] Although the results of our study were positive and were also validated and supported by previous related literature this current study recommends that future researchers conduct further studies using a larger sample size and humans as target samples to obtain more conclusive and generalized results. This would add more reliability and authenticity to the literature for using honey as an alternative for formalin in laboratories.

CONCLUSION

From the discussion mentioned above this current study concludes that honey is a natural fixative and is readily available with no known toxicity therefore it can be used as a nontoxic substitute for formalin. This current study also concludes that the tissues fixed in Carnoy's solution also showed results comparable to that of formalin. Bouin's solution when employed as a fixator also showed good results. Although Zenker's solution showed negligible histological changes among some tissues and showed poor results in some tissues.

DECLARATIONS

Consent to participate: All methods were performed following the relevant guidelines and regulations.

Availability of data and materials: Data will be available on request. The corresponding author will submit all dataset files.

Competing interests: None

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REFERENCES

1. Özkan N, Şalva E, Çakalağaoğlu F, Tüzüner B. Honey as a substitute for formalin? Biotechnic & Histochemistry 2012; 87(2): 148-53. https://doi.org/10.3109/10520295.2011.590155

2. Paavilainen L, Edvinsson Å, Asplund A, et al. The impact of tissue fixatives on morphology and antibody-based protein profiling in tissues and cells. Journal of Histochemistry & Cytochemistry 2010; 58(3): 237-46. DOI: 10.1369/jhc.2009.954321

3. Sabarinath B, Sivapathasundharam B, Sathyakumar M. Fixative properties of honey in comparison with formalin. Journal of

Histotechnology 2014; 37(1): 21-5. https://doi.org/10.1179/2046023613Y.0000000037
4. Rosai J. Why microscopy will remain a cornerstone of surgical pathology. Laboratory investigation 2007; 87(5): 403-8. https://doi.org/10.1038/labinvest.3700551
5. Melville R, Lippmann M. Influence of data elements in OSHA air sampling database on occupational exposure levels. Applied Occupational and Environmental Hygiene 2001; 16(9): 884-99. DOI: 10.1080/10473220117850
6. Zanini C, Gerbaudo E, Ercole E, Vendramin A, Forni M. Evaluation of two commercial and three home-made fixatives for the substitution of formalin: a formaldehyde–free laboratory is possible. Environmental Health 2012; 11: 1-14. DOI: https://doi.org/10.1186/1476-069X-11-59
7. Rutland CS. Histological and histochemical methods 4th edition. Journal of Anatomy 2008; 213(3): 356. DOI: 10.1111/j.1469-7580.2008.00957.x
8. Bancroft JD, Gamble M. Theory and practice of histological techniques: Elsevier health sciences; 2008. https://books.google.com/books?hl=en&lr=&id=Dhn2KispfdQC&oi=fnd&pg=PR13&dq=Theory+and+practice+of+histological+techniques&ots=JBqFdCRvBb&sig=nhQF0AdIKe1zB8YZIV5gM46qBVc
9. Luz D, Ribeiro Jr U, Chassot C, De Salles Collet e Silva F, Cecconello I, Corbett CEP. Carnoy's solution enhances lymph node detection: an anatomical dissection study in cadavers. Histopathology 2008; 53(6): 740-2. https://doi.org/10.1111/j.1365-2559.2008.03148.x
10. Marx RE, Stern D. Oral and maxillofacial pathology: a rationale for diagnosis and treatment: Quintessence Publishing Company Hanover Park; 2012. http://www.quintpub.com/display_detail.php3?psku=B0007
11. Avwioro G, Bankole J, Iyiola S, Avwioro T, Akinola G. One of the properties of honey in wound healing is prevention of autolysis. Der Pharmacia Lettre 2010; 2(3): 321-5. http://scholarsresearchlibrary.com/archive.html
12. Patil S, Premalatha B, Rao RS, Ganavi B. Revelation in the field of tissue preservation–A preliminary study on natural formalin substitutes. Journal of international oral health: JIOH 2013; 5(1): 31. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3768083/
13. Muddana K, Muppala JNK, Dorankula SPR, Maloth AK, Kulkarni PG, Thadudari D. Honey and olive oil as bio-friendly substitutes for formalin and xylene in routine histopathology. Indian Journal of Dental Research 2017; 28(3): 286. https://www.ijdr.in/text.asp?2017/28/3/286/210669
14. Li Y, Li Q, Zhu S, et al. The effect of strontium-substituted hydroxyapatite coating on implant fixation in ovariectomized rats. Biomaterials 2010; 31(34): 9006-14. https://doi.org/10.1016/j.biomaterials.2010.07.112
15. Gurcan MN, Boucheron LE, Can A, Madabhushi A, Rajpoot NM, Yener B. Histopathological image analysis: A review. IEEE reviews in biomedical engineering 2009; 2: 147-71. DOI: 10.1109/RBME.2009.2034865
16. Meloan SN, Puchtler H. "Harris hematoxylin," what harris really wrote and the mechanism of hemalum stains. Journal of Histotechnology 1987; 10(4): 257-61. https://doi.org/10.1179/his.1987.10.4.257
17. Costa GM, Araujo SL, Xavier FAF, et al. picrosirius red and masson's trichrome staining techniques as tools for detection of collagen fibers in the skin of dogs with endocrine dermatopathologies. Ciência Animal Brasileira 2019; 20. https://doi.org/10.1590/1089-6891v20e-55398

18. Yamabayashi S. Periodic acid—Schiff—Alcian Blue: A method for the differential staining of glycoproteins. The Histochemical Journal 1987; 19: 565-71. DOI: https://doi.org/10.1007/BF01687364
19. Gillespie JW, Best CJ, Bichsel VE, et al. Evaluation of non-formalin tissue fixation for molecular profiling studies. The American journal of pathology 2002; 160(2): 449-57. https://doi.org/10.1016/S0002-9440(10)64864-X Get rights and content
20. Cox ML, Schray CL, Luster CN, et al. Assessment of fixatives, fixation, and tissue processing on morphology and RNA integrity. Experimental and molecular pathology 2006; 80(2): 183-91. https://doi.org/10.1016/j.yexmp.2005.10.002 Get rights and content
21. Williams JM, Duckworth CA, Vowell K, Burkitt MD, Pritchard DM. Intestinal preparation techniques for histological analysis in the mouse. Current protocols in mouse biology 2016; 6(2): 148-68. https://doi.org/10.1002/cpmo.2
22. Al-Maaini R, Bryant P. The effectiveness of honey as a substitute for formalin in the histological fixation of tissue. Journal of Histotechnology 2006; 29(3): 173-6. https://doi.org/10.1179/his.2006.29.3.173
23. Pereira MA, Dias AR, Faraj SF, et al. Carnoy's solution is an adequate tissue fixative for routine surgical pathology, preserving cell morphology and molecular integrity. Histopathology 2015; 66(3): 388-97. https://doi.org/10.1111/his.12532