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A Comparative Study on Rapid, Simple and Cost-Effective Method in Determining the Decalcification Agent in Bone Tissue Processing in Hematoxylin and Eosin Staining Procedure

M. Preethi[1]*, R. Ajitha[2], Roshan.A[3], Neha Niranjan[4]

[1]*Department of Pathology, Sree Balaji Medical College and Hospital, Chromepet, Chennai-44, Tamil Nadu, India*

[2]*Department of Pathology, Sree Balaji Medical College and Hospital, Chromepet, Chennai-44, Tamil Nadu, India*

[3]*Department of Pathology, Chettinad Academy of Research and Education, Kelambakkam, Chennai-603103 , Tamil Nadu, India*

[4]*Department of Pathology, Chettinad Academy of Research and Education , Kelambakkam, Chennai- 603103, Tamil Nadu, India*

Abstract

Vinegar is most commonly used as a condiment with food. however, there are also different types of vinegar that are very effective for cleaning. In addition to household vinegar, it is mainly produced as a precursor to polyvinyl acetate and cellulose acetate.In our present invention we have used vinegar as decalcification agent in bone specimen in the hematoxylin and eosin staining . The present study, apple vinegar used as decalcifying agent in processing bone specimens for hematoxylin and eosin staining.A study was conducted from apiril 2022 to September 2022 , 30 bone specimens were taken from department of pathology . Bone Sections were made into the block and sent for the Hematoxyline and eosin staining .In H&E staining two groups were separated Group A , Group B, group C and group D . Group A used vinegar as decalcifying agent ,Group B as 10% HCL, group C as 2% NO and sodium bicarbonate solution as decalcifying agent ,thin section were made 3-4 micron and bone sections were subjected to hematoxyline and eosin staining All group slides were given to the pathologist for they opinion and for reporting the slides. The results showed that Group A and group C had showed superior staining properties ,.cost effective decalcification agent was sodium bicarbonate and vinegar when compared to the other two. The study suggest that vinegar is consider as ecofriendly decalcifying agent in hematoxyline and eosin staining process.

Keywords: *Decalcification Agent, Histopathology Examination, Vinegar.*

Introduction

Vinegar is used most commonly used as a condiment with food. There are many different types of vinegar used for this, the most popular being 'brown' vinegar. However, there are also different types of vinegar that are very effective for cleaning. White wine vinegar, apple cider vinegar and distilled white vinegar can all be used. Acetic acid is produced industrially both synthetically and by bacterial fermentation. About 75% of acetic acid made for use in the chemical industry is made by the carbonylation of methanol. The most popular vinegar for cleaning is distilled white vinegar. Unlike the brown vinegar used with food, white vinegar is clear and will not stain materials. Vinegar is no less than 2% acetic acid by volume, making acetic acid the main component of vinegar apart from water. Acetic acid has a distinctive sour

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Corresponding Author: drpreethi1290@gmail.com

taste and pungent smell obtained from esterfication of grains, sugarcane juice, apple juice, grape juice etc . In addition to household vinegar, it is mainly produced as a precursor to polyvinyl acetate and cellulose acetate. It is classified as a weak acid since it only partially dissociates in solution, but concentrated acetic acid is corrosive and can attack the skin. Soaking chicken bones in vinegar for several days leaves bones soft and rubbery. The acid component of vinegar reacts with calcium compounds in bones, making the calcium soluble so that the water component of vinegar can then dissolve the calcium from the bones, leaving the bone less rigid and able to bend. When the chicken bone was placed in the glass of vinegar, the acid in the vinegar dissolved the calcium carbonate so that only collagen was left. Calcium (the mineral in calcium carbonate) is needed to make our bones strong. When there isn't enough calcium, our bones become soft and are more likely to break. In our present invention we have used vinegar as decalcification agent in bone specimen in the hematoxylin and eosin staining . The present study relates to the field of Histopathology. More particularly the present study relates to a biological source, apple vinegar used as decalcifying agent in processing bone specimens for hematoxylin and eosin staining.

Objectives

1. The main object of the present study is to find, cost effective, rapid and simple decalcification material for decalicification of bone.
2. To compare chemical decalcification with eco-friendly decalcification agent, for decalcification of bone samples.
3. To compare the staining characteristics of chemical decalcification agent with natural decalcification agent as decalcifying agent.

Materials and Methods

A cross-sectional study was conducted in Sree Balaji medical college and hospital from April 2022 to September 2022 . 30 bone specimens were taken from the department of pathology . Bone Sections were made into the block and subjected for Hematoxylin and eosin staining. In H&E staining four groups were seperated Group A , Group B, group C and group D . Group A used vinegar as decalcifying agent, Group B as 10% Hydrochloric acid, group C as 2% nitric acid and Group D with sodium bicarbonate solution as decalcifying agent . In group A bony sections were immersed in vinegar for 3 days for decalcification, thin sections were made 3-4 microns and bone sections were subjected to hematoxylin and eosin staining. In group B, bone sections taken from the amputated great toe specimens were immersed in hydrochloric acid for 4 days for decalcification, thin sections were made 3-4 microns and bone sections were subjected to hematoxylin and eosin staining. In group C, bony sections were immersed in nitric oxide for 3 days for decalcification , thin sections were made 3-4 microns and bone sections were subjected to hematoxylin and eosin staining. In group D, bony sections were immersed in sodium bicarbonate solution for 3 days for decalcification , thin sections were made 3-4 microns and bone sections were subjected to hematoxylin and eosin staining. All group slides were given to the pathologists for their opinion and for reporting the slides.

Result

Two sections of 3- 4 microns thickness were prepared. They were stained with hematoxylin and eosin stain, considered in to 4 groups where vinegar (Group A), hydrochloric acid solution (Group B), nitric acid (group c) and sodium bicarbonate solution (group D) were used as decalcifying agents. The stained sections were graded based on the parameters of intensity of bone softness, thin bone section in the slide, Nuclear staining, Cytoplasmic staining, Clarity of staining, Uniformity of staining, Crispness of staining. The results showed that Group A and group C had showed superior staining

properties in all the parameters when compared to hydrochloric acid and sodium bicarbonate solution. Cost effective decalcification agent was sodium bicarbonate and vinegar when compared to the other two. Nitric acid and hydrochloric acid were considered as faster decalcification agents when compared to other two. The overall study reveals that the safer, hazardous free, biological source, less expensive material, natural, ecofriendly agent is Vinegar when compared to chemical decalcification agents in decalcifying bone.

Morphometric Analysis

After reviewing, the sections were further subjected to morphometric analysis. The images were captured using microscope with a 100X objective. The final image captured on the monitor at magnification of 1000X [Table no:1]. For each specimen, intensity of bone softness, thin sections of bone, distinct cellular and nuclear outlines were seen, avoiding overlapping to know the uniformity, clarity and crispiness of staining (Figure 1, 2, 3, 4). The intensity of softness of the bone was 70% for vinegar and 60% for 10%HCL, 2% nitric acid 70% and sodium bicarbonate solution 50% thin sections were made from vinegar 3-5micron(80%) and 10% HCL 4-6 micron (75%) , cellular details for both vinegar, nitric acid and HCL had 80% , nuclear details were 75% for all the agent , overlapping of cells , 80% in vinegar and 75% in HCL, nitric acid 78% and sodium bicarbonate 72% the crispiness of the cells was 85% in vinegar and nitric acid and 10% HCL and sodium bicarbonate is 80%[Table no:2]. The overall study reveals that the safer, harzardous free, biological source, less expensive material is vinegar when compared to all the chemical decalcification agents.

Table 1. Comparision of Vinegar, 10% HCL, 2% Nitric Oxide and Sodium Bicarbonate as Decalcification Agent by Pathologist

S.No	Decalcification Agent %	Intensity of the Softness of the Bone%	Thin Section Slide%	Cellular Details%	Cellular Debris%	Nuclear Details%	Overlapping of Cells%	Crispiness of the Cell%
1	Vinegar	70	80	80	Not seen	75	80	85
2	10% Hcl	60	75	80	Not seen	75	75	80
3	2% Nitric Oxide	70	78	80	Not seen	75	78	85
4	Sodium Bicarbonate	50	68	70	Not seen	75	72	80

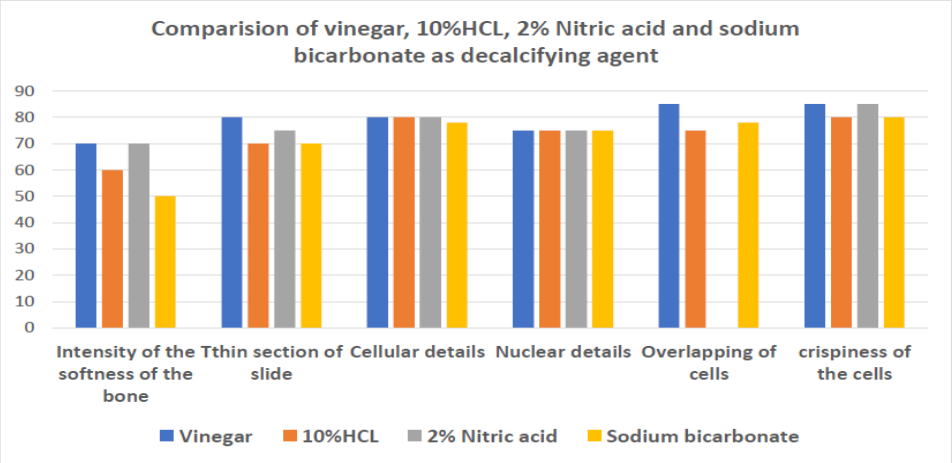

Figure 1. Comparision Chart of Vinegar, 10%HCL, 2% Nitric Oxide and Sodium Bicarbonate as Decalcification Agent

Table 2. Comparison of Decalcification Agent According to Cost and Time Duration of Stain Process

S.no	Decalcification agent	Cost	Time taken for decalcification
1	vinegar	Rs 100 (100ml)	3 days
2	10% HCL	Rs 250 (100ml)	2 days
3	2% nitric oxide	Rs 180 (100ml)	2 days 6hrs
4	Sodium bicarbonate	Rs 140 (100ml)	3 days

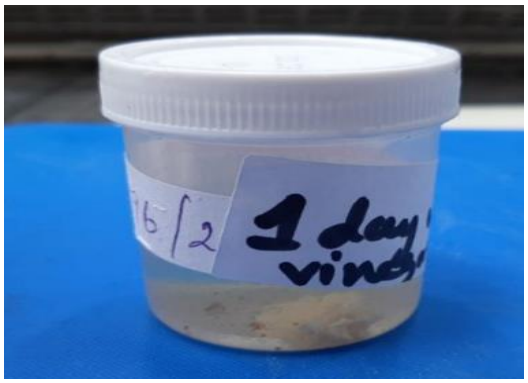

Figure 2. Day 1 of Decalcification Showing Bone Piece Immersed in the Vinegar

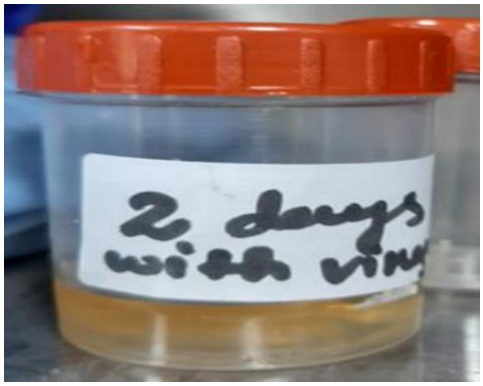

Figure 3. Day 2 of Decalcification Showing Bone Piece Immersed in Vinegar

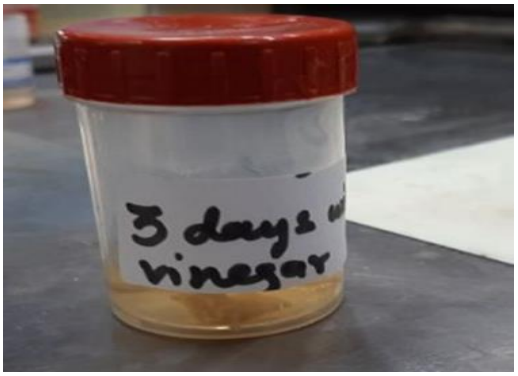

Figure 4. Day 4 of Decalcification Showing Bone Piece Immersed in Vinegar

Discussion

The process of decalcification is done to study the histology of the tissue and also to evaluate the biological response to bone materials.[1, 2, 3, 4] For many years, scientists have tried to introduce new decalcifying substances or tried to modify known decalcification agents [5, 6] in order to meet the criteria of a good decalcifying agent like Ensures complete removal of calcium; Causes minimal damage to cells and tissues; Causes non impairment to subsequent staining and; Decalcifies at reasonable speed [7]. Most authors have compared two to four decalcifying agents, sometimes varying the methods used and by employing a lot of permutations and combinations of the methods and agents, mainly to decalcify bone [8, 9, 10].

In the present study, we attempted to compare the efficacy of six decalcifying agents, its rate of decalcification, its effect on organic and inorganic components of bone, and staining characteristics.

The speed factor of the decalcifying agents was the highest with 10% HCL and lowest by lowest vinegar decalcifying solution, which was in accordance with literature. Also noted that chalky white depost are seen in the initial rate of decalcification 2 days after the start decalcification procedure. [Fig 5, 6]. Contrary to the overall increased time taken by the vinegar, it was noted that decalcified bone softness was more when compared to the 10% HCL [11, 12].

When sectioning it was noted that there was crumbling of tissue decalcified with 10% HCL and in vinegar crumbling of tissue was less when observed under microscope as also noted by Zappa et al [13] with respect to 10% HCL. Bone decalcified with neutral vinegar responded the best to microtome knife, hence deceiving the physical and radiological methods of testing end point of decalcification with respect to HCL [14].

Soft-tissue attachment and soft-tissue shrinkage, as reported by Zappa et al., [15] suggest that formic acid and nitric acid produce

worst results in contrast to the results obtained from our study, wherein formic acid gave good results as it showed minimal soft-tissue shrinkage and minimal loss of tissue [Figure 7, 8]. The bone organization with its extra-cellular matrix and histological zones were clearly distinct and excellent in decalcified bone with vinegar.

The overall superior results obtained with vinegar may be attributed to the mechanism of capturing metallic ions like calcium which binds to the chelating agent. This means that the calcium ions from the external layer of the apatite crystals will be removed. When all calcium ions from the outer layer of apatite crystals are removed, they will be replaced by ions from the deeper layers. In this way, the crystal size decreases gradually, producing an excellent preservation of tissue components [16].

The quality of decalcified sections and rate of decalcification depends on factors like fixation concentration of decalcifying agent used, temperature, pressure, agitation, electric current, microwave radiation, tissue suspension, and size and type of tissue [17].

In a study by Waerhaug, bone were decalcified rapidly under vacuum [8, 9]. The time taken for decalcification was reduced to one-tenth [10]. Changes in temperature at which decalcification occurs also varies the time taken for complete decalcification. Vongsavan et al., in his study on cat and rat teeth, reported a faster process of decalcification in microwave oven at 37±2°C than in room temperature or conventional oven [18]. Another study by Pitol et al. showed that microwave-aided decalcification showed to be more effective than the traditional methods in aspects like reduction of time period for decalcification, good morphology of bone tissue, and an increase of calcium release using microwaving [19].

Regardless of the solution used, methods of decalcification share their characteristic of being accelerated when additional factors are employed [20]. The present study was done with respect to different decalcifying agents only and none of the factors were employed, thus standardizing the procedure [17, 18]. Future studies in which the factors can be varied and decalcifying agent can be kept constant, might bring us to the near ideal decalcifying agent [21].

Being a Pathologist, it is essential to decrease the price and hazardous of unsafe chemical agents used in histopathological laboratories [22]. The quality of eco-friendly decalcifying agents is more efficient than hydrochloric acid in H & E staining procedure. In addition to it, these are harmless, quicker and price effective [23]. The knowledge of using vinegar as natural replacements to high concentrated hydrochloric acid is a small step to the future acid free histopathological laboratories.

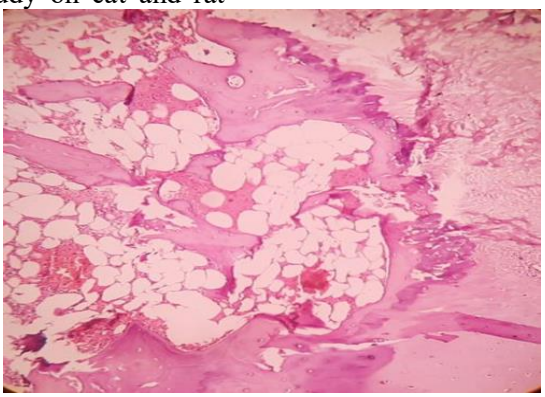

Figure 5. Bony Fragments With Bone Marrow Elements in Sections Made From Vinegar As Decalcification

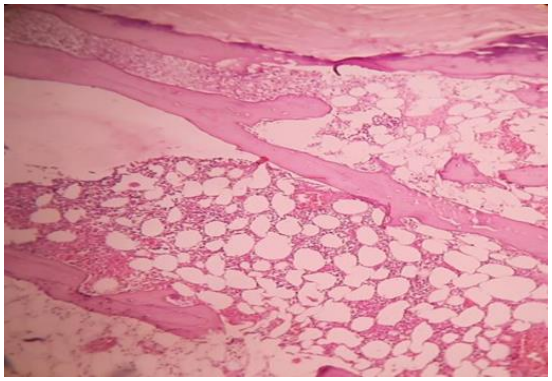

Figure 6. 10x Bone Marrow Elements in Sections made from 2% Nitric Acid As Decalcification Agent

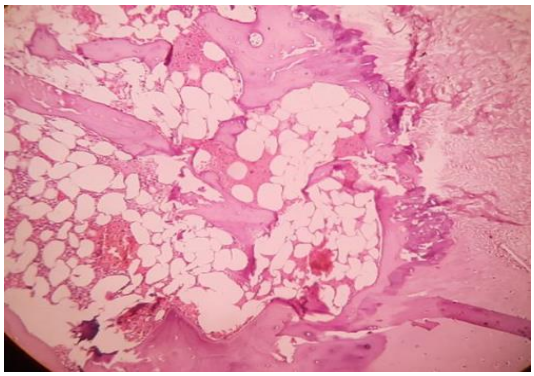

Figure 7. 100x Show Bone Marrow Elements & Soft Tissue Fragments in Sections Made from 10% HCL

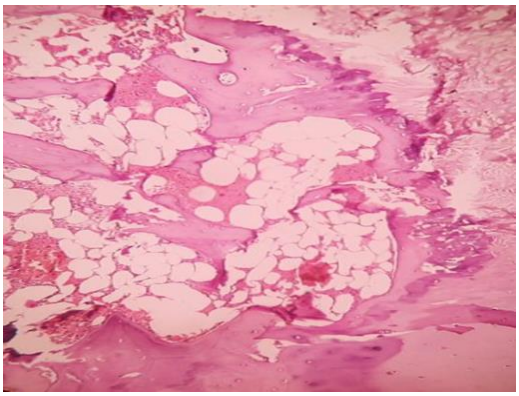

Figure 8. 10x Bony Fragments and Sounding Soft Tissue Fragments in Sections Made From Sodium Bicarbonate as Decalcification Agent

Conclusion

The study suggests that vinegar is considered as an eco-friendly decalcifying agent, Nitric acid is considered the best, safe, hazard free agent. Hydrochloric acid is considered the fastest decalcification agent in decalcifying bone in hematoxylin and eosin staining process.

Limitations

The study duration was limited to three months, and the sample size was small.

Acknowledgement and Declaration of Patient Consent

The authors certify that they have obtained all appropriate patient consent forms. In the form the patient(s) has/have given their consent for their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published, and due efforts will be made to conceal their identity.

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Nil.

Conflicts of Interest

There are no conflicts of interest.

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