Comparison of Conventional and Microwave Bone Decalcification Methods by Using 10% Nitric Acid: A Research Protocol

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ABSTRACT

Background: In bony specimens, elimination of calcium is obtained by a method known as “Decalcification”. It is completed through the means of chemical agents such as acids, chelators etc that combine with ions of calcium. Decalcifying agent is used in regular conventional method where the hard tissue is placed at a room temperature (20-25°C) with modifications of the solution at orderly intervals until the final cutoff point is obtained. Usage of microwave oven for the process of decalcification is a new and fast method in contrast to the routine conventional method of decalcification. In this study, an attempt has been made to regulate and compare the conventional procedure of decalcification with decalcification done by microwave oven of hard tissue specimens by using nitric acid of 10% concentration with regards to decalcification speed, conservation of tissue architecture as well as productiveness of staining.

Objectives: The study will made a comparison of Conventional and Microwave Bone Decalcification Methods by using 10% Nitric Acid.

Methodology: This prospective analytical study, will include decalcification of 30 hard tissue specimens by microwave method and conventional method. The results will be compared in terms
of decalcification speed, conservation of tissue architecture and staining productiveness. Necessary tests will be applied to analyze the data.

**Expected Results:** Significant advantages of microwave method are expected over conventional method of decalcification.

**Conclusion:** The conclusion will be drawn based on careful analysis of the results.

**Keywords:** Microwave; bone; decalcification; collagen; paraffin; architecture; staining.

1. INTRODUCTION

Preservation of bony tissue near to viable state is truly crucial for the comprehension of tissue information and functions. Sections which are cut by regular procedures to obtain fine sections is not possible in instances of specimens which include bones, odontomas, teeth and lesions which have undergone calcification. In these kind of bony specimens, elimination of calcium is obtained by a method known as “Decalcification” which construct the tissue smooth with the use of an instrument named microtome [1].

Decalcification of bony specimens is a maximum touchy procedure within side of the histopathology laboratory [2]. This procedure is usually accomplished by chemical retailers, such as acids which are inorganic to form salts of calcium which are soluble and chelator’s that combine with calcium ions [3].

The average duration taken for routine procedure of processing of tissue by Conventional Processing (CP) and routine staining is roughly 6-9 h and time duration taken for Conventional method is 24-41 h (based on the measurements of the specimen). In malignancy cases, as the issuing of report has a time limit, the necessity of a faster process of tissue is required [4].

For faster and accurate diagnosis several methods have been proposed with the usage of automated tissue processor. The standard of final specimen is improved, but the extent of time span stays constant i.e; 6-9 hours [4]. Temperature acceleration decreases the thickness of the fluid, which is used for processing, permitting high speed entrance into the tissue. With the application of conventional heat to the processing fluid, it will result in irregular tissue penetration leading to in dappled staining [3].

Some studies have suggested that the use of microwave oven cause a temperature elevation which results in enhancement of decalcification by the process of diffusion of the solution which is used for decalcification. Also, few studies have suggested that the use of microwave oven do not expand the process of diffusion of the decalcifying solution but rather aid a greater deposition of calcium attributable to the already established magnetic field [5].

Diffusion process inside the system will be increased by rise in the temperature. But a highly elevated temperature (55-60°C) is harmful to tissue morphologically. If the calcium deprivation in the tissue happens too quick, it results in hydrolysis and swelling of calcified matrix. A great rise of temperature generated in microwave can be rectified by ice bath while fixing the samples [5].

Decalcifying agent is used in regular conventional method where the hard tissue is placed at a room temperature (20-25°C) with modifications of the solution at orderly intervals until the final cutoff point is obtained. Usage of microwave oven for the process of decalcification is a new and fast method in contrast to the routine conventional method of decalcification. In microwave method, bony tissues are placed in a microwave oven in the decalcifying agent for irregular periods with orderly modifications of the solution till the final cutoff point is arrived. Irradiation of the bony specimens in a microwave has appeared to fasten up the process of decalcification from few days to h [6].

In 1970, Mayers proposed usage of microwave in laboratory. He promoted usage of microwave to speed up the hour span of tissue fixation [4]. Aside from reducing the time of diagnosis of tissue specimen, Microwave Decalcification (MD) has also eliminated the disclosure to possible toxins. Several workups have revealed that processing in the microwave results in minor amount of alteration in the nucleic acid [7,8].

Microwave oven produces steady heat and increases the charge of tissue piercing maintaining the good calibre of the bony tissue specimen [4,7,8,9]. Altering electromagnetic fields are produced by non-ionizing radiation of
microwaves which leads to the revolving of molecules which are dipolar like proteins as well as water. This produces molecular dynamics which leads to the causation of energy flux that will be continued till radiation come to an end [8]. Analysis showed that at ambient temperature, decalcification of bone by microwave technique is hastened approximately for 10 times in contrast with conventional method of decalcification [10].

Various decalciﬁying agents can be used for decalcification process. The prime agent to be used will be based upon the emergency of technique. When various chemicals are used they may result in deformation and maceration of tissues. The tissue appears unchanged grossly, but under microscopy it shows shrinkage, disruption, swelling and vacuolization which are not related to pathological conditions [11].

The decalciﬁcation agents which can be use are formal nitric acid of 10%, ethylenediaminetetraacetic acid, formal nitric acid of 8%, perenyi’s fluid, etc. Ethylenediaminetetraacetic acid is the slowest agent, but the sections can be taken easily and are also easy to handle and not fragile. Formal nitric acids of 8 and 10% are usually the fastest decalciﬁying agents, easy to handle and dissections can be taken easily. The formic acid 8% may result in friability of the tissue. The specimen decalciﬁed with perenyi’s fluid may result in over staining and under staining of sections, diﬃculty in sectioning and the effect of chromic acid and nitric acid may result in tissue friability [3].

A perfect decalciﬁying agent must ‘be quick’, ‘be superior’ and should be satisfactory. An ideal agent must make certain of total detachment of calcium, and should result in mild harm to tissue, and should not lead to any staining impairment and should decalciﬁy the tissue at reasonable speed [6]. Some decalciﬁying agents may take out the ions of calcium completely but they can damage these tissue components and also affect the staining characteristics. The rate of decalciﬁcation and the quality of sections which are decalciﬁed relies upon various factors such as temperature, ﬁxation concentration of the agent used for decalciﬁcation, pressure blending microwave radiation, electric current, tissue suspension, size of tissue and type of tissue [3,12,13].

The cutoff point for the process of decalciﬁcation can be assessed through various methods as follows:

1) Radiographically where the opacity suggests incomplete decalciﬁcation.
2) Physical method of probing the bony specimen with a needle.
3) Chemical methods by using the strong liquor ammonia [3,14].

In this study, an attempt has been made to regulate and compare the conventional procedure of decalciﬁcation with decalciﬁcation done by microwave oven of hard tissue specimens by using nitric acid 10% with regards to decalciﬁcation speed, conservation of tissue architecture as well as productiveness of staining.

1.1 Aim and Objectives

The aim and objective of present study is to regulate and compare the conventional procedure of decalciﬁcation with decalciﬁcation done by microwave oven of hard tissue specimens by using nitric acid 10% with regards to decalciﬁcation speed, conservation of tissue architecture as well as productiveness of staining.

2. MATERIALS AND METHODS

Place of study - Division of Histopathology, Department of Pathology, Jawaharlal Nehru Medical College, Sawangi, Wardha.

Study duration - 2 years.

Study design - It is a prospective analytical study.

Sample Type - Hard tissue specimens received in Division of Histopathology.

Techniques:

1. Steps for conventional method of decalciﬁcation are as follows

   i. Hard tissue specimens are to be collected.
   ii. The specimens will be labeled properly.
   iii. These bony specimens will be immersed in 10% nitric acid for decalciﬁcation process.
   iv. The solution will be changed periodically till the bony tissue gets softened.
v. The duration of the entire process will be recorded.
vi. These specimens will be exposed to routine tissue processing as well as respective staining.

2. Steps for microwave method of decalcification are as follows
   i. Hard tissue specimens will be washed with water for 10 minutes.
   ii. These specimens are then put in a container consisting of 10% nitric acid.
   iii. This container is kept in domestic microwave oven and set for one-minute cycle at 700 W.
   iv. After 1 min of cycle, the bony specimen will be taken off outside and settled to cool off for 45 min.
   v. This entire process is repeated for several times (average - 7 cycles/day).
   vi. The nitric acid solution is changed every 3 h. till decalcification is achieved.

Inclusion Criteria: All hard tissue specimens received in Division of Surgical Pathology.

Exclusion Criteria: All soft tissue specimens received in Division of Surgical Pathology.

3. EXPECTED OUTCOME/ RESULTS

In this study, as we are comparing and contrasting two different decalcification methods with respect to speed of the procedure for completion of decalcification process, the ability to preserve the tissue architecture, the staining productiveness and good morphology under microscope, and also as microwave oven is a novel method and also has all advanced characteristics for decalcification process, we are expecting a better outcome with use of microwave oven when compared to conventional method.

4. DISCUSSION

Decalcification process is a time taking process. Many specimens contain numerous calcified areas which needs to be decalcified before the tissue processing and sectioning. Usually it takes several weeks to decalcify a hard tissue specimen. This process is based on the quality and rate of demineralization, the application of microwave energy in the form of nonionizing radiation results in rotation of water and proteins (polar side chains). This molecular kinetics result in production of energy variability [15].
R Sangeetha et al. (2013) did a study and have concluded that decalcification done in microwave is quicker than conventional method. And also, the tissue conservation and staining productiveness was better in microwave procedure of decalcification compared to conventional method of decalcification method [15].

A novel method using a domestic microwave oven has been found to speed up the decalcification method. The microwave has been proved to be functional in decreasing the time which is necessary for decalcification. The energy which is produced by microwave combines with molecules which are dipolar by transmitting kinetic energy as well as alteration of electric field [5].

A Thirumal Raj et al. (2016) in a study comprising of 240 hard tissue specimens have concluded that a combination of both microwave and conventional decalcification is affective to speed up the time spent in laboratory with minute settlement over quality of the tissues [16].

Archana Srivasyaiah et al. (2016) in a study comprising 72 premolars and the decalcifying agent used were trichloroacetic acid, nitric acid, and formic acid with concentrations of 5% and 7%. They have concluded nitric acid of 5% concentration used in microwave method demonstrated as the perfect agent due to its speed. This also resulted in fine histological details and characteristics if various stains used [17].

Ahmad Danish Rehan et al (2017) did a study comprising 30 hard tissue specimens and the decalcifying solutions used were diluting 5% of formic acid, 5% of nitric acid, 14% of EDTA. The study concluded that usage of nitric acid of concentration 5% was the quick agent when used in microwave oven for the decalcification process. The results also demonstrated that the structural features and better staining productiveness were good when 5% nitric acid is used for decalcification as when compared to other solutions [18].

Sanjay k et al. (2012) conducted a study using neutral EDTA, 10% of formic acid, 5% of nitric acid, and other acids. They have concluded that
neutral EDTA showed better soft tissue coherence and good staining of hard bony tissues as well as soft tissue. They also concluded that minimal shrinkage of soft tissue and minute tissue loss is shown by 10% formic acid [19].

Singh S and Sicar K (2010) conducted a study and they have demonstrated that preservation of morphological characteristics is based on staining uniformity. The various decalcifying agents used in this study were as follows - 10% of formal formic acid, 10% of formal nitric acid, and 5% of formal EDTA. They have concluded that EDTA is found to be much better when compared to others with respect to uniformity of staining. They also stated that the decalcification time is reduced when microwave was used [20]. Few of the related articles were reported [21-37].

In the present study, for decalcification of hard tissue specimens, microwave method, and conventional method will be done. The results obtained by these methods will be compared in regards to decalcification speed, conservation of tissue architecture as well as productiveness of staining.

5. LIMITATIONS

Few limitations which will be there in the study are improper biopsy technique, inadequate biopsy material and improper tissue processing and staining.

6. CONCLUSION

It will be discussed and conclusion will be drawn in correlation with observation and results.

CONSENT

It is not applicable.

ETHICAL APPROVAL

This will be drawn after the proper assessment of study conducted after the clearance from the institutional ethics committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Lynch SR. 4th ed. London: W B Saunders. Lynch’s Medical Laboratory Technology. 1983;937–44.
2. Bhaskar SN. Orban’s Oral Histology and Embryology .10th ed. New Delhi: CBS Publishers and Distributors. 1990;349-54.
3. Callis GM, Bancroft JD. Theory and Practice of Histological Methods. 6th ed. Edinburgh: Churchill Livingstone. 2008; 338-60.
4. Amrutha N, Patil S, Rao RS. Microwaves: A revolution in histoprocessing. J Contemp Dent Pract. 2014;15(2):149-52.
5. Pitol DL, Caetano FH, Lunardi LO. Microwave induced fast decalcification of rat bone for electron microscopic analysis: An ultrastructural and cytochemical study. Braz Dent J. 2007;18:153–7.
6. Roncaroli F, Mussa B, Bussolati G. Microwave oven for improved tissue fixation and decalcification. Pathological. 1991;83: 307-10.
7. Mathai AM, Naik R, Pai MR, Rai S, Baliga P. Microwave histoprocessing versus conventional histoprocessing. Ind J Pathol Microbiol. 2008;51:12-16.
8. Ralph L, Rohr A. Comparison of routine and rapid microwave tissue processing in surgical pathology laboratory quality of histologic sections and advantages of microwave processing. Am J ClinPathol. 2001;115:703-08.
9. Babu TM, Malathi N, Magesh KT. A comparative study on microwave and routine tissue processing. Indian J Dental Research. 2011;22(1):51–55.
10. Vongsavan N, Mathews B, Harrison GK. Decalcification of teeth in a microwave oven. Histochem J. 1990;22:377–80.
11. Morse A. Formic acid-sodium citrate decalcification and butyl alcohol dehydration of teeth and bones for sectioning in paraffin. J Dent Res. 1945;24: 143-53.
12. Mattuella LG, Bento LW, Vier-Pelisser FV, Araiyo FB, Fossati AC. Comparative analysis of two fixating and two decalcifying solutions for processing of human primary teeth with inactive dentin carious lesion. Rev Odonto Ciênc. 2007; 22:99–105.
13. Birkedal-Hansen H. Kinetics of acid demineralization in histologic technique. J Histochem Cytochem. 1974;22:434–41.
14. Drury RA, Wallington EA. 5th ed. Oxford: Oxford University Press. Carleton's Histological Technique. 1980:199–205.

15. Sangeetha R, Uma K, Chandavarkar V. Comparison of routine decalcification methods with microwave decalcification of bone and teeth. J Oral Maxillofac Pathol. 2013;17:386–91.

16. Thirumal Raj, Shankar Gouda Patil, Roopa S. Rao. Journal of Clinical and Diagnostic Research: JCDR. 2016;10(10):ZC121.

17. Archana Srinivasayia, Priyanka Nitin, Usha Hegde. Journal of Laboratory Physicians. 2016;8(2):106.

18. Ahmad Danish Rehan, Sonal Saigal, Ankur Bhargava, Uddipan Kumar, Pragya Thakur, Tasnim Kausar. Comparison of microwave decalcification with conventional decalcification method by using different decalcifying agents. International Journal of Research in Medical Sciences. July 2017;5(7).

19. Sanjai K, Kumarswamy J, Patil A, Papaih L, Jayaram S, Krishnan L, et al. Evaluation and comparison of decalcification agents on the human teeth. Journal of Oral and Maxillofacial Pathology. 2012;16(2):222-227.

20. Singh S, Sircar K. Evaluation of efficacy of various chemicals for decalcification of dental hard tissues - An in-vitro study. Journal of Orofacial and Health Sciences. 2010;5-10.

21. James, Spencer L, Chris D. Castle, Zachary V. Dingels, Jack T. Fox, Erin B. Hamilton, Zichen Liu, Nicholas L. S. Roberts, et al. Estimating global injuries morbidity and mortality: Methods and data used in the Global Burden of Disease 2017 Study. Injury Prevention. October 2020; 26(Suppl 1):i125–53. Available:https://doi.org/10.1136/injuryprev-2019-043531

22. James, Spencer L, Chris D. Castle, Zachary V. Dingels, Jack T. Fox, Erin B. Hamilton, Zichen Liu, Nicholas L. S. Roberts, et al. Global injury morbidity and mortality from 1990 to 2017: Results from the Global Burden of Disease Study 2017. Injury Prevention. October 2020;26(Suppl 1):i96–114. Available:https://doi.org/10.1136/injuryprev-2019-043494

23. Murray, Christopher JL, Cristiana Abbafati, Kaja M. Abbas, Mohammad Abbasi, Mohsen Abbasi-Kangevari, Foad Abd-Allah, Mohammad Abdollahi, et al. Five insights from the Global Burden of Disease Study 2019. The Lancet. October 2020;396(10258):1135–59. Available:https://doi.org/10.1016/S0140-6736(20)31404-5

24. Murray, Christopher JL, Aleksandr Y. Aravkin, Peng Zheng, Cristiana Abbafati, Kaja M. Abbas, Mohsen Abbasi-Kangevari, Foad Abd-Allah, et al. Global burden of 87 risk factors in 204 countries and territories, 1990–2019: A systematic analysis for the Global Burden of Disease Study 2019. The Lancet. October 2020;396(10258):1223–49. Available:https://doi.org/10.1016/S0140-6736(20)30752-2

25. Manchanda A, Agrawal A, Vagha S, Singh K. Spectrum of central nervous system tumors on histopathology at Tertiary Health Care Hospital. International Journal of Pharmaceutical Research. 2019;11(4):2060–64. Available:https://doi.org/10.31838/ijpr/2019.11.04.512

26. Dhar R, Singh S, Talwar D, Mohan M, Tripathi SK, Swarnakar R, Trivedi S, Rajagopala S, D'Souza G, Padmanaban, Baburao A. Bronchiectasis in India: Results from the European multicentre bronchiectasis audit and research collaboration (EMBARC) and respiratory research network of India registry. The Lancet Global Health. 2019 Sep 1;7(9):e1269-79.

27. Acharya S, Shukla S, Mahajan SN, Diwan SK. Acute dengue myositis with rhabdomyolysis and acute renal failure. Annals of Indian Academy of Neurology. 2010 Jul;13(3):221.

28. Gadbail AR, Chaudhary M, Patil S, Gawande M. Actual proliferating index and p53 protein expression as prognostic marker in odontogenic cysts. Oral Diseases. 2009 Oct;15(7):490-8.

29. Prasad N, Bhatt M, Agarwal SK, Kohli HS, Gopalakrishnan N, Fernando E, Sahay M, Rajapurkar M, Chowdhary AR, Ratni M, Jeloka T. The adverse effect of COVID pandemic on the care of patients with kidney diseases in India. Kidney International Reports. 2020 Sep 1;5(9):1545-50.

30. Walia IS, Borle RM, Mehendiratta D, Yadav AO. Microbiology and antibiotic sensitivity of head and neck space
infections of odontogenic origin. Journal of Maxillofacial and Oral Surgery. 2014 Mar 1;13(1):16-21.

31. Lohe VK, Degwekar SS, Bhowate RR, Kadu RP, Dangore SB. Evaluation of correlation of serum lipid profile in patients with oral cancer and precancer and its association with tobacco abuse. Journal of Oral Pathology & Medicine. 2010 Feb;39(2):141-8.

32. Korde S, Sridharan G, Gadbail A, Poornima V. Nitric oxide and oral cancer: A review. Oral Oncology. 2012 Jun 1;48(6):475-83.

33. Gondivkar SM, Gadbail AR. Gorham-Stout syndrome: A rare clinical entity and review of literature. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology. 2010 Feb 1;109(2):e41-8.

34. Gadbail AR, Chaudhary M, Gawande M, Hande A, Sarode S, Tekade SA, Korde S, Zade P, Bhowate R, Borle R, Patil S. Oral squamous cell carcinoma in the background of oral submucous fibrosis is a distinct clinicopathological entity with better prognosis. Journal of Oral Pathology & Medicine. 2017 Jul;46(6):448-53.

35. Gadre PK, Ramanojam S, Patankar A, Gadre KS. Nonvascularized bone grafting for mandibular reconstruction: Myth or reality?. Journal of Craniofacial Surgery. 2011 Sep 1;22(5):1727-35.

36. Sorte K, Sune P, Bhake A, Shivkumar VB, Gangane N, Basak A. Quantitative assessment of DNA damage directly in lens epithelial cells from senile cataract patients. Molecular Vision. 2011;17:1.

37. Basak S, Rajurkar MN, Mallick SK. Detection of Blastocystis hominis: A controversial human pathogen. Parasitology Research. 2014 Jan;113(1):261-5.