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A Comparative Study of Decalcification using three Different Agents and their Quantitative End-point Estimation

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Abstract

The present study on decalcification includes following aims & objectives:

1) To compare the efficacy of modified 5 % EDTA solution (neutralized with 2 % ammonium hydroxide) with routinely used 5 % HNO_3 & 5 % formic acid as decalcifying agents.
2) To quantitatively evaluate the optimum end-point with each of the above decalcifying agents & the total time required for decalcification.
3) To recommend the best decalcifying agent and determine its optimum end-point.

Total 90 samples were selected randomly from different patients, divided in slots of 30, with age group 13-20 years, independent of sex. Modified EDTA solution (neutralized with 2% ammonium hydroxide instead of sodium hydroxide) was found to be the most effective decalcifying agents as compared to 5 % HNO_3 & 5 % HCOOH. The decalcification time was considerably reduced (33-35 days). 5 % nitric acid was faster in its action (11-12 days) while 5 % formic acid was the intermediate one (16-18 days). The choice of decalcifying agent & method preferably should be largely dictated by the urgency of procedure i.e. whether biopsy report is awaited by the surgeon or purpose of study, time, & equipment available, etc. The method of end-point determination described in the study is a tool for quantitative evaluation of decalcification methods & an objective assessment of the methods and/or tissue-specific factors. Decalcification of teeth is not dependent on the sex of patient, arch trait, & type trait.

Key words: Decalcification; 5 % HNO_3; 5 % HCOOH; 5 % EDTA.

Abbreviations: OCPC = Ortho-cresolphthalein complexone;
EDTA = Ethylene di-amine tetra-acetic acid

INTRODUCTION

Various decalcifying agents and methods have been described in literature, but none of them proved to be effective enough to obtain superior quality tooth sections. This is because tissues of the teeth are of different consistencies. Few studies have actually standardized the procedure. Everyday practice of mechanical and radiological methods doesn't allow the course of procedure to be followed strictly. Thus, the need of quantitative analysis of calcium determination from the decalcifying solution arose. The present in-vitro study on decalcification process is a culmination of three different decalcifying agents and their optimum end-point estimation. It doesn't in any way contradict the voluminous matter published since the time of inception of histotechnique. The study is a step forward in establishing the decalcification dynamics. An attempt has been made in this study to standardize the procedure. A number of papers on methods of decalcification have been published, but only few on end-point determination. The present study attempts to do so.

MATERIAL AND METHODS

The present study was carried out in Department of Oral Pathology and Microbiology, Government Dental College, Aurangabad, Maharashtra, India. Total 90 samples were selected randomly from different patients, divided in slots of 30, with age group 13-20 years, independent of sex. Maxillary/Mandibular, I/II premolars were selected. The decalcifying agents used were 5 % nitric acid, 5 % formic acid, and chelating agent EDTA (neutralized with 2% ammonium hydroxide). For quantitative analysis of end-point determination calcium-kit OCPC, provided by "Accurex Biomedical Pvt. Ltd.", was used. Instruments employed were semi-auto analyzer (Trans-Asia) for calcium estimation, de-ionized distill water plant (Trans-Asia), and balance (K-Roy Classics).

The samples were dropped in 10% buffered neutral formalin jar for 4 days to ensure adequate fixation. After fixation samples are ready for decalcification. After proper labeling of the samples, they were kept in horizontal coplin jar and then decalcifying agent was poured (approx 100 ml for each sample). The procedure was carried out at room temperature (32-35 °C). In case of 5 % HNO_3 and 5 % HCOOH, the solution was changed after every 24 hr, whereas for 5% EDTA it was changed after every 72-96 hr interval.

End-point determination

The decalcifying solution after being saturated with calcium ions was taken for quantitative calcium estimation. Readings were noted at the end of every 4 days and subsequently strict surveillance was done after every 24 hrs for 5% HNO_3, 48 hrs for HCOOH, and 72 hrs for 5% EDTA. Calcium was then quantitatively measured on semi-auto-analyzer at 577-578 nm.

RESULTS AND DISCUSSION

According to the present study nitric acid was the fastest decalcifying agent needing just about 11-12 days for decalcification. This was in accordance with Culling (1985).[5] The next rapid decalcifying agent was 5% formic acid that took 16-18 days for decalcification. Chatterjee and Gadewar (1977) used formic acid to decalcify fetal mandible and it required around 20-33 days.[2] Thus it is proved that the actual time for decalcification dependent on mineralization of the specimen to be decalcified and so the disparity in decalcification times was observed in different studies [Cohen (1983)].[3]

The high dissociation constant or low pKa of 5 % nitric acid makes it that much quicker in its action of removing the calcium from the teeth as compared to 5 % formic acid.

In the studies reported by [Preece (1972), Culling (1985), and Chatterjee-Gadewar (1997)][7, 5, 2] the time taken for decalcification using EDTA was 6-12 weeks, depending on size of the specimen and degree of mineralization. The time taken for decalcification in the present study using 5 % EDTA was considerably reduced (33-35 days) because the decalcifying solution was modified as per the study of Sanderson Radley, *et al.,* (1995).[8]

Ammonium hydroxide is a weakly ionize base where as sodium hydroxide is a strongly ionized base. This chemical property makes Na^+ to compete with Ca^{++} that is being extracted from the tooth undergoing decalcification, for forming a bond

Table 1: Compatibility of Samples:
Table 1.1: Arch & Type Triat

Decalcifying Agents	Mandible	Maxilla	Total	Type I	Type II	Total
5% HNO3	21	09	30	21	09	30
5% HCOOH	07	23	30	16	14	30
5% EDTA	14	16	30	19	11	30
Total	42	48	90*	56	34	90**

* $p < 0.01$ (significant); ** $p > 0.1$ (insignificant)

with EDTA. EDTA forms chelate with calcium ions at a neutral pH. Thus, when sodium and calcium ions compete with each other to bind with EDTA, the amount of calcium extracted from the specimen automatically slows down and ultimately, the time required for decalcification is more.

Sanderson *et al.,* (1995) showed that decalcification took around 6 days, whereas according to the present study that employed same solution it took about 33-35 days. This vast variation is attributed to the method of decalcification. The present study doesn't deny the universally accepted fact that fixation is certainly necessary and a prime requisite if the cellular components of pulp, dentin, cementum, and supporting soft tissues are to be well preserved and accurately visualized.

Fixation in formalin helps the nucleic acid to become resistant to the hydrolytic action of acids as stated by Sielly J. (1982).[9] Studies have confirmed that formalin exerts a clear inhibitory effect upon the acid hydrolysis of the hard tissue proteins. [Preece, Culling, Bancroft and Stevens][7, 5, 1]. The present study proposes that even the occasional re-precipitation artifact can be avoided if the decalcification procedure is strictly monitored by quantitative analysis of end-point determination method described in the study. It can be hypothesized that it is only when secondary calcium phosphate survives the decalcification process that artifacts arise. The proposed method of end-point determination by a semi-auto analyzer allows a quantitative assessment of the decalcification process. The method described in the study makes it comparable to required precision of end-point determination, and is comparable to studies of Van Wyk (1987).

The method of end-point determination described in the study is a tool for quantitative evaluation of decalcification methods and an objective assessment of the methods and/or tissue-specific factors. This is not feasible with the present mechanical, radiological, or qualitative chemical methods described in the literature by Preece (1972), Culling (1985), Chatterjee-Gadewar (1977).[7, 5, 3]

The quantitative calcium estimation allows one to observe the dynamic course of the procedure to be observed which isn't possible with older qualitative tests. The literature has reported different methods of quantitative assessment using a colorimeter, flame-photometer (Van Wyk 1987), a continuous auto-analyzer and atomic absorption spectrophotometer (Muller (1990). However, the present study has employed a semi-auto analyzer

Table 2: End-point estimation, mg % decalcification after a periodicity & total number of days required for decalcification:

Day	5% HNO3	5% HCOOH	5% EDTA
1	36.12	38.44	00
4	26.91	25.10	4.31
8	16.82	10.71	13.11
12	0.05	5.52	20.85
16		1.86	13.60
18		0.13	-
20			11.67
25			2.50
30			2.06
33			00.75
Group Mean	19.975	13.62	8.685
Grand Mean			12.425*
Days Required	11-12	16-18	33-35

* p (0.01) = 3.59 (significant in all the three groups); F (2, 7) = 196.57

because it has high degree of precision and accuracy as compared to colorimeter and flame photometer. Additionally, it is compared in terms of method, i.e. colorimetric analysis to a continuous auto analyzer and an atomic absorption spectrophotometer. Semi-auto analyzer was also used for the simple reason that it was easily available. All the above equipment mentioned work on the same principle i.e. estimation of the calcium at a specific wavelength (577 nm in the present study)[11]. The intensity of colored-complexone produced was directly proportional to the calcium concentration, which was measured at 577 nm. For deciding the end-point, absorption factor was kept consistent at 15. Table number 2 shows reading of 0.05 for 5 % HNO_3 acid was considered as the end point since the absorption factor 15 had been reached. Similarly, end-point for 5 % HCOOH (=00.13) and 5 % EDTA (=00.75) was decided. These findings were in consistence with those of Van Wyk (colorimeter and flame-photometer), Muller (auto analyzer and spectrophotometer), with end point 0.07 mg % and 0.61 mg % respectively.[10, 6] whereas the present study (semi-auto analyzer) determined endpoints for 5 % HNO_3 = 0.05 mg %, 5 % HCOOH = 0.13 mg %, and 5 % EDTA = 0.75 mg% respectively. Estimation of near zero reading of calcium decided the timings of decalcification of the three groups. Muller (1990) using ultrasonic treated nitric acid reported the timings after quantitative end-point estimation as approximately 48 hours. But as pointed as earlier, the disparity in the timings of decalcification in this study and that of Muller can be attributed the method of decalcification. Since the selection of teeth was done at random and from different patients, it is believed that no two samples were alike. The teeth sample, though all premolars, and of particular age group, had different apatitic structures, various sized and arrangement of the apatitic crystals.

CONCLUSION

Modified EDTA solution (neutralized with 2% ammonium hydroxide instead of sodium hydroxide) was the most effective agent as compared to 5 % HNO_3 and 5 % HCOOH. The decalcification time was considerably reduced.

5% nitric acid although faster in its action (11-12 days). 5% formic acid was the intermediate one (16-18 days), with 5% nitric acid and EDTA (33-35 days) being the two extreme ends of the spectrum. Decalcification of the teeth is not dependent on the sex of the patient, arch trait, and type trait.

It can be comprehensively stated that the decalcification procedure depended largely on the size of the specimen (macroscopically). The choice of the decalcifying agent and method would be largely dictated by the urgency of the procedure i.e. whether biopsy report is awaited by the surgeon, purpose of the study (either research or diagnostic), time and equipment available, etc. Although there is a generally positive correlation between quantitative end-point methods, additional data and larger canvas are needed to confirm the reliability of the method and predictive value concerning correct interpretation of hard tissue structures.

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