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Comparison of decalcifying agents and techniques for human dental tissues

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Abstract

Teeth are among the hardest animal tissues, because they are composed of large amounts of inorganic compounds. Consequently, teeth are difficult to prepare for microscopic examination. Acids and chelating agents traditionally have been used to remove calcium ions. We compared decalcifying agents including strong acids, weak acids, chelating agents, techniques using electric current, agitation and heat. Freshly extracted teeth were fixed and decalcified using formic acid-formalin, formal-nitric acid, formalin-EDTA, Von Ebner's fluid and Perenyi's fluid. Three additional techniques including formic acid with agitation, formic acid with heat and formic acid with electric current also were evaluated. Decalcified teeth were evaluated histologically for tissue preservation and staining characteristics. Formic acid with gentle agitation produced the best decalcification overall based on time required for decalcification, ease of sectioning, hard and soft tissue staining and tissue preservation. Our findings support the use of agitation with formic acid decalcification, because it reduces significantly both the time required and the deleterious effects of prolonged immersion.

Key words: acid decalcification, agitation decalcification, EDTA decalcification, electric current decalcification, heat decalcification, histology, pulp, teeth

Thin sections of hard tissues are difficult to obtain by ordinary methods, which makes histologic study of tissues such as teeth problematic (Sangeetha et al. 2013). Histological examination of hard dental tissues and pulp is imperative for diagnosis of developmental disorders, pulp pathologies, forensic odontology and research (Sanjai et al. 2012).

Strong acids, weak acids and chelating agents traditionally have been used to remove calcium ions from hard tissues (Drury and Wallington 1980). Although decalcification by strong acids is rapid, it damages tissue and affects tissue stainability. Decalcification with weak acids, such as formic acid, preserves tissue details, but is time-consuming. Chelating agents, such as EDTA, do not damage tissues or affect stainability, but they require a long time (Callis 2008). Decalcification can be accelerated by applying heat, agitation, vacuum and electric current (Verdenius and Alma 1958). Microwave energy (Sangeetha et al. 2013) and ultrasonic cleaners (Hatta et al. 2014) also have been used to accelerate decalcification.

Traditionally, physical, chemical and radiographic methods have been used to determine the end point of decalcification. Apart from these methods, end point determination by precise calculation of residual calcium in the tissues deserves mention. Colorimetric (Muller et al. 1990), flame photometric (Van Wyk 1987) and spectrophotometric techniques (Kiviranta et al. 1980) have

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been used to detect residual calcium in tissue after decalcification.

We attempted to develop an ideal decalcifying agent and technique that balances rapid decalcification with preservation of staining characteristics to facilitate the study of hard dental tissues.

Material and methods

We used 80 freshly extracted, periodontally compromised molar teeth. The study groups comprised 10 samples each for decalcification by one of five conventional decalcifying agents including formic acid-formalin, formal-nitric acid, formalin-EDTA, von Ebner's solution, Perenyi's fluid and three techniques including application of agitation, electric current or heat (Table 1) (Drury and Wallington 1980, Callis 2008).

For fixation of the pulp tissue, a coronal access to the pulp chamber was prepared on freshly extracted teeth using an endodontic air rotor. Approximately 2 mm of the apical root was cut using a rotary disc to access the radicular pulp. Formalin was injected using a 24 gauge syringe into the root canals for fixation of pulp tissue.

All tooth specimens were immersed in 10% buffered formalin for 24 h. After fixation, each freshly extracted tooth was suspended with thread tied to glass rods so that the specimen was in uniform contact with the decalcifying agent. The decalcifying agent was contained in a 1 l glass beaker at room temperature, except for the heating technique. The volume for all decalcifying agents was 300 ml and the bath was changed every 3 days.

Electric current, agitation and heat were used to accelerate decalcification by conventional formic acid-formalin. For decalcification with electric current, a customized electrolytic cell was constructed using formic acid-formalin solution as the electrolyte and carbon rods as electrodes (GeIfand and Richman 1952) with electric current supplied by a 6 V DC adapter. The temperature range of the electrolyte solution was 25 – 35° C.

For decalcification with agitation, a glass beaker filled with formic acid-formalin was placed on a magnetic stirrer with a 9 × 22 mm magnetic bar revolving continuously at 400 rpm (2MLH; Remi Elektrotechnik Ltd., Vasai, India). The tooth samples were suspended in formic acid-formalin as described above. We detected no change in the temperature of the decalcifying agent during agitation.

For decalcification with heat, a glass beaker containing formic acid-formalin, with tooth samples suspended in it, was placed in a water bath (Alco, Ambala, India) maintained at 40° C.

All specimens were checked periodically and the end point of decalcification was determined by radiographic (Fig. 1) and chemical methods (Culling 1974, Drury and Wallington 1980). After decalcification, the specimens were washed under running tap water overnight, then processed in an automatic tissue processor (Leica Biosystems, Buffalo Grove, IL). The specimens were dehydrated through a series of alcohols from 70% to absolute. Specimens then were cleared with xylene and embedded in paraffin wax. The blocks were sectioned at 6 µm. The sections were stained with hematoxylin and eosin (H & E) (Callis 2008). Ease of sectioning was evaluated and scored for each tooth specimen as easy, difficult or very difficult, according to the criteria reported by Prasad et al. (2013). Staining characteristics of the H & E stained

Table 1. Composition of decalcification agents and techniques (Drury and Wallington 1980, Callis 2008)

Serial no.	Study groups	Composition
1	Formic acid-formalin	5 – 10 ml 90% concentrated formic acid diluted with 5 ml 37–40% formaldehyde and distilled water to form 100 ml solution
2	Formal-nitric acid	5 ml formalin, 7.5 – 15 ml nitric acid, distilled water to 100 ml
3	Formalin-EDTA	5.5 g EDTA, disodium salt, 90 ml distilled water, 10 ml 37 – 40% formaldehyde
4	Von Ebner's	15 ml conc. hydrochloric acid, 175 g sodium chloride, distilled water to 1,000 ml
5	Perenyi's Fluid	40 ml 10% nitric acid, 30 ml absolute ethanol, 30 ml 0.5% chromic acid
6	Formic acid with electric current	5 – 10 ml 90% stock formic acid, 5 ml 37–40% formaldehyde, distilled water to 100 ml
7	Formic acid with agitation	5 – 10 ml 90% stock formic acid, 5 ml 37 – 40% formaldehyde, distilled water to 100 ml
8	Formic acid with heat	5 – 10 ml 90% stock formic acid, 5 ml 37 – 40% formaldehyde, distilled water to 100 ml

Ten samples/group.

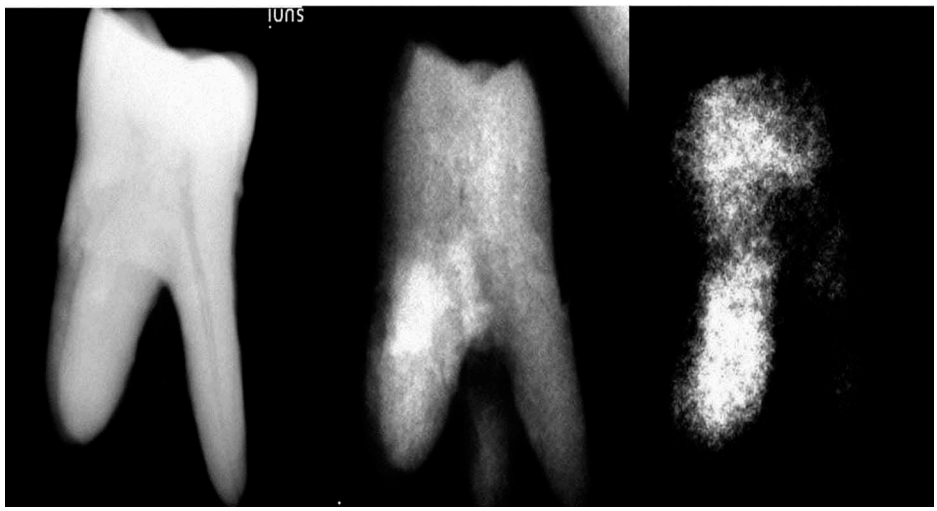

Fig. 1. Radiograph showing end point determination of decalcification of teeth.

sections were evaluated and graded subjectively as adequately stained, understained or overstained.

The presence or absence of histopathology including dentin destruction, cementum destruction, pulp organization and tissue artifacts was recorded according to Prasad and Donoghue (2013).

Statistical analysis

The data compiled were analyzed using SPSS software, version 17 (Chicago, IL). Results are reported as means ± SD or as number and percentage. The times required for decalcification were subjected to one-way ANOVA (F-test) to determine whether there was an overall difference between the groups. To confirm which specific groups differed from others, we used the Tukey *post hoc* test. Inter- and intragroup comparisons were performed for nonparametric data. The Chi-square test was used for analyzing categorical data. Values for $p \leq 0.05$ were considered statistically significant.

Table 2. Time required for complete decalcification

Study groups	Time required for decalcification (days)
Formic acid-formalin	55.4 ± 4.03
Formal-nitric acid	5.3 ± 1.70
Formalin-EDTA	83.8 ± 12.89
Von Ebner's solution	28.3 ± 3.74
Perenyi's Fluid	6.0 ± 0.94
Formic acid with electric current	26.5 ± 3.62
Formic acid with agitation	22.7 ± 2.90
Formic acid with heat	20.4 ± 4.57
F value	229.815
p value	0.000

Data are means ± S.D of all eight groups and are compared by F test (ANOVA) to yield significance. Turkey *post hoc* test was applied to compare each group separately with the others for significance.

Results

The time required for complete decalcification was least in formal-nitric acid and required 5.3 ± 1.7 days, while formalin-EDTA decalcification required the longest time, 83.8 ± 12.89 days. The difference was statistically significant ($p = 0.000$) (Table 2).

Differences among formol-nitric acid and Perenyi's fluid, von Ebner's and formic acid with electric current, von Ebner's and formic acid with heat, formic acid with electric current and formic acid with agitation, formic acid with electric current and formic acid with heat, formic acid with agitation and formic acid with heat were not significantly different.

All teeth decalcified in formic acid with heat were easy to section. Sectioning was most difficult for teeth decalcified in formalin-EDTA and Perenyi's fluid (30% each). Differences among all groups were statistically significant ($p = 0.0160$) (Table 3).

Ninety percent of hard tissue sections decalcified in formic acid with agitation exhibited adequate H & E staining, 80% each for formic acid-formalin, formalin-EDTA and Perenyi's fluid, and 50% for formol-nitric acid and formic acid with heat. Differences in hard tissue staining among

Table 3. Comparison of ease of sectioning

Study groups	Easy	Difficult	Very difficult	*p*
Formic acid-formalin	7 (70%)	3 (30%)	0	0.205
Formal-nitric acid	7 (70%)	3 (30%)	0	0.205
Formalin-EDTA	3 (30%)	4 (40%)	3 (30%)	0.904
Von Ebner's solution	8 (80%)	2 (20%)	0	0.057
Perenyi's fluid	3 (30%)	5 (50%)	2 (20%)	0.496
Formic acid with electric current	5 (50%)	5 (50%)	0	1.000
Formic acid with agitation	6 (60%)	3 (30%)	1 (10%)	0.149
Formic acid with heat	10 (100%)	0	0	0.000

Intragroup comparison for easy, difficult and very difficult sections after chi-square analysis (three values) is given for each group. Intergroup comparison after Chi-square analysis (24 values), $p = 0.0160$.

the acid treated groups were statistically significant ($p = 0.0472$) (Table 4, Fig. 2a, b).

Formic acid-formalin decalcification caused no destruction of dentin. (Table 5, Fig. 2c, d); formal-nitric acid caused the greatest destruction. (Table 6, Fig. 2e, f)

Pulp morphology was preserved best by decalcification with formalin-EDTA (Table 7, Fig. 3a, b). Formic acid with electric current produced the most sections with artifacts. (Table 8, Fig. 4c, d)

When formic acid-formalin and formic acid with electric current were compared individually for each parameter, the time required for decalcification was significantly less in the formic acid with electric current group, ($p = 0.000$), but the formic acid-formalin sections were considerably easier to cut ($p = 0.003$). Similarly, formic acid-formalin decalcified sections exhibited significantly better hard tissue staining ($p = 0.002$) and pulp organization ($p = 0.004$) with significantly less dental tissue destruction for dentin and cementum destruction, and artifacts ($p = 0.000$) (Table 9).

Formic acid with agitation decalcification was significantly more rapid than conventional formic acid-formalin ($p = 0.000$). These two groups were equivalent with regard to ease of sectioning, hard tissue staining, dentin destruction, pulp organization and occurrence of artifacts. Cementum destruction was significantly greater in the sections decalcified with formic acid with agitation ($p = 0.047$) (Table 10).

We also found statistically significant differences between conventional formic acid-formalin decalcification and the heat technique (Table 11). Although decalcification with formic acid with heat was completed in a significantly shorter time ($p = 0.000$), formic acid-formalin samples were easier to section ($p = 0.000$), exhibited better hard tissue staining ($p = 0.000$) and pulp organization ($p = 0.004$). Also, sections decalcified with formic acid-formalin exhibited less destruction of dentin and cementum ($p = 0.001$ and 0.000, respectively) and fewer artifacts ($p = 0.000$).

Discussion

Many decalcifying agents have been developed in search of a balance between rapid decalcification and preservation of morphology. We found that

Table 4. Comparison of hard tissue staining

Study groups	Adequately stained	Understained	Overstained	*p*
Formic acid-formalin	8 (80%)	2 (20%)	0	0.057
Formal-nitric acid	5 (50%)	2 (20%)	3 (30%)	0.496
Formalin-EDTA	8 (80%)	1 (10%)	1 (10%)	0.007
Von Ebner's solution	7 (70%)	3 (30%)	0	0.205
Perenyi's fluid	8 (80%)	2 (20%)	0	0.057
Formic acid with electric current	6 (60%)	4 (40%)	0	0.527
Formic acid with agitation	9 (90%)	0	1 (10%)	0.011
Formic acid with heat	5 (50%)	5 (50%)	0	1.000

Intragroup comparison for adequately stained, understained, and overstained sections after Chi-square analysis (three values) is given for each group. Intergroup comparison after Chi-square analysis (24 values), $p = 0.0472$.

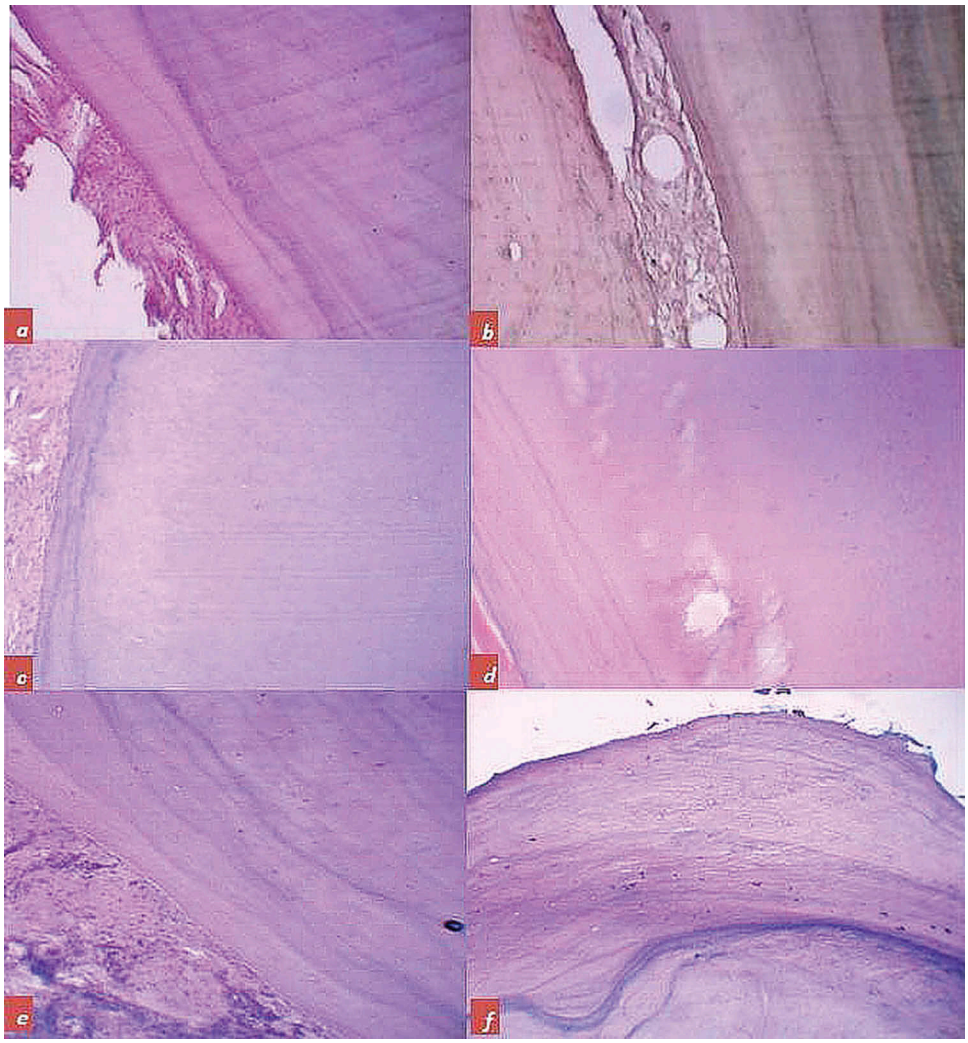

Fig. 2. Top row) Left to right: adequately (a) and understained (b) hard tissue section. H & E stain. Middle row) Left to right: absence (c) and presence (d) of dentin destruction. Bottom row) Left to right: absence (e) and presence (f) of cementum destruction.

formol-nitric acid required the least time for complete decalcification, whereas formalin-EDTA required the most time, which is consistent with the literature (Sanjai et al. 2012, Prasad and Donoghue (2013). Strong acids decalcify most rapidly followed by weak organic acids and chelating acids (Culling 1974). Application of electric current, agitation or heat reduced the time required for formic acid-formalin to effect decalcification. The effect of agitation on the rate of decalcification has been reported earlier (Verdenius and Alma 1958, Birkedal-Hansen 1974). The effect of electric current on the rate of decalcification that we observed was consistent with previous reports in which the acceleration was due to increased temperature owing to the electric current (Dolan 1951, GeIfand and Richman 1952, Verdenius and Alma 1958, Coleman and Desalva 1966). Similarly, heat increased the diffusion of reaction products, which decreased the decalcification time (Verdenius and Alma 1958).

We found that samples decalcified in formic acid with heat were easiest to section. Formalin-EDTA sections were friable and were the most difficult to section. Our findings were contrary to earlier reports that formalin-EDTA decalcified tissues were easiest to section (Sanjai et al. 2012, Prasad and Donoghue 2013). Our findings may have been due to the long exposure of the tissue to slow EDTA chelation. Complete decalcification of the center of hard tissues of teeth using a slow decalcification agent means that the superficial tissues are in contact with the agent for a long period. This may lead to over-

Table 5. Comparison of dentin destruction

Study groups	Present	Absent	*p*
Formic acid-formalin	0	10 (100%)	0.000
Formal-nitric acid	7 (70%)	3 (30%)	0.205
Formalin-EDTA	2 (20%)	8 (80%)	0.057
Von Ebner's solution	3 (30%)	7 (70%)	0.205
Perenyi's fluid	5 (50%)	5 (50%)	1.000
Formic acid with electric current	9 (90%)	1 (10%)	0.011
Formic acid with agitation	2 (20%)	8 (80%)	0.057
Formic acid with heat	7 (70%)	3 (30%)	0.205

Intragroup comparison for destruction and no destruction of dentin after Chi-square analysis (Two values) is shown for each group. Intergroup comparison after Chi-square analysis (16 values), $p = 0.0002$.

sections (Zappa et al. 2005, Prasad and Donoghue 2013). To the contrary, Gupta et al. (2014) reported superior hard and soft tissue staining results after formal-nitric acid decalcification compared to formic acid-EDTA. We found that the use of heat to accelerate decalcification with formic acid with electric current and formic acid with heat produced poor staining.

We found that formic acid-formalin decalcification damaged dentin least followed by formic acid with agitation and formalin-EDTA. Formic acid with electric current, formal-nitric acid and formic acid with heat caused the most damaged to the dentin. Heat and strong acids caused fraying of

Table 6. Comparison of cementum destruction

Study groups	Present	Absent	*p*
Formic acid-formalin	2 (20%)	8 (80%)	0.057
Formal-nitric acid	8 (80%)	2 (20%)	0.057
Formalin-EDTA	4 (40%)	6 (60%)	0.527
Von Ebner's solution	1 (10%)	9 (90%)	0.011
Perenyi's fluid	3 (30%)	7 (70%)	0.205
Formic acid with electric current	6 (60%)	4 (40%)	0.527
Formic acid with agitation	1 (10%)	9 (90%)	0.011
Formic acid with heat	6 (60%)	4 (40%)	0.527

Intragroup comparison for destruction and no destruction of cementum (two values) is shown for each group. Intergroup comparison after Chi-square analysis (16 values), $p = 0.006$.

Table 7. Comparison of pulp organization

Study groups	Present	Absent	*p*
Formic acid-formalin	6 (60%)	4 (40%)	0.527
Formal-nitric acid	5 (50%)	5 (50%)	1.000
Formalin-EDTA	8 (80%)	2 (20%)	0.0577
Von Ebner's solution	6 (60%)	4 (40%)	0.527
Perenyi's fluid	4 (40%)	6 (60%)	0.527
Formic acid with electric current	4 (40%)	6 (60%)	0.527
Formic acid with agitation	7 (70%)	3 (30%)	0.205
Formic acid with heat	4 (40%)	6 (60%)	0.527

Intragroup comparison for intact and altered pulp organization after chi-square analysis (two values) is shown for each group. Intergroup comparison after Chi-square analysis, $p = 0.4866$.

decalcification of superficial tissues of teeth, and crumbling during sectioning.

We found excellent staining results after decalcification with formic acid with agitation; staining was poorest after formal-nitric acid decalcification. More rapid decalcification by strong acids caused greater tissue injury and the effects were evident in H & E dentinal tubules and disruption of odontoblast architecture (Prasad and Donoghue 2013).

We observed that formal-nitric acid decalcification caused the greatest damage to the cementum followed by formic acid with electric current and formic acid with heat; the lytic effects of strong acids and heat on the

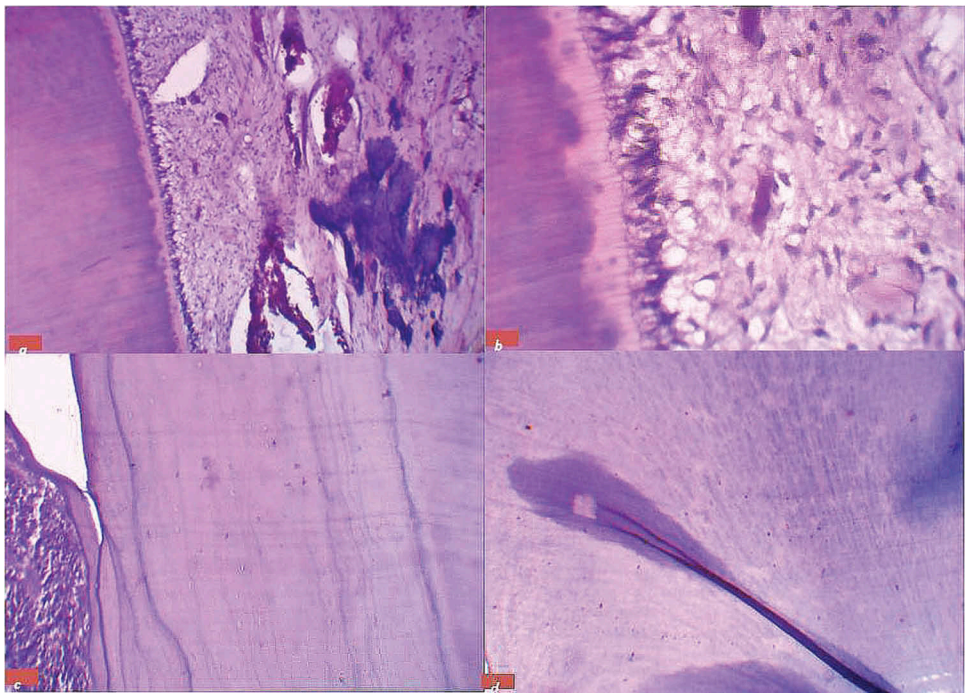

Fig. 3. Upper row) Left to right: best (a) and worst (b) pulp organization. Lower row) Left to right: tissue separation (c) and tissue folding (d) artifacts. H & E stain.

Table 8. Comparison of artifacts

Study group	Present	Absent	*p*
Formic acid-formalin	6 (60%)	4 (40%)	0.527
Formal-nitric acid	9 (90%)	1 (10%)	0.011
Formalin-EDTA	8 (80%)	2 (20%)	0.057
Von Ebner's solution	7 (70%)	3 (30%)	0.205
Perenyi's fluid	7 (70%)	3 (30%)	0.205
Formic acid with electrophoresis	10 (100%)	0	0.000
Formic acid with agitation	6 (60%)	4 (40%)	0.527
Formic acid with heat	9 (90%)	1 (10%)	0.011)

Intragroup comparison for presence and absence of artifacts after chi-square analysis (two values) is shown for each group. Intergroup comparison after Chi-square analysis (16 values), $p = 0.0445$.

superficial tissues of the teeth could be an explanation.

Differences in pulp organization among the eight groups were not statistically significant. We found that pulp histology was equivalent for all eight groups. Prasad et al. (2013) reported similar findings.

Obtaining good histological results for calcified tissues frequently is not possible without some damage to the soft tissues. Rapid and complete decalcification of hard tissues using strong acids and heat usually compromises the soft tissue histology; therefore, it is difficult to fulfill the requirements for simultaneous analysis of mineralized and non-mineralized tissues (Fernandes et al. 2007). We found that artifacts such as soft tissue separation from hard tissue, and tissue tears and folds were most common after formic acid with electric current decalcification and next most common after decalcification with formic acid with heat. We observed the fewest artifacts after decalcification using formic acid-formalin and formic acid with agitation.

We used carbon rods as electrodes for the formic acid with electric current technique. We observed carbon precipitation superficially, which blackened the surface of the teeth; this was evident also in histologic sections of the superficial tissues.

Weak acids such as formic acid generally are best for decalcification. We found that formic acid

Table 9. Comparison of decalcification with formic acid-formalin with formic acid with electric current

Characteristic		Formic acid-formalin	Formic acid with electric current	*p*
Time required for decalcification (mean days ± SD)		55.4 ± 4.03	26.5 ± 3.62	0.000
Ease of sectioning	Easy	7 (70%)	5 (50%)	0.003
	Difficult	3 (30%)	5 (50%)	
	Very difficult	0	0	
Hard tissue staining	Adequately stained	8 (80%)	6 (60%)	0.002
	Understained	2 (20%)	4 (40%)	
	Overstained	0	0	
Dentin destruction	Present	0	9 (90%)	0.000
	Absent	10 (100%)	1 (10%)	
Cementum destruction	Present	2 (20%)	6 (60%)	0.000
	Absent	8 (80%)	4 (40%)	
Pulp organization	Present	6 (60%)	4 (40%)	0.004
	Absent	4 (40%)	6 (60%)	
Artifacts	Present	6 (60%)	10 (100%)	0.000
	Absent	4 (40%)	0	

To compare two particular groups for each criterion, the *p* value was derived individually for each. The *p* value was obtained after Tukey *post hoc* comparison for the first criterion, i.e., time required for decalcification. The *p* values shown were obtained after chi-square analysis for the remaining parameters, i.e., ease of sectioning, hard tissue staining, dentin destruction, cementum destruction, pulp organization and artifacts.

Table 10. Comparison of formic acid-formalin with formic acid with agitation

Characteristic		Formic acid-formalin	Formic acid with agitation	*p*
Time required for decalcification (mean days ± SD)		55.4 ± 4.03	22.7 ± 2.90	0.000
Ease of sectioning	Easy	7 (70%)	6 (60%)	0.621
	Difficult	3 (30%)	3 (30%)	
	Very difficult	0	1 (10%)	
Hard tissue staining	Adequately stained	8 (80%)	9 (90%)	0.1560
	Understained	2 (20%)	0	
	Overstained	0	1 (10%)	
Dentin destruction	Present	0	2 (20%)	0.136
	Absent	10 (100%)	8 (80%)	
Cementum destruction	Present	2 (20%)	1 (10%)	0.047
	Absent	8 (80%)	9 (90%)	
Pulp organization	Present	6 (60%)	7 (70%)	0.138
	Absent	4 (40%)	3 (30%)	
Artifacts	Present	6 (60%)	6 (60%)	1.000
	Absent	4 (40%)	4 (40%)	

To compare two particular groups for each criterion, the *p* value was derived individually for each. The *p* value was obtained after Tukey *post hoc* comparison for the first criterion, i.e., time required for decalcification. The *p* values shown were obtained after chi-square analysis for the remaining parameters, i.e., ease of sectioning, hard tissue staining, dentin destruction, cementum destruction, pulp organization and artifacts.

with agitation was equivalent to conventional formic acid-formalin for preserving normal histology while minimizing decalcification time. Chelating agents such as EDTA preserve morphological integrity and stainability, but required a long decalcification period and sections of this tissue were difficult to handle. Strong acids such as nitric acid and hydrochloric acid decalcified tissue rapidly, but caused considerable impairment of staining and significant tissue destruction. Therefore, we recommend formic acid with agitation for decalcification of teeth.

Table 11. Comparison of formic acid-formalin formic acid with heat

Parameter		Formic acid-formalin	Formic acid with heat	*p*
Time required for decalcification (mean days ± SD)		55.4 ± 4.03	20.4 ± 4.57	0.000
Ease of sectioning	Easy	7 (70%)	10 (100%)	0.000
	Difficult	3 (30%)	0	
	Very difficult	0	0	
Hard tissue staining	Adequately stained	8 (80%)	5 (50%)	0.000
	Understained	2 (20%)	5 (50%)	
	Overstained	0	0	
Dentin destruction	Present	0	7 (70%)	0.001
	Absent	10 (100%)	3 (30%)	
Cementum destruction	Present	2 (20%)	6 (60%)	0.000
	Absent	8 (80%)	4 (40%)	
Pulp Organization	Present	6 (60%)	4 (40%)	0.004
	Absent	4 (40%)	6 (60%)	
Artifacts	Present	6 (60%)	9 (90%)	0.000
	Absent	4 (40%)	1 (10%)	

To compare two particular groups for each criterion, the *p* value was derived individually for each. The *p* value was obtained after Tukey *post hoc* comparison for the first criterion, i.e., time required for decalcification. The *p* values shown were obtained after chi-square analysis for the remaining parameters, i.e., ease of sectioning, hard tissue staining, dentin destruction, cementum destruction, pulp organization and artifacts.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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