ORIGINAL PAPERS

Dent. Med. Probl. 2005, **42**, 1, 21–26
ISSN 1644-387X

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Comparison of Different Decalcification Methods to Hard Teeth Tissues Morphological Analysis

Porównanie różnych metod odwapniania do analizy morfologicznej twardych tkanek zębów

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Abstract

Background. There is a number of decalcification methods, however, each of them – apart from series of advantages – has its own drawbacks.
Objectives. In this elaboration, several various methods have been used in different time intervals, so to determine which of them should be used while standard histological examination is performed, to achieve the most favourable morphological image in the shortest decalcification time.
Material and Methods. The examination was carried out for 288 teeth coming from routine extractions. All extracted teeth were fixed in 10% neutralised formalin solution and then the teeth were divided into six groups, each of them was divided into sub-categories containing teeth of normal structure and those affected by decay. Each of the sub-categories contained 8 teeth: incisors and canines, premolars and molars. Individual groups were subjected to decalcification as follows: 1. 10% disodium versenate solution – EDTA. 2. 10% disodium versenate solution and TRIS. 3. Electrolytic in Romeis fluid (hydrochloric and formic acid solution) with the aid of PW27 bone decalcifying agent and the current of 0.5 A. 4. Electrolytic in Romeis fluid enriched with TRIS with the aid of PW27 bone decalcifying agent and the current of 0.5 A. 5. 7% nitric acid solution (HNO_3). 6. Formic acid solution (HCOOH) with 70% ethylic alcohol in 1:1 ratio. Some of the decalcifications were performed in two temperature variants – at the room temperature and at 37°C.
Results. It appears that the best method to be employed for routine histopathology diagnostics is to decalcify teeth in the Romeis fluid. For educational purposes and scientific research requiring preparations of a very high quality, when especially fast decalcification is not necessary, the disodium versenate has proved to serve best for this application (**Dent. Med. Probl. 2005, 42, 1, 21–26**).

Key words: decalcification methods, hard tooth tissues.

Streszczenie

Wprowadzenie. Istnieje wiele metod odwapniania, ale każda z nich, oprócz wielu zalet, ma swoje wady.
Cel pracy. W pracy zastosowano kilka metod w różnym przedziale czasowym, aby ustalić, którą z nich należałoby stosować podczas przeprowadzania standardowych badań histologicznych, aby uzyskać najlepsze obrazy morfologiczne przy najkrótszym czasie odwapniania.
Materiał i metody. Badania przeprowadzono na 288 zębach uzyskanych podczas rutynowych ekstrakcji. Wszystkie zęby po ekstrakcji były utrwalane w 10% zobojętnionym roztworze formaliny, a następnie zostały podzielone na 6 grup. W obrębie każdej z nich wyróżniono podgrupy, do których zakwalifikowano zęby o budowie prawidłowej i ze zmianami próchniczymi. W każdej z podgrup znajdowało się 8 zębów: zęby sieczne wraz z kłami, zęby przedtrzonowe i trzonowe. Poszczególne grupy były odwapniane w: 1. 10% roztworze wersenianu dwusodowego – EDTA. 2. 10% roztworze wersenianu dwusodowego i TRIS. 3. Elektrolitycznie w płynie Romeisa (roztwór kwasu solnego i mrówkowego) przy użyciu odwapniacza kości PW27 i natężeniu prądu 0,5 A. 4. Elektrolitycznie w płynie Romeisa wzbogaconym TRIS przy użyciu odwapniacza kości PW27 i natężeniu prądu 0,5 A. 5. W 7% roztworze kwasu azotowego (HNO_3). 6. W roztworze kwasu mrówkowego (HCOOH) z 70% alkoholem etylowym w stosunku 1:1. Część odwapnień dokonano w dwóch wariantach temperaturowych – w temperaturze pokojowej i w 37°C.
Wyniki. Wyniki przeprowadzonych badań wskazują na to, że najlepszą metodą do zastosowania w rutynowej diagnostyce histopatologicznej jest odwapnianie zębów w płynie Romeisa. Do celów dydaktycznych i badań naukowych, wymagających otrzymania preparatów o bardzo dobrej jakości bez konieczności ich szybkiego odwapniania, najlepiej stosować wersenian dwusodowy (**Dent. Med. Probl. 2005, 42, 1, 21–26**).

Słowa kluczowe: metody odwapniania, twarde tkanki zęba.

Teeth belong to the hardest tissues due to tooth enamel, which is denser and chemically more inert than other body tissues. Because of big contents in teeth, calcium and phosphorus, from which teeth inorganic substance is composed – biological apatite is very hard to prepare for microscopic examinations.

On the other hand, complex teeth structure and first of all extraordinary enamel hardness makes this organ very difficult to observe under microscope.

Diamond knifes make possible cutting hard tooth tissues, but due to the costs, other methods of hard teeth tissue decalcification are used. Electron microscope allows for indirect estimation of tooth surface. Dynamic development of electronics and visualization techniques will probably make watching hard tooth tissue possible in a very precise way without using decalcificative substances.

The aims of this experiment were:

1. Composition of teeth morphological images after using different decalcification methods, obtained in microscope.

2. Establishment of the fastest teeth decalcification methods.

3. Determination of the fastest way of teeth decalcification in accordance with their morphological images.

Material and Methods

The examinations were carried out for 288 teeth coming from routine extractions and from orthodontic, prosthetic reasons. All extracted teeth were washed in normal salt solution and then fixed in 10% neutralized formalin solution. The teeth were divided into 6 groups depending on the substance in which teeth were decalcified:

1. 10% disodium versenate solution – EDTA.

2. 10% disodium versenate solution and TRIS.

3. Electrolytic in Romeis fluid (hydrochloric and formic acid solution) with the aid of PW27 bone decalcifying agent and the current of 0.5 A.

4. Electrolytic in Romeis fluid enriched with TRIS with the aid of PW27 bone decalcifying agent and the current of 0.5 A.

5. 7% nitric acid solution (HNO_3).

6. Formic acid solution (HCOOH) with 70% ethylic alcohol in 1:1 ratio.

Each group was divided into subgroups containing teeth of normal structure and those affected by decay. Each subcategory contained 8 teeth: incisors including canines, premolars and molars.

Teeth are decalcified in room temperature and incubated at 37°C. Teeth in concentrated nitric acid and in Romeis fluid with or without this were not decalcified in incubator. For every tooth, 100% ml substances for decalcification were used. Decalcification time which is indispensable for getting tissues for further examinations was observed depending on decalcificative substance. Efficacy of decalcification was checked by specimen picture in 8-hour distance. When needle passed through whole specimen thickness, decalcification process was over. After decalcification, teeth were cut in vertical axis, revealing the pulp. Specimens prepared this way were lead by alcohols, acetone, xylene and then embedded in paraplast. Received blocks were cut on microtome for 4-6 microns slices and were stained with hematoxylin-eosin method. All specimens were closed by cover glasses and were observed in microscope in 40–400 magnification. The pictures of teeth enamel, dentin, cement, pulp and surrounding tissues were compared. Changes which appear because of the caries were also estimated. It was assumed that basing on morphological analysis it was possible to establish the best teeth decalcification methods, which were indispensable for routine histopathological diagnostics and experiments. Elements of the teeth structure were estimated qualitatively, considering conduction of their microscopical structure after decalcification. That is why 3–1 points score was used, where 3 means the best conducted structure, and 1, the worst. 8-point score for estimation of time decalcification also was created, where 1 means the longest decalcification time, and 8, the shortest one – 10-day time.

Results

The best results – clear morphological images – were obtained after decalcification in 10% EDTA and in 10% EDTA + TRIS and in both temperature ranges (Fig. 1). Pulp structure and organic dentin matrix, cement and periodontium ligaments were very well preserved. Teeth type and their health condition did not matter in results of decalcification process and morphological pictures which were electrolytic decalcificated in Romeis fluid, and in the teeth decalcified in Romeis fluid with TRIS had very well preserved structure of pulp elements with very good odontoblasts layer visibility (Fig. 2). Slightly connective fibrous tissue was observed in the pulp (Fig. 3). In teeth with advanced caries, electrolytic decalcification method in Romeis fluid showed very well preserved teeth structure with caries (Fig. 4).

Groups which were decalcified in 7% nitric acid had destroyed or blurred teeth structure. Especially, there were teeth with caries (Fig. 5).

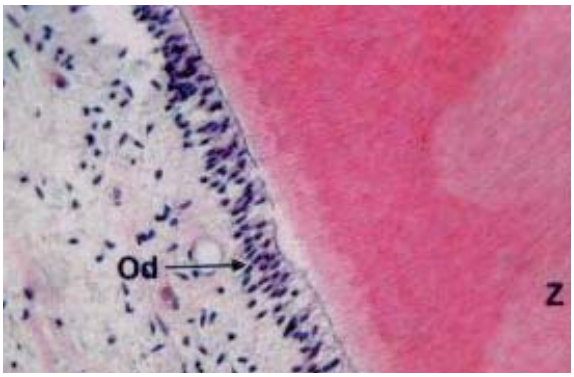

Fig. 1. Incisor, 10% EDTA, temp. 37°C, ×200, z – dentin, od – odontoblasts

Ryc. 1. Ząb sieczny,10% EDTA, temp. 37°C, 200×, z – zębina, od – odontoblasty

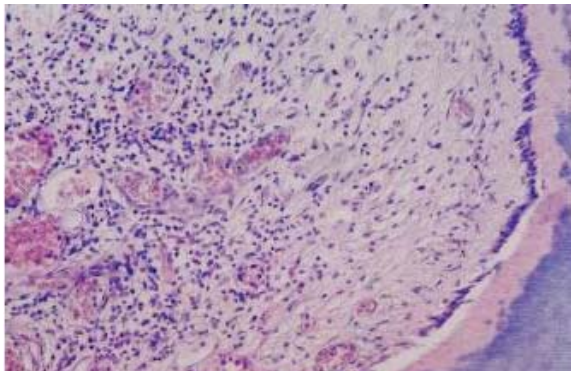

Fig. 4. Electrolytic decalcification method in Romeis fluid enriched with TRIS, ×200

Ryc. 4. Metoda elektrolityczna w płynie Romeisa wzbogacona TRIS, 200×

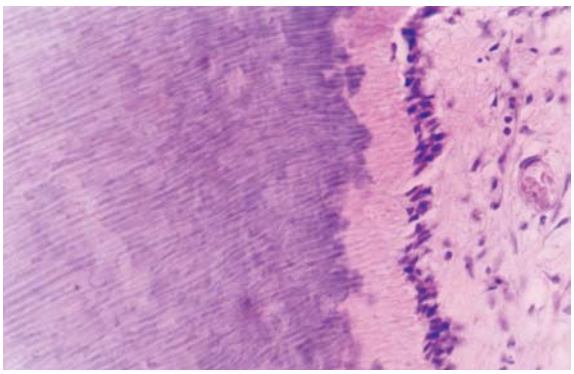

Fig 2. Pulp and odontoblasts, electrolytic decalcification method in Romeis fluid enriched with TRIS, ×400

Ryc. 2. Miazga i odontoblasty, metoda elektrolityczna w płynie Romeisa wzbogacona TRIS, 400×

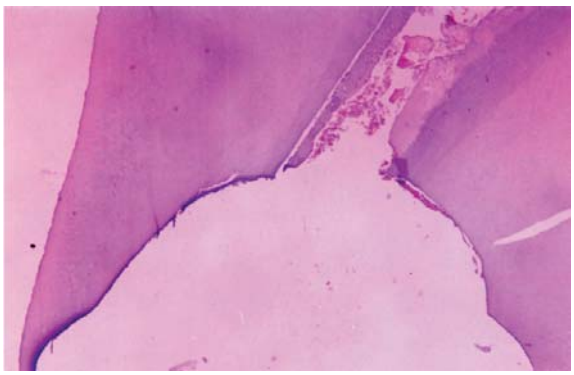

Fig. 5. Root pulp, nitric acid, ×100

Ryc. 5. Miazga korzeniowa, kwas azotowy, 100×

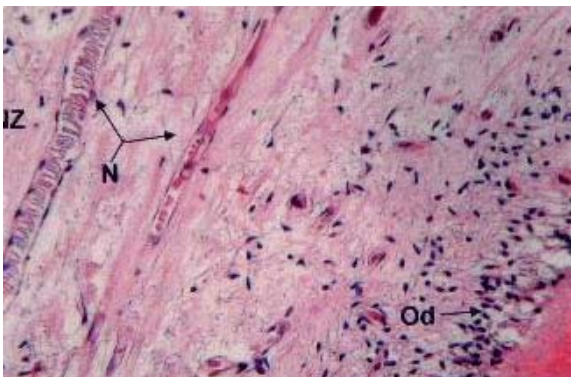

Fig. 3. Incisor, EDTA + TRIS, z – dentin, od – odontoblasts, n – vessel, ×200

Ryc. 3. Ząb sieczny, EDTA + TRIS, z – zębina, od – odontoblasty, n – naczynia, 200×

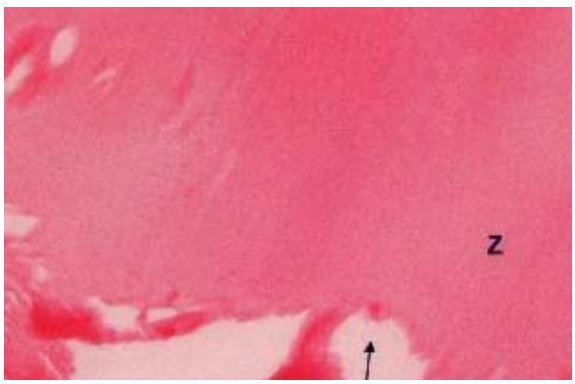

Fig. 6. Molar tooth with caries, formic acid, ×200

Ryc. 6. Ząb trzonowy z próchnicą, kwas mrówkowy, 200×

Root pulp was much destroyed. Image of individual components was blurred and invisible, like odontoblasts layer. Surrounding tissue image was also blurred and indistinct. Pulp organization in healthy and ill teeth was worse after formic acid decalcification than after in EDTA and EDTA + TRIS method (Fig. 6). Decalcification times in particular teeth groups after using different decalcification methods are showed in Table 1 and 2. Points estimation of decalcification teeth time shows that the fastest decalcification is with using electrolytic method and Romeis fluid (7 points)

Table 1. Conditions of investigation

Tabela 1. Warunki przeprowadzania badań

Decalcification condition (Warunki odwapniania)	Lesion in teeth (Zmiany w zębach)	Kind of teeth (Rodzaj zębów)	Decalcification methods and time – days (Metody i czas odwapniania – doby)					
			EDTA	EDTA+TRIS	ROMEIS	ROMEIS+TRIS	HNO_3	HCOOH
Room temperature (Temperatura pokojowa)	no lesions (bez zmian)	INCISORS PREMOLARS MOLARS	77 77 77	65 65 65	14 14 17	10 10 11	25 26 26	41 41 42
	caries (próchnica)	INCISORS PREMOLARS MOLARS	76 76 77	63 64 66	14 14 15	10 10 11	25 25 26	39 39 41
37°C	no lesions (bez zmian)	INCISORS PREMOLARS MOLARS	71 74 77	64 64 64				37 37 38
	caries (próchnica)	INCISORS PREMOLARS MOLARS	71 71 73	61 62 64				33 33 34

Table 2. Decalcification time in days

Tabela 2. Czas odwapniania w dobach

Decalcification method (Metoda odwapniania)	Room temperature (Temperatura pokojowa)		37°C	
	teeth without lesions (zęby bez zmian)	caries (próchnica)	teeth without lesions (zęby bez zmian)	caries (próchnica)
EDTA	77	76–77	71–77	71–73
EDTA+TRIS	65	63–66	64	61–64
ROMEIS	14–17	14–15	–	–
ROMEIS+TRIS	10–11	10–11	–	–
HNO_3	25–26	25–26	–	–
HCOOH	41–42	39–41	37–38	33–34

Table 3. Points estimation of teeth decalcification time

Tabela 3. Ocena punktowa czasu odwapniania zębów

Decalcification method (Metoda odwapniania)	Decalcification time (Czas odwapniania)	
	days (doby)	points (pkt)
10% EDTA	71–77	1
10% EDTA+TRIS	61–66	2
ROMEIS	14–17	7
ROMEIS + TRIS	10–11	7–8
HNO_3	25–26	6
HCOOH	33–42	4–5

> 10 days/dni	8 points/pkt
11–20 days/dni	7 points/pkt
21–30 days/dni	6 points/pkt
31–40 days/dni	5 points/pkt
41–50 days/dni	4 points/pkt
51–60 days/dni	3 points/pkt
61–70 days/dni	2 points/pkt
< 70 days/dni	1 point/pkt

and Romeis fluid and TRIS (7–8 points) (Table 3). Points estimation of teeth elements structure after decalcification shows that enamel, dentin, cementum, pulp and surrounding tissues structure were preserved the best after using 10% EDTA decalcification and 10% EDTA with TRIS (Table 4). It seems that the best method which could be used in routine histopathological diagnostics is teeth decalcification in Romeis fluid. For scientific experiments in which one needs very good quality specimens and fast decalcification is not a must, disodium versenate is the best.

Discussion

Preparation of soft or hard tissues is done to observe them under microscope process. For showing micromorphology of teeth, cervix for example, method using scanning electron microscope is very good [1]. On using this method, one cannot estimate whole teeth structure because during observing them under this kind of microscope epoxydous model reflects only dark images of normal tooth surfaces [2]. Preparation procedure of hard teeth tissues is started from its fixation. To achieve this, for example, buffered 4% glutaraldehyde is used [3].

Table 4. Points estimation of teeth elements structure after decalcification

Tabela 4. Ocena punktowa elementów budowy zębów po odwapnieniu

Decalcification method (Metoda odwapniania)	Teeth elements structure (Elementy budowy zęba)				Total – points (Łącznie – pkt)
	enamel and dentin (szkliwo i zębina)	cementum (cement)	pulp (miazga)	surrounding tissues (tkanki otaczające)	
10% EDTA	3	2	3	3	11
10% EDTA+TRIS	3	2	3	3	11
ROMEIS	2	2	3	2	9
ROMEIS+TRIS	2	2	2	3	9
HNO_3	2	2	1	1	6
HCOOH	2	1	1	1	5

Arber estimated mRNA hybridization in situ (ISH) hold teeth in 10% formalin for 6, 24, 72 hours. He showed that time of teeth tissues staying in formalin had no influence on ISH results [4]. For many years, scientists have tried to introduce new decalcificative substances or tried to modify known decalcification methods. Hence liquid temperature elevation, which not always provides the scientists with good effects [5, 6]. The authors have made the experiments in two temperatures: room temperature and 37°C. Decalcification process was slightly accelerated in teeth which were in incubator. Different temperatures did not influence the quality of histological specimens. Myers showed that 600°C temperatures are very destructive for teeth tissues. These teeth after decalcification had changes in the form of shrinkage of tissues or decalcification [7]. During decalcification, worse results were received during nitric acid decalcification and during formic acid decalcification. Decalcification lasted here for 25–42 days, which was not the longest time, but decalcificated teeth structures were destroyed [5]. Menzel, for examination of pulp diseases, removed enamel and then put pulp and dentin to methyl polimethacrylan and he cut specimens by microtome [8].

Described experiments confirm other authors' examinations, that the best morphological images can be obtained after teeth decalcification with using 10% EDTA and 10% EDTA with TRIS at 37°C and at room temperature [9]. In specimens which are decalcified with EDTA, one can examine mRNA and DNA [10]. The basic staining which is used for electronic microscope specimens estimation is hematoxylin and eosin [7]. In the experiments, the authors used just this kind of staining and obtained very good results. Some scientists try to modify this kind of staining [11, 12]. Experiments, which the authors performed, should attract attention to some preparations which are used for decalcification.

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Received: 17.03.2004
Revised: 31.03.2004
Accepted: 7.09.2004

Praca wpłynęła do Redakcji: 17.03.2004 r.
Po recenzji: 31.03.2004 r.
Zaakceptowano do druku: 7.09.2004 r.