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THE INFLUENCE OF DIFFERENT AGITATION TECHINQUES ON THE RESULTS OF TEETH DEMINERALIZATION BY ALKALINE SOLUTION OF EDTA

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In order to analyze biomineralized microscopic structures on the microscopic level, they must be previously subjected to histological in vitro demineralization process, which removes the inorganic component. The speed of demineralization and the influence on the preservation of tissue morphology are the two most important parameters for the choice of decalcifying solution. The solution of Na_2-EDTA (ethylenediaminetetraacetic acid - disodium salt), madein different concentrations, is considered to bethe slowest, but alsothe most reliable in means of demineralization. The aim of the study was to investigate the possible acceleration of tooth samples demineralization with alkalinized solution of Na_2-EDTA,using different conditions of agitation, as well as periodic renewal of the solution for demineralization. Twelve wisdom teeth were used for the purposes of research, extracted for orthodontic reasons. The process of demineralization was performed in the 18.6% aqueous solution of Na_2-EDTA. During 45 days, the decline of the specimen'sweight was measured for three physical conditions: the irradiation of the tooth halves samples in the demineralizing agent with the microwaves was used as an agitating technique, 24-hour stirring of the demineralizing agent with the tooth samples on the magnetic stirrer was applied, and the demineralization of the material on the room temperature. Each of these conditions was further subdivided into two groups: in one the demineralizing agent was constant during 45 days of the experiment, and in the second the demineralizing agent was replaced by the same volume of new solution every three days. Demineralized samples were routinely processed to microscopic slides, stained with hematoxylin and eosin. The preservation of dental structures up to cytological details of different cell types in dental pulp was evidenced on microscopic slides. Demineralization of teeth with alkalinized solution of Na_2-EDTA runs independently of examined types of agitation and/or renewal of chelating solution. It is possible to make a significant laboratory rationalization for in vitro tissue demineralization by excluding physical agitation, and frequent renewal of demineralizing solution. *Acta Medica Medianae 2015;54(4):24-31.*

Key words: *di-sodium EDTA, demineralization, mineralized tissues, histochemistry*

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Introduction

The problem of the preparation for microscopic analysis of mineralized connective tissues represents one of the greatest challenges ofhistological laboratory methodology. The mineralized tissues are naturally harder than "unmineralized", soft tissue structures, and therefore the process of histological preparation, cutting and analysis of these tissues meets many difficulties in routine laboratory work. Moreover, the consistency of mineralized tissues causes the uneven cutting speed of the knife through its harder and softer structures, leading to deformation of sliced material, thus diminishing the quality of morphological documentation analysis (1, 2).

Many mineralized tissues as well as structures that become mineralized during the ageing process or due to the pathological alterations, along with inorganic components of the matrix, possess also organic portion of the extracellular matrix that functions as a support and organizes their space orientation. The organic part of the extracellular matrix is always present, even in the highly mineralized tissues such as enamel (3). In vitro demineralization is a widely used technique in routine experimental laboratory work with the

purpose to equalize the consistencies of the hard and soft tissues, and to "expose" the organic matrix for future analysis. The process itself is based on the application of different reagents that remove the inorganic component from the biomineralized structures (4).

Bone demineralization is often an essential step in routine histopathology during the histological processing of bone samples, and has the important impact on the diagnostic process (5). The two most important criteria for choosing the appropriate demineralization technique are the speed of demineralization, and the degree of morphology and reactivity preservation in the examined tissues (2). Although some demineralizers remove the inorganic component quickly and completely, they may causethe decreased histochemical reactivity of the examined tissue or may damage itsorganic components (2, 4). The most common chemicals used with the purpose of demineralization are chelatingsubstances (EDTA) and various organic and inorganic acids. Inorganic acids, such as nitric acid (HNO_3), are very fast demineralizers, and they significantly diminish the quality of the morphology of the soft tissues as well as the quality of the histochemical staining of examined structures (5, 6). This is dependent on the acidity of the solution and the time it will take to decalcify. Thus, the quicker the decalcifications, the greater will be the damage and its effects on hematoxylin and eosinestaining. It was reported that the decalcificationprocess causes important molecular and morphological alterations in the tissues such as edema, vacuolation and ruptures not attributable to the pathologic condition (1).

On the other hand, EDTA solutions, made in different concentrations and/or in combinations with fixatives, are considered to be the slowest, but the most reliable demineralizers for a wide range of delicate morphological methods, including in situ hybridization (6, 7) and immunohistochemistry because of its antigen-preserving properties (8, 9).

The factors that should be taken in consideration when choosing the appropriate solution for demineralization are the wanted degree of preservation of tissues and its influence on the quality of staining. If the priority is fast demineralization, the nitric acid is the best agent to use.However, EDTA is the agent of choice if we want to achieve the best preservation of tissue and to have the maximum quality staining.

Aim

Bearing in mind that numerous papers emphasize the beneficial role of Na_2-EDTA in tissue morphology preservation during the demineralization process, but also point at the slow decalcination time, the aim of this research was to investigate the possibilityof accelerating the processof toothsamplesdemineralization with alkalinized solution ofEDTA, using different conditions of agitation, as well as byperiodicrenewal of demineralizationsolution.

Material and methods

Twelve impacted molars without signs of caries or other damage were obtained from the patients of both sexes (6 male and 6 female), aged 19-27 years. The teeth were extracted under local anesthesia during treatment. All the teeth were extracted at Department of Oral Surgery of ClinicofDentistry in Niš, in accordance with the principles of good dental practice and ethical principles. All the patients signed the written consent and were properly informed about the purpose of the research.

After the extraction, the teeth were fixated in 10% formaldehyde for 60 minutes, before being cracked open in two halves in buccolingual direction with the use of the autopsy chisel. The teeth halves were additionally fixated in 10% formaldehyde for another 24-48 hours.

Each chosen tooth half for the experiment was given a unique code and was treated as a separate object of analysis during the experimental procedure (Figure 1). After the fixation, all the teeth halves were measured on the analytical balance (KERN EG-620-3NM, Kern & Sohn GmbH, Balingen, Germany) with the precision of 0.1 mg, at the Institute for Biomedical Research of the Faculty of Medicine in Niš. The tooth halves were then rinsed in a tap water for 24 hours and were subjected to demineralization process in 18.6% Na_2-EDTA water solution, equilibrated to pH=7.75 by addition of NaOH pellets.

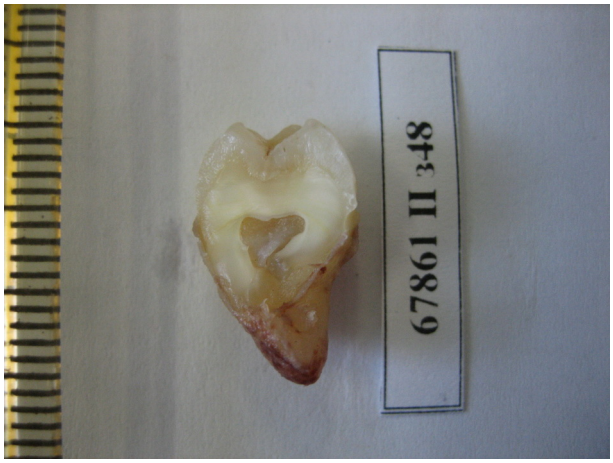

Figure 1. Sample of the halved third molar, calibrated and marked as a separate object of analysis.

Twelve tooth halves, divided into six groups, underwent the process of demineralization. They were exposed to a combination of three different physical conditions, and two states of demineralizing solution renewal (Table 1). Namely, the examined physical conditions were:1) the microwave irradiation of the tooth halves samples in the demineralizing agent, 2) stirring of the demineral-

Table 1. Experimental groups and methods of demineralization with Na_2-EDTA

	RT	RTR	MW	MWR	SM	SMR
Number of samples	2 molars	2 molars	2 molars	2 molars	2 molars	2 molars
Physical condition	Room temperature	Room temperature	Microwave oven	Microwave oven	Magnetic stirrer	Magnetic stirrer
Renewal of solution	No	Every 3 days	no	Every 3 days	no	Every 3 days

zing agent with the tooth samples on the magnetic stirrer, and 3) the demineralization of the material on the room temperature. In each of these three conditionsthere were two groups, in one the demineralizing agent was constant during 45 days of experiment, and in the second the demi-neralizing agent was replaced by the same volume of new solution on every three days. In total, six experimental groups were defined: 1) room temperature group without demineralizing solution renewal (RT), 2) room temperature group with demineralizing solution renewal (RTR), 3) microwave group without demineralizing solution renewal (MW), 4) microwave group with demineralizing solution renewal (MWR), 5) magnetic stirrer group without demineralizing solution renewal (SM), 6) magnetic stirrer group with demineralizing solution renewal (SMR) (Table 1). Each subgroup containedtwo halves of two different teeth that were subjected to demineralization in separate closed vessels, emerged into 50 ml of the demineralizing agent.

Microwave agitation was performed in a microwave oven (Samsung CE2717N) with the use of the time switch that turned on the magnetotron for 1 minute every 60 minutes during 16 hours (and 8 hours of break). The working power of the microwave agitation was 150 W on 100 ml of demineralizing agent and on a resulting temperature of 45°C. The stirring agitation had for a goal the constant moving of the demineralizing agent in the closed vessel with the tooth sample during 24 hour cycle, which was achieved by insertion of the magnetic pill into the vessel that was placed on a magnetic stirrer (AREHeatingMagneticStirrerC-16, VELPScientifica, EU). After 32 days of the demineralization of the tooth samples, we stopped measuring the samples weight due to the limited resolution power of the used analytical balance. The tooth samples were left in demineralizing agent until the day 45 from the beginning of the procedure with daily checking of sample consisten-cy. After 45 days, the samples had the consistency similar to the cartilage and were easily cut with the sharp blade in two pieces, parallel to the plane of the tooth samples fracture.

After 45 days of demineralization, the specimens were rinsed under the tap water, each in its own plastic cassette, and were separately and automatically processed in increasing series of alcohol, cleared in xylene and embedded in paraffin. The paraffin tissue samples were cut on the LeicaRM2255 microtome (LeicaMicro-Systems, Reuil-Malmaison, France) to get 4 μm thick sections. All the obtained tissue sections were stained routinely with hematoxylin and eosin.

Microscopic tissue specimens were examined on the light microscope LeicaDMR (LeicaMicro-Systems, Reuil-Malmaison, France) at the Institute for Biomedical Research of the Faculty of Medicine in Niš (University of Niš). We assessed the quality of demineralization process based on the morphological characteristics of the tissue, with special consideration of the cytological details.

Beside the weight measurement of the teeth halves on the daily level, we introduced additional relative value for the analysis of efficiency of demineralization process in different examined groups: the ratio of the daily weight of each tooth compared to its starting weight values, translated in percentages. The obtained values of the weight of specimens, belonging to the appointed experimental groups, were then compared by using: descriptive statistical analyses (determining the maximal and minimal values, median value, average values and standard deviations), the Kruskal–Wallis one-way analysis of variance by ranks (ANOVA) and Dunn's multiple comparison test. For all the used methods, the significant statistical importance was considered when p was less than 0.05. The program Sigma Stat 2.0 (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis.

Results

All examined groups achieved the satisfying mechanical properties of the tissues, which allowed the cutting of the treated teeth with a sharp blade, in order to pursue the further demineralization or the proper orientation of the material for further processing to the paraffin blocks. The paraffin blocks, showed light resistance to microtome trimming in the group of tooth agitated by stirring, which made difficult to make serial sections, and thus reduce the number and quality of sections suitable for microscopic analysis. In the same group, the focal nacreous coloration in demineralized tissue was noticed which might be regarded as the fields of lower demineralization, having in mind that these parts of the tissue were making the cutting process more difficult. The obtained microscopic slides that were stained with

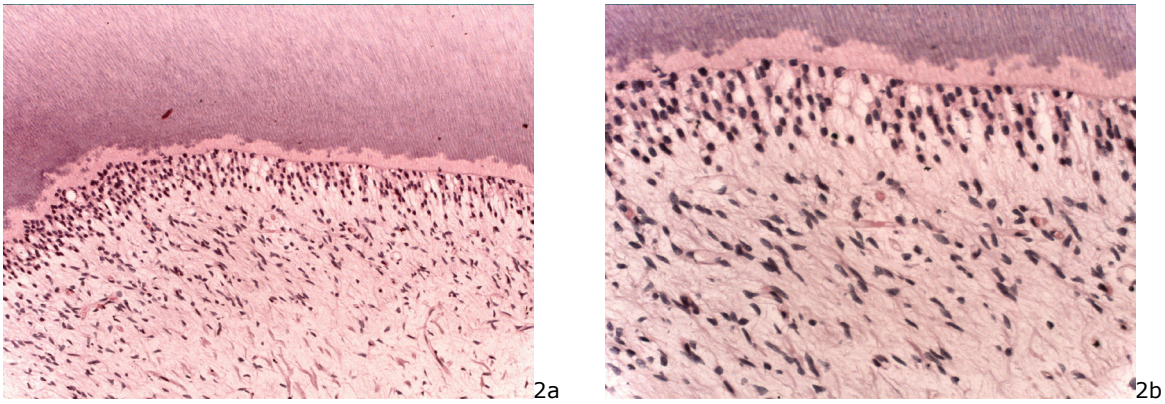

Figure 2. The microscopic result obtained after demineralization in alkaline EDTA that shows the good preservation of pulp-dentin junction and the morphology of the pulp (left), dentin, predentin, as well as cytological details of different cellular types (right) (hematoxylin eosin, left x200, right x630).

hematoxylin and eosin showed normal dentin and pulp architectonics as well as the preservation of the cytological details of different cellular types; even the texture of chromatin was clearly visible in the nuclei (Figure 2).

The quantitative evaluation of the obtained values for teeth halves weight in all groups showed that a maximum of demineralization oc-curs between 3rd and5th days, and after 15th day there is the significant reduction of the demineralization rate, nearly reaching a plateau (Chart 1). It is interesting to note that the values of daily variations of efficiency of the demineralization process have very similar graphical form, regardless of which group is concerned (Chart 1). In the first few days of the experiment, intensive demineralization was observed (showed as percentage weight loss of teeth on a daily basis) in groups agitated by microwaves; however, about the 15th day (Chart 1), teeth weights were approaching a plateau of other groups.

When observing the final effects of the demineralization process through the variable of their weight expressed in percentages, RT, RTR and MW groups showed better results compared to other experimental groups (SM, SMR and MWR) (Chart 1). However, there were fewer statistically significant differences when comparing mean and medium value among the examined groups. It was found between the group with the lowest efficiency (SMR) compared to the groups with the highest efficiency of demineralization (RTR, MW),

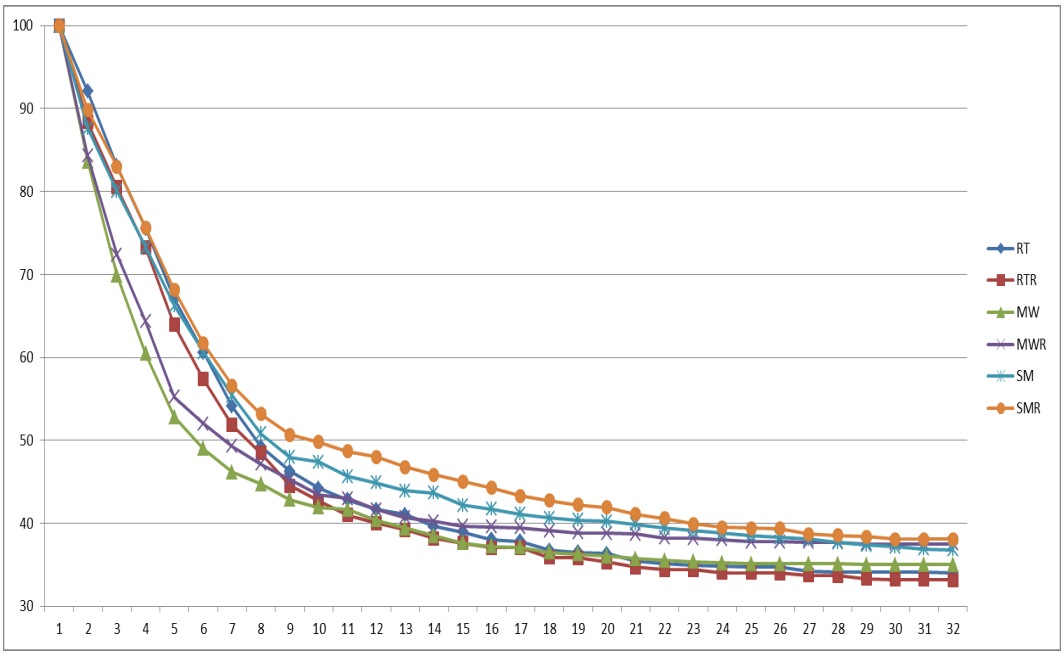

Chart 1. The influence of the process of demineralization on the teeth halves' weight in all groups, presented as the median of the daily percentage loss of samples' weight

Table 2. The percentage loss of the weights of the tooth halves samples in the examined groups daily and their statistical comparison

Column	Duration (days)	Range of starting weights of tooth samples	Mean	±SD	Median
RT	31	0.614-0.931	42.44	±15.5	36.50
RTR	31	0.543-1.174	41.10	±14.8	35.81 *
MW	31	0.761-1.087	39.99	±11.7	36.28 #
MWR	31	0.75-1.04	42.37	±11.6	38.82
SM	31	1.255-1.415	45.15	±13.3	40.38
SMR	31	1.08-1.367	46.91	±13.6	42.24 *,#

*, # - ANOVA-Kruskal-Wallis One Way Analysis of Variance on Ranks, Dunn's multiple comparison test, SD-standard deviation

where p value was 0.001 (Table 2). The outcome of the final 10 days of decalcination in different groups does not show statistically significant differences among them.

Discussion

If taking in consideration the differences of the starting weights of the teeth samples among the examined groups, it seems that the none of conditions with agitation nor the renewal of the demineralization solution has much influence on the acceleration of the demineralization process. The statistically significant difference noticed between the group agitated by stirring and other groups is most probably due to the higher starting weights of the teeth samples treated with stirring. A nearly constant concentration of the active substance of demineralization, achieved by periodical renewal of decalcifying agent, does not influence the speed of demineralization, which becomes clearer if we consider the absence of statistically important difference between the values of the similar experimental groups (Group 1 and 2, Group 3 and 4 and Group 5 and 6).

It seems that the speed of demineralization is neither under the influence of physical agitations, nor the available concentration of decalcinator´s active molecules, but that it is more dependent on the starting weight of the tooth sample. Our results show the more pronounced overall weight loss within the teeth with the higher starting weight. However, when comparing the percentage loss of the tooth mass it is observed that these teeth are finally less demineralized. The sharp fall of tooth weight in the first days of experiment is probably due to the demineralization of the tooth enamel, the highest mineralized structure of human body which contains 96% of hydroxyapatite crystals, as well as of the dentin and cemenum. The enamel is removed around day 15, and then continues the phase of demineralization of the tertiary dentin and of cementum, with a slower decrease of tooth weight on the daily level and with tendency of the graphic curves for each group to form the plateau. From earlier investigations it has become evident that the time needed for bone decalcification is primarily dependent on the minimum diffusing distance of the specimen (10), and that the process is rapid in the beginning, becoming gradually slower towards the end of demineralization (11).

There is a possibility of saving the demineralization agent during the process of hard tissue demineralization by knowing relatively constant nature of demineralization performed with alkaline solution of EDTA, having in mind that it does not change its course when combined with the abovementioned physical conditions nor under influence of maintaining of its high concentration. Taking in consideration a relatively high concentration of Na_2-EDTA of 18.6%, as well as a relatively high volume consumption of Na_2-EDTA of 50 ml per tooth sample, it becomes clear that it is not necessary to apply any additional procedures, because the sole exposition of the tooth to the demineralization solution is sufficient for proper demineralization in time. Besides the factor of the starting tooth weight, the influence of the „volume interaction" of the decalcifier should be also taken in consideration, becuse chelation (as demineralization process) is a slow reaction, and even a higher kinetics of molecules in the solution (at least for the examinedtemperature range) does not accelerates such an interaction, which, on the other side, opens the question of accessability of mineralized parts of the tissue in context of its porosity for the molecules of decalcifier agent.

Similar remarks about relative constancy of the course of the demineralization that was performed with various types of EDTA solutions can be found in other publications. Calliset al. (5) reported that microwave radiation does not influence the acceleration of the demineralization because the process of demineralization lasted for 40 days in group exposed to additional agitation, but also in group without agitation. Similar to our results, they found that the renewal of the demineralization solution and redemineralization in the microwave does not accelerate the demineralization. They emphasize that only the volume ratio is important for the chelation of all accessible calcium. Very similar to our work, Kiviranta et al. (10) reported that the agitation on the temperature of 60°C has no impact on the speed of demineralization, nor the volume of EDTA solution,

except in the case of very small volumes where the chelation saturation of the decalcifier solution was observed. However, the material used by Kiviranta was much smaller compared to ours and was subjected to preparation for electron microscopy.

Microwave agitation that can contribute to the acceleration of the demineralization process on the higher temperatures can have unfavorable impact on the tissue antigeneicity by diminishing it (12–14). Therefore, Keithlyand al. (15) recommend 45°C as an optimum for microwave demineralization. These authors examined the demineralization on the tibia of guinea pig on different temperatures. They reported that the process of demineralization lasts 23 days on the temperature of 20°C, only 3 hours on the temperature of 100°C, and approximately 17 hours when the tissue is exposed to 45°C. Kaithley et al. (15) speculate that volume of the EDTA solution influences the time of demineralization after noticing that the demineralization process takes longer for samples that were exposed to lower volume of EDTA solution. They also suggest the renewal of the demineralization solution every six hours. Their experiment was based on the demineralization with the limited volume of EDTA solution in the microwave or by using stirring. Tinling et al. (16) emphasized the importance of the microwave irradiation in relation to the temperature for the agitation during the demineralization process when using EDTA. They reported that the demineralization of the samples in the microwave oven was achieved in 33 hours, while those samples that were exposed to the stirring took 45 hours to decalcify. Both experiments were performed on the constant temperature of 20°C, and their results show the positive effect of microwaves on the degree of demineralization, independently of the temperature.

Our results imply an interesting characteristic of Na_2-EDTA as a decalcifying agent. Namely, its independence of the used agitation technique and its volume change during demineralization process that can have important practical applications in the rationalization of the laboratory resources, having in mind that the groups in which the decalcifying agent was changed every three days didnot show superior demineralization of the teeth samples compared to the groups with no renewal of Na_2-EDTA, although the applied quantity of decalcifying agent was 10 times bigger.

Finally, if we consider the groups with the best and worse results in demineralization, RTR and SMR respectively, it is noticeable that, although there is a statistically significant difference between them, their medians differ by about 5%. Having in mind that the good mechanical properties of the examined tissue for further histological analysis were obtained in all of the examined groups, we therefore propose the use of Na_2-EDTA on the room temperature and without the renewal of the decalcifying solution as the most economic and suitable way of the hard tissue demineralization.

Conclusion

The process of tooth demineralization with the alkaline solution of Na_2-EDTA is independent of agitation process (in the microwave oven or with constant mechanical stirring) or the renewal of the chelating solution. It is possible to make important rationalization of the material in the routine laboratory practice, using empirically determined optimal volume and concentration of the alkaline solution of EDTA and by excluding other procedures such as agitation techniques (and solution renewal).

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UTICAJI RAZLIČITIH AGITACIONIH USLOVA NA REZULTATE DEMINERALIZACIJE ZUBA UPOTREBOM BAZNOG RASTVORA EDTA

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Za potrebe mikroskopske analize biomineralizovanih struktura, u histološkim tehnikama upotrebljava se proces *in vitro* demineralizacije, kojima se iz njih odstranjuje neorganska komponenta. Brzina demineralizacije i uticaj na očuvanje morfologije tkiva dva su najvažnija parmetra pri odabiru rastvora za dekalcinaciju, a rastvori EDTA (etil-endiamin-tetra-sirćetne kiseline), načinjeni u različitim koncentracijama, smatraju se najsporijim, ali i najpouzdanijim sredstvom za dekalcinaciju. Cilj rada bio je ispitivanje mogućnosti ubrzanja procesa dekalcinisanja uzoraka zuba baznim rastvorom EDTA, upotrebom različitih fizičkih uslova, kao i primenom periodičnog obnavljanja rastvora za dekalcinaciju. Za istraživanje je korišćeno12 trećih molara ekstrahovanih iz ortodontskih razloga. Tokom demineralizacionog procesa, upotrebom 18,6%-tnog vodenog Na_2-EDTA primenjenog na uzorcima zuba, u trajanju od 45 dana, meren je pad težine uzoraka kod tri različita fizička stanja: agitacija mikrotalasima, rotacija tečnosti magnetnom mešalicom i sobna temperatura. Svako od navedenih stanja bilo je podeljeno na dve grupe, jednu sa promenom demineralizacionog rastvora na treći dan i jednu bez promene demineralizacionog rastvora. Demineralizovani uzorci zuba su bili podvrgnuti rutinskoj obradi do hematoksilinom i eozinom obojenih mikroskopskih preparata. Mikroskopski preparati pokazali su neizmenjeni pulpo-dentinski kompleks, kao i očuvanost citoloških detalja različitih ćelijskih tipova zubne pulpe. Proces demineralizacije zuba baznim rastvorom EDTA nije pod uticajem ispitivanih procesa, kao ni pod uticajem dopremanja nove količine hejlatorskog sredstva. Moguće je učiniti značajne uštede u rutinskom laboratorijskom demineralizovanju tkiva isključivanjem drugih postupaka, kao što su agitacione tehnike, kao i učestalo obnavljanje demineralizacionog rastvora. *Acta Medica Medianae 2015;54(4):24-31.*

Ključne reči: *di-natrijum EDTA demineralizacija, mineralizovana tkiva, histohemija*