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Assessment of the effects of decontamination and storage methods on the structural integrity of human enamel

Avaliação dos efeitos de métodos de descontaminação e armazenamento sobre a integridade estrutural do esmalte humano

Adriana Rodrigues de FREITAS[a], Fábio Duarte da Costa AZNAR[a], André Luís da SILVA[b], Arsenio SALES-PERES[a], Silvia Helena de Carvalho SALES-PERES[a*]

[a]Faculdade de Odontologia de Bauru, USP – Universidade de São Paulo, Bauru, SP, Brasil

Resumo

Introdução: O armazenamento de dentes para utilização em pesquisas é uma questão controvérsia e não há consenso sobre o método de tratamento mais apropriado para esta finalidade. **Objetivo:** O objetivo deste estudo foi analisar a efetividade e a influência de diferentes métodos de descontaminação e armazenamento do esmalte dentário humano, a fim de manter sua integridade. **Material e método:** A amostra foi constituída por 124 molares distribuídos aleatoriamente em três grupos, de acordo com o método: controle – água destilada, soluções de timol a 0,1% e de azida de sódio a 0,02%. Os testes realizados foram fluorescência a laser, microdureza de superfície e análise de perfilometria (0, 15 e 30 dias) e teste microbiológico (7, 15 e 30 dias). Os dados foram analisados por meio dos testes ANOVA e Tukey ($p<0,05$). **Resultado:** No teste de fluorescência a laser, a solução de timol a 0,1% demonstrou ser mais viável na manutenção da integridade do órgão dentário, uma vez que este não apresentou variações significativas nos valores entre os períodos avaliados ($p<0,05$). A análise de microdureza de superfície evidenciou perda de estrutura dentária em todos os métodos, sendo que a azida de sódio promoveu menor perda dentária. Na análise de perfilometria observou-se perda de estrutura em todos os grupos, com maior perda no grupo azida de sódio. Nenhum dos métodos conseguiu inibir o crescimento bacteriano. **Conclusão:** Dentre os métodos de processamento analisados nenhum foi capaz de aliar a efetividade na descontaminação ao armazenamento com manutenção da integridade estrutural do esmalte dentário humano.

Descritores: Esmalte dentário; infecção; dureza; fluorescência.

Abstract

Introduction: The storage of teeth for use in research is a controversial issue with no consensus on the most appropriate treatment method for this purpose. **Objective:** The aim of this study was analyze the effectiveness and the influence of different methods of decontamination and storage of human enamel samples, in order to maintain their integrity. **Material and method:** The sample consisted of 124 molars distributed randomly into three groups according to the method: control - distilled water, 0.1% thymol and 0.02% sodium azide. The tests performed were laser fluorescence, surface microhardness and profilometry analysis (0, 15 and 30 days) and Microbiological test (7, 15 and 30 days). Data were analyzed by the ANOVA and Tukey tests ($P < 0.05$). **Result:** In the laser fluorescence test, thymol proved to be more feasible for maintaining the integrity of the dental organ, since it did not show significant variations in values among the analyzed periods ($P > 0.05$). The surface microhardness analysis showed loss of tooth structure in all methods, and sodium azide led to a lower level of tooth loss. Profilometry analysis showed loss of mass in all groups whereas sodium azide showed the greatest loss. None of the methods was able to inhibit bacterial growth. **Conclusion:** Among the processing methods analyzed none was able to combine effective decontamination and storage with maintenance of the structural integrity of the human enamel.

Descriptors: Dental enamel; infection; hardness; fluorescence.

INTRODUCTION

The use of extracted teeth in *in vitro* and *in situ* experiments contributes to the development of new techniques and dental materials[1,2].

Teeth can be considered a potential source of cross infection and contamination[3,4], and efficient methods of decontamination should be adopted prior to their use, in addition to storage conditions

that allow the maintenance of their physical properties[5-7]. In order to preserve teeth, a variety of storage methods have been used, including chloramines, formalin, sodium hypochlorite, thymol, alcohol, glutaraldehyde[8] and the autoclaving process[3,4].

The majority of cariogenicity and erosive tests are conducted intraorally in an effort to use the natural environment of the tooth and *in situ* conditions[9-11], consequently, in many experiments enamel specimens from human or bovine sources find their way into the volunteer's mouth[5].

Enamel, the hardest tissue in nature, is a highly mineralized non-regenerative tissue with a well-organized microstructure[12]. The decontamination method must not affect the structural integrity of dental enamel and hard tissue specimens, in order to maintain the baseline condition of specimens[13].

The method of decontamination, conditions and time of storage of teeth seem to be important variables in studies that use this type of substrate[4,14,15]. Elucidating this question is crucial to understanding the maintenance of the structural integrity of tooth enamel under storage conditions.

The aim of this study was to analyze the effectiveness and the influence of different methods of decontamination and storage of human teeth, in order to maintain their properties and the structural integrity of the enamel.

MATERIAL AND METHOD

One hundred and twenty-four freshly extracted and non-carious human third molars were selected for this study. Teeth were provided by a Human Tooth Bank, which collects, cleans and stores teeth in filtered water under refrigeration until their use. The study protocol was approved by the Research Ethics Commission of the Institution and was conducted in compliance with the norms of the Declaration of Helsinki.

An in vitro study was conducted to assess the effectiveness and influence of different methods of decontamination and storage of human teeth, in order to maintain their properties and structural integrity. The teeth were immersed in solutions of 0.1% thymol, 0.02% sodium azide and distilled water (control) (solutions previously prepared by the biochemical laboratory of BSD-USP) and subjected to a microbiological test, laser fluorescence, microhardness and profilometry analyses (Figure 1).

For the Laser Fluorescence analysis, we used 30 molars, ten for each group (distilled water – control, thymol and sodium azide). The teeth were analyzed by means of the laser fluorescence device DIAGNOdent ® (KAVO, Biberach, Germany). According to the manufacturer's instructions, the instrument was calibrated to its ceramic standard before the measurement of each tooth, and the teeth were left at room temperature for 5 minutes. After this, they were immersed in deionized water and dried on sterile gauze. Five sites on the buccal surface of each tooth were selected and identified for analysis, acquisition and recording of the values, which were subsequently used to calculate the mean value for each tooth. After these initial analyses, the teeth were randomly distributed, placed in jars with lid, immersed in the test solutions and stored under refrigeration (4 °C). New records of laser fluorescence were obtained for each group after 15 and 30 days of storage.

For the Surface Microhardness analysis, enamel fragments obtained from 12 molars. The crowns were sectioned from the roots with a diamond disk (Isomet 1000; Buehler, Lake Bluff, IL, USA). The enamel surface of the blocks was ground flat with water-cooled carborundum discs (320, 600 and 1200 grit Al2O3 papers; Buehler, Lake Bluff, IL, USA), and polished with felt pads with diamond

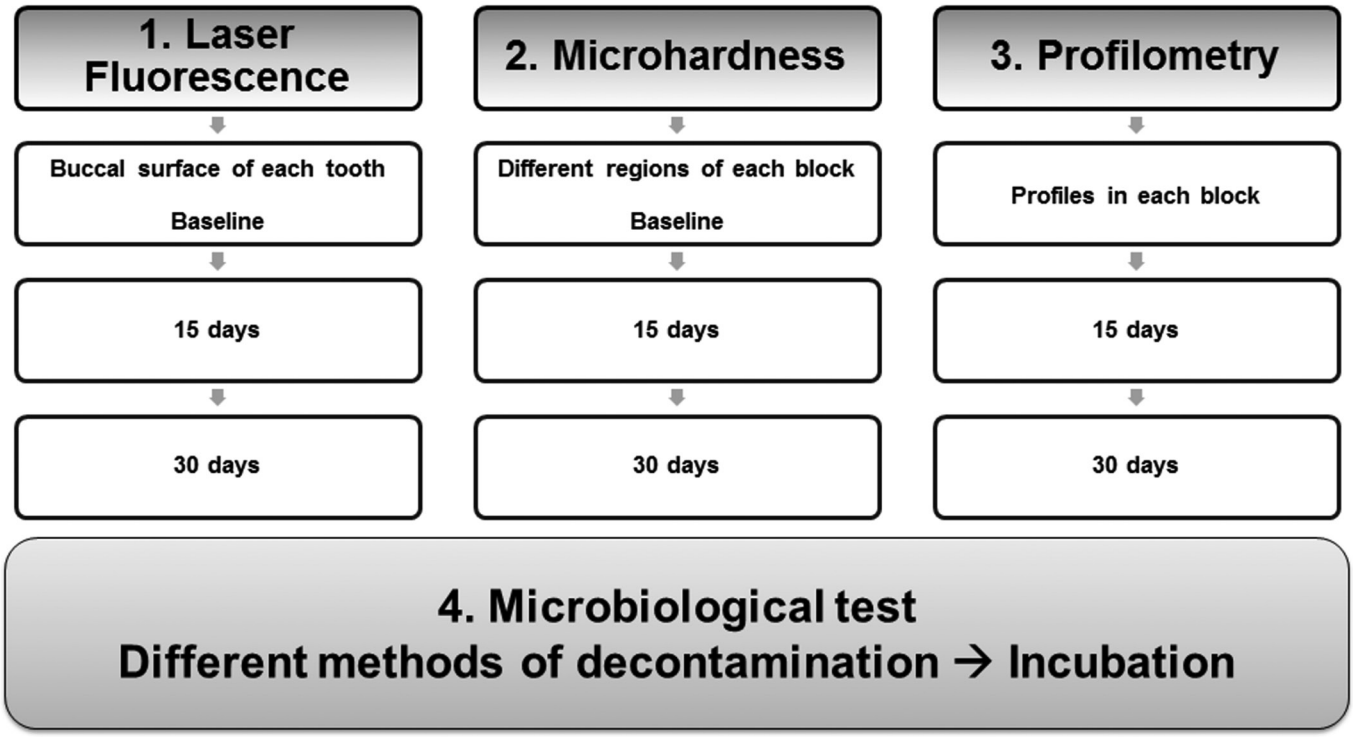

Figure 1. Different methods applied to analyze the efficacy and maintenance of solutions tests.

spray (1 lm; Buehler, Lake Bluff, IL, USA). To standardize the blocks, specimens were previously selected to measure the initial KNOOP microhardness (Five indentations made in different regions of the blocks, 25 g, 10 s, HMV-2000; Shimadzu Corporation, Tokyo, Japan). Blocks that presented 10% below and 10% above the mean value were excluded from the sample. Twelve specimens with a mean surface microhardness of between 417.40 and 464.80 KHN (mean=441.10) were randomly divided into groups, and immersed in the following solutions: distilled water- control, thymol and sodium azide. After time intervals of 15 and 30 days of storage, new microhardness analyses were performed in each group.

For profilometry analysis, enamel fragments obtained from 12 molars and prepared in the same way as those used for the microhardness analysis were used. The purpose of this analysis was to highlight enamel loss, thus it was not performed at time interval 0 (full surface), only in the subsequent periods. In order to maintain reference surfaces for determining lesion depth, two layers of nail varnish (Risque, Niasi, Taboão da Serra, São Paulo, Brazil) were applied on half of the specimen surfaces. Enamel blocks were randomly divided into groups and immersed in the following solutions: distilled water- control, thymol and sodium azide. At the time interval of 15 days the blocks were remove from the solutions, washed with deionized water and dried with gauze. Surface profiles of the enamel specimens were obtained with a contact profilometer (Hommel Tester T 1000, Hommelwerke, VS, Schwenningen, Germany). To determine enamel loss, the nail varnish was removed from the specimens and five profiles were recorded. The profile scans were performed in the centre of each specimen at intervals of 250 μm. Treatment scans was superimposed and the average depth of the area under the curve in the eroded area was calculated with a specially designed software program (Turbo Datawin-NT, Version 1.34, Copyright© 2001). The results of the five scans were averaged for each specimen. Then the nail varnish was applied again on half of the specimen surfaces. The blocks were stored for the time of 30 days, when new profilometry analyses were performed at exactly the same sites as for the measurement at the time interval of 15 days.

Prior to the Microbiological test, a pilot study was conducted, using a laminar flow chamber to assess the initial contamination of the teeth. For this purpose, 10 molars were selected (5 sterilized-control and 5 test), and placed in two sterilized jars with lid containing 40 mL of Brain Heart Infusion (BHI) each. The test group was inoculated with 40 μL of broth containing 3.5×10^4 spores of *Bacillus subtilis* (Microbiology Laboratory of BSD-USP), while the other group remained uncontaminated (control). Both samples were stored at 37 °C for 24 hours. After the incubation period the samples were removed from the broths, the contents deposited on glass slides, and submitted to Gram stain. The slides were examined under an optical microscope and strains of microorganisms identified for each group.

To conduct the experiment 60 molars were used, and were divided into the groups: 15 distilled water (positive control), 15 thymol, 15 sodium azide and 15 autoclave (negative control). All teeth were immersed in sterilized test tubes containing 40 ml of BHI broth, kept in an incubator at 37 °C, for 24 hours. After this stage the teeth from the Autoclave group were wrapped in gauze, placed in self-sealing envelopes, and submitted to a 15-minute sterilization cycle at 121 °C, with the drying process of the device being excluded.

In the next step all teeth were immersed in their solutions: distilled water (positive and negative controls), thymol and sodium azide. Each group was stored under refrigeration (4 °C) for time intervals of 7, 15 and 30 days (5 teeth in each group and t each time). After the mentioned time intervals, the tubes were removed from the refrigerator and left for 5 minutes at room temperature. Then the teeth were removed from solution, dried on sterile gauze and put into new tubes containing BHI broth, which were stored in an incubator at 37 °C for 6 hours.

The tubes containing broth + tooth were initially analyzed by the turbidity of the medium. Originally the culture medium was shown to be yellow and clean; this condition was considered a negative result for bacterial growth, whereas the presence of turbidity in samples was considered positive for bacterial growth. In order to confirm the results of this analysis (positive or negative) serial decimal dilutions (10^{-1} to 10^{-3}) of the broth contained in the tubes was performed, using 9 mL of sterile saline solution in each tube. Aliquots of 50μl of the 10^{-2} and 10^{-3} dilutions were seeded on the surface of culture plates containing BHI agar, by means of an automatic pipette, always working from the more diluted solution to the less diluted. The plates were incubated in an incubator at 37 °C for 24 hours. After this period the culture plates were analyzed for the presence/absence of Colony Forming Units (CFU).

Data were entered into Excel® spreadsheets (Microsoft, Redmond, WA, USA, 2010), and analyzed using the software program STATISTICA® statistical package version 10.0 (Statsoft, Tulsa, OK, USA, 2011). The laser fluorescence, surface microhardness and profilometry analysis data were checked for the assumptions of equality of variances and normal distribution of errors. Since the assumptions were satisfied, data were analyzed by two and one-way analysis of variance, ANOVA. For individual comparisons among the groups, the Tukey test was used. The level of significance was set at 5% ($P<0.05$) and Interval of Confidence of 95%. The microbiological test was analyzed qualitatively, according to the bacterial growth.

RESULT

The main results of the laser fluorescence, surface microhardness and profilometry analysis are described in Table 1. In all tests performed we observed significant relations between the groups, storage time and interaction of these two variables (P>0.05).

In the pilot study of the microbiological test, no differences were observed in morphological forms of bacterial strain that were found in the both control and experimental group.

The culture medium of the tubes was analyzed after 6 hours of incubation in an incubator. The positive control, thymol and sodium azide showed bacterial growth in all the time intervals analyzed (7, 15 and 30 days). The negative control (autoclave) was the only group that did not show initial turbidity in any of the periods, but after 24 hours in an incubator there was turbidity in 40% of the samples.

Table 1. Mean, standard deviation and association among processing method and time of storage in Laser fluorescence, Microhardness and Profilometry analysis

Tests/Groups	Baseline*	15 days*	30 days*
Laser fluorescence			
Thymol	1.70 ± 0.41^{Aa}	1.60 ± 0.41^{Ba}	1.48 ± 0.41^{Ba}
Sodium azide	1.84 ± 0.55^{Aa}	2.00 ± 0.57^{Aa}	1.72 ± 0.49^{Bb}
Control	1.74 ± 0.51^{Aa}	1.96 ± 0.57^{Aa}	2.14 ± 0.56^{Ab}
Microhardness			
Thymol	446.80 ± 21.35^{Aa}	225.93 ± 36.51^{Bb}	172.93 ± 53.57^{Bb}
Sodium azide	440.20 ± 19.75^{Aa}	247.40 ± 21.09^{Bb}	276.93 ± 13.37^{Bb}
Control	456.53 ± 12.29^{Aa}	118.53 ± 10.01^{Bb}	97.53 ± 11.89^{Ab}
Profilometry			
Thymol	-	15.39 ± 2.89^{Aa}	10.55 ± 1.04^{Ba}
Sodium azide	-	20.75 ± 6.00^{Ba}	20.25 ± 3.26^{Ba}
Control	-	10.49 ± 0.93^{Ba}	12.56 ± 3.60^{Ba}

*Mean ± S.D. Different lower case letters in the same column indicate statistical significance between the groups. Different upper case letters in the same line indicate statistical significance among the experimental conditions ($P < 0.05$).

The culture plates produced from dilutions of the contents of the tubes (culture medium and teeth) were qualitatively analyzed. Since there was great proliferation of microorganisms on the plates, Colony Forming Unit counts proved to be unfeasible. The positive control, thymol and sodium azide plates showed bacterial growth in all the time intervals analyzed. The autoclave culture plates (negative control) showed negative results in this analysis, however after 24 hours of incubation there was turbidity in 40% of the samples.

DISCUSSION

In laboratory conditions, many variables such as temperature, humidity and storing solutions need to be considered[14]. The storage method can be used to prevent dehydration of the specimens, but may also incorporate anti-microbial substances in order to prevent the growth of microorganisms[4,16]. The use of extracted teeth requires the adoption of measures for cross contamination prevention, such as the use of personal protective equipment[3,4].

The 0.1% thymol solution was investigated and showed decline in comparison with the laser fluorescence values during the time intervals evaluated (0, 15 and 30 days), however without significant differences (P<0.05). Storage solutions may reduce the concentration of fluorophores in the samples by the dilution, resulting in decreased response to laser fluorescence[17]. In this case the thymol solution may have favored a decrease in concentration of fluorophores by its bacteriostatic potential. In the surface microhardness and profilometry analysis this group presented loss of enamel structure (Table 1) with significant differences between the analyzed time intervals (P<0.05). The microbiological test showed the inefficiency of this solution for the inhibition of bacterial growth in samples of teeth stored for 7, 15 and 30 days. Thymol solution has been largely used as storage method in *in vitro*[18] and *in situ* studies[9,19,20]. The use of this solution has not determined significant influence on shear resistance[21] and microleakage[22] tests, however studies have described the use of thymol solution without standardization, probably due to insufficient parameters to enable the choice of the most appropriate method[23]. However, this agent did not appear to offer useful properties, since it promoted significant loss of enamel structure and it was not able to inhibit bacterial growth in the samples analyzed.

The 0.02% sodium azide solution presented an increase in laser fluorescence values at 15 days of storage, and later showed significant decrease in these values between 15 and 30 days (P=0.045). Two hypotheses can be raised, the first being the bacteriostatic effect after 15 days of storage, and the second that it allowed dispersal of the mineral content of the teeth[17]. In the microhardness analysis this solution presented the lowest loss of structure (–37.09%), however in the profilometry analysis it showed the highest loss of mass values (Table 1). It was observed that sodium azide promoted loss of tooth structure from enamel specimens in the time interval of 15 days, and hardened these structures after 30 days of storage (Table 1). Sodium azide presented no activity in the inhibition of bacterial growth in all samples and periods analyzed. This solution showed some instability in the tests and different storage times, which seems to agree with the findings shown in the scientific literature[14,15]. Gamma irradiation is an effective method for tooth decontamination[5,24] but has an unfavorable cost-benefit ratio for its application in this type of research. Bovine enamel specimens were disinfected by microwave irradiation without prejudice to the property of hardness[25] and other properties, such as laser fluorescence and the profilometry should be analyzed in future studies.

The control group, distilled water showed linear performance throughout the laser fluorescence test (Table 1), with a significant increase in these values between the time intervals (P=0.002).

With regard to the surface microhardness and profilometry analyses it promoted the loss of structure on the enamel block in both cases, with the greatest loss of hardness (–78.58%). Distilled water was used as control in this study and somehow favored the loss of structure of the enamel specimens. In view of this finding, this matter should be investigated in the future, which refers to the possibility of replacing the deionized water with fluoridated water to store enamel specimens, in an attempt to minimize these losses.

Storage in distilled water was used as positive control in the microbiological test, while the sterilization of teeth in an autoclave was used as negative control. The autoclave is described as a simple, inexpensive and accessible method for decontamination of dental elements used for didactic and scientific purposes[3]. As expected distilled water allowed bacterial growth in all samples, while the autoclave sterilization showed the opposite performance. Although the sample of tubes for the autoclave group did not show bacterial growth in the stipulated period of 6 hours after the initial analysis of these samples, they were incubated for a period of 24 hours in order to assess possible changes in an extended period, which was observed in 40% of the tubes. This late contamination of the samples can be attributed to the presence of viable microorganisms in dentinal tubules or due to the presence of cracks on the tooth surface[26].

In this study, the storage of specimens in different conditions was shown to be capable of influencing the results, and the time was less favorable in the storage of 30 days in all groups. The chemical and optical changes in dental properties are important factors to be considered when choosing the solution and the storage time[14,15,27,28].

The limitation of this study was due to the previous contamination of the teeth used. The results of pilot test showed no difference between the groups. An alternative to this bias would be the prior sterilization of the teeth by immersion in 10% formalin for seven days[3,4] or processing in an autoclave[3]. Nonetheless, this procedure would not meet the requirements of laboratory analysis, since these processes could influence the results of tests to which the teeth would be submitted.

CONCLUSION

The processing methods analyzed showed no effectiveness in the decontamination and the maintenance of the structural integrity of the human enamel. The performance of sodium azide solution seems to invalidate its use in laboratory tests that require the use of tooth structures, due to the perceived changes it causes in these structures. The 0.1% thymol solution seems to be the storage method most accepted, however it needs to be applied in combination with another effective decontamination method.

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CONFLICTS OF INTERESTS

The authors declare no conflicts of interest.

*CORRESPONDING AUTHOR

Silvia Helena de Carvalho Sales Peres, Departamento de Odontopediatria, Ortodontia e Saúde Coletiva, Faculdade de Odontologia de Bauru, USP - Universidade de São Paulo, Al. Octávio Pinheiro Brisolla, 9-75, 17012-901 Bauru - SP, Brasil, e-mail: shcperes@usp.br

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