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Effect of disinfection and storage media on surface microhardness of human enamel blocks

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ABSTRACT

Protocolized disinfection and storage are critical to ensuring the quality of dental specimens used in in vitro studies. However, few evaluations have addressed the effect of different combinations of disinfection and storage media on the surface microhardness of human enamel blocks. ***Aim:*** *To evaluate the effect of different disinfection and storage media on the microhardness of human enamel blocks over four time periods.* ***Materials and Method:*** *The study received ethical approval. The surface microhardness of human enamel blocks was measured at baseline (T0). Subsequently, three blocks were assigned to each of six different disinfection-storage media combinations: acetic acid (5%) – chloramine (1%) [*AA-CT*], hypochlorite (5.25%) – chloramine (1%) [*NaOCl-CT*], chloramine (0.5%) – chloramine (1%) [*CT-CT*], acetic acid (5%) – thymol (0.02%) [*AA-TH*], hypochlorite (5.25%) – thymol (0.02%) [*NaOCl-TH*], and chloramine (0.5%) – thymol (0.02%) [*CT-TH*]. After two weeks (T1), one month (T2), three months (T3) or six months (T4) of storage at 4°C, microhardness was reassessed. Percentages of microhardness variation (mineral gain/loss) from baseline were calculated for each time. Data were analyzed using one-way ANOVA and two-way ANOVA to identify differences among disinfection-storage medium combinations and storage times, respectively.* ***Results:*** *A total 72 enamel blocks were analyzed. The microhardness variation percentages showed that the CT-TH combination (T1: 0.00%; T2: 4.05%; T3: 2.78%; T4: -4.74%; $p>0.005$) had the least variation, and that the microhardness values in the six groups at T3 were the most stable and did not differ significantly ($p= 0.630$).* ***Conclusion****: Combinations of chloramine-thymol and chloramine-chloramine generated the fewest changes in microhardness in enamel blocks, and are suitable for preserving extracted teeth.*

Keywords: *tooth - enamel - disinfection - storage*

Efecto de los medios de desinfección y almacenamiento sobre la microdureza superficial en bloques de esmalte humano.

RESUMEN

La desinfección y el almacenamiento protocolizados son procesos fundamentales para garantizar la calidad de las muestras dentales necesarias en los estudios in vitro; sin embargo, son escasas las evaluaciones sobre el efecto que tienen las distintas combinaciones de medios de desinfección y de almacenamiento sobre la microdureza superficial de bloques de esmalte humano. ***Objetivo:*** *Evaluar el efecto de diferentes medios de desinfección y almacenamiento sobre la microdureza de bloques de esmalte humano durante cuatro periodos de tiempo.* ***Materiales y método:*** *Con la aprobación ética, se midió la microdureza superficial de bloques de esmalte humano en el momento inicial (T0). Posteriormente, se asignaron tres bloques a seis grupos de combinaciones de medios de desinfección y almacenamiento: ácido acético (5 %) - cloramina (1 %) [AA-CT], hipoclorito (5,25 %) - cloramina (1 %) [NaOCl-CT], cloramina (0,5 %) - cloramina (1 %) [CT-CT], ácido acético (5 %) - timol (0,02 %) [AA-TH], hipoclorito (5,25 %) - timol (0,02 %) [NaOCl-TH] y cloramina (0,5 %) - timol (0,02 %) [CT-TH]. Tras dos semanas (T1), un mes (T2), tres meses (T3) o seis meses (T4) de almacenamiento a 4 °C, se volvió a evaluar la microdureza. Se calcularon los porcentajes de variación de la microdureza (ganancia/pérdida mineral) con respecto al valor inicial para cada momento. Los datos se analizaron mediante las pruebas de ANOVA de una vía y un ANOVA de dos vías para identificar las diferencias entre los grupos de combinación de desinfección-medio de almacenamiento y los tiempos de almacenamiento, respectivamente.* ***Resultados:*** *Se analizaron un total de 54 bloques de esmalte. Los porcentajes de variación de la microdureza mostraron que la combinación CT-TH (T1: 0,00 %; T2: 4,05 %; T3: 2,78 %; T4: -4,74 %; $p > 0,005$) presentaba la menor variación, y que los valores de microdureza obtenidos en los seis grupos en T3 fueron los que se mantuvieron más estable y por tanto no diferían significativamente ($p = 0,630$).* ***Conclusión:*** *Las combinaciones de cloramina-timol y cloramina-cloramina generaron los menores cambios en la microdureza de los bloques de esmalte y son adecuadas para la conservación de piezas dentales.*

Palabras clave: *dientes - esmalte - desinfección - almacenamiento*

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INTRODUCTION

Dental research often uses natural teeth to investigate pathologies and conditions of hard tissues, as well as to evaluate dental materials. Although human teeth are the most suitable substrate, freshly extracted samples are not always readily available[1], making it necessary to collect and store them for variable periods of time until they are used.

Extracted teeth are disinfected and stored in different media which prevent microbial growth, dehydration, and mineral structure changes. It is essential to choose the right medium to ensure the quality of these tissues when the time comes to analyze and evaluate them[2]. Disinfection media include sodium hypochlorite[2–4], acetic acid[3,5], formalin[4], 0.5% chloramine T[6,7], and thymol T T[7], among others [8], while storage solutions include saline solutions, chloramine T, formalin and thymol[1,2,9,10], which have been used at different concentrations with varying degrees of success.

Sodium hypochlorite is a non-specific proteolytic agent that effectively removes organic components at room temperature[11]. However, it is not recommended as a storage medium because increases the permeability of dental enamel and decreases the adhesion strength of tissue[4,12,13].

Acetic acid (5%) has antimicrobial properties related to its ability to alter bacterial cell membranes, denature proteins and cause cell death[3,5], and has been proposed as a disinfectant for extracted human teeth[5].

Formalin (10%) has antimicrobial and protein-binding properties; however, it should be used with caution due to its carcinogenic properties[14].

Chloramine T (0.5%) is a disinfectant activity, and optimum capacity, like that of the oral environment, to preserve the properties the dental structure[15]. Despite its similarity to sodium hypochlorite, chloramine T does not degrade the collagen structure[10]. Its effect on adhesive resistance in teeth is comparable to that obtained with freshly extracted teeth[16].

Thymol (0.1%) is not considered the most effective disinfectant; however, it is a good storage medium that adequately preserves tooth color and adhesive strength. However, it may interfere with methacrylate polymerization[17].

Despite the availability of isolated information for each medium, few evaluations have addressed the combined effect of disinfection and storage media on the surface microhardness of teeth exposed for different periods of time. In this context, it is critical to define the influence of different combinations of disinfection and storage media on tooth specimens in order to manage the protocols applied for all stages, from tooth collection to experimental use, thereby ensuring the quality of the results. Thus, the aim of this study was to evaluate the effect of different disinfection and storage media combinations on the microhardness of human enamel blocks over four time periods.

MATERIALS AND METHOD

This exploratory experimental study was approved by the Institutional Research Ethics Committee (Approval number 012-2017). Teeth extracted for therapeutic reasons unrelated to this study were collected from the university's oral surgery clinics through voluntary donation after the donors signed an informed consent form. Sample size was established as 18 recently-extracted sound teeth. Any teeth with caries lesions, coronal/radicular restorations, enamel development defects, or endodontic treatments were excluded. Specimens were collected by dentists in high-density polyethylene containers, immersed in 0.5% chloramine T solution, and transferred to the laboratory. Teeth were cleaned and organic tissue remains removed with Gracey 11/12 curettes and endodontic files.

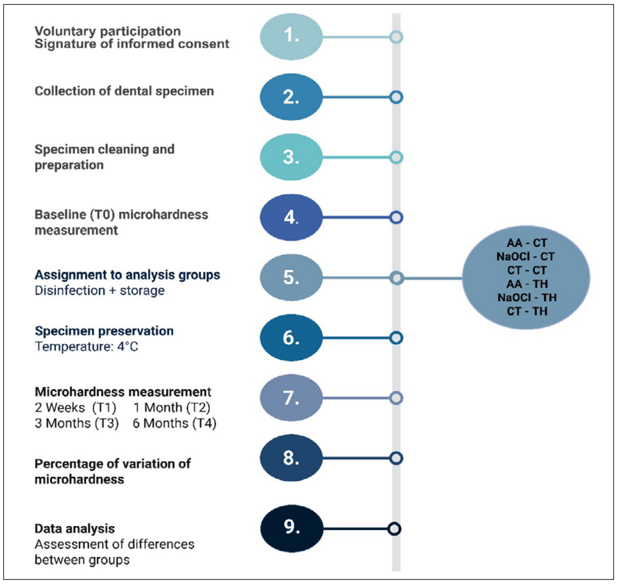

Fig. 1: Flowchart: The processing of enamel blocks exposed to different disinfection-storage media combinations.

A flowchart of the enamel block processing is shown in Fig. 1. Enamel blocks (4x4 mm) were made from each surface of the tooth crowns (IsoMet 1000 - Buehler) and polished to obtain a flat surface (Bühler Metaserv 250). At baseline, transverse microhardness was assessed using microhardness tester (Wilson Hardness Model 1202) coupled with the FM-ARS analysis program. A Knoop-type diamond indenter with a load of 25 g for 5 seconds was used to make five indentations in each block. Values were expressed as Knoop Hardness Numbers (KHN) in kg/mm^2.

Then, three enamel blocks were randomly assigned to each of the following disinfection-storage media combination groups: acetic acid (5%) – chloramine (1%) [**AA-CT**], hypochlorite (5.25%) – chloramine (1%) [**NaOCl-CT**], chloramine (0.5%) – chloramine (1%) [**CT-CT**], acetic acid (5%) – thymol (0.02%) [**AA-TH**], hypochlorite (5.25%) – thymol (0.02%) [**NaOCl-TH**], and chloramine (0.5%) – thymol (0.02%) [**CT-TH**].

Each group of three blocks was immersed in the assigned disinfection medium for five minutes. Subsequently, the blocks were washed thoroughly with deionized water and transferred to the corresponding storage medium. Samples were stored at 4 °C and evaluated at four storage times: two weeks (T1), one month (T2), three months (T3), and six months (T4). At the end of each time, microhardness was reassessed. Change in microhardness was determined by calculating the difference between the baseline microhardness values and those obtained at each time point (T1–T4). The percentage of change was calculated through the MicroHardness Analysis (MHA) equation[18] and reported as a positive or negative value, indicating mineral loss or gain, respectively:

$$MHA = \frac{MHAbaseline - MHAfinal}{MHAbaseline} \times 100.$$

Statistical analysis

The percentage variations in the microhardness of the blocks exposed to different combinations of disinfection-storage media were recorded, organized in an Excel database, and analyzed using GraphPad Prism version 11.0.0 for Windows (GraphPad Software, Boston, Massachusetts USA). First, the normality of the data was assessed using the Shapiro-Wilk test. One-way ANOVA analysis of variance was used to evaluate the difference between groups, followed by two-way ANOVA with Tukey's post hoc test, to identify differences in the microhardness of enamel blocks exposed to disinfection-storage media combinations at four different times.

RESULTS

A total 72 enamel blocks were analyzed. Nineteen indentations were analyzed on the enamel blocks exposed to each disinfection-storage media combination, excluding any atypical microhardness measurements with values less than 100 kg/mm^2. Results of the analysis regarding the effect of the disinfection–storage medium combination on surface microhardness are shown in Fig. 2 as percentages of variation.

The Chloramine-Thymol (**CT-TH**) media combination (T1: 0.00%, T2: 4.05%, T3: 2.78%, T4: -4.74%, $p = 0.6301$) showed the most stable values of microhardness compared to the other combinations. Statistically significant differences ($p<0.05$) were observed in the microhardness variation of the blocks exposed to **NaOCl-CT** (T1: 0.00%, T2: 0.75%, T3: 21.93%, T4: 88.72%, $p<0.0001$) with respect to those exposed to **AA-TH** (T1: -2.77%, T2: 32.79%, T3: 17.11%, T4: 22.54%; $p = 0.0055$) and to **NaOCl-TH** (T1: 0.00%, T2: 3.31%, T3: 22.18%, T4: -7.55%; $p = 0.0055$) at the four analyzed times (Fig. 2). In addition, differences in microhardness were found, considering two factors – treatment and time, p<0.0001 (Table S1). Multiple comparisons test showed differences in microhardness measurements at 1 month (T2) and six months (T4) (Table S2).

The comparisons of the six disinfection-storage media combinations at each evaluated time point are shown in Fig. 3. No differences were found between the disinfection-storage media groups at T1 ($p=0.2227$), while significant differences were identified at T2 ($p=0.0060$). **AA-CT** (26.67%) and **NaOCl-CT** (0.75%) had statistically significant differences ($p=0.0099$), besides the microhardness observed for **AA-CT** (26.67%) and **CT-TH** (4.05%) ($p=0.0046$), and for **NaOCl-CT** (0.75%) and **AA-TH** (32.79%) ($p=0.0333$). The most stable values of dental samples microhardness were observed at T3 for all the combinations ($p = 0.2878$). At T4, the main differences were observed when comparing **AA-CT** (10.49%) and **NaOCl-CT** (88.72%) to the other analyzed combinations ($p<0.0001$) (Table 1).

Analysis based on disinfection-storage media combinations.

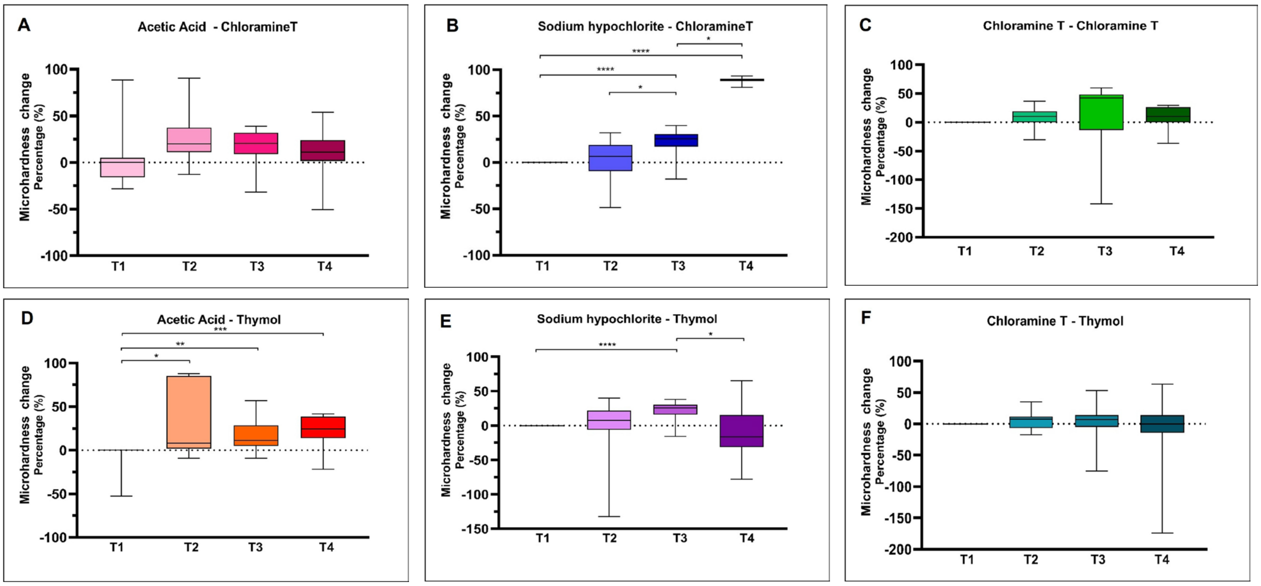

Fig. 2: Change in microhardness in enamel blocks exposed to disinfection-storage media. Colors represent the groups of analysis; color intensity increases over time. **A.** *Acetic Acid – Chloramine T (Pink),* **B.** *Sodium hypochlorite – Chloramine T (Blue),* **C.** *Chloramine T – Chloramine T (Green),* **D.** *Acetic Acid – Thymol (Orange),* **E.** *Sodium hypochlorite – Thymol (Purple),* **F.** *Chloramine T – Thymol (Bluish green). Differences between four times were evaluated for each treatment, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).*

Analysis based on evaluation times.

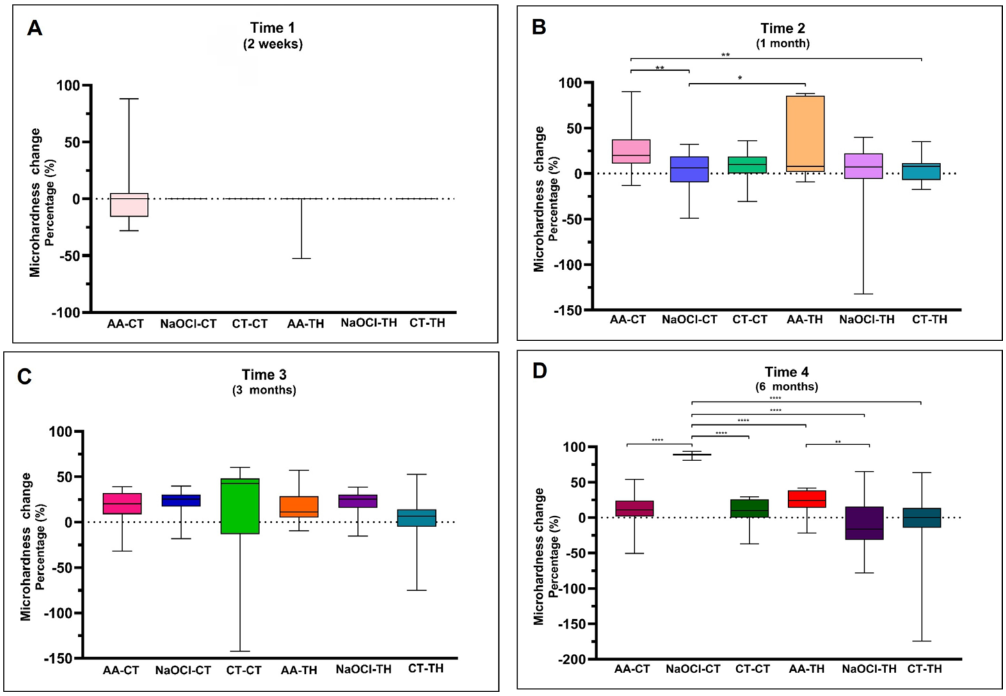

Fig. 3: Change in microhardness in enamel blocks exposed for four times (T1 to T4), to disinfection-storage media. Colors represent the groups of analysis; color intensity increases over time) Acetic Acid – Chloramine T (Pink); Sodium hypochlorite – Chloramine T (Blue); Chloramine T – Chloramine T (Green); Acetic Acid – Thymol (Orange); Sodium hypochlorite – Thymol (Purple); Chloramine T – Thymol (Bluish green). **A.** *Time 1 (2 weeks),* **B.** *Time 2 (1 Month),* **C.** *Time 3 (3 Months),* **D.** *Time 4 (6 Months). Multiple comparison tests were used to evaluate differences between combinations for each time. Statistical significances were presented $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).*

Table 1. Comparison of microhardness between treatments and times based on one-way ANOVA

Time	Disinfection-Storage Medium 1	%	Disinfection-Storage Medium 2	%	*Adjusted p value*
T1 2 weeks	NaOCl – CT	0.0	AA - CT	11.8	*0.8018*
	CT – CT	0.0	AA - CT	11.8	*0.8018*
	AA – TH	-2.77	AA - CT	11.8	*0.7604*
	CT – TH	0.0	AA - CT	11.8	*0.8018*
	NaOCl – TH	0.0	AA - CT	11.8	*0.8018*
	CT – CT	0.0	NaOCl - CT	0.0	--
	AA – TH	-2.77	NaOCl - CT	0.0	*0.9120*
	CT – TH	0.0	NaOCl - CT	0.0	--
	NaOCl – TH	0.0	NaOCl - CT	0.0	--
	AA – TH	-2.77	CT - CT	0.0	*0.9120*
	CT – TH	0.0	CT – TH	0.0	--
	NaOCl – TH	0.0	CT - CT	0.0	--
	CT – TH	0.0	AA - TH	-2.77	*0.9120*
	NaOCl – TH	0.0	AA - TH	-2.77	*0.9120*
	NaOCl – TH	0.0	CT - TH	0.0	--
T2 1 month	NaOCl – CT	0.75	AA - CT	26.67	***0.0099***
	CT – CT	8.36	AA - CT	26.67	*0.2218*
	AA – TH	32.79	AA - CT	26.67	*0.9947*
	CT – TH	4.05	AA - CT	26.67	***0.0046***
	NaOCl – TH	3.31	AA - CT	26.67	*0.2262*
	CT – CT	3.31	NaOCl - CT	0.75	*0.8610*
	AA – TH	32.79	NaOCl - CT	0.75	***0.0333***
	CT – TH	4.05	NaOCl - CT	0.75	*0.9938*
	NaOCl – TH	3.01	NaOCl - CT	0.75	*0.9999*
	AA – TH	32.79	CT - CT	8.36	*0.0834*
	CT – TH	4.05	CT - CT	8.36	*0.9694*
	NaOCl – TH	3.31	CT - CT	8.36	*0.9902*
	CT – TH	4.05	AA - TH	32.79	*0.1964*
	NaOCl – TH	3.31	AA - TH	32.79	*0.1503*
	NaOCl – TH	3.31	CT - TH	4.05	*>0.9999*
T3 3 months	NaOCl – CT	21.93	AA - CT	16.59	*0.8553*
	CT – CT	11.84	AA - CT	16.59	*0.9992*
	AA – TH	17.11	AA - CT	16.59	*>0.9999*
	CT – TH	2.78	AA - CT	16.59	*0.4226*
	NaOCl – TH	22.18	AA - CT	16.59	*0.8441*
	CT – CT	11.84	NaOCl - CT	21.93	*0.9704*
	AA – TH	17.11	NaOCl - CT	21.93	*0.9362*
	CT – TH	2.78	NaOCl - CT	21.93	*0.0584*
	NaOCl – TH	22.18	NaOCl - CT	21.93	*>0.9999*
	AA - TH	17.11	CT - CT	11.84	*0.9980*
	CT - TH	2.78	CT - CT	11.84	*0.9715*
	NaOCl - TH	22.18	CT - CT	11.84	*0.9775*
	CT - TH	2.78	AA - TH	17.11	*0.4514*
	NaOCl - TH	22.18	AA - TH	17.11	*0.9206*
	NaOCl - TH	22.18	CT - TH	2.78	*0.1395*

Table 1. continued

T4 6 months	NaOCl - CT	88.72	AA - CT	10.49	***<0.0001***
	CT - CT	8.45	AA - CT	10.49	*0.9990*
	AA - TH	22.54	AA - CT	10.49	*0.2937*
	CT - TH	-4.74	AA - CT	10.49	*0.7716*
	NaOCl - TH	-7.55	AA - CT	10.49	*0.4648*
	CT - CT	8.45	NaOCl - CT	88.72	***<0.0001***
	AA - TH	22.54	NaOCl - CT	88.72	***<0.0001***
	CT - TH	-4.74	NaOCl - CT	88.72	***<0.0001***
	NaOCl - TH	-7.55	NaOCl - CT	88.72	***<0.0001***
	AA - TH	22.54	CT - CT	8.45	*0.3274*
	CT - TH	-4.74	CT - CT	8.45	*0.8287*
	NaOCl - TH	-7.55	CT - CT	8.45	*0.6408*
	CT - TH	-4.74	AA - TH	22.54	*0.2873*
	NaOCl - TH	-7.55	AA - TH	22.54	***0.0073***
	NaOCl - TH	-7.55	CT - TH	-4.74	*>0.9999*

Differences between groups were assessed using one-way ANOVA. Multiple comparisons test was then performed, followed by Tukey's post-test. P-values less than 0.05 were regarded as significant, Not calculated --

DISCUSSION

This study evaluated the effects of disinfection and storage media on the microhardness of human enamel blocks evaluated after four different time periods. The combination of chloramine T and thymol showed the most stable surface microhardness values compared to the rest of the combinations at all evaluated times.

Disinfection and preservation processes need to be defined effectively in order to ensure the quality of dental samples to be used in research or academic processes. Two essential aspects are limiting cross-contamination and causing the least possible damage to the mineralized structure, since teeth that are not disinfected and are kept dry, even for short periods, have been found to be unsuitable for academic or research projects[10,18,19].

Extracted teeth should be immersed in a medium that (a) preserves their moisture, to reduce any structural changes, and (b) disinfects them, to reduce any potential health risks caused by blood-borne pathogens to people handling specimens during academic and research activities[20]. The latter is stated in the ISO guidelines for studies with dental materials (tooth adhesion tests)[21], and is the basis for the aim of this study.

This research helps highlight the importance of protocolized management of extracted teeth, which are used in different processes – mainly preclinical practices. The processes used by students to acquire extracted teeth are not currently regulated, exposing the dental school community to hazards that could easily be addressed through community awareness and protocol implementation[22].

The result of this study showed that the most stable microhardness values for the surface structure were observed with the chloramine T-thymol combination, in agreement with previous reports[7]. Thymol has been described as effective in preventing cross-contamination and sample dehydration, which supports its use as storage medium. However, using thymol to preserve extracted teeth should consider their intended use, as it has been reported to be suboptimal in adhesion studies[1,7,16,23]. Thymol has been described as an inhibitor of methacrylate polymerization[1], as well as causing some alterations in the calcium, potassium, sodium, and phosphorus content of enamel[24] and dentin[25].

The second option found in this study to be adequate for disinfection-storage was the combination of chloramine T at 0.5% and 1%, as it did not generate considerable structural changes. This agrees with previous reports on the use of chloramine as a disinfectant and storage medium for extracted teeth[7,26]. ISO guidelines and Colombian Technical Standard (NTC 4882) consider the use of chloramine T 0.5% as a storage medium for dental samples for a period not exceeding one week, considering that other chemical agents can be absorbed and generate alterations[21,27]. This alternative has been shown to have no significant effects on the hardness, modulus of elasticity, or morphological characteristics of dental samples[26]. However, there are some contradictory reports regarding its effect on adhesion[7,9,26].

Not all combinations with chloramine T produced favorable results. The combination hypochlorite-chloramine T displayed the greatest variation in surface microhardness, especially at the longest exposure time. Although hypochlorite has been widely used in protocols for disinfecting and inactivating dental samples due to its antimicrobial properties, it affects dental hardness[28] and collagen content[29], making it an unsuitable storage medium for preserving dental samples to be used in research.

Although this study did not find optimal results with acetic acid, it has been described not only as having high efficacy as a disinfectant, but also for the preservation of the morphological properties of dental specimens[30]. Further studies are needed to determine the ideal disinfection and storage medium for different applications and the maximum storage time for human tooth banks.

CONCLUSION

This study found that the chloramine-thymol and chloramine-chloramine combinations of disinfection-storage media resulted in the most stable enamel surface microhardness values, suggesting that they are appropriate for maintaining tooth integrity during storage.

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CONFLICT OF INTERESTS

The authors declare no potential conflicts of interest regarding the research, authorship, and/or publication of this article.

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Table S1. Two-way ANOVA analysis

ANOVA table	SS	DF	MS	F (DFn, DFd)	*P value*
Time x T Disinfection-Storage Medium	106419	15	7095	F (15, 324) = 10.36	*p <0.0001*
Time	20566	3	6855	F (3, 324) = 10.01	*p <0.0001*
Disinfection-Storage Medium	38826	5	7765	F (5, 108) = 11.95	*p <0.0001*

Table S2. Comparison of microhardness between treatments and times based on two-way ANOVA

Time	Disinfection-Storage Medium 1	(%)	Disinfection-Storage Medium 2	(%)	*p value*
T2 (1 month)	NaOCl – CT	26.67	AA - CT	0.75	***0.0272***
	CT – CT	26.67	AA - CT	8.36	*0.2537*
	AA – TH	26.67	AA - CT	32.79	*0.9788*
	CT – TH	26.67	AA - CT	4.05	*0.081*
	NaOCl – TH	26.67	AA - CT	3.31	*0.0645*
	CT – CT	0.75	NaOCl - CT	8.36	*0.9459*
	AA – TH	0.75	NaOCl - CT	32.79	***0.0023***
	CT – TH	0.75	NaOCl - CT	4.05	*0.9988*
	NaOCl – TH	0.75	NaOCl - CT	3.31	*0.9997*
	AA – TH	8.36	CT - CT	32.79	***0.0456***
	CT – TH	8.36	CT - CT	4.05	*0.9957*
	NaOCl – TH	8.36	CT - CT	3.31	*0.9911*
	CT – TH	32.79	AA - TH	4.05	***0.0093***
	NaOCl – TH	32.79	AA - TH	3.31	***0.0069***
	NaOCl – TH	4.051	CT - TH	3.31	*>0.9999*
T4 (6 months)	NaOCl - CT	10.49	AA - CT	88.72	***<0.0001***
	CT - CT	10.49	AA - CT	8.453	*0.9999*
	AA - TH	10.49	AA - CT	22.54	*0.7098*
	CT - TH	10.49	AA - CT	-4.74	*0.4630*
	NaOCl - TH	10.49	AA - CT	-7.55	*0.2694*
	CT - CT	88.72	NaOCl - CT	8.45	***<0.0001***
	AA - TH	88.72	NaOCl - CT	22.54	***<0.0001***
	CT - TH	88.72	NaOCl - CT	-4.74	***<0.0001***
	NaOCl - TH	88.72	NaOCl - CT	-7.55	***<0.0001***
	AA - TH	8.45	CT - CT	22.54	*0.5522*
	CT - TH	8.453	CT - CT	-4.739	0.6229
	NaOCl - TH	8.453	CT - CT	-7.547	0.4056
	CT - TH	22.54	AA - TH	-4.739	**0.0165**
	NaOCl - TH	22.54	AA - TH	-7.547	**0.0053**
	NaOCl - TH	-4.739	CT - TH	-7.547	0.9995

Differences between groups were assessed using two-way ANOVA. A multiple comparisons test was then performed. followed by Tukey's post-test. P-values less than 0.05 were regarded as significant