Rev. Mus. Argentino Cienc. Nat., n.s.
24(2): 241-248, 2022
ISSN 1514-5158 (impresa)
ISSN 1853-0400 (en línea)

Effects of bleaching techniques used in osteological preparation in biological collections and their implications for dental microwear analysis

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Abstract: Dental microwear analysis is the study of enamel marks produced by ingested elements, allowing dietary inference in fossil groups. To generate these extrapolations, it is necessary to study reference specimens from biological collections. Observations on teeth of specimens treated with hydrogen peroxide (H_2O_2) and/or sodium hypoclorite (NaClO), a preparation technique used in some institutions, reveal patterns on the enamel surface inconsistent with dietary scars. To understand how these chemicals could be affecting teeth enamel and microwear patterns we ran controlled experiments using a *Hydrochoerus hydrochaeris* tooth. Distal portion was treated with NaClO 1.25% for 1 minute and then with H_2O_2 10% from intervals of 30 to 120 minutes. Mesial portion was submerged in NaClO 1.25% during the same intervals. Casts were made for control and treatment stages and examined in scanning electron microscope (SEM) at 400x magnification. H_2O_2 progressively softens shallow traces and at longer exposure erodes deep scars. NaClO deepens the scars, changing its shape. Both chemicals homogenize enamel surface at longer exposure. Based on these results we highly recommend avoiding these chemicals and emphasize the importance of reporting their use in vertebrate collections as the inclusion of treated teeth in microwear analysis could result in erroneous dietary inferences.

Keywords: cleaning process, dental enamel, diet, mammal collection

Resumen: Efectos de las técnicas de blanqueamiento utilizadas en la preparación osteológica en colecciones biológicas y sus implicaciones sobre el análisis de microdesgaste dental. El análisis de microdesgaste dental es el estudio de cicatrices en el esmalte producidas por el procesamiento de alimentos en el aparato bucal, permitiendo la inferencia dietaria en grupos fósiles. Para generar esas extrapolaciones es necesario el estudio de especímenes de referencia depositados en colecciones biológicas. Observaciones sobre dientes de especímenes tratados con peróxido de hidrógeno (H_2O_2) y/o hipoclorito de sodio (NaClO), una técnica de preparación utilizada en algunas instituciones, revela patrones en la superficie del esmalte inconsistentes con cicatrices dietarias. Para comprender cómo esos compuestos podrían estar afectando el esmalte dental y los patrones de microdesgaste realizamos experimentos controlados utilizando un diente de *Hydrochoerus hydrochaeris*. La porción distal fue tratada con NaClO 1.25% por 1 minuto y en seguida con H_2O_2 10% por intervalos de 30 a 120 minutos. La porción mesial fue sumergida en NaClO 1.25% durante los mismos intervalos de tiempo. Copias de las etapas de control y tratamiento fueron confeccionadas y evaluadas en microscopio electrónico de barrido (MEB) con aumento de 400x. El H_2O_2 suaviza progresivamente las marcas superficiales y en exposiciones más largas erosiona cicatrices profundas. El NaClO profundiza las cicatrices, cambiando su forma. Ambos químicos homogeneízan la superficie del esmalte en exposiciones más largas. Basado en esos resultados recomendamos fuertemente evitar esos químicos y enfatizamos la importancia de reportar su uso en colecciones de vertebrados, ya que la inclusión de un diente tratado con H_2O_2 y/o NaClO en un análisis de microdesgaste podría resultar en inferencias dietarias erróneas.

Palabras clave: proceso de limpieza, esmalte dental, dieta, colección de mamíferos

INTRODUCTION

Microwear analysis is a technique frequently used in paleontology to infer the dietary preferences of vertebrates (Solounias & Semprebon, 2002; Williams *et al*., 2009; Winkler *et al*., 2019). It consists of the classification and quantification of wear traces on the surface of the teeth produced by ingested elements, allowing to assign a specimen to a dietary category, as for example, grazer, browser, frugivore or granivore (Corona *et al*., 2019; Semprebon *et al*., 2004; Townsend & Croft, 2008). To generate these inferences, it is resorted to the comparative study of voucher specimens preserved on biological collections.

As virtually all the studies on microwear are performed using casts of the original teeth (Corona *et al*., 2019; Solounias & Semprebon, 2002; Townsend & Croft, 2008), some works have attempted to the correct use of silicones and resins, so they do not affect the resolution of the study leading to errors in the analysis (Galbany *et al*., 2004; Mihlbachler *et al*., 2019; Rose, 1983). Studies have also investigated the effects of exogenous grit and dust (Burgman *et al*., 2016; Merceron *et al*., 2016; Sanson *et al*., 2007) or taphonomic process (Böhm *et al*., 2019; King *et al*., 1999) on tooth microwear patterns. The development and comparison of different techniques for microwear analysis were also explored by several researchers (Grine *et al*., 2002; Merceron *et al*., 2005; DeSantis *et al*., 2013; Scott *et al*., 2006, Solounias & Semprebon, 2002; Ungar *et al*., 2003).

Besides all the above-mentioned subjects we must consider while working with microwear, any effort was directed to the selection of materials of extant specimens used for comparisons. Working with teeth of *Lagostomus maximus* of a few mammal collections - Museo Argentino de Ciencias Naturales 'Bernardino Rivadavia' (MACN), Museo de La Plata (MLP) and Fundación Felix de Azara (CFA-MA) - we could notice that in some samples the texture observed on tooth enamel was really different (Fig. 1a and b) from the observed in dental microwear studies (Fig. 1c) for dietary signature, being difficult or even impossible to identify the typical structures that are generally related to dietary microwear (scratches, gouges and pits).

These observations led us to question if some preparation techniques could be playing an important role in the observed tooth surface relief. Here we focus on two chemical compounds that were largely used in the past, and even nowadays are sometimes used, for cleaning or bleaching: hydrogen peroxide (H_2O_2) and sodium hypochlorite (NaClO) (Díaz *et al*., 1998; Holden, 1914; Williams *et al*., 1977). Dentistry research shows how hydrogen peroxide affects human teeth, as this product is used as bleaching agent with esthetical purposes (Elfallah *et al*., 2015; Rodrigues *et al*., 2017). The effect of sodium hypochlorite, another bleaching agent largely used in vertebrate collections, was also showed in some experiments with human teeth as it is used for sterilization and enamel deproteinization in some dentistry procedures (Amaechi *et al*., 1998; Ekambaram *et al*., 2017).

Even though many authors now indicate not to use these compounds in osteological preparations due to their deleterious effects (Hendry, 1999; Simmons & Muñoz-Saba, 2005) there are not studies demonstrating their effects on dental microwear of specimens housed in biological collections. In this work we point out some altered materials found in Mammalogy collections, and we run some short experiments to check the effects of hydrogen peroxide and sodium hypochlorite on the occlusal micro relief. The aim is to check qualitatively the effects of these chemical compounds on tooth enamel surface and contribute to the recognition of altered materials in biological collections. This could improve the selection of samples that will constitute the reference database for tooth microwear analysis, avoiding misinterpretation in dietary inferences in fossil species.

MATERIAL AND METHODS

We used SEM images of three specimens of *Lagostomus maximus* from the mammal collections of MACN and CFA (MACN-Ma 3928, MACN-Ma 3971, CFA-Ma 8706) to compare enamel surface textures (Fig. 1).

For the purpose of this study, a tooth was selected for analysis from a skull of an adult *Hydrochoerus hydrochaeris* (Rodentia, Caviidae) (didactic collection, catalog number not assigned) from the mammal collection of MACN. This species was selected for the experiment as it was abundant in the didactic collection and because the chosen specimen presented no chemical treatment. These caviomorph rodents have continuously growing teeth (elodont), with enamel ridges parallel to dentin, meaning no occlusal wear on enamel could expose dentin and lead to a microwear analysis in a structure other than enamel. A third left upper molar was sepa-

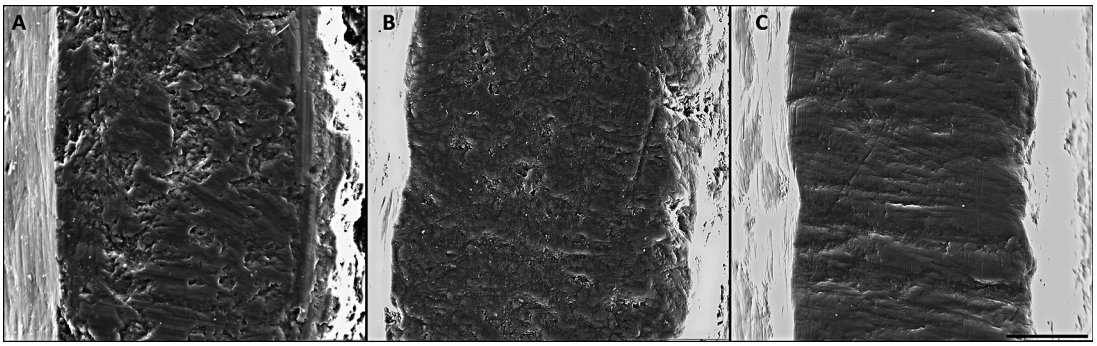

Fig. 1. Occlusal surface of different specimens of *Lagostomus maximus* at 400x magnification SEM. A, MACN-Ma 3928. B, MACN-Ma 3971. C, CFA-Ma 8706. A and B have altered texture of the enamel surfaces and the original microwear features are indistinct or hard to distinguish. In C the enamel surface is unaltered and shows many scratch marks, a pattern usually seen on grazer mammals. Scale bar = 100μm.

rated and divided in three parts, where distal and mesial regions were treated with chemical compounds (H_2O_2 and NaClO) and a third portion (central) remained with no treatments (control). The concentrations of hydrogen peroxide and sodium hypoclorite selected followed protocols indicated in the literature dealing with mammal collections (Díaz *et al*., 1998).

Molds of the occlusal surface of tooth samples were made before the treatments and after each interval of submersion using polyvinyl siloxane (*President MicroSystem Light Body* Coltène®), to track possible modifications on teeth microwear patterns. To obtain casts, the molds were then filled with liquid epoxy resin (*CRISTALTACK*®) prepared with 2 parts of resin (component A) to 1 part of catalyst (component B) and let dry for 24 hours. Casts of teeth were mounted on pin stubs using cyanoacrylate adhesive and coated with 200 Å gold-palladium film using a sputter-coater 'Termo VG Scientific SC 7620'. To evaluate the effects of each chemical compound, microphotographies were obtained from the same region of the occlusal surface on control and treated samples using a SEM Philips series XL model 30 at the MACN under 20kV, 5mm distance work. There is not a standard magnification for microwear studies in rodents; so we defined a 400x magnification. This magnification was based on the width of the enamel ridges of *Lagostomus maximus* specimens; the same magnification was then applied to *Hydrochoerus hydrochaeris*.

Experiment 1

The distal portion of the tooth was treated following protocol indicated by Díaz *et al*. (1998): 1 minute of submersion in 1.25% solution of NaClO of domestic use (2.5%) and then submerged in a 10% solution of H_2O_2 for intervals of 30, 60, 90 and 120 minutes. Díaz *et al*. (1998) were not clear about the interval of submersion in H_2O_2, so we tested different intervals to identify the effects of short and long exposures.

Experiment 2

The mesial portion of the tooth was submerged in 1.25% solution of NaClO (2.5%) during intervals of 30, 60, 90 and 120 minutes. The exposition intervals were selected by us knowing that intervals indicated in protocols are not correctly respected in many preparations and also considering the not neutralized reactions that allows chemicals to continue acting even after the exposure intervals.

RESULTS

In *Lagostomus maximus* SEM images (Fig. 1) it is possible to observe that specimens MACN-Ma 3928 and MACN-Ma 3971 have microwear features (scratches, pits and gouges) hard to distinguish by having an altered texture of the enamel surfaces. CFA-Ma 8706 presents an unaltered enamel surface and shows several scratch marks.

Experiment 1

In the sample treated with 1-minute NaClO and then with H_2O_2, the superficial scars of the enamel are progressively hard to distinguish, as their limits become more diffuse and there is a homogenization of the surface (see Fig. 2). For deeper structures it is possible to identify they become more visible under longer exposure time (e.g., gouge indicated by an arrow at left superior margin of the images at Fig. 2). At exposure

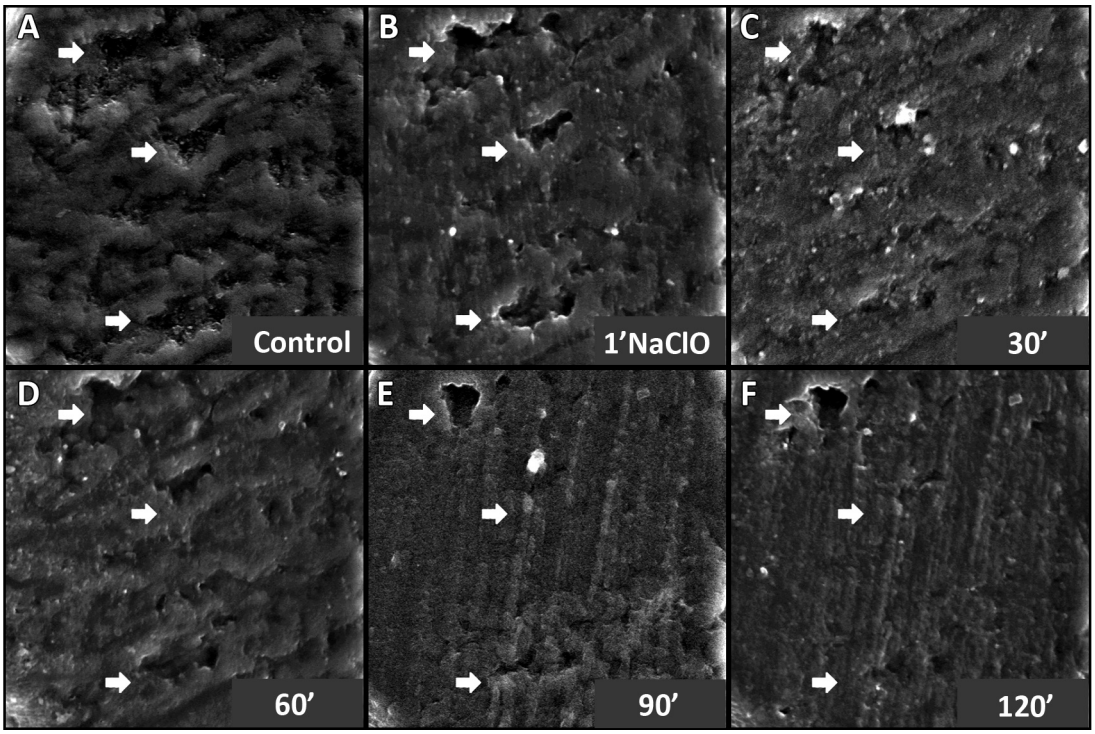

Fig. 2. SEM images (100x100μm) of a third upper molar of *Hydrochoerus hydrochaeris* showing the enamel surface before treatments (A) and the changes in microwear patterns after a 1-minute submersion in NaClO (B) and different exposition intervals to H_2O_2: C) 30 minutes, D) 60 minutes, E) 90 minutes, F) 120 minutes. White arrows indicate the same surface structures changing along intervals. 400x magnification.

intervals of 90 (Fig. 2 E) and 120 minutes (Fig. 2 F), the generation of a new pattern like parallel scratches can be observed on occlusal enamel surface.

Experiment 2

The sample treated only with NaClO shows progressive changes in the size and shape of the scars (Fig. 3), resulting in a shift on the general morphology of the scars. At shorter intervals of submersion in this solution (30 and 60 minutes), there is a deepening of the scratches on the enamel and the entire teeth surface seems to be splitting into layers (Fig. 3 B and C). At higher exposure intervals (Fig. 3 D and E), the microwear patterns start to be obscured and there is an increased corroded surface, but it is possible to identify some new depressions with a gouge or pit morphology.

DISCUSSION

The preparation techniques of samples to be deposited in osteological collections has evolved along the years with the practice of cleaning with chemicals to remove tissues (*e.g*. sodium hypochlorite) and bleaching specimens using sodium hypochlorite and/or hydrogen peroxide (Díaz *et al*., 1998; Holden, 1914; Williams *et al*., 1977). Nowadays, maybe as a result of increased interest in natural history conservation (Cato *et al*., 2001), some authors recommend avoiding their use, applying it only if the specimen is for exhibition purposes and, even in these cases, just if it is really necessary (Hendry, 1999; Simmons & Muñoz-Saba, 2005).

Alterations caused by bleaching agents on dental material go beyond the superficial enamel, preventing teeth material to be used for many studies. The H_2O_2 is an oxidant agent with high capacity of forming free radicals like O_2 and HO_2 which can damage biomolecules preventing teeth to be used for DNA and other molecular studies (Hu *et al*., 1995; Ribeiro *et al*., 2006). Some studies concluded also that hydrogen peroxide and sodium hypochlorite alter isotopic values in bioapatite carbonate fractions, causing its dissolution (H_2O_2) and incorporating exogenous car-

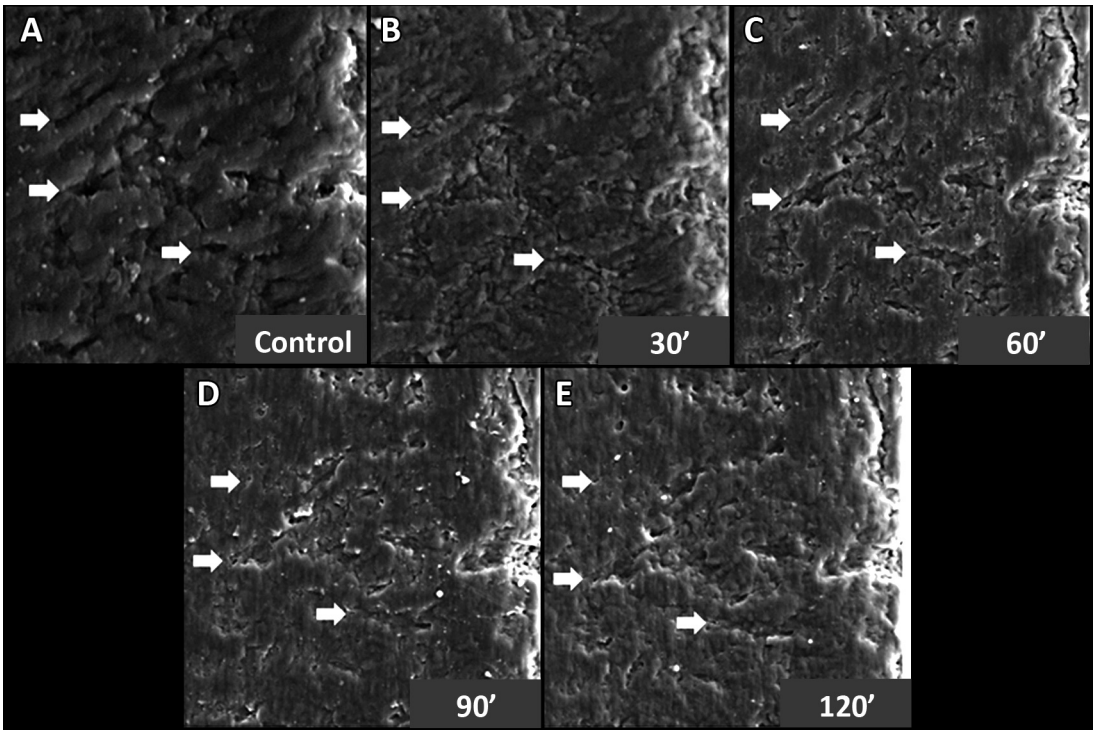

Fig. 3. SEM images (100x100μm) of a third upper molar of *Hydrochoerus hydrochaeris* showing the enamel surface before treatments (A) and the changes in microwear patterns in different exposition intervals of NaClO: B) 30 minutes, C) 60 minutes, D) 90 minutes, E) 120 minutes. White arrows indicate the same surface structures changing along intervals. 400x magnification.

bonates to it (NaClO) (e.g., Pellegrini & Snoeck, 2016).

There are different conclusions in dentistry research about the use of hydrogen peroxide concerning the effect on teeth enamel surface (Alqahtani, 2014; Joiner, 2007). Some works show it can alter the microhardness and superficial texture of teeth enamel, due to demineralization of the teeth (Jiang *et al*., 2008; Rodrigues *et al*., 2017). The effect seems also to be directly related to the concentrations and exposure time of the teeth to this bleaching agent (Bistey *et al*., 2007). This conclusion is supported by our results, where we identify that this chemical compound not only deepens dietary scar present at the teeth but also generates a new micro relief at longer exposure time.

As in most biological collections the hydrogen peroxide used is generally the commercial one, the treated materials could be suffering the effects of not only hydrogen peroxide but also of the acids used to stabilize these solutions as more changes seems to be produced on enamel surface in an acid environment (Rodrigues *et al*., 2017; Xu *et al*. 2011). Commercial hydrogen peroxide generally has the addition of an acid to lower the pH values and ensure the stability of the product. The one we used, for example, has phosphoric acid, used on dentistry for acid etching and could be directly affecting the teeth surface (Cerci *et al*., 2012; Kodaka *et al*., 1993). At longer intervals of exposure to H_2O_2, 90 and 120 minutes, some parallel grooves become evident, which could reflect the alignment of the enamel microstructure that becomes visible after the removal of the superficial enamel (Koenigswald & Clemens, 1992).

Sodium hypochlorite is also used in dentistry but not as a bleaching agent, as in the case of hydrogen peroxide, but for teeth sterilization and deproteinization (Amaechi *et al*., 1998; Ekambaram *et al*., 2017). When applied on teeth, NaClO increases dentinal tubules and enamel pores, turning the tissue more permeable and enhancing, for example, the bond between teeth and resins (Abdelmegid, 2018; Silva *et al*., 2018). It is possible to see in our images, mainly at longer exposure times, that NaClO deepens many scars and creates new ones mainly with a gouge or pit aspect, what could be a result of enhanced

corrosion due to larger permeability and a larger surface exposed to the action of this chemical compound.

The diversity of textures observed on altered samples is a combination of the chemical compound and the exposure intervals chosen. For shorter intervals (30-60 minutes), H_2O_2 seems to cause fewer modifications on general teeth microwear pattern when compared do NaClO. Any case, it is crucial to notice that even shorter periods of submersion in any of these chemical compounds altered the original microwear pattern of the teeth, disabling irreversibly the treated materials for microwear studies as they affect natural wear patterns produced by specimen dietary behaviors, leading to erroneous dietary inferences when using these samples.

CONCLUSIONS

The chemical attack by H_2O_2 and NaClO used for cleaning and bleaching skeletons and skulls continues to be used in some biological collections, even when its negative impacts were previously adverted for DNA and isotopic studies. It is important to highlight here that the chemical compounds, mainly sodium hypochlorite, may not be neutralized when the skulls and skeletons are washed with water as this procedure only dilutes the compounds, meaning the chemical can continue acting a longer period even on a lower concentration. Specimens in biological collections are stored by many years, so time plays an important role on materials treated with bleaching agents and the effects of this longer exposure has to be studied as a particular case when compared to dentistry research.

Summing up, we show the effects of H_2O_2 and NaClO on teeth surface with special attention to dental microwear, bringing evidence for the use of alternative preparation and conservation techniques that allow the recovery of information. New experiments in other groups and using different concentration and/or exposure times for any chemical compound used in biological collections could contribute to the knowledge about the effects of some practices on osteological and teeth samples.

ACKNOWLEDGEMENTS

This work was supported by grant CONICET PUE 2017-2021 to MACN. We thank to the editor Agustín Martinelli and two reviewers, Marina Lozano (Institut Català de Paleoecologia Humana i Evolució Social, España) and Patrícia Braunn (Museu de Ciências Naturais do Rio Grande do Sul, Secretaria do Meio Ambiente e Infraestrutura, Porto Alegre, Brasil), for their insightful comments that helped in clarifying a previous version of this manuscript.

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Doi: 10.22179/REVMACN.24.789

Recibido: 7-VII-2022
Aceptado: 17-XI-2022