THE USE OF OZONE TO LIGHTEN TEETH. AN EXPERIMENTAL STUDY

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ABSTRACT
Tooth-whitening agents are available for therapeutic use in the dental office or at home. However, whitening more severe stains, such as those caused by systemic ingestion of tetracycline, constitutes a challenge. The aim of this study was to evaluate, in an experimental model of growing rats, the efficacy of using ozone to lighten tetracycline-stained incisors. At weaning, male Wistar rats (n = 40) were randomly assigned to one of three groups. Two control groups, C21 and C60 (n = 8, each) were used to document the usual age-related color. The third group (n = 24) received 0.25 g% of oxytetracycline (O) until 60 days of age. These rats were subsequently divided into three further groups: O0, O3 and O5 (n = 8, each). These rats were anesthetized; O3 and O5 groups received ozone application to the lower incisors for 3 (group O3) or 5 minutes (group O5), respectively; while O0 did not receive the ozone treatment. Teeth were then photographed and the incisors from the control (C60) and treatment groups (O0, O3 and O5) were cut, and compared to a standard color guide (there were eight shades numbered 0 to 7, lightest to darkest) to assess the hue visually. The teeth were then placed in phosphoric acid to quantify the color by spectrophotometry. The data (mean ± SD) were analyzed by One-Way Analysis of Variance (ANOVA) followed by Tukey’s test or Dunnett test. The visual observation, analyzed blindly by one investigator, showed that O3 and O5 groups had diminished yellowing of the teeth as compared to the untreated O0 group (P<0.001). The color quantified by spectrophotometry also detected significant differences among groups (O3 < O5, P<0.01; O3 < O6, P < 0.001 and O5 < O6, P<0.01). C21 and C60 were significantly different among groups (P<0.001). This is the first experimental study to show that ozone can be successfully used for lightening the yellowish tinge of tetracycline-stained rat incisors. Further studies are required for its potential use in the dental clinic.

Key words: ozone, tetracycline, bleaching-agent.

USO DEL OZONO PARA BLANQUEAR DIENTES. ESTUDIO EXPERIMENTAL

RESUMEN
Los agentes blanqueadores dentales están disponibles para tratamiento en el consultorio odontológico o en el domicilio. Sin embargo, aclarar manchas severas, como las causadas por la ingestión sistémica de tetraciclina, constituyen un desafío. El objetivo de este estudio fue evaluar, en un modelo experimental de ratas en crecimiento, la eficiencia del uso de ozono para aclarar los incisivos oscurecidos por el uso de tetraciclina. Ratas macho Wistar al destete (n=40) fueron asignadas aleatoriamente a uno de tres grupos. Dos control groups, C21 y C60 (n=8, cada uno) fueron asignados a documentar el color usual de los incisivos, correspondiente a la edad del animal. El tercer grupo (n=24) recibió 0.25 g% de oxitetraciclina (O) hasta los 60 días de edad. Estas ratas fueron luego divididas aleatoriamente en tres más grupos: O0, O3 y O5 (n=8, cada uno). Estas ratas fueron anestesiadas; O3 y O5 recibieron ozono aplicado a los incisivos inferiores para 3 (grupo O3) o 5 minutos (grupo O5), respectivamente; mientras que O0 no recibió tratamiento. Se tomaron fotografías de los incisivos de C60 y tratamiento grupos (O0, O3 y O5) y luego se compararon con una guía estándar de ocho colores (ordenados de 0 a 7, desde el más claro a más oscuro) para cuantificar visualmente el color de los incisivos. Luego, se colocaron en ácido fosfórico para cuantificar el color por espectrofotometría. Los datos (media ± SD) fueron analizados por medio de ANOVA y prueba de Tukey o Dunnett (a = 0.05) para determinar el efecto del tratamiento. El análisis visual de las imágenes mostró que O3 y O5 disminuyeron el color amarillo intenso respecto a O0 (P<0.001). La coloración cuantificada por espectrofotometría también mostró diferencias significativas entre grupos (O3 < O5, P<0.01; O3 < O6, P < 0.001 y O5 < O6, P<0.01). C21 y C60 fueron significativamente diferentes entre grupos (P<0.001). Esta es la primera investigación experimental para mostrar que el ozono puede ser exitosamente utilizado para aclarar los tonos amarillos de los incisivos de ratas infectadas con tetraciclina. Se requieren estudios adicionales para su uso en la clínica odontológica.

Palabras claves: ozono-tetraciclina- agente blanqueador.
INTRODUCTION
Tooth-whitening agents are available for use in the office or at home. The demand for tooth bleaching dental treatments is increasing as patients wish to improve their appearance. The efficacy of tooth whitening is a major concern in dental practice, since its cosmetic result is immediately noticeable, though it may not be the only aspect involved in good facial appearance.

However, whitening more severe stains, such as those caused by systemic ingestion of tetracycline, constitutes a challenge. Different bleaching agents have been utilized, e.g. carbamide peroxide or hydrogen peroxide plus carbamide peroxide. There are also variations in the way these agents are applied, such as exposure time, e.g., months of carbamide peroxide treatment; or concentration, e.g. 10%, 15% or 20% carbamide peroxide. Additionally the mode of activation or methods of application of the whitening compound may differ.

In dentistry, ozone has proved to be successful in the treatment of root caries, non-cavitated fissure carious lesions, early carious lesions in teeth, dental surgery or following tooth extraction processes and reduction of pathogenic micro-organisms of carious dentine. Ozone has also been used to whiten teeth in individuals with dental sensitivity and mucosal ulcerations; in those who consume tobacco, coffee or chocolate; and in those who have extrinsically stained teeth due to brown-colored melanoids or chlorhexidine use. However, ozone has not been used to bleach intrinsically stained teeth. The aim of this study was to evaluate the efficiency of ozone to lighten tetracycline-stained incisors in an experimental model of growing rats.

MATERIALS AND METHODS
This study was performed in accordance with the Code of Ethics in Research of the University of Buenos Aires and the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocol was approved by the University of Buenos Aires.

At weaning (21 days of age), 40 male Wistar rats from the animal laboratory of the Department of Biochemistry, Faculty of Dentistry, University of Buenos Aires, Argentina, were studied. The rats were housed in galvanized cages with meshed floors in order to maintain hygienic conditions and to avoid coprophagia. They were exposed to a 12-h light, 12-h dark cycle throughout the study. Room temperature was maintained at 21±1°C with a humidity of 50-60%. Rats were fed a rodent stock diet ad libitum.

Animals were randomly assigned to one of three groups. Two control groups (n=8, each) (C21 and C60) remained untreated in order to document the spontaneous evolution of the color of the jaw incisors at age 21 and 60 days, respectively. Tap water was provided ad libitum. At 21 days of age, when the rats were weaned (group C21), their lower incisors were cut and saved to determine the baseline color. At age 60 days, the lower incisors from the C60 group were photographed and cut in order to assess their color as described below.

The third group of rats (O, n=24) received 0.25 g% of oxytetracycline (Holliday, Scott) in drinking water and were fed ad libitum from the time of weaning to age 60 days.

At 60 days of age, group O was randomly divided into three further groups (n=8, each) O0, O3 or O5. The rats were anesthetized with ketamine (70 mg/kg) and xylazine (13 mg/kg), administered intraperitoneally. The anesthesia persisted for at least 60 minutes and allowed sufficient time to administer the ozone application. O0 was given no ozone treatment while in groups O3 and O5, ozone was applied to the jaw incisors for 3 (group O3) or 5 minutes (group O5), respectively. The ozone system used for the application was the HealOzone 2130C (KaVo Dental, Biberach, Germany). This is a self-contained device that produces ozone at a fixed concentration of 2100 p.p.m. ± 5% at a flow rate of 615 cc min⁻¹.

Visual Analysis
Once the experimental period was completed, the lower incisors (C60, O0, O3, O5) were photographed (Digital Camera Nikon D80, lent AF-S VR Micro-Nikkor 105 mm f/2.86, Nikon Corporation, Japan and electronic flash Macro Sigma EM-140D6, Carl-Zeiss, Rodermark, Germany) to assess the color of the teeth. The visual evaluation was carried out using a modification of the Vita Classic shade guide (Vita Classic, Lumin Vacuum Shade Guide, Vita Zahnfabrik H. Rauter GmbH & Co.KG, Germany), which includes
shades arranged by value, and was developed for human teeth. In the present study, the shades were modified to conform to the range of colors of rat teeth, ranging from the slightly yellow color of the rats at weaning (C21) to the darkest yellow tinge available. There were eight shades numbered 0 to 7, lightest to darkest, using a standard color guide. The color difference was calculated by subtracting the tab number corresponding to the experimental group (O0, O3 and O5) from that of C60 (paired by age). The data were analyzed statistically.

The color of the teeth of each rat was determined by a single investigator, in order to avoid individual variations in the estimate. The colors of the incisors were analyzed blindly as the investigator did not know the source of the teeth of the rats in each of the groups studied.

Spectrophotometric Analysis

The incisors of C21, C60, O0, O3 and O5 groups were cut and prepared for quantitative assessment of tooth color. They were placed in one milliliter of 37% phosphoric acid. The solutions, tested in duplicate, as well as the blank solution (37% phosphoric acid) were vortexed and then incubated at room temperature for 1 hour. In order to obtain the best readability and accuracy, the scale was set to read zero absorbance with a blank. Each sample was transferred to a cuvette for reading at wavelength 425nm (spectrophotometer Metrolab-1600 plus, Metrolab Argentina). This peak was chosen because it was the most prominent when the solutions were scanned for absorption from 400nm to 800nm. The data were presented as mean absorbance units (AU) ± standard deviation, where absorbance is a measure of the quantity of light absorbed by a sample, which is proportional to the amount of chromophores present.

Statistical analysis

Color difference by age (experimental groups minus C60) obtained from visual assay was analyzed by One-Way Analysis of Variance (ANOVA) followed by Tukey’s test. Comparison of readings between control (C60) and experimental O0, O3 or O5 groups were performed using a One-Way Analysis of Variance (ANOVA) followed by Dunnett test. In order to detect differences between the five groups, One-Way Analysis of Variance (ANOVA) followed by Tukey’s test was used. The significance level was set at 5%. The Statistical Product and Service Solutions for Windows 9.0 (SPSS, Inc., Chicago, IL) were used for statistical analyses.

RESULTS

At weaning, the lower incisors acquired a slight yellow color and by 60 days of age, this color was more intense, as expected (P< 0.001). After tetracycline treatment (age 60 days), the lower incisors in the O0 group were dark yellow-orange. The results of the visual observation of tooth color showed that the ozone procedure lightened the pigmentation of the incisors of groups O3 and O5. The teeth of groups O3 and O5 were lighter than those of group O0 but darker than those of C60 (Fig. 1). Additionally, using the color scale developed from the standard shades, the color difference between C60 and O0, O3 and O5 was significantly higher for O0 (5.25±0.46) and decreased from O3 (3.25±0.50) to O5 (1.75±0.39) (p<0.01); with significant differences between O5 and O3 (p<0.01). C21 numbered as zero was significantly different among groups (p<0.001).

Fig. 1: Color comparison of the lower incisors shown in photographs of one rat of each group tested.

C60: Rat 60 days of age to document the natural age-related color (yellow).
O0: Rat 60 days of age that received oxytetracycline from weaning. The antibiotic used in this study stained teeth yellow-orange.
O3: Rat 60 days of age that received oxytetracycline from weaning and ozone treatment for 3 minutes.
O5: Rat 60 days of age that received oxytetracycline from weaning and ozone treatment for 5 minutes.
The color quantified by spectrophotometry showed significant differences between groups C60 and O3, O5 and O0. C60 was lower than experimental groups (P<0.01). Additionally, differences were observed for O3, O5 and O0 groups (O3 < O0, P<0.01; O5 < O0, P<0.001). Five minutes of ozone exposure resulted in better lightening (O5 < O3, P<0.01). C21 color was significantly lower than all groups (P<0.01) (Figure 2).

DISCUSSION

This is the first experimental study designed to evaluate the use of ozone to lighten tetracycline stained incisors. The whitening response to ozone of the lower incisors of the rats was impressive, clearly evident by visual evaluation. After three minutes of ozone exposure, the incisors were whitened and the tetracycline staining was reduced to a more suitable color. Furthermore, after five minutes' treatment the incisors continued to lighten. The quantitative spectrophotometric analysis of tooth color confirmed the visual impression of tooth whitening. Three minutes of ozone exposure reduced the yellow color by 28%, while five minutes achieved a 56% reduction. The mechanism of this process may involve the ozonation of double bond systems, which contribute to the chromophoric properties of the product23.

Most of the clinical studies that have used bleaching agents to whiten teeth have been based on visual assessments; this methodology is subjective and potentially influenced by a number of factors. In the present study, the blind use of a standard visual color guide allowed quantification of differences in color of the incisors as natural evolution with advancing age as well as with the ozone treatment employed. The quantitative spectrophotometric assessment of tooth color confirmed the differences detected visually and provided further details of the degree of coloring among groups.

The specific model selected for assessing tooth color and the response to ozone application was the rat incisor which, unlike most teeth, is continually erupting. This characteristic makes these teeth suitable for evaluating the whitening properties of ozone. Despite some small differences between rat and human incisor dentine24, a close resemblance in enamel morphology has been established25. Additionally, the relatively thick enamel layer in the mandible incisor makes it suitable for chemical and biochemical analyses26.

It has been shown that rat incisors start out white in the young rat, but by age 21 days (initial time of the present study) the upper incisors have a slight yellow tinge. By 25 days these teeth are distinctly yellow while the lower incisors have acquired a slight yellow shade. By 38 days of life, these colors are more intense, though the upper incisors remain with more color than the mandible teeth. The relationship between more pigmented upper incisors
and less pigmented lower incisors remains true throughout the rat’s life. In adult rats, the upper teeth are dark yellow-orange and the lower incisors are yellow\textsuperscript{27}. Since the color of the rat incisors changes throughout life, we performed baseline biochemical measurements, at weaning (C\textsubscript{21}) and at the end of the experimental period (C\textsubscript{60}). In the experimental groups given the antibiotic in the drinking water, we demonstrated that their teeth were stained yellow-orange in group O\textsubscript{0}. Tetracycline interferes with odontogenesis by attaching irreversibly to calcified tooth structures\textsuperscript{28}. It has been suggested that the color is derived from photo-oxidation of tetracycline molecules bound within the tooth structures\textsuperscript{29}. In infants and children, administration of tetracycline may cause not only permanent staining of teeth, but enamel hypoplasia and decreased linear skeletal growth\textsuperscript{30}. Although it is known that certain types of stains can be eliminated by a number of methods\textsuperscript{31}; tetracycline staining is more difficult to bleach\textsuperscript{32}. Conventional whitening of tetracycline-stained teeth may require longer sessions at the dental office\textsuperscript{33} due to the resistance of tetracycline stains to bleach\textsuperscript{34}.

An important advantage of the ozone procedure utilized in our experimental model was the short time needed to achieve successful bleaching. Future studies will be needed to elucidate the ozone effects on morphology and/or substance of the enamel that could result from a limited application to the teeth. Other bleaching agents that have been used to treat tetracycline-stained teeth have been shown to produce morphological alterations in the enamel or on the subsurface of enamel\textsuperscript{35}. Carbamide peroxide bleaching induces surface erosion, depressions, porosity and increased depth of enamel grooves and partial removal of enamel prisms. However, most bleaching agents differ from ozone since they are acidic, which is not favorable to enamel, dentin and cement\textsuperscript{2}.

**CONCLUSION**

This is the first study to demonstrate that tetracycline-stained incisors can be successfully lightened by the use of ozone. A simple, safe and non-invasive ozone treatment would provide an alternative therapeutic agent to current methods; however, further morphological assessments will be required for its use in the dental clinic.

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