STUDY PROTOCOL

CLINICAL PROTOCOL

1. Informative leaflets, containing the expected benefits and risks of the study, were provided to all parents/guardians. The latter handed in a written informed consent.
2. The clinical history of the child was filled out by interviewing their parents and children´s examination was carried out by an experienced pediatric dentist.
3. Caries lesions were recorded using a mirror and a WHO probe in conformity with the International Caries Detection and Assessment System criteria (ICDAS II).
4. Patients who met the inclusion criteria were randomly assigned to one of the three experimental groups.
5. At the beginning of the check-up, 3.5 ml of unstimulated saliva was collected for 5 minutes in a sterile polyethylene tube. Children had not ingested water or food 1 hour before the examination. Saliva samples were stored at –20^{o}C until measurement.
6. Plaque index measurement.

   After collecting saliva sample, we measured plaque index using a three tone disclosing agent. Next, we took photographic registries and carried out a prophylaxis without toothpaste.
7. Apply the assigned varnish.

   The teeth were dried with dry compressed air and isolated with cotton rolls and saliva ejector. Then, 0.25 ml of varnish was applied to the surface of the teeth and allowed to dry for 30 seconds. In the placebo group, distilled water was applied with a brush identical to that used to apply the varnishes
8. Check-up in 3 months, till 12 months.

PROTOCOL OF SAMPLES MEASUREMENTS

pH and Latic acid

Saliva samples were thawed and shaken for 10 seconds at 20°C (ClassicVortex Mixer, Velp Scientifica, Italy) and the saliva sample was poured onto a pH test strip (range 4.0–9.0; Code. 1.16996.0001; Reflectoquant™ Merck, Darmstadt, Germany) which was introduced in a RQflex®10 reflectometer (Merck Millipore, Darmstadt, Germany) to provide the pH value. Another saliva sample was poured onto a lactic acid test strip (range 1.0-60.0 mg/L; Code. 1.16127.0001; Reflectoquant™ Merck, Darmstadt, Germany) which was introduced in a RQflex® 10 reflectometer (Merck Millipore, Darmstadt, Germany) to provide the lactic acid value.

Fluoride

Fluoride concentrations were measured using an ion-specific fluoride electrode (Orion 9609 BNWP, Thermo Fisher Scientific Inc. Waltham, USA) coupled to an ion analyzer (Orion EA-940 Thermo Fisher Scientific Inc. Waltham, USA).

Trace elements

We analyzed 2 ml of the homogenized sample using mass spectrometry with inductively coupled argon plasma (ICP-MS Agilent 7900; Agilent Technologies Inc.; CA; USA).

Bacterial load in saliva

The quantitative analysis of bacterial load was made by qPCR using universal primers on the 16S ribosomal gene with high coverage rate in all bacteria PCR (Polymerase Chain Reaction). The DNA extraction was performed with the Maxwell AS1290 LEV Blood DNA Kit (Promega Biotech Ibérica S.L, Madrid, Spain). DNA was read by fluorimetry on the Prusga Quantus kit with the Quantifluor ONE dsDNA System (Promega Biotech Ibérica S.L, Madrid, Spain).

Caries index

The prevalence of caries was recorded using ICDAS II scores (range 0-6), which were transformed into the components of the dmfs/DMF-S indexes, and were assigned to each caries lesion, to calculate the caries experience.

Plaque index

A three-tone plaque disclosing gel (Triplaque, GC, Leuven, Belgium) was applied to identify new, mature and acid producing biofilm. The Turesky modification of the quigley-hein plaque index scale and Plaque Maturity and Acidity Index (PMAI), which we developed, was used to measure the plaque index.

STATISTICAL ANALYSIS

The analysis was carried out using the SigmaStat 3.5 statistical software package (Systat Software Inc., Point Richmond, CA, USA).

The Kokmogorov-Smirnov test was used to determine sample normality and the Levene test was used for equality of variance.

Pearson's chi-square test was used to determine between-group differences in sex and a one-way ANOVA test for differences in age. To determine within-group differences in age by sex we used the Mann-Whitney test. To detect between-group differences in DMFS and dmfs values a Kruskal-Wallis test was performed and within the same group between the T0 and T4 a Wilcoxon test was performed.

Differences in concentrations of trace elements, pH, lactic acid and bacterial load between baseline and 3, 6, 9 and 12 months were determined by simple variance analysis of repeated measures. When there were differences between the times, two-by-two comparisons were made using the Holm–Sidak test.

A paired t-test was used to analyze the within-group evolution of fluoride, Turesky modification of the Quigley-Hein plaque index scale and Plaque Maturity and Acidity Index concentrations between baseline and 12 months when there was normality and a Wilcoxon test when there was no normality. One-way ANOVA was used to detect between-group differences in the same period.

A value of $p<0.05$ was considered significant.