Study Protocol and Statistical Analysis Plan

Study design: single-blinded, 2-arm, randomized controlled clinical trial comparing an ultrasonic activation as the test protocol and a non-activated irrigation protocol as the control regarding their effectiveness in reducing bacteria and endotoxin from root canals after chemo-mechanical preparation. The study is not operator blinded because of the various devices used during the irrigation protocols, but the data analysis is blind.

Participants:

Criteria for inclusion: patients with necrotic pulps and apical periodontitis in single rooted teeth or in 1 root with a single canal from multi-rooted teeth. **Criteria for exclusion**: patients who had received antibiotics during the previous 3 months or had any general disease; non-restored teeth; periodontal pockets depths greater than 4 mm; and radiographic evidence of previous endodontic treatment, open apex, crown/root fracture, root resorption or calcifications.

Sample size: sample size was estimated based on the means and standard deviations of the pilot study using BioEstat 5.3 software (Mamiraua, AM, Brazil). Calculation of statistical significance was estimated using the Wilcoxon test (power of 80% and significance level of 5%). A sample size of 20 patients per group was calculated based on the results of the pilot study. Considering the dropouts, a final sample size of 25 patients per group was decided.

Randomization: block randomization method generated by an electronic online randomizer (Research Randomizer, www.randomizer.org).

Interventions: final irrigation protocols after root canal preparation: ultrasonic irrigation (UI) and needle irrigation (NI) groups. In the UI group, the irrigant was activated by a smooth wire with 0.2 mm diameter and .01 taper (Irrisonic - Helse, Ribeirão Preto, SP, Brazil), driven by an piezoelectric ultrasonic device set at 10% power following the manufacturer's recommendations. The studied protocol consisted of 2 cycles of refreshment/ activation of 2.5% NaOCl (30 s each), followed by 2 cycles of refreshment/ activation of 17% EDTA and 2 cycles of refreshment/ activation of 2.5% NaOCl. In the NI group, the volume of the irrigants, their sequence, the depth and time of irrigation were similar to the UI group but with no-ultrasonic activation.

Primary outcome measures: bacteria quantification by qPCR using universal primers for the conserved regions of the 16S rRNA gene of the Bacteria domain.

Secondary outcome measures: quantification of endotoxin by the quantitative kinetic turbidimetric LAL assay (Pyrogent 5000, Lonza Group Ltd, Walkersville, MD, USA) following the manufacturer's instructions.

Statistical analysis plan: data will be summarized and statistically analyzed with SPSS Statistics Desktop software (IBM Corporation, Armonk, NY, USA) using Wilcoxon test for related samples for intra-group analyses; Mann-Whitney test for quantitative analysis of bacteria and endotoxins between groups; and chi-squared tests for qualitative analysis (incidence of positive

samples for bacterial DNA and endotoxin). All analyses will be performed with a significance level of 5%.