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EFFECT OF DIFFERENT METHODS OF STERILIZATION ON THE INACTIVATION OF BACTERIAL ENDOTOXIN (LPS) IN ENDODONTIC FILES

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ABSTRACT

This study evaluated the presence of lipopolysaccharide (LPS) in endodontic files after *in vitro* contamination with *E. coli* LPS and sterilization in dry heat or wet heat, with or without previous immersion in a $Ca(OH)_2$ suspension. LPS was quantified using the Kinetic QCL™ test and data were analyzed statistically by the t-test. LPS was quantified only in the contaminated group, not submitted to any immersion or sterilization procedure (0.262±0.1296 endotoxin units/mL) (p=0.0003). In conclusion, both wet heat and dry heat sterilization were effective in inactivating LPS without the need of previous immersion of the files in a $Ca(OH)_2$ suspension.

Key-words: endotoxin; LPS; Endodontics; dry heat; wet heat

It is currently known that anaerobic microorganisms (14,18), especially Gram negative (1), are highly prevalent in root canals of primary and permanent teeth with apical periodontitis. Gram-negative microorganisms not only have different virulence factors and generate products and by-products that are toxic to tissues, but also present an endotoxin in their cellular wall known as lipopolysaccharide (LPS). The bacterial endotoxin is released during bacterial multiplication or death and triggers a series of important biological events that lead to an inflammatory response (16) and bone resorption (6).

During endodontic treatment, including neutralization of the necrotic content and biomechanical preparation, the released endotoxin may adhere to the metallic portion of the endodontic files and may be transferred from one root canal to another and even to different patients when files are reused after sterilization. This hypothesis is supported by the findings of studies that have demonstrated that bacterial endotoxin adheres to mineralized tissues, prostheses (8), orthodontic brackets (9) and implants (12).

Although it is well known that sterilization causes the death of bacteria, viruses, fungi and spores, the effect of sterilization of endodontic files in autoclave (wet heat) or Pasteur oven (dry heat) on the inactivation of bacterial endotoxin has not yet been shown. It is important to assess whether these instruments should undergo some kind of pretreatment prior to sterilization in order to eliminate endotoxin. Therefore, the purpose of this study was to evaluate the presence of endotoxin on endodontic files contaminated *in vitro* with *E. coli* LPS after wet heat or dry heat sterilization, with or without previous immersion in a calcium hydroxide suspension.

A total of 63 endodontic stainless steel K-files (size 35, 21 mm; Maillefer, Ballaigues, Switzerland) were assigned to 7 groups (n=9). The files in groups I to VI were sterilized in a dry oven (Lab-Line Imperial V Gravity Conventional Ovens, Barnstead International, Boston, MA, USA) and subsequently contaminated with endotoxin by immersion in a solution of lyophilized *Escherichia coli* endotoxin (Lipopolysaccharide B *E. coli* 055:B5, Sigma Aldrich Corporation, St. Louis, MO, USA)

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suspended in pyrogen-free water (Bio Whittaker; Cambrex Co., Walkersville, MD, USA) in a concentration of 25 ng/mL, at 37ºC for 1 h with agitation (126 rpm).

Several LPS concentrations have been used to investigate the effect of sterilization on endotoxin in the medical and pharmaceutical fields, ranging from 0.01 ng/mL to 10 mg/mL (2,4,10,11). Due to the lack of studies assessing the amount of endotoxin retrieved from endodontic instruments, a pilot study was carried out using different LPS concentrations: 25 ng/mL, 5, 50 and 500 µg/mL. Based on the findings of this pilot study, the concentration of 25 ng/mL was chosen for the present investigation because it presented values within the standard curve required by the test.

After the 1-hour stirring period, the files were placed on pyrogen-free Petri plates and submitted to the following procedures: **Group I:** after contamination with endotoxin, the files were sterilized for 1 h in dry heat (Pasteur oven; Olidef CZ, Ribeirão Preto, SP, Brazil) preheated at 170ºC; **Group II:** after contamination with endotoxin, the files were sterilized for 1 h in wet heat (Autoclave Bios 12L; Dabi Atlante, Ribeirão Preto, SP, Brazil) at 121ºC for 20 min; **Group III:** the files were contaminated with endotoxin and placed in a saturated suspension of calcium hydroxide (0.05 g/mL; Merck, Darmstadt, Germany) in pyrogen-free water at 37ºC for 2 h with agitation. They were subsequently sterilized as described in Group I; **Group IV:** the files were contaminated with endotoxin, placed in the saturated calcium hydroxide suspension at 37ºC for 2 h with agitation and subsequently sterilized as described in Group II; **Group V:** the files were contaminated with endotoxin but were neither immersed in the calcium hydroxide suspension nor sterilized; **Group VI:** the files were contaminated with endotoxin and immersed in the saturated calcium hydroxide suspension at 37ºC for 2 h with agitation. No sterilization was done; **Group VII:** the files were removed directly from the original packaging and no endotoxin contamination, immersion or sterilization procedures were undertaken.

The files were then individually placed in 10 x 75 mm pyrogen-free tubes with lids (BioWhittaker; Cambrex Co.), containing 1 mL of pyrogen-free water (recovery solution) at 37ºC for 1 h under agitation. The quantification of endotoxin released in the recovery solution was done using the Kinetic-QCL® kinetic assay (Lot 4L2830, BioWhittaker; Cambrex Co.) according to the manufacturer instructions. Kinetic-QCL® is a quantitative kinetic test for the detection of endotoxin in solutions, based on the reaction time required by the sample to reach a specific absorbance. A short reaction time indicates a high concentration of endotoxin in the sample (7). The Kinetic-QCL™ test is used for the evaluation of small volumes of parenteral solutions, vaccines, antibiotics and biological products because it is less affected by products that can cause false-positive or false-negative results. It has been used in the medical and pharmaceutical fields (5,17) due to its high dependability and precise results. The Kinetic-QCL® test was chosen for the present study because of its high sensitivity for detection of bacterial endotoxin at concentrations between 0.0005 and 50 EU/mL (3).

A total of 100 µL of each recovery solution and solutions to construct the standard curve were placed on 96-well pyrogen-free cell culture cluster plates (Corning Inc., New York, USA). The plates were individually placed in a Bio Tek Eix 808™ Incubator Reader (BioWhittaker; Cambrex Co.) for 10 min for pre-incubation. After this, 100 µL Limulus Amebocyte Lysate (LAL) reconstituted with 2.6 mL pyrogen-free water was added to each well for reading at 405 nm absorbance. The results were obtained using WinKQCL™ software (BioWhittaker, Cambrex Co.) and were recorded as endotoxin units *per* mL (EU/mL). Data were analyzed statistically by the Student's t-test using Prism 4.0 program (GraphPad Inc., San Diego, CA, USA). The level of significance was set at 5%.

According to the results of endotoxin quantification 100% of the files of Groups I (dry heat sterilization), II (wet heat sterilization), III (calcium hydroxide + dry heat sterilization), IV (calcium hydroxide + wet heat sterilization), VI (calcium hydroxide) and VII (taken from original packaging, no previous LPS contamination, immersion or sterilization) were free of LPS. Only Group V (no immersion or sterilization) showed LPS contamination in the recovery solution, ranging from 0.1105-0.5313 EU/mL (0.262 ± 0.1296). There was a statistically significant difference (p=0.0003) between group V and the other groups.

The results of Group V showed that bacterial endotoxin adhered to the metallic surface of endodontic files and was released into the recovery solution. This observation is clinically relevant because if files are not adequately sterilized by a method that inactivates endotoxin, there is the possibility that it can be transferred from one root canal to another, and even between different patients when files are reused after sterilization.

In the medical and pharmaceutical fields, the kinetics of endotoxin inactivation by dry heat (10,11) and wet heat (2,4,13,19) has been studied in solutions and medical devices with different levels of efficacy, depending on the temperature and time used. In the present study, endodontic files were contaminated with *E. coli* endotoxin at 25 ng/mL concentration. The results showed that bacterial endotoxin released in the recovery solution after retrieval from the files was eliminated upon dry and wet heat sterilization.

Bamba *et al*. (2) observed that the level of LPS inactivation by wet heat was concentration-dependent and that concentrations lower than 10 ng/mL in aqueous solutions can be effectively inactivated by wet heat. Likewise, Ogawa *et al*. (13) reported that this method was effective in inactivating endotoxin at low concentrations (10 ng/mL). In the present study, wet heat sterilization (121ºC, 20 min) successfully inactivated *E. coli* LPS, as shown by the quantification of endotoxin in a recovery solution in which endodontic files contaminated with 25 ng/mL LPS were immersed.

Because the effect of wet and dry sterilization on the inactivation of bacterial endotoxin on endodontic files is unknown, the methodology of the present study included immersion of files contaminated with LPS in a calcium hydroxide suspension before sterilization in order to assess whether this procedure (immersion) would actually be necessary from a clinical standpoint. Calcium hydroxide was chosen based on the findings of a previous study (15), which showed that it was able to hydrolyze the lipid A molecule that is the toxic portion of bacterial endotoxin and is responsible for LPS harmful effects. In the present study, after contamination with endotoxin, the files of group VI were immersed in a calcium hydroxide suspension and were not submitted to either of the sterilization methods. The analysis of endotoxin quantification showed no *E. coli* LPS in the recovery solution of all files of this group. This result was statistically similar (p=0.0003) to those of the groups in which wet and dry sterilization was performed and confirms the capacity of calcium hydroxide to inactivate endotoxin as shown in previous *in vitro* and *in vivo* studies (6,16). Nevertheless, the immersion of endodontic files in a calcium hydroxide suspension before sterilization is not necessary after clinical use because files are routinely washed and sterilized by wet or dry heat, which are effective methods for elimination of microorganisms and endotoxin.

Although this investigation showed *in vitro* that the endotoxin present on endodontic files can be inactivated by both wet and dry sterilization, further research should be done with different concentrations of endotoxin and tests for endotoxin detection. Clinical trials should also be conducted.

In conclusion, the contaminated endodontic files that were not submitted to any method of sterilization presented bacterial endotoxin. Sterilization of the contaminated endodontic files using either wet or dry heat eliminated bacterial endotoxin. Immersion in a calcium hydroxide suspension before sterilization was not necessary to inactivate bacterial endotoxin.

RESUMO

Efeito de diferentes métodos de esterilização na inativação da endotoxina bacteriana (LPS) presnete em limas endodônicas

Este estudo avaliou a presença de endotoxina (LPS) em limas endodônticas após contaminação *in vitro* com LPS de *E. coli* e esterilização em autoclave ou forno de Pasteur, com ou sem imersão prévia em suspensão de hidróxido de cálcio. A quantificação do LPS foi efetuada pelo teste Kinetic QCL™, e os resultados submetidos à análise estatística (teste t). LPS foi quantificado apenas no grupo contaminado e não submetido a nenhum procedimento de imersão ou esterilização (0,262±0,1296 unidades de endotoxina/mL) (p=0,0003). Concluiu-se que a esterilização das limas em autoclave ou forno de Pasteur foi eficaz na inativação do LPS, não sendo necessária a sua imersão prévia em suspensão de hidróxido de cálcio.

Palavras-chave: endotoxina; LPS; endodontia; forno de Pasteur; autoclave

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