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Are bovine teeth a suitable substitute for human teeth in *in vitro* studies to assess endotoxin load in root canals?

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Abstract: The present study aimed to determine the feasibility of using bovine teeth as a suitable alternative for human teeth, in experiments involving *in vitro* endotoxin contamination. Twenty bovine central incisors and 20 human single-root premolars had their dental crowns removed and root lengths set at 16 mm. Root canals were prepared up to #60 K-file size and sterilized with cobalt-60 gamma irradiation (20 kGy, 6 h). The teeth were randomly divided into four groups: G1-bovine teeth (bovine negative control, n = 10), G2-human teeth (human negative control, n = 10), G3-bovine teeth, inoculated with *Escherichia coli* (055:B55) LPS, and G4-human teeth inoculated with *E. coli* LPS. The G1 and G2 groups were exposed to apyrogenic water. After the teeth had been incubated at 37 °C and atmospheric humidity for 24 h, the samples of solutions in the main canals were collected with apyrogenic absorbent paper tips. LPS levels were quantified using Limulus Amebocyte Lysate assay. The data obtained were statistically analyzed using one-way ANOVA, with a significance level of 5%. A high amount of endotoxin was detected in the inoculated human teeth (G4) when compared to the sterilized teeth (G2), as well as in the inoculated bovine teeth (G3) when compared to the inoculated human teeth (G4). However, there was no statistical difference between bovine teeth before and after the *E. coli* endotoxin inoculation. Therefore, under the mentioned experimental conditions, the use of bovine teeth should not be a choice for laboratory research on endotoxin contamination.

Keywords: Dental Pulp Cavity; Endotoxins; Decontamination; Humans; Cattle.

Introduction

The use of human teeth in *in vitro* studies has decreased over time because of ethical issues, difficulty in obtaining samples of the correct size, and difficulty in standardizing the sample characteristics.[1] Thus, animal teeth, such as teeth from pigs,[2] rodents,[3] and bovines,[4] have been increasingly employed in laboratorial studies and have consequently become a substitute for human teeth.

Bovine teeth can be easily acquired and handled due to their size.[1] Moreover, the age and acquisition of intact bovine teeth can be controlled, which is an advantage.[5] When compared, human and bovine teeth have

few differences with regard to composition and tissue structure. Some studies report a similarity in their radiodensity,[6] enamel thickness, and dentin surface hardness.[7] However, the enamel prisms are higher and harder in bovine teeth.[1] Both human and bovine teeth show similar dentinal tubule numbers and diameters in the crown. However, the average dentinal tubule diameter, particularly in the root, is larger in bovines than in humans.[8] Moreover, the thickness of the peritubular dentin is greater in bovine teeth.[9]

Several studies have successfully used bovine central incisors as a substitute for human teeth, particularly tests investigating adherence and dental material micro-infiltration,[10] resistance to fracture, shear bond strength,[11] and microbiological tests.[12]

However, *in vivo*[13] and *in vitro*[14] experiments that assess the presence and levels of endotoxin in root canals have generally been carried out using human teeth So far, there is no other study that examines contamination by endotoxins in bovine teeth in order to validate this experimental model.

Thus, the present study aimed to verify the feasibility of using bovine teeth, sterilized with the cobalt-60 (20 kGy, 6 h) protocol, as an alternative for human teeth in studies employing samples contaminated with bacterial lipopolysaccharide (LPS).

Methodology

The present study was approved by the Ethics Committee in Research of the *Pontifícia Universidade Católica do Rio Grande do Sul* – PUCRS (PUCRS, Protocols numbers 5859 and 811207).

Sample selection and preparation

Twenty bovine central incisors and 20 human single-root premolars were selected. They were sectioned transversally to stardardize the root length at 16 mm. The working length was set at 15 mm. All the canals were prepared manually using the serial technique up to #60 hand K-files (Dentsply/Maillefer Instruments S.A., Ballaigues, Switzerland), and irrigated with 2% sodium hypochlorite solution (Iodontosul, Porto Alegre, Rio Grande do Sul, Brazil).

The smear layer of the root canal was obtained using 17% EDTA (Iodontosul, Porto Alegre, Brazil) for 5 min, and then shaking with file #60 for one minutes. The final irrigation was performed using 2 mL of 2% sodium hypochlorite solution. The root canals were dried with sterilized paper points (Dentsply/Maillefer Instruments S.A., Ballaigues, Switzerland).

Samples were divided into four groups according to species (human and bovine) and presence or absence of LPS contamination (negative and positive): G1-bovine teeth (bovine negative control, n = 10); G2-human teeth (human negative control, n = 10); G3-bovine teeth, inoculated with *Escherichia coli* (055:B55) LPS; and G4-human teeth, inoculated with *E. coli* LPS. All teeth were stabilized on cell-culture plates (12 wells, TPP®, Trasadingen, Switzerland), using Durepoxi® (Henkel, Düsseldorf, Germany).

The plates with the teeth and material used in this study were apyrogenized by cobalt-60 gamma irradiation (20 kGy, for 6 h) (Empresa Brasileira de Radiações - EMBRARAD, Cotia, Brazil), as previously described.[15]

Specimen contamination

The sample contamination protocol was in accordance with Signoretti *et al*.[16] The positive bovine and positive human teeth groups were inoculated inside a laminar air flow cabinet, with 30 µL of a solution containing *E.coli* O55:B5 endotoxin, (Lonza, Walkersville, USA) using a micropipette.

The LPS solution (80 EU/mL) was previously diluted in apyrogenic water to approximately 50.37 EU/mL, for standardization of contamination and use.

In G1 and G2 (groups without LPS inoculation), the root canal was filled with 30 µL of apyrogenic water.

Apyrogenic cotton pellets were placed in the cervical portion of the root canals and the plates with the samples were sealed and incubated for 24 h at 37 °C, and humidity.

Determination of LPS levels

All root canals were filled with 10 µL of apyrogenic water before collection of samples. The fluid from the main root canal was collected with three #60 absorbent, apyrogenic paper points (Tanari®, Manaus, Brazil) that were held in position for 10 seconds. They were then transferred into apyrogenic glass tubes, sealed, and kept at −20 °C until the quantification of LPS levels could be carried out. The LPS content was

also assessed in the apyrogenic water samples and in the paper tips employed during the experiment.

The glass tubes containing the paper tips were filled with 1 mL of apyrogenic water, heated to 37 °C for one h, and vortexed (1 min, 3.000 RPM, Vortex AP56, Phoenix, Araraquara, Brazil).

The chromogenic-kinetic test of turbidimetric Limulus Amoebocyte Lysate (LAL) assay (Pyrogent 5000®, BioWhitaker, Cambrex Co., Walkersville, USA) was used to quantify LPS levels in the root canals, as described by Xavier *et al*.[17]

The samples collected from the canals were mixed with LAL reagent and automatically monitored by a microplate reader/WinKQCL™ Software. Using the initial absorbance reading of each well as its own blank, the reader determines the time required for the absorbance to increase 0.03 absorbance units. This is termed as reaction time. The WinKQCL™ Software automatically performs a log/log linear correlation of the Reaction Time of each standard and its corresponding endotoxin concentration. Increase in optical density is measured by the reaction time, which is inversely proportional to the quantity of LPS present in the sample.

A standard curve was drawn using known concentrations of the endotoxin, supplied by the kit, for use as a parameter for the quantification of LPS levels. All the samples collected for the purpose of analysis and quantification were diluted 10 times.

The assays were duplicated in different wells, in a 96 well micro-plate (Corning Costar, Cambridge, USA). For the negative control, 100 µL of apyrogenic water were added, 100 µL standard endotoxin at different concentrations for the curve, and 100 µL of each sample for quantification. LPS levels were measured according to the manufacturer's instructions.

The microplate was incubated at 37 ± 1 °C for 10 min in the enzyme immunoassay reader (Ultramark, Bio-Rad Laboratories Inc., Hercules, USA) that was coupled with a computer that had the Wink QCL version 4 (BioWittaker, Cambrex CO., Walkersville, USA) software installed.

After the incubation period, 100 µL of LAL chromogenic-kinetic reagent (Sigma Chemical Company, Saintt Louis, USA) was added to each well, thus commencing the reading and quantification of LPS levels.

Statistical analysis

EU/mL measures were logarithmically transformed in order to reduce asymmetry and heteroscedasticity. The data were described using geometric mean, maximum, and minimum values. The groups were compared by carrying out one-way variance analysis (ANOVA one-way) on the logarithms. Significance level was 5% ($p \leq 0.05$). Statistical analysis was performed using SPSS 13.0 software (SPSS Inc., Chicago, USA).

Results

In order to validate the turbidimetric LAL assay, the standard curve followed the linearity criteria ($r = 1$).

Endotoxin contamination levels (< 0.0100 EU/mL) were not detected in the material used (apyrogenic water and absorbent paper tips) during the collection process.

The results of the analysis of the experimental groups are shown in Figure. A large amount of endotoxin was detected in inoculated human teeth (G4) when compared to the sterilized teeth (G2) and in inoculated bovine teeth (G3) when compared to inoculated human teeth (G4) ($p < 0.01$). However, no statistical difference was detected in the bovine teeth before and after *E. coli* endotoxin inoculation ($p > 0.05$).

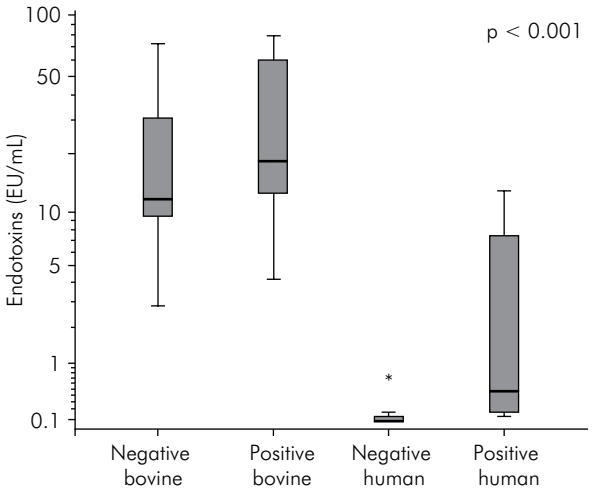

Figure. Each experimental group boxplot distribution of the endotoxin concentration in EU/mL.

Discussion

Studies on the actions of irrigants, intracanal medicaments, and new technologies have been frequently performed as it is possible to inactivate and eliminate bacterial LPS from inside the root canal system.

LPS is an immunological mediator that is released during bacterial multiplication and death. It plays an important role in apical periodontitis pathogenesis. Besides its harmful biological effects, LPS can penetrate the dentin tubules four times deeper than the bacterium itself.[18] In endodontics, the most reliable experimental model for assessment of LPS behavior inside the root canal system, involves extracted teeth. The most widely used experimental model employs human teeth,[13,14] which has its limitations. Studies investigating endotoxins in animal models (*e.g.*, dogs) have been carried out,[19] even though approval and authorization for the use endotoxins have been restricted. Therefore, extracted bovine teeth may be a suitable substitute for human teeth; however, it is necessary to know whether the experimental model of bovine teeth is a feasible option for studies on endotoxin quantification.

The use of human or bovine extracted roots for *in vitro* studies, instead of *in vivo* models, has some advantages such as achievement of sample apyrogenization, followed by introduction of a standardized amount of endotoxin into the sample. The cobalt-60 gamma irradiation protocol has been employed in several studies[13,14] to produce tooth samples without endotoxins The materials and teeth used in this study were sterilized using the same protocol [cobalt-60 irradiation (20 kGy for 6 h)], in order to eliminate pre-existing endotoxins.[20] The sterilization and decontamination process, using ionized energy, consists of exposing the products to electromagnetic short waves generated from the sealed sources of cobalt-60. When those waves meet the live organisms present in the sample being treated, they cause DNA rupture which leads to failure or incapacity to reproduce. Due to the high penetration power of the electromagnetic waves, live organisms can be reached wherever they are irrespective of the manner of packaging used. However, the protocol used in this study was not effective for bovine teeth. It was observed that the samples from G1 (sterilized bovine teeth) had the same level of endotoxins as the teeth from G3 (sterilized bovine teeth exposed to endotoxin contamination). There was a tendency for increased LPS load in the positive group (G3), possibly due to death of the bacteria already present in the canal, or the identification of endotoxins that were already present in the sample. It might be necessary to test different disinfection protocols with different irradiation doses (kGy), in order to make the bovine dental pieces apyrogenic different irradiation doses (kGy), in order to achieve sterilization of bovine dental pieces. However, according to Soares *et al*.,[21] high irradiation power increased the chances of damage to the dentin structure. This damage may be in the form of cracks, particularly in the peritubular dentin, and can compromise the use of that sample in certain kinds of experiments.

The results obtained from human teeth (negative and positive groups) are in accordance with the results previously reported by Maekawa *et al*.[22] and Signoretti *et al*.,[16] who employed the same detoxification protocol for human teeth.

Considering the possibility of pre-existent contamination in bovine teeth, some studies have observed an association between *E.coli* O157:H7 and its endotoxins and cattle and bovine by-products, leading researchers to look for ways to solve this zoonosis by means of specific slaughtering practices and carcass decontamination protocols.[23,24] Many factors, such as age,[25,26] season of the year,[27] diet,[28] animal stress when slaughtered,[29] and confined environments, can affect contamination[30] The bovine dental carcass used in this study may have already been contaminated with endotoxins during transportation or storage. However, even if the dental sample had been contaminated during handling, the results obtained in the negative control group cannot be justified as the pieces had been apyrogenized before being used. Furthermore, some microbial species from bovine teeth may not have been eliminated by the irradiation dose applied in the study. Therefore, the sterilization process employed in the present study (cobalt-60 irradiation, 20 kGy, for 6 h) did not have the same efficacy as that observed with human teeth.

Conclusions

According to the results obtained in the present study, the use of bovine teeth sterilized with cobalt-60 20 kGy irradiation for 6 h was not a suitable alternative for human teeth in *in vitro* research on *E.coli* LPS. Further studies should be carried out to determine an ideal protocol for preparation and apyrogenization of bovine dental specimens.

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