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Antimicrobial efficacy of cryotreatment against *Enterococcus faecalis* in root canals.

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Running title. Cryotreatment and NaOCl against *Enterococcus faecalis*

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Significance and Impact of Study

The cryotreatment seems to have a greater effect on the reduction of bacteria compared to a standard NaOCl irrigation. It remains unknown the effect on the clinical outcome of root canal treatment by the statistically bacterial load reduction after cryotreatment. The interesting potential of cryotreatment should be further investigated through clinical studies aimed to establish a correct irrigation protocol.

Abstract

The purpose of this investigation was to evaluate intracanal bacterial reduction by cryotreatment using a dental instrument equipped with a duct and connected to a cryogenic fluid source. A total of 86 roots were infected with *Enterococcus faecalis* and incubated. After incubation, the contaminated roots were divided into 3 study groups: 35 roots irrigated with 2 ml of a 5% sodium hypochlorite (NaOCl) solution, 35 roots irrigated with 2 ml of a 5% NaOCl solution and further treated with cryo and 10 roots irrigated with 2 ml of saline solution, plus positive and negative controls. Subsequent to each irrigation treatment, the residual bacterial colonies were counted. The use of cryo-instrumentation in association with NaOCl irrigation significantly reduced the number of *Ent. faecalis* ($p<0.01$) in the root canal compared with controls.

The interesting potential of cryotreatment should be further investigated through clinical studies aimed to establish a correct irrigation protocol. Within the limits of the study, the cryotreatment seems to have a greater effect on the reduction of bacteria compared to a standard NaOCl irrigation.

Key words: Cryotreatment, endodontic irrigants, *Enterococcus faecalis*, root canal disinfection, sodium hypochlorite.

1 # Introduction

2 Bacteria are the main factor of pulpar and periapical inflammation. As *Enterococcus faecalis* is the
3 most commonly species isolated from the canals of teeth presenting post-treatment disease, it was
4 used in several previous studies on the efficacy of endodontic irrigants (Gomes *et al.* 2003). It has
5 been widely reported that bacteria can remain viable within the canal system even after
6 chemomechanical preparation (Oliveira *et al.* 2007): hence, a variety of irrigant solutions have been
7 used in endodontics to eliminate or reduce bacterial amount. Sodium hypochlorite (NaOCl) is
8 currently the most commonly irrigant used in endodontics (Clegg *et al.* 2006, Dewsnup *et al.* 2010).
9 However, residual bacteria are readily detectable in approximately one-half of teeth at the time of
10 placement of a filling material, despite extensive irrigation with NaOCl (Soukos *et al.* 2006).
11 Numerous irrigation methods have been proposed to enhance the effectiveness of NaOCl in
12 disinfecting the root canal system and then in killing the microorganisms (Huque *et al.* 1998, Oliveira
13 *et al.* 2007).
14 Since cryosurgery has been successfully used as a treatment for surface skin lesions in case of
15 cutaneous tumors (Capon *et al*. 1998), this study aimed to test the potential microbicidal properties of
16 cryotherapic treatment against *Ent. faecalis*, associated with NaOCl (5%) intracanal irrigant.
17 Cryotreatment was done using a dental instrument (Fig. 1) equipped with a duct and connected to a
18 cryogenic fluid source (liquid nitrogen), with a cooling needle receiving the cryogenic fluid (Patent
19 number IT1331875 of October 30, 2001).
20

21
Results and Discussion

22
We have evaluated the intracanal bacterial reduction using a dental instrument equipped with a duct
23
and connected to a cryogenic fluid source (Fig. 1).
24
The results of the post irrigation bacterial counts are summarized in Tab. 1. After 24 h infection with
25
Ent. faecalis, a significant improvement of root canal disinfection was obtained with NaOCl + cryo

treatment ($p<0.01$): a significant reduction in the bacterial number in the root canal lower than 2 logs was detected compared to positive controls, and lower than 1 log compared to NaOCl alone (Tab. 1). All roots in the negative control group showed no bacterial growth. No statistically significant difference was detected between the specimens treated with 0.9% NaCl solution and the positive controls.

Ent. faecalis is the species most often implicated in persistent root canal infections because of its several virulence factors that make it difficult to eradicate from the canals (Pinheiro *et al.* 2003, Oliveira *et al.* 2007, Dewsnup *et al.* 2010).

It has been recently shown that although an irrigant can penetrate into the dentinal tubules, its concentration could not be sufficient to kill all types of present bacteria (Miller and Baumgartner 2010, Pasqualini *et al.* 2010). Previous studies have shown that disinfection of root dentin is not achieved by chemomechanical preparation alone. Deep bacteria in dentinal tubules are apparently protected from instrumentation and irrigation, making their removal or eradication difficult (Kho and Baumgartner 2006). A variety of irrigant solutions has been used in endodontics to reduce or eliminate bacteria: NaOCl is currently the most commonly irrigant used.

Previously, we reported the efficacy of subsonic agitation of NaOCl with a device known as the EndoActivator (EA): thirty seconds of NaOCl subsonic agitation with EA appear to be slightly more effective in reducing bacterial load in the root canal (Pasqualini *et al.* 2010).

The results of this study indicate that cryo-instrumentation after NaOCl irrigation significantly reduced the number of bacteria in the root canal compared to NaOCl alone, without the total elimination of *Ent. faecalis*. The cryogenic fluid (liquid nitrogen), by suitably varying the duration of the treatment, can reach the desired depth, with the immediate freezing of bacterial cells and their subsequent cryodestruction. These data corroborate results obtained by other authors (Kho and Baumgartner 2006, Dewsnup *et al.* 2010): in fact, disinfection of root dentin is not achieved by

1 chemomechanical preparation alone. The significant increment in the cleansing, induced by cryo
2 application, can be explained related with the previous study by Yamamoto *et al.* (2001). The process
3 of freezing and thawing induces injury in microorganisms, in part, through membrane or cell wall
4 disruption, leakage of intracellular constituents, and changes in protein conformation (Yamamoto *et*
5 *al.* 2001). Our data are in agreement with those reported by Yamamoto *et al.* (2001) that showed that
6 in a test executed applying the freezing and thawing technique (30 s "ON", 30 s "OFF" and 30 s
7 "ON") using liquid nitrogen, the bacteria "*in vitro*" are significantly reduced, with respect to the
8 situation before the treatment. The microorganisms stored under frozen conditions are often
9 accompanied by an increased level of sensitivity to selective ingredients such as NaCl, surfactants
10 and bile salts, which may result in an inability of the microorganism to multiply on selective media
11 (Yamamoto *et al.* 2001).
12
13 The removing of the bacteria and the death and/or a serious damage of a lot of them produce a
14 significantly better sterilization of the dental channel. After a cryotreatment in the dental channel, we
15 do not have any medium that could help the damaged bacteria to recover their health and to
16 reproduce themselves. These *in vitro* data acquire greater importance since referred to a 24 h of
17 bacterial infection that approaches the clinical situation *in vivo*. Currently there are no data available
in literature for a comparison.

18

19 ## Material and Methods

20 A total of 86 roots with a fully formed apex was used. Each tooth was radiographed to confirm the
21 presence of a single canal. Specimens were immersed in a 5% solution of NaOCl (Niclor
22 5;OGNA,Muggiò,Italy) for 1 h and then stored in saline solution until preparation.
23 Each specimen was sectioned to obtain a residual root length of 17 mm. Each root canal was
24 preflared using K-Flexofiles (Dentsply Maillefer, Ballaigues, Switzerland) up to #20 followed by
25 Protaper NiTi instruments (Dentsply Maillefer) at the working length. The working length was

1 established under microscopic vision (OPMI Pro Ergo; Carl Zeiss, Oberkochen, Germany) at 10X
2 magnification when the tip of the instrument was visible at the apical foramen. Irrigation was
3 performed with a 33-gauge needle syringe using 25 ml of 5% NaOCl at 50°C (Niclor 5; OGNA,
4 Muggiò, Italy) and alternating with 10 ml of 17% EDTA (Tubuliclean, OGNA); the total irrigation
5 time was 10 min per specimen (Zehnder 2006). After drying with paper points, the roots were
6 inspected under the microscope at 10X magnification to verify the absence of cracks and canal
7 cleanliness. Root surfaces were sealed with varnish and sticky wax; each specimen was fixed with
8 cyanoacrylic cement onto an Eppendorf tube, which was placed on special silicone-made stands to
9 allow easier manipulation, then packed with double sheet and finally sterilized with ethylene oxide
10 gas. The sterilized roots were placed under a laminar flow biohazard cabinet (CLANLAF VFR 1206;
11 Capriolo, Brescia, Italy). Pure culture of *Ent. faecalis* (ATCC 29212) was previously grown in brain-
12 heart infusion (BHI) (Oxoid, Milan, Italy) medium broth for 24 h and adjusted
13 spectrophotometrically to an optical density of 0.15 at 620 nm (Genesys 20 Spectrophotometer;
14 Thermo Electron Corporation, Madison, WI) to obtain a final concentration of $3x10^7$ colony-forming
15 unit (CFU)/ml as confirmed by colony counts in triplicate. The 80 specimens and 4 positive control
16 roots were each contaminated with 30 µl of a pure culture of *Ent. faecalis* followed by incubation at
17 37°C for 24 h to allow bacteria penetration into the root canal dentine. Positive controls (n = 4) were
18 irrigated for 1 min with 2 ml of BHI broth. The 2 negative controls of roots were inoculated with
19 sterile BHI medium broth. After incubation, the infected roots were randomly divided into 3 study
20 groups: NaOCl group (n = 35) irrigated with 2 ml of a 5% NaOCl solution for 1 min; cryo group (n =
21 35) irrigated with 2 ml of a 5% NaOCl solution for 1 min and then treated with cryo for 30 s; NaCl
22 group (n = 10) irrigated with 2 ml of 0.9% NaCl solution for 1 min. The dental instrument used for
23 cryotherapic treatment is equipped with a conduit of conveyor capable of being connected to a source
24 of fluid and a needle cryogenic cooling, which receives the fluid from the refrigeration pipe
25 conveyor. The needle is made of a flexible material and has a smaller outer diameter (0.25 mm) than

the size of the entrance inside a tooth canal, so that it can be inserted into the canal tooth itself, where the cryogenic fluid is blown (Fig. 1).

Subsequent to each irrigation treatment, the microbiological sampling was performed by flooding the canal with sterile saline followed by placing a size 50 Hedstrom file into the canal to scrape the dentin during the process. A sterile absorbent paper point was placed into the lumen for 1 min, transferred into a test tube containing 1 ml of 0.9% saline solution and then it was shaken vigorously for 1 min in a vortex mixer. After 10-fold serial dilutions, aliquots of 0.1 ml were plated onto BHI agar and incubated at 37°C under aerobic conditions for 24 h. The CFU grown were counted and then transformed into actual counts based on the known dilution factors.

T test was used to determine if there was a significant difference in CFU/ml between NaOCl group and NaOCl + cryo group. Independent *t* test was used to compare NaOCl group to the positive controls and NaOCl + cryo group to the positive controls. Differences were considered statistically significant when $p<0.05$.

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1 **Figure 1** A dental instrument for treating teeth, provided with a needle cryogenic cooling, which receives the
2 fluid from the refrigeration pipe conveyor. The needle cryogenic is made of a flexible material so that it can
3 be inserted into the canal tooth itself, where the cryogenic fluid is blown.
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6
7 **Table 1** Efficacy of different treatments (NaOCl, NaOCl + cryo, NaCl) used for endodontic disinfection
8 compared with positive control against *Ent. faecalis* ATCC 29212 after 24 h infection. Error bars denote the
9 standard error of the mean.
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1 **Figure 1**.

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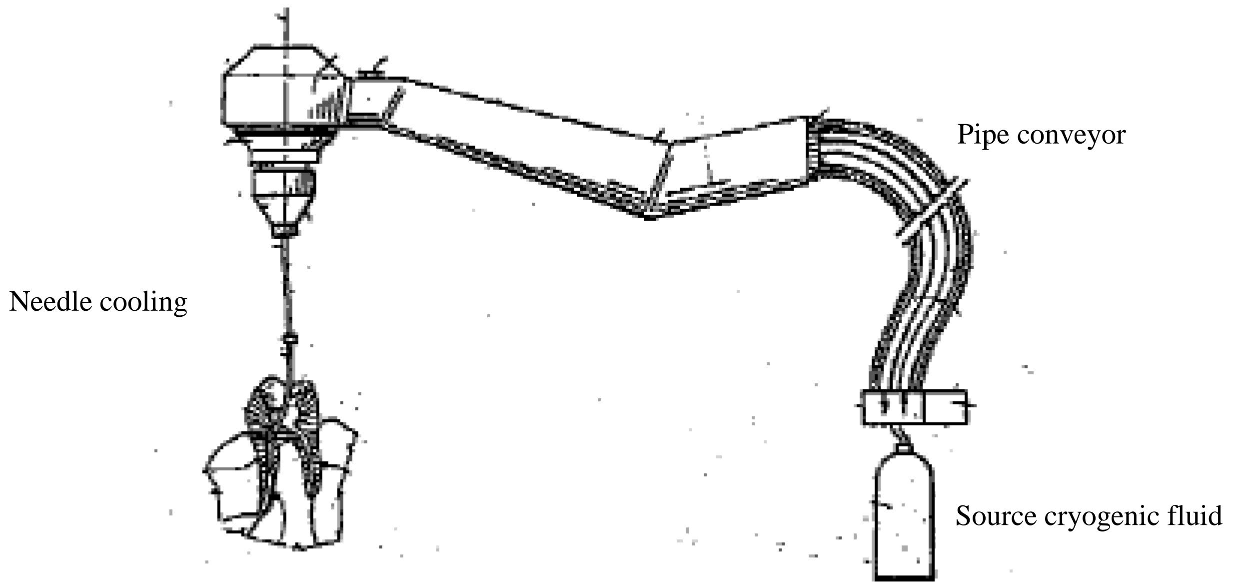
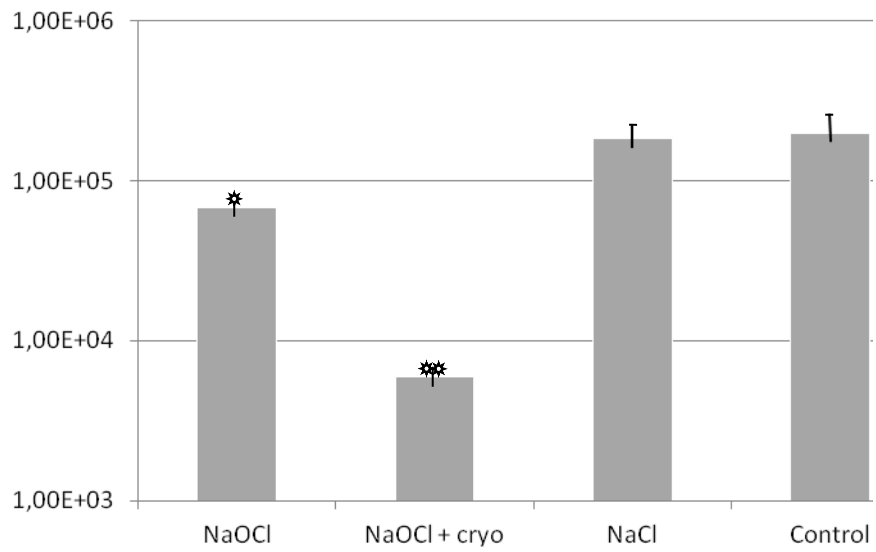


3

4 **Table 1**.



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8 ✵ Significantly different from control ($p<0.05$)

9 ✵✵ Significantly different from control ($p<0.01$)

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