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INFLUENCE OF INTRA-CANAL CRYOTHERAPY ON PRO- AND ANTI-INFLAMMATORY MEDIATORS EXPRESSION USING DIFFERENT IRRIGATION PROTOCOLS A RANDOMIZED CLINICAL TRIAL

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KEYWORDS

Cryotherapy, Interleukins , PCR , Sodium hypochlorite

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ABSTRACT

Aim: To evaluate the effect of intra-canal cryotherapy on interleukin-1β and interleukin-10 expression in teeth with symptomatic apical periodontitis. **Methodology:** Forty-eight single rooted teeth in patients with symptomatic apical periodontitis were randomly divided into 4 groups according to the irrigation protocol. Group I (control); 5% sodium hypochlorite at room temperature. Group II; 5% cold sodium hypochlorite (2-5°C). Group III; final rinse with 20 mL of 5% cold sodium hypochlorite (2-5°C) for 5 minutes. Group IV; final rinse with 20 mL of cold saline (2-5°C) for 5 minutes. Paper point passing 2 mm beyond the apex was used to collect periapical fluid before, immediately after mechanical instrumentation and after one week to characterize the mRNA expression of IL-1β and Il-10 using real-time polymerase chain reaction (PCR). Statistical analysis was performed using one-way ANOVA to compare between all groups. **Results:** Our results revealed statistically significant (at P≤ 0.05) down regulation of IL-1β gene expression levels in all groups after one week with no statistically significant difference between the four groups and statistically significant (at P≤ 0.05) up regulation of IL-10 gene expression levels in all groups after one week. **Conclusion:** All the cryotherapy irrigation protocols showed lower levels of expression of the pro-inflammatory mediators (interleukin-1β) and higher levels of expression of the anti-inflammatory mediator (interleukin-10) than the control group after one week.

INTRODUCTION

Apical periodontitis (AP) occurs initially as an inflammatory reaction to micro-organisms colonizing the root canal system, then as an infectious disease when bacteria exit the root canal into the periapical tissue. It is like any other connective tissue inflammation with the same sequence of inflammatory events including the vasodilatation of blood vessels, increase in vascular permeability, migration of the inflammatory cells such as lymphocytes, macrophages and neutrophils to the inflamed site [(1)].

The principal immunologically effector cells of apical periodontitis was macrophages and T helper cells (Th1) [(1,2)]. Release of cell signaling molecules called cytokines helps in communication between cells in immune responses and enhance cell recruitment towards the infected

and inflamed sites. Cytokines are present in many forms, such as peptides, proteins and glycoproteins. Development of apical periodontitis is usually mediated by many cytokines like Interleukin -1β (IL-1β), Interleukin -6 (IL-6) and tumor necrosis factor (TNF) [3,4].

Interleukins representing the functional cytokines are the main mediators of inflammatory reactions. Local effects include increased adherence of leukocytes to endothelial walls, lymphocytes stimulation, neutrophil potentiation, increased production of prostaglandin and proteolytic enzymes and bone resorption mediation [4].

IL-1β as a prototypic proinflammatory cytokine having a pleiotropic consequence on a variable number of cells and a major action in both acute and chronic inflammatory and autoimmune diseases[5]. IL-1β is the preformed dominant form found in human apical periodontitis. The endothelial, fibroblasts, neuronal, keratinocytes, and immune cells e.g., macrophages and mast cells are sources of release of IL-1β. It exerts several local effects including the enhancement of adhesion of leukocytes to endothelial walls, lymphocytes stimulation, potentiating neutrophils, activating prostaglandins and proteolytic enzymes production, improvement the resorption of bone and its formation inhibition[6].

Interleukin 10 (IL-10) is a potent anti-inflammatory cytokine playing a critical function in the prevention of autoimmune and inflammatory diseases [7]. IL-10 deficiency or impaired expression results in the enhancement of inflammatory response to microbial challenge but also contributing to the development of inflammatory bowel disease and a variety of autoimmune disorders [8]. Thus, impairment of IL-10 expression or signaling during an acute infection enhances pathogens removal, but also leads to exaggerated inflammatory response with exacerbated immunopathology and damage of tissues [9].

The fundamental objective of root canal therapy is to alleviate the patients' pain and to render the tooth functioning. To control pre-operative and post-operative inflammation and pain, various strategies have been suggested. One of the simplest and effective strategies for pain control in dentistry is "cryotherapy" [10].

Cryotherapy has been proved to effectively reduce edema, pain, inflammation, the time of tissues recovery after short term applications in orthopedics and other surgeries [11,12]. For therapeutic purposes, cryotherapy does not mean the application of cold but rather the extraction of heat to minimize or decrease the tissue temperature [13].

Employing cryotherapy during root canal treatment has recently gained popularity. This study was the first to be carried out to measure the effectiveness of intra-canal cryotherapy on decreasing post-operative inflammation in patients suffering from symptomatic apical periodontitis in terms of measuring the levels of periapical expression of pro-inflammatory (IL-1β) and anti-inflammatory (IL-10) mediators.

The rationale of this study was extracting heat from tissues by cold application to minimize post-operative inflammation.

The null hypothesis was no significant difference in the levels of pro- and anti-inflammatory mediators' expression between the control group without cryotherapy and the three experimental groups utilizing cryotherapy.

PATIENTS AND METHODS

Approval was purchased by the Research Ethics Committee (REC), Faculty of Dentistry, Suez Canal University (56/2017). Before starting the treatment procedures, all participants were discussed about the nature and objectives of the study, along with obtaining an authored consent.

A total sample size of forty-eight patients (12 for each group) suffering from symptomatic apical periodontitis as defined by the American Association of Endodontists were sufficient to detect this high effect size of f=0.5, a power of 80% and a 5% significance level (Calculation of sample size was done by IBM™, Sample Power™ Version 3.0.1, based on previous studies [14,15].

Study group

Forty-eight patients were chosen from the of the Department of Endodontics' outpatient clinic, Faculty of Dentistry, Suez Canal University. Inclusion criteria were patients aging from 20-50 years suffering from symptomatic apical periodontitis in single rooted teeth with a preoperative pain score >7 on the visual analog scale (VAS) scoring [16]. The exclusion criteria were patients presenting with periapical abscess, patients under immune-suppressive chemotherapy, anti-inflammatory drugs or antibiotic medications for the last 2 months, medically compromised patients and pregnant or lactating females (fig.1).

Randomization

The patients were randomly assorted into four groups (12 each) according to irrigation protocols. They were instructed to choose from forty-eight opaque and tightly sealed envelopes containing the patients' coding.

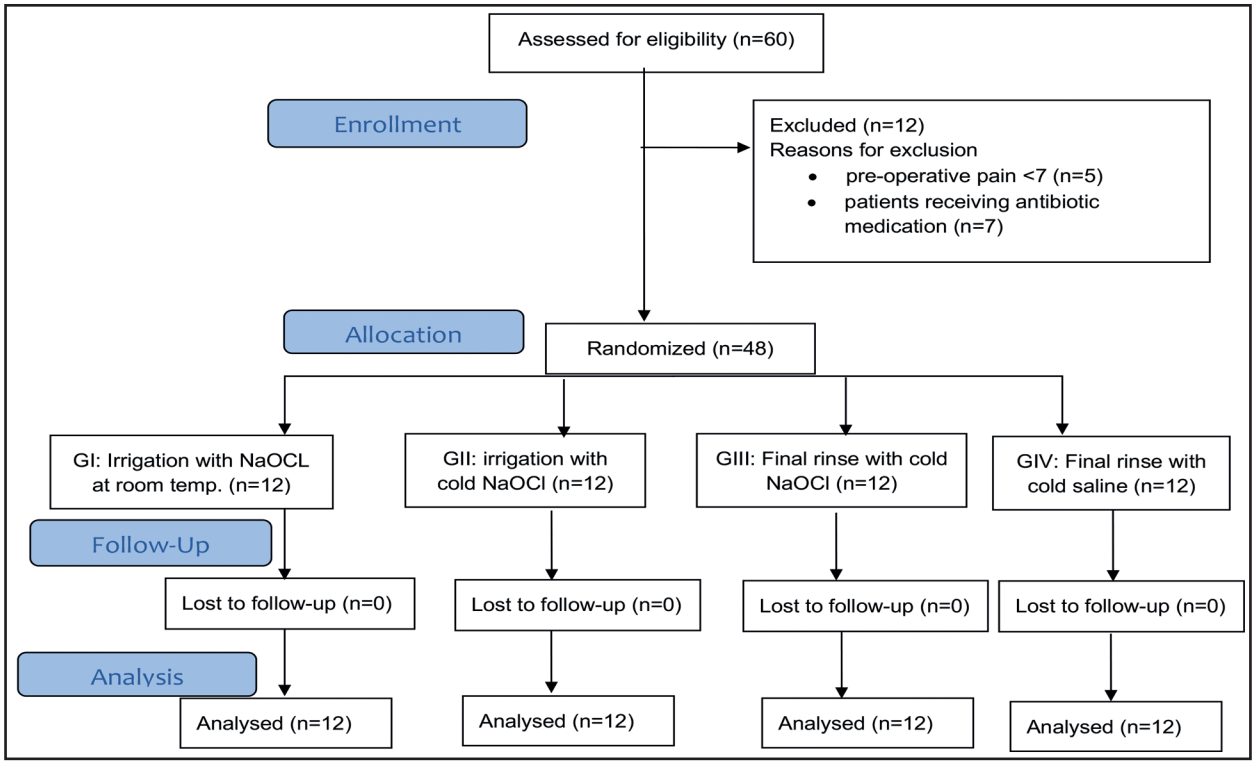

Fig. (1) The consolidated standards of reporting trials flow diagram.

Patients' grouping and evaluation methods

Forty-eight single rooted teeth in forty-eight patients were randomly into four groups (12 each) according to irrigation protocols. **Group I (control);** irrigation with 5% sodium hypochlorite (NaOCl) at room temperature from the beginning of cleaning and shaping, between each file and till the final rinse. **Group II;** irrigation with 5% cold sodium hypochlorite (2-5°C) from the beginning of cleaning and shaping and between each file. Final rinse was done by 20 mL of 5% cold sodium hypochlorite (2-5°C) for 5 minutes. **Group III;** irrigation with 5% sodium hypochlorite at room temperature from the beginning of cleaning and shaping and between each file. Then final rinse with 20 mL of 5% cold sodium hypochlorite (2-5°C) for 5 minutes. **Group IV;** irrigation with 5% sodium hypochlorite at room temperature from the beginning of cleaning and shaping and between each file. Then final rinse with 20 mL of cold saline (2-5°C) for 5 minutes. Each root canal received a total of 30 mL irrigation.

Clinical procedures of root canal treatment

Root canal therapy was conducted in two appointments. At the first appointment, all patients were anaesthetized using one carpule of articaine hydrochloride 40 mg/mL + 1/100 000 Epinephrine bitartrate solution. (Inibsa Dental, Barcelona, Spain). Cases in which additional anesthesia was needed, another carpule of Articaine hydrochloride 40 mg/ml + 1/100 000 Epinephrine bitartrate solution was administrated.

Access cavity was performed under absolute rubber dam isolation using sterile round carbide bur under copious water cooling, then #10 K-file (Micro Méga, Besançon, France) was used to achieve the initial glide path.

Coronal flaring was done using SX rotary file (Protaper, Dentsply Maillefer, Ballaigues, Switzerland), then root canals irrigation with 2 mL 5% NaOCl. Electronic apex locator (Root ZX Mini; J. Morita Co, Tustin, CA) was utilized to determine the working length at 0.5 reading which was confirmed by periapical x-rays. Bio-mechanical preparation was carried out by the rotary file ProTaper Next (Dentsply Maillefer, Ballaigues, Switzerland) according to instructions of the manufacturer till X3 file.

Sample collection for evaluation of gene expression of cytokines

In each group, samples collection was done before, immediately after root canal cleaning and shaping and after a week to characterize the mRNA expression profile of IL-1β and IL-10. Root canals were dried followed by inserting 3 paper points into the root canal to pass 2 mm beyond the apical foramen into the periapical tissues and hold in place for 1 minute to be soaked by the periapical interstitial fluid. Four mm from the tip of each paper point was cut and dropped into a micro-centrifuge tube then stored at -70°C.

Extraction of purified RNA was done utilizing total RNA purification kit according to the procedure of the manufacturer (Thermo Scientific, Fermentas, #K0731). Complementary DNA (cDNA) synthesis was performed using Revert Aid H minus Reverse Transcriptase to convert RNA into cDNA.

Absorption of ultraviolet (UV) light purines and pyrimidines ring structure for pure samples (least contaminated with free nucleic acids, phenols, proteins, carbohydrates, and organic solvents, etc.) was used to calculate the number of nucleic acids in order to estimate RNA and cDNA concentrations to be sure the presence of enough pure concentrations to perform real time PCR. The spectrophotometer

Q5000 (UV-VIS/USA) was used for all the measurements and calculations [17].

Real-time polymerase chain reaction (PCR)

The mRNAs expression of target genes in periapical interstitial fluid was measured by using Real-time PCR with SYBR Green and β-actin as an internal reference. Amplification of the isolated cDNA was done utilizing 2X Maxima SYBR Green/ROX qPCR Master Mix according to the instructions of the manufacturer (ThermoScientific, USA, # K0221) and gene specific primers (Table 1).

Final reaction mixture was inserted into a Step One Plus real-time thermal cycler (Applied Biosystems, Life Technology, USA) and the PCR program was carried out in 45 cycles under the conditions of PCR: initial denaturation 95 C/10 min, Denaturation 95 C/15 sec, annealing 60 C/30 sec, Extension 72 C/30 sec. Temperature was raised from 60 to 95 at the end of the final cycle, to develop a melting curve. The housekeeping gene (β-actin) expressed as normalized was utilized to estimate relative gene expression or fold change in target gene. Consequently, the quantities critical threshold (Ct) of target gene were normalized to Ct of housekeeping gene using the $2^{-\Delta\Delta Ct}$ [18].

Table (1): *Forward and reverse primers used in qPCR.*

Gene	Forward primer (/5 ------ /3)	Reverse primer (/5 ------ /3)
IL-1β	ATGCACCTGTACGATCACTG	ACAAAGGACATGGAGAACACC
IL-10	GCC TAA CAT GCT TCG AGA TC	TGA TGT CTG GGT CTT GGT TC
β -actin	CACCAACTGGGACGACAT	ACAGCCTGGATAGCAACG

Statistical analysis:

Data of PCR analysis for gene expression of cytokines were collected, tabulated and statistically analyzed. Results were expressed as mean, standard deviation (SD), median and range values. ANOVA one-way was applied to assess difference among groups at 0.05 significance level. Statistical analysis was performed using IBM® SPSS® Statistics version 20 for Windows.

RESULTS

1. Comparison of demographic data regarding age and sex between groups (table 2):

There was no statistically significant difference regarding mean age values (P=0.394) or gender distributions (P = 1.000) between the different groups.

Table (2): *Comparison of demographic data regarding age and sex between groups*

	Group I (n = 12)	Group II (n = 12)	Group III (n = 12)	Group IV (n = 12)	*P*-value
Age (Years)					
Mean (SD)	31.3 (4.9)	27.9 (4.9)	29.9 (5.3)	29.8 (4.2)	0.394
Gender [n (%)]					
Male	6 (50)	6 (50)	6 (50)	6 (50)	1.000
Female	6 (50)	6 (50)	6 (50)	6 (50)	

**: Significant at P ≤ 0.05*

2. Comparison regarding time effect on IL-1β gene expression (fig.2):

The results revealed a statistically significant ($p \leq 0.05$) down regulation of IL-1β gene expression levels in all groups after one week with no significant difference among the four groups. Group II showed the lowest levels of IL-1β expression after one week followed by group III then group IV while group I showed the highest levels of IL-1β expression.

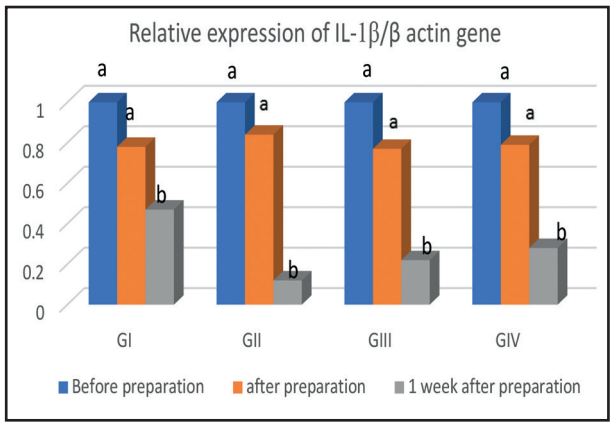

Fig. (2) Bar chart representing relative expression of IL-1β/β actin gene.

3. Comparison regarding time effect on IL-10 gene expression (fig.3):

Results of this study showed statistically significant (at $p \leq 0.05$) up regulation of IL-10 gene expression levels in all groups after one week. Group II showed statistically significant highest levels of IL-10 expression after one week than the other groups.

Group I showed statistically significant difference with group III and no statistically significant difference with group IV. While there was non-significant difference between groups III and IV.

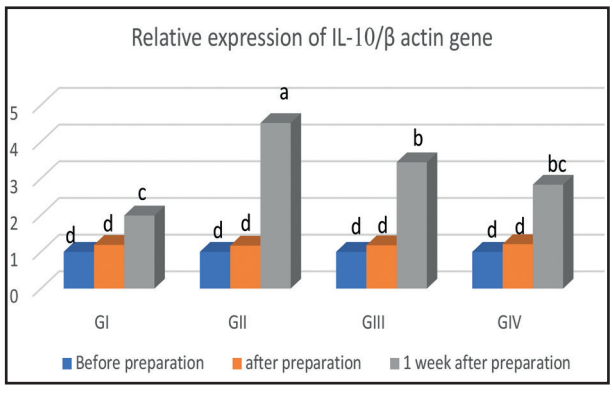

Fig. (3) Bar chart representing relative expression of IL-10/β actin gene.

DISCUSSION

Apical periodontitis is usually triggered by microorganisms residing in the root canal and invading the periapical tissues through the apical part of the root canal. Inflammation can also occur due to accidental trauma, injury following instrumentation of the root canal or inflammation from chemicals and endodontic materials, any of which may cause an extreme short-term tissue reaction. Apical periodontitis is usually manifested clinically in terms of pain, tooth elevation in is socket and tenderness to pressure. these initial, symptomatic lesions are classified as symptomatic apical periodontitis [19].

Apical periodontitis (AP) is an inflammatory condition featured by the recruitment of members of both innate and adaptive immune systems in periapical tissues as a result of infection from root canal [20]. To avoid unnecessary tissue damage, pro-inflammatory pathways must be strictly counter regulated [21]. A balance exists between the pro- and anti- inflammatory cytokines in chronic inflammatory processes which regulates the degree of host responses to antigens stimulation [22].

Interleukin-1β, IL-6 and IL-10 were the most frequently studied analytes for many reasons; firstly, their pathophysiological roles in periradicular disease which has been reviewed by Nair et al.[23]. Secondly, they are considered the most important for human osteoclastic activity [24] and third, they present in apical lesions and have been demonstrated with their level is proportional to the lesions size[25,26]. This renders them ideal biomarker for the activity of periradicular disease.

Interleukin-1β, as a pyrogenic cytokine is produced by blood monocytes following an infection, injury or immunologic reaction. At lower concentrations, it can cause fever, hypotension and

producing more pro-inflammatory cytokines, e.g., IL-6 [27].

Interleukin-10 is a soluble protein responsible for immune and nonimmune cells communication and is referred as cytokine synthesis inhibitory factor. The immune system cells (dendritic cells, natural, killer cells, macrophages, eosinophils and neutrophils, B- and T-cells including Th1, Th2 and T-regulator) express and release it [28,29].

Interleukin-10 activates Th1 (T helper) clones, because of inhibitory action on monocyte and macrophages[30]. It particularly regulates the cytokine expression of myeloid origin, thereby affect maintenance and stimulation of immune responses in periodontitis [31]. It is classified as an anti-inflammatory cytokine capable of inhibiting various pro-inflammatory cytokines synthesis, such as IL-1, IL-6, IL-8 and TNF-α [32].

In this study, expression of 1β and IL-10 were investigated. Polymerase chain reaction (PCR) was used to detect inflammatory mediators' expression because of its high accuracy and reliability [33] which was in agreement with Arikawa et al. [34] who checked the validity and accuracy of real time PCR for evaluating the expression of multiple cytokine mRNA. Their data showed that changes in the levels of cytokine mRNA identified by PCR Arrays precisely predict protein quantities change measured by ELISA. Hereafter, PCR Array is a sensitive, simple and reliable tool for profiling of multiple cytokines.

In the present study, although non-statistically significant, all the experimental groups (cryotherapy) revealed reduction in the expression of the pro-inflammatory cytokines IL-1β after one week than the control group.

All the groups showed high levels of expression of anti-inflammatory cytokine IL-10 after one week. Group II showed statistically significant higher levels of IL-10 than the other groups. This might be greatly attributed to the longer time of cold application than the other groups which reduced tissue edema and inflammation and the degree of tissue injury which subsequently helped to improve the recovery of the tissues. The marked elevation in numbers of the anti-inflammatory cytokine IL-10, classified as a cytokine synthesis inhibitory factor, helped in suppressing the levels of the pro-inflammatory mediator IL-1β [35].

This was in agreement with Panichi et al. [36] who considered IL-6 to be one of three pro-inflammatory cytokines and IL-10 to be anti-inflammatory cytokine capable of suppressing IL-6 production.

Pro-inflammatory reactions must be tightly reversed to avoid excessive destruction of tissues[22]. Pro-inflammatory and anti-inflammatory cytokines balance usually regulates degree of host responses to stimulation of antigens during chronic inflammation. In AP, TH1 cells, macrophages and neutrophils are responsible for the production of the proinflammatory cytokines involved in the phases of lesion expansion because of bone destruction. In contrast, TH2 is responsible for the production of anti-inflammatory cytokines which have crucial role the healing and restriction of the immune reactions [37].

This was in agreement with Neto et al. [38] who checked the pro-inflammatory (IL-6, IL-1β, IFN-γ and TNF-α) and anti-inflammatory (TGF-β and IL-4) cytokines expression in apical periodontitis lesions and found a equilibrium in expression between both pro- and anti-inflammatory cytokines. Also, de Oliveira et al. [39] reviewed the effect of cytokines in pain and described the effect of pro-inflammatory mediators (IL-1, IL-2, IL-6, IL-7 and TNF) and the anti-inflammatory mediators (IL-4, IL-10 and IL-13) on the pathophysiology of pain syndromes. They concluded that anti-inflammatory

cytokines could break the hyper-excitability cycle of sensory neurons by promoting a new non-opioid therapeutic approach to pathological pain caused by inflammation or peripheral nerve damage.

The mechanism of action of cold application (cryotherapy) can be divided into three basic actions: decrease in blood flow, metabolic activity and inhibition in the neural receptors of skin and subcutaneous tissues. This renders it efficient in reducing inflammation, pain, edema and recovery time in the short-term application [40].

The optimum application time of cryotherapy has not been determined. However, different application time according to the depth of tissues was recommended; 3 to 5 minutes of cryotherapy was advised when there is minimum fat and muscle, while approximately 20 minutes was recommended for deeper tissues, such as the hip [41].

The transmission of cold to the periodontal ligament in the root's apical and coronal portions is not the same because of differences in thickness of dentin and the numbers and direction of dentinal tubules between the two levels. Apical dentin has fewer and more mineralized tubules which encourage more efficient cold transmission than the cervical dentin which exhibits larger and increased number of dentinal tubules [42]. Also, the apical third of teeth with single root usually exhibits 1 to 7 pulp ramifications which increase the rate of cold transmission compared to multi-rooted teeth [43].

Patients suffering from symptomatic apical periodontitis and preoperative visual analog scale (VAS) score higher than 7 were selected, because postoperative pain and inflammation is mostly anticipated when preoperative pain is present [22,44].

This might be inferred to cryotherapy reduced the count of leukocytes adhering to the capillary endothelial walls; thereby, reducing their migration into affected tissues and reducing endothelial dysfunction. Also, decreasing cell metabolism; thus, decreasing the oxygen demand of cells and restricting free radicals' production in tissues and minimizing the recruitment of pro-inflammatory mediators to inflammation site [45].

CONCLUSION

It can be concluded that all the cryotherapy irrigation protocols yielded lower levels of expression of the pro-inflammatory mediators (interleukin-1β) and higher levels of expression of the anti-inflammatory mediator (interleukin-10) as compared with the control group. Cryotherapy proved to be an effective, practical and cheap method to control post-operative inflammation. However, further studies regarding anti-microbial efficacy of cold NaOCl should be conducted.

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