Determination of endotoxins in the vital pulp of human carious teeth: Association with pulpal pain

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Objectives. The aims of this investigation were to determine the presence or absence of endotoxins in the pulp of symptomatic and symptom-free human carious teeth, to quantify the amount of endotoxins present, and to associate the presence of endotoxins with the acute pulpal pain.

Material and methods. Pulpal tissue was sampled from 28 single-rooted carious teeth (15 symptomatic, 13 symptom-free) derived from 28 patients. Samples were also taken from the pulp of 5 noncarious control teeth. During sampling an effort was made to collect an equal weight of pulpal tissue in all cases (approximately 8 mg). The extraction of endotoxins was performed with the use of phenol-water. The assay and quantitative determination of endotoxins was performed with the use of a limulus lysate test. The data were analyzed statistically by using the independent *t* test.

Results. Endotoxins were detected in pulpal tissues of all carious teeth in the symptomatic (mean average, 0.15773 ng/mL; SD = 0.045811) and symptom-free group (mean average, 0.10723 ng/mL; SD = 0.010925). In noncarious control teeth, endotoxins were not detected. The presence of endotoxins was significantly higher in the group of symptomatic teeth than in the group of symptom-free teeth ($P < .001$).

Conclusions. The presence of endotoxins in the pulpal tissue of all the carious teeth indicates that they may play a major role in the pathogenesis of human pulpal diseases. Since a significantly higher level of endotoxins was detected in the pulp of symptomatic carious teeth than in that of symptom-free carious teeth, an association of endotoxins levels with severity of pulpal pain is probable.

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As long as enamel and cementum protect the dentine, the dental pulp will remain healthy, unless an external factor such as trauma disturbs its blood microcirculation. The removal of these protective barriers as a result of caries, trauma, or iatrogenic exposure will create a communication gate between the dental pulp and the microorganisms of the oral cavity through dentinal tubules or through an immediate contact after a pulp exposure.[1,2] The influence of bacterial invasion in the dental pulp in animals was studied by Kakehashi et al in 1965. Their research demonstrated that bacteria produced an extensive inflammation.[3] In human beings a histologic examination revealed that whenever bacterial growth was observed on cavity dentinal walls of restored teeth, an inflammatory reaction was present in the pulp. No inflammatory reactions were observed in cavities without bacterial presence, even when silicate cement was placed directly on the exposed pulp.[4]

The cell wall of gram-negative bacteria such as *Prevotella* contains endotoxins that can be either secreted in vesicles by growing organisms or released into the environment after the death of the cell. Endotoxins are a major virulence factor capable of initiating various biological responses, such as complement activation, fever induction, macrophage activation, cytotoxicity, and bone resorption.[5,6] Studies have shown that microbial byproducts (eg, endotoxins) may produce an inflammatory reaction in the dental pulp.[7-9]

Symptoms originating from carious teeth with vital pulp vary considerably, ranging from simple discomfort to spontaneous pain of extreme intensity.[10] Endotoxins may evoke pain through activation of the Hageman factor[11] or through neurotoxic properties when acting on presynaptic nerve terminals.[12-14]

Despite considerable interest in the mechanisms of inflammation and pain in the dental pulp of carious teeth, no direct evidence has been presented to show the presence of endotoxins in the vital and in the inflamed pulp of human beings.

Therefore, the purposes of this investigation were (1) to determine the presence or absence of endotoxins in the pulp of symptomatic and symptom-free carious teeth diagnosed as irreversible and reversible pulpitis, respectively, (2) to quantify the amount of endotoxins

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present in the above pulps, and (3) to study the association between the presence of endotoxins with the acute pulpal pain present in symptomatic teeth.

MATERIAL AND METHODS

Patient selection

Included in this study were 28 single-rooted teeth (3 maxillary central incisors, 1 maxillary lateral incisor, 3 maxillary canines, 10 maxillary second premolars, 5 mandibular first premolars, 6 mandibular second premolars) with vital pulps diagnosed as having irreversible and reversible pulpitis from 28 patients, 15 to 62 years of age, who presented at the Clinic of Conservative Dentistry at the Dental School of the University of Athens. The teeth were divided into 2 groups: Group 1, symptomatic carious (n = 15); and group 2, symptom-free carious (n = 13). Moreover, a third group of single-rooted sound noncarious teeth (n = 5) was included as a control, constituted from 4 patients, 11 to 13 years of age (Table I).

The following information was noted for each patient: Age, gender, tooth, pulp vitality (electrical and thermal), nature of pain (duration, location, sharp, or dull), history of previous local treatment, periodontal status, and radiographic findings.

All the teeth in groups 1 and 2 had deep interproximal or occlusal carious lesions or secondary caries under a restoration, without exposure of the dental pulp as evaluated clinically and radiographically (at least 0.5 mm of dentine protected the pulp). None of the teeth showed radiographic evidence of periapical inflammation. Group 1 (symptomatic carious) consisted of 15 single-rooted teeth with the clinical diagnosis of irreversible pulpitis. The teeth were placed into this group on the basis of the following criteria: The teeth were currently causing spontaneous pain or had a recent history of causing severe pain that was diffused and lasted for minutes or hours when exposed to thermal stimuli; all the teeth responded to ice or electric pulp testing; and a severe, prolonged pain reaction was elicited with ice. Group 2 (symptom-free carious) consisted of 13 deeply carious and symptom-free single-rooted teeth that needed a post or dowel for their final restoration. The teeth were placed into this group on the basis of the following criteria: No history of moderate or severe pulpal pain and no pain at the time of sampling; and clinical and radiographic examination determined the presence of caries without pulpal exposure, no signs of periapical pathosis, and normal to slight reaction to the vitality test. Group 3 consisted of 5 intact teeth from 4 patients. The teeth were placed into this group on the basis of the following criteria: A verbal history confirmed no previous pulpal pain; a clinical and radiographic examination assured that these teeth had no caries, restoration, wear facets, or periodontal disease; and all the teeth were found normal after being tested by both ice and electric pulp test. Teeth in this group included premolars extracted because of orthodontic considerations.

Teeth with a history of previous local medication to manage pulpal pain, with periodontal pockets more than 3 mm, with root caries, or with an exposure of the pulp were not included in the study.

The protocol was approved and supported by a grant from the University of Athens. All patients agreed to participate in the study, and each patient signed a detailed informative consent form.

Pulp sampling

After the diagnosis of reversible or irreversible pulpitis, local anesthesia was administered and the crown of the tooth to be sampled was thoroughly washed with air-water spray and dried. A rubber dam was placed and the operation field including the outer surface of the tooth, the dam, and the clamp was disinfected with tincture of iodine. Patients were excluded from the study if the application of the rubber dam was not effective. All carious dentine was removed with a sterile bur at low speed and without water spray. This was followed by a wide penetration of the pulp chamber with a new, sterile long-shanked round bur. The pulpal tissue was engaged and removed with a new, sterile barbed broach and then transferred to a preweighed pyrogen-free tube (Whittaker Bioproducts, Walkersville, Md). All the tubes were reweighed with the specimens. During the sampling an effort was made to collect an equal quantity of the pulpal tissue in all cases (approximately 8 mg). All the samples were stored at –30°C, as recommended by the manufacturer, until the next step of the procedure.

Release of endotoxins

The extraction of endotoxins was performed by the phenol-water method.[15] Three millilters of a 65% phenol solution were added to the tubes containing the specimens. The samples were vortexed at room temperature for 1 minute and centrifuged at 3500 rpm for 50 minutes. The solution then formed 2 phases: The upper phase, which contained endotoxins, and the lower phase, which contained mostly phenol. The upper phase (supernatant) was removed with a pyrogen-free pipette and placed in a pyrogen-free tube for 10 minutes at 60°C to inactivate proteins that might cause false-positive results. Heating does not affect the activity of endotoxins since they are heat stable.[5,6]

Measurement of endotoxins

To determine the amount of endotoxins present in the collected supernatant, the LAL method, a modified

limulus amebocyte lysate and a synthetic color-producing substrate to detect endotoxins chromogenically (Quantitative Chromogenic LAL-1000, Whittaker Bioproducts), was used. With this method a yellow color will develop if endotoxins are present in the sample.

Reagent preparation. From the endotoxins supplied in the kit (*E. coli* 0111:B4) with a known concentration (23 EU/mL), 4 standard endotoxin solutions were prepared with concentrations of 0.1, 0.25, 0.5, and 1.0 EU/mL, to be used as positive controls. By reading the absorbency of the above known concentrations of endotoxin in a phasmatophotometer (Spectronic 70; Bausch & Lomb) at 405 nm, the corresponding absorbance values were recorded. As a result, a standard curve was constructed from which the best fit was calculated. The absorbance at 405 nm is linear in the concentration range used (Fig 1).

Test method. The kit used was supplied with LAL reagent water, *E. coli* endotoxin (23 EU/mL), chromogenic substrate, and chromogenic limulus amebocyte lysate (LAL). Acetic acid (25%) was used as the stop reagent.

Different solutions were needed for the quantification of endotoxins in the samples: (1) 4 tubes with 50 μL of the above-mentioned endotoxin standards, (2) 1 blank tube containing 50 μL of LAL reagent water instead of sample to be used as a negative control, and (3) samples tubes with 50 μL of supernatant from each specimen.

At the beginning (time 0), 50 μL of LAL was added to each of the aforementioned tubes (4 tubes of endotoxin standards, one blank tube, and tubes with samples). Thorough mixing was performed, and the tubes were incubated for 10 minutes at 37°C. After 10 minutes, 100 μL of chromogenic substrate solution were added, mixed, and incubated for 6 minutes at 37°C, after which 100 μL of stop reagent (acetic acid) was added, and samples were vortexed. All samples were independently examined in a phasmatophotometer by 2 persons who were blinded to the samples (Fig 2).

Calculation of endotoxic concentration. The mean absorbance value of the blank was subtracted from the mean absorbance value of the standards and the value of samples to calculate the mean Δ absorbance. Since this absorbance value is in direct proportion to the amount of endotoxins present, the endotoxic concentration can be calculated graphically from the standard curve.

Statistical analysis. To correlate the amount of endotoxins found in the pulp with the presence or absence of pulpal pain, we analyzed the data on the presence or absence of severe and spontaneous pulpal pain and the quantitative determination of endotoxins in the pulpal tissue statistically by means of independent *t* test.

Table I. Distribution of various tooth types, patient age, endotoxic concentration, and tooth group

Patient	*Tooth type*	*Age*	*Endotoxic concentration (ng/mL)*	*Tooth group*
1	11	45	0.148	Symptomatic
2	11	28	0.150	Symptomatic
3	35	19	0.180	Symptomatic
4	12	30	0.150	Symptomatic
5	13	62	0.170	Symptomatic
6	13	40	0.143	Symptomatic
7	23	25	0.130	Symptomatic
8	15	33	0.150	Symptomatic
9	15	15	0.310	Symptomatic
10	25	18	0.135	Symptomatic
11	15	24	0.160	Symptomatic
12	45	32	0.170	Symptomatic
13	35	43	0.120	Symptomatic
14	25	58	0.120	Symptomatic
15	25	26	0.130	Symptomatic
16	11	54	0.113	Symptom-free
17	44	37	0.112	Symptom-free
18	15	27	0.110	Symptom-free
19	25	22	0.115	Symptom-free
20	25	44	0.115	Symptom-free
21	44	28	0.113	Symptom-free
22	45	32	0.098	Symptom-free
23	35	50	0.110	Symptom-free
24	44	43	0.110	Symptom-free
25	45	35	0.095	Symptom-free
26	25	19	0.113	Symptom-free
27	44	23	0.080	Symptom-free
28	34	27	0.110	Symptom-free
29	34	12	0	Control
30	44	12	0	Control
31	34	13	0	Control
32	34	12	0	Control
33	44	11	0	Control

RESULTS

Endotoxins were detected in all pulpal tissues of the teeth in groups 1 (symptomatic carious) and 2 (symptom-free carious). No endotoxins were detected in group 3 (control; intact noncarious teeth). The mean concentration of endotoxins in the experimental groups is shown in Table II.

The concentration of endotoxins in the pulpal tissue of symptomatic teeth was significantly higher ($P < .001$) than that of the symptom-free teeth (Table III).

DISCUSSION

The objectives of this investigation were the determination and quantification of the amount of endotoxins present in the pulp of symptomatic and symptom-free human carious teeth and the association of their presence with acute pulpal pain.

To achieve our purpose, we used the LAL method, which is the same or similar to methods used in earlier

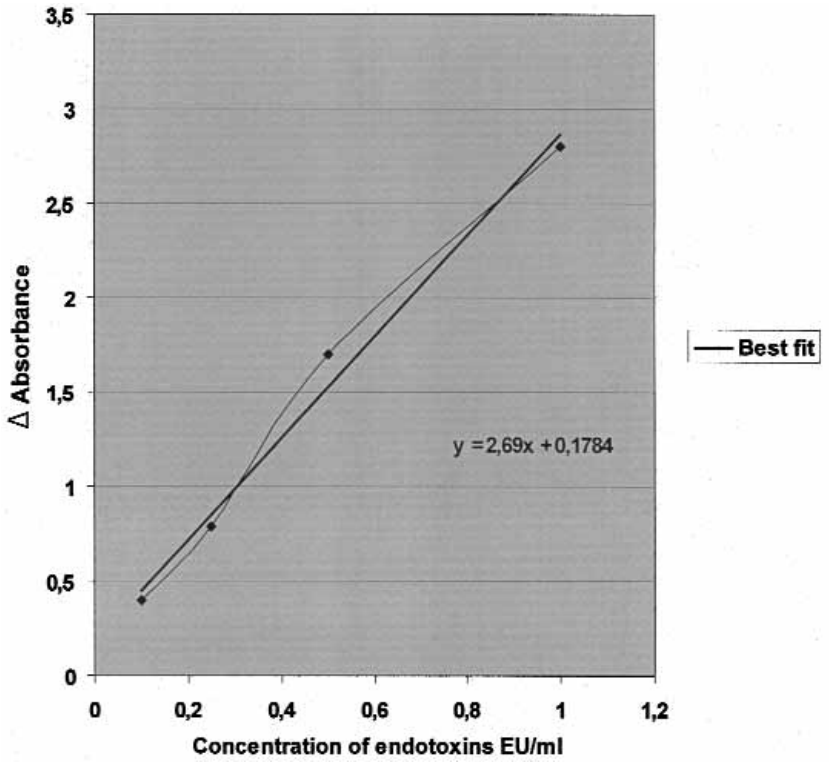

Fig 1. Standard curve used for the calculation of endotoxic concentration.

Table II. Mean concentration of endotoxins in the pulpal tissue of different experimental groups

Group	*Mean ng/mL*	*N*	*SD*	*Max ng/mL*	*Min ng/mL*
Symptomatic carious teeth	0.15773	15	0.045811	0.31	0.12
Symptom-free carious teeth	0.10723	13	0.010925	0.115	0.08
Control noncarious teeth	0	5	0	0	0

Table III. Statistical analysis between symptomatic and symptom-free teeth

	Independent t test	*df*	*P value*
Pulp symptomatic/symptom-free	3.871	26	<.001

studies of endotoxins. A survey of the literature shows that Dahlen and Bergenholtz used the LAL method to identify endotoxic activity in teeth with necrotic pulps[16]; samples (10-15 μL) with gram-negative organisms could be diluted 10^4 to 10^6 times and still give a positive reaction. Horiba et al[17] used a chromogenic substrate that reacted specifically with endotoxins in a chromogenic method to quantify endotoxins in the dentinal wall of infected root canals, using a spectrophotometer at 405 nm. They found endotoxins at the level of picograms. Thus, with the LAL method it is possible to identify a low concentration of endotoxins.[18] Fukui et al[19] found endotoxins in the plasma of different patients. Endotoxins were recently detected by the LAL method in middle ear effusions.[20] Several other studies have used the LAL method for demonstration of endotoxins.[21-24]

Our results demonstrated that the mean value of endotoxins detected in approximately 8 mg of the inflamed pulpal tissue of symptomatic teeth was 0.15773 ng/mL, whereas it was 0.10723 ng/mL in symptom-free teeth. In the literature the average value of detected endotoxins ranged from approximately 1 to 100 μg/mL.[16-18,21,22] An exact comparison between studies was difficult because of differences in tissue samples, sampling technique, and other methodological aspects. For example, Schein and Schilder,[21] using the limulus lysate test, deposited 0.1 mL of saline solution in the pulp chamber and the same amount of fluid was aspirated. In this volume of fluid endotoxins originating from both pulpal tissue and pulpal wall were dissolved, leading to the detection of a concentrated solution of endotoxins in a microgram

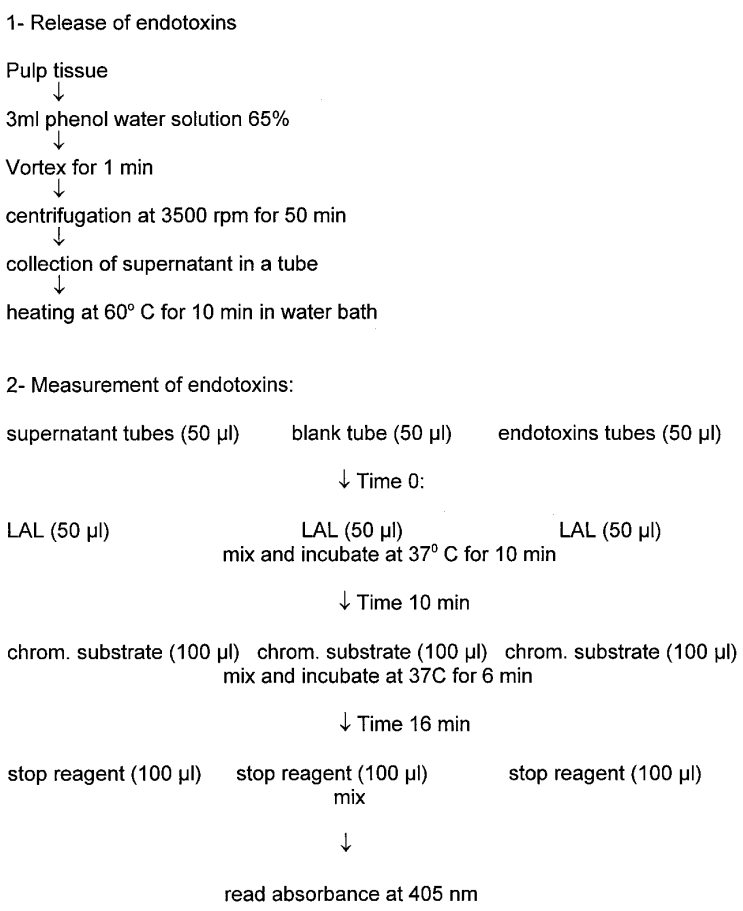

Fig 2. The method used for the release and the quantification of endotoxins.

range. In our study the amount of endotoxins contained in the removed pulp only was dissolved in 3 mL of phenol-water solution, thus resulting in a weaker concentration measured in nanograms.

Other substances may induce a false-positive LAL reaction (ie, blood products). This was not the case in our study, as all these substances were inactivated through heating. With our technique the normal noncontaminated pulps were negative to endotoxic detection.

Many studies have demonstrated that the major etiologic factor for the initiation and progression of caries and pulp inflammation in human teeth is the microorganisms present in the oral cavity.[3,25,26] In recent years there has been considerable interest in the role of anaerobic microorganisms plays in inflammation and pain. It has been established that application of crude bacterial extracts or specific bacterial components to exposed dentin can induce inflammation in the underlying pulp, indicating that bacterial products are probably capable of diffusing to the pulp.[8] Such a hypothesis is supported by the finding that bacterial endotoxins were found to be capable of passing through 0.5 mm of dentin.[27] Inflammation of the pulpal tissue may also be induced, under dental restorations by secondary caries or by marginal leakage. The results following Class V cavity preparation in monkey teeth and restoration with a poor marginal seal strongly supported the importance of bacteria as a major factor in the development of pulpal inflammation and necrosis.[28] However, scanning and transmission electron microscopic examinations of human vital pulps under deep carious lesions has rarely revealed the presence of microorganisms.[29,30] Moreover, bacterial penetration of noncarious dentin to the pulp has been a rare finding even after long-term exposure of the dentin to the oral microflora.[31]

Hoshino et al[32] used an anaerobic technique to examine the microflora of the pulps of freshly extracted teeth with deep carious lesions without pulpal exposure. They detected anaerobic bacteria in 6 of 9 teeth and postulated that bacteria penetrated to the pulps through

the infected dentinal tubules. Endotoxins producing gram-negative cocci were among the microorganisms found.[32]

In our study endotoxins were detected in the pulp of all carious teeth. Since there was no pulpal exposure in our cases, it seems logical that the origin of endotoxins is gram-negative bacteria situated in the deep carious dentin, transported to the pulp through dentinal tubules. However, endotoxins may have been produced by bacteria already existing in the pulpal tissue since, within a necrotic area of vital pulp, bacteria may be present when no or slight amounts of irregular dentin forms in a limited area under deep carious lesions.[1]

In a previous study we found that endotoxins are present in the carious dentin of symptomatic and symptom-free teeth with vital pulp. A greater amount of endotoxins was present in painful teeth than in those without symptoms.[23] In the present study we found that the amount of endotoxins was significantly greater in the pulps of symptomatic carious teeth with irreversible pulpitis than in the painless carious teeth. Therefore, there appeared to be a strong association between the level of pain and the concentration of endotoxins in the pulp of carious teeth. A probable explanation for this phenomenon is that endotoxins activated the Hageman factor, which in turn led to the production of bradykinin, a potent pain mediator and inducer of increased vascular permeability.[11] In addition, endotoxins possess neurotoxic properties that act on presynaptic nerve terminals.[12-14] Matsushita et al[33] recently reported that endotoxin from black-pigmented bacteria may be involved in multilesional periapical periodontitis by inducing particular cytokines and/or humoral immune responses.

Moreover, the relationship between bacterial endotoxins and pain has been demonstrated in teeth with infected root canals and periapical lesions; more endotoxins were found in the periapical areas of symptomatic teeth than in those of symptom-free teeth.[21,24] In his classic work on the bacteriology of pulps rendered nonvital by trauma, Sundqvist found that a greater number of different bacterial strains were isolated from patients with pain symptoms.[34] There was a significant relationship between clinical symptoms and the presence of *Bacteroides melaninogenicus* (*Prevotella*). This anaerobic endotoxin producing, gram-negative rod was isolated only from symptomatic teeth and was absent from those that were asymptomatic.[34] The association of *B melaninogenicus* (*Prevotella*) and clinical symptoms was confirmed by Griffee et al[35] in their study of 33 teeth with nonvital pulps. *B melaninogenicus* was isolated from 12 teeth, and its presence was found to correlate with pain, foul odor, and the formation of sinus tracts.[35] Thus, it seems that endotoxins play a major role in the pathogenesis of the pulp and periapical tissues, since their presence in caries, inflamed pulp, infected root canals, and periapical lesions is associated with clinical symptoms.

In the present investigation it was evident that although endotoxins were detected in all pulpal tissues of all carious teeth, pain was not found in all patients but only in those who had a greater concentration of endotoxins in the pulp. The same occured in our previous study, where pain was not evident in all patients despite the detection of endotoxins in carious dentin.[23] Our results have been in agreement with the results of others who have found that there is a higher level of endotoxins in symptomatic teeth than in those without symptoms.[21,24,34,35] These findings suggest that the concentration of endotoxins in the pulp must exceed a certain level for pain to be clinically apparent. It is generally believed that pulp fibroblasts are able to respond to low levels of endotoxins (5 to 125 μg/mL) by increased cell division and synthesis of connective tissue matrix. In contrast, a high level of endotoxins (625 μg/mL) has been shown to be obviously toxic to connective tissue containing fibroblasts and would result in tissue necrosis.[36] Nakane et al[37] investigated the influence of endotoxins purified from *Porphyromonas gingivalis, Porphyromonas endodontalis, Fusobacterium nucleatum*, and *E coli* on the production of DNA and protein from human dental pulp cells. They found that a concentration of 1 μg/mL endotoxins caused no change in the production of DNA or protein. But at 10 μg/mL the amount of DNA was increased, and at 100 μg/mL it was inhibited.[37] Thus, infections associated with low concentrations of endotoxins may actually stimulate repair mechanisms.[11] In clinical practice, one might find that symptom-free teeth with deep carious lesions have an amount of endotoxins in their pulps that is insufficient to excite the intradental nerves or to produce an acute inflammation.

Although the cause of pulpal pain is multifactoral, our study suggests that the concentration of endotoxins in the pulp is one factor. A certain threshold level of endotoxins must be reached to have an effect on the inflamed dental pulp such that clinical pain may be observed.

CONCLUSION

Endotoxins were demonstrated in the inflamed pulp tissue of symptomatic and asymptomatic carious teeth. The amount of endotoxins in the inflamed pulp of carious teeth was significantly greater in symptomatic teeth than in symptom-free teeth. A hypothesis has been presented, suggesting that a certain threshold level of endotoxins is required for the development of pain in the inflamed pulp.

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