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Evaluation Of Interleukin 6, Interleukin 8, TNF Alpha as Biomarkers For Pulpitis - In Vivo Study

Research Article

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Abstract

Introduction: Dental pulpitis occurs due to the inflammation of pulpal tissue. Inflammation of the pulp does not start when the bacteria reach the pulp rather the bacterial products may reach the pulp and initiate an inflammatory response.
Aim: To evaluate the levels of interleukin 6, interleukin 8 and tumor necrosis factor alpha as biomarkers for reversible and irreversible pulpitis.
Materials and Method: Saliva samples were collected from reversible and irreversible pulpitis and control patients. The samples were frozen until analysis. All samples were centrifuged for 15 minutes. The saliva supernatant was separated and stored at low temperatures. Cytokine profiling was determined using a sandwich immunoassay ELISA kits with 96- well plate. The specific cytokines- Interleukin 6, Interleukin 8 and Tumor necrosis factor Alpha were measured according to the manufacturer's protocol.
Results: Data was tabulated in excel sheets and multiple anova tests was done. Samples collected from reversible and irreversible pulpitis showed higher cytokine levels than control levels. Samples collected from reversible pulpitis cases have shown to be associated with higher cytokines levels than the samples collected from irreversible pulpitis cases.
Conclusion: Cytokine measurements may help in knowing the exact status of pulp and thereby predict the long-term prognosis of vital pulp therapies. Interleukin 6, interleukin 8, and tumor necrosis factor can be used as a biomarker for dental pulpitis.

Keywords: Reversible Pulpitis; Irreversible Pulpitis; Biomarkers; Interleukins; Cytokine.

Introduction

Pulpitis is typically caused by an opportunistic infection of the pulp space by commensal oral microorganisms [1-3]. The most common route of entry for the microorganisms is dental caries [4-11]. Other potential pathways for pulpal microbial infection include trauma, dentinal cracks, exposed dentinal tubules or the main apical foramen [12-14]. The microorganism and their by-products induce TLR expressing dental pulp cells to produce immune responses. This group includes odontoblasts [15], endothelial cells [16] as well as macrophages and dendritic cells [17].

Based on the patient's signs and symptoms the pulpal condition can be classified into four types- normal, reversibly inflamed, irreversibly inflamed or necrotic [18, 19]. Histological assessment is the gold standard to accurately assess the inflammatory state of dental pulp. Reversible pulpitis is recognized by the absence of bacteria and localized coagulation, liquefaction necrosis immediately surrounding the irritant, whereas irreversible pulpitis is characterized by the presence of the bacteria or their metabolites in the dental pulp tissue and by preponderance of acute inflammatory cells predominantly neutrophils in the tissue beneath the lesion suggesting chemotactic activity. Lysosomal enzymes discharged by neutrophils result in widespread tissue damage and suppuration [20].

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Acute pulpitis can be an extremely painful condition and is one of the main causes for patients to seek emergency dental treatment. The main clinical difference between reversible and irreversible pulpitis is in the pulp's response to thermal stimulus. Reversible pulpitis will present an exaggerated, non-lingering pain to cold stimulus. Irreversible pulpitis is characterized by constant, spontaneous pain, lingering response to cold stimulus. However, forty percent of teeth with irreversible pulpitis can be painless [21]. In reversible type, the pulp is expected to recover after removal of the triggering stimulus. In contrast, pulp is not expected to recover in irreversible cases and it is fully removed by pulpectomy. The outcome of pulpal insult is a dynamic process that depends on both the invading microorganisms and host responses to them, which include inflammation and immunity. Evidence from experimental pulpitis models has clearly demonstrated that bacterial antigens and/or metabolic by-products can diffuse through dentinal tubules to elicit immune responses in the dental pulp [22-25]. Immune complexes and by-products from immune responses, such as extracellular proteolytic enzymes released by phagocytosis, can further aggravate pulpal inflammation [26, 27], making the problem worse.

Cytokines are the guiding factors of inflammation and its progression to tissue necrosis [28]. The ultimate goal should then be to develop an inexpensive chairside test for non-invasive molecular pulp diagnostics. There are numerous well designed clinical trials and lab studies done. But, only few studies are done for cytokine level assessment and its association with dental pulpitis. So the aim of this study is to evaluate the levels of interleukin 6, interleukin 8 and tumor necrosis factor alpha as biomarkers for reversible and irreversible pulpitis.

Materials and Method

Study Design

It is a Randomized Controlled Trial.

Ethical Approval

Approval for the project was obtained from the Institutional Review Board of Saveetha Institute of Medical and Technical Sciences, Chennai, India. SRB/MDS/ENDO/18-19/0040.

Eligibility Criteria

Inclusion criteria

Teeth with reversible pulpitis,Teeth with irreversible pulpitis and Teeth with deep caries.

Exclusion criteria

Teeth with complex anatomies, Teeth with abscess, Patients with medically compromised conditions, Pregnant patients.

Setting and Location

The volunteer patients fitting the inclusion criteria described above were included in the study. The study participants for the study were recruited from the pool of patients in the Department of Conservative and Endodontics atSaveetha Dental College, Saveetha Institute of Medical and Technical Sciences, Chennai, India.

Groups

Group I – Reversible pulpitis
Group II – Irreversible pulpitis
Group III-Control

Methodology

Patients reported with pain were first distinguished into three groups: Reversible, Irreversible pulpitis and control group. Patients who reported with pain were divided into reversible and irreversible pulpitis groups based on their response to cold pulp sensibility test and Electric pulp test. 2ml of saliva was collected in sterile containers from each. For the control group, Saliva was collected from patients with zero caries and clinically healthy gingiva. A total of 10 samples from each group were sent to the lab within 48 hours. The saliva samples were frozen (−70°C), until analysis. All samples were centrifuged at 4500 g for 15 min. The saliva supernatant was separated and stored at low temperature in an "ultra-cool temperature storage unit" at −80°C so as to prevent microbial growth and avoid degeneration of cytokines.

Outcome Measure

Cytokine profiling was determined using a sandwich immunoassay ELISA kit was done with a 96-well plate. The specific cytokines that were studied include: Interleukin 6 (IL-6), Interleukin 8 (IL-8) and Tumor Necrosis Factor alpha (TNFα) according to the manufacturer's protocol.

Estimation of IL 6

Briefly, IL-6 present in the samples or standard binds to anti-IL-6 monoclonal antibody adsorbed to the microwells. A biotin-conjugated monoclonal anti-IL-6 antibody was added and binds to IL6 captured by the first antibody. Following incubation unbound biotin-conjugated anti-IL-6 is removed during a wash step. Streptavidin-HRP was added and binds to the biotin-conjugated antiIL-6; following incubation unbound Streptavidin-HRP was removed during a wash step, and substrate solution reactive with HRP was added to the wells. A colored product was formed. This was in proportion to the amount of IL-6 present in the sample. The reaction was terminated by addition of acid and absorbance was measured at 450 nm.

Estimation of IL 8

In brief, plates were coated with anti-human IL-8 in PBS (pH 7.3) and nonspecific binding was blocked with 500 µl of 1% bovine serum albumin. Samples and reconstituted IL-8 standard in concentrations ranging from 15 -1000pg/ml were added to the wells. Biotinylated polyclonal IL-8 was used as detection Ab, followed by addition of streptavidin-HRP in dilution buffer. Colour was developed using Tetramethylbenzidine as substrate and optical density was taken at 450nm.

Estimation of TNF Alpha

The wells of microtiter plates were coated with 100µl of saliva and mixed with carbonate buffer. The plates were kept at 4°C overnight and then washed thrice with phosphate buffer saline (pH 7.4) containing 0.05% Tween20 and blocked with 3% BSA in phosphate buffered saline (100µl/well), kept at 37°C for 1 hr. After washing 100µl of serially diluted purified TNF-alpha antibody was added and incubated at 37°C for 1hr. This was followed by washing with PBS and Tween 20. The wells were then coated with 100µl of anti rabbit IgG-conjugated with horseradish peroxidase (diluted 1:5000). The plates were incubated at 37°C for 1hr and washed. The activity of bound horseradish peroxidase was monitored by addition of 50µl of substrate (1µl of hydrogen peroxide, 0.5 mg OPD in 1ml of citrate phosphate buffer, pH 5.0). After 20 min in dark at room temperature, the reaction was arrested by the addition of 50µl of 2N sulfuric acid. The intensity of the colour was recorded on ELISA reader at 490 nm.

Results and Discussion

Data was tabulated in excel sheets and statistical analysis was done. Multiple anova tests were performed. Samples collected from reversible and irreversible pulpitis showed higher cytokine levels than control levels. Samples collected from reversible pulpitis cases have shown to be associated with higher cytokines levels than the samples collected from irreversible pulpitis cases. The results were statistically significant with P value less than 0.05.

The levels of IL 6, IL 8 and TNF alpha levels in irreversible pulpitis were higher than reversible and control groups. The results were statistically significant with p value less than 0.5. When the levels of IL 6, IL 8 and TNF alpha were compared between reversible and irreversible pulpitis, the irreversible pulpitis group demonstrated higher values than the reversible group. This could be attributed to the fact that irreversible pulpitis is more severe form inflammation characterized by edema, pain etc.

IL-6 displays multiple biological effects and acts as a major mediator of the host response following tissue injury and infection as well as inflammation. It increases the levels of acute-phase proteins, C-reactive protein, serum amyloid A and fibrinogen [29, 30]. IL-6 causes up-regulation of adhesion molecules and induces angiogenesis leading to increase in vascular permeability and inflammatory oedema. In addition, it induces osteoclast differentiation and bone resorption [31]. Although significantly higher levels of IL-6 have been detected in pulpal tissue from irreversible pulpitis and from periapical lesions [32] as well as higher mRNA levels in irreversible pulpitis compared with healthy teeth, it was not detected in pulpal blood [33].

In the present study, IL-6 was significantly higher in the caries with irreversible pulpitis as compared to normal pulps. As pulpal symptoms are generally explained by increase in intra-pulpal pressure due to oedema [34], levels of IL-6 can be correlated to the extent of inflammation and oedema in the pulp in addition to its role as a mediator of host response following tissue injury and infection.

IL-8 is a potent chemokine with strong chemoattractive activity for neutrophils. In addition to recruiting neutrophils, IL-8 stimulates neutrophils to a higher state of activation. It is rapidly synthesized at local sites of inflammation where it fulfils its function of recruiting and activating acute inflammatory cells [35]. In dis-

Figure 1. Addition of Saliva samples to the Elisa wells.

Figure 2. Addition of HRP Conjugate.

Figure 3. Elisa Well.

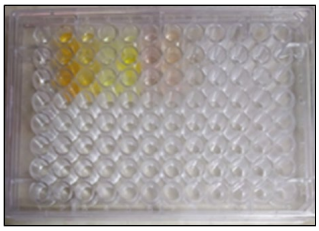

Figure 4. Showing Levels of IL6, TNF Alpha in Group I (Reversible pulpitis), Group II(Irreversible pulpitis), Group III (Control group). Il6 and Tnf alpha levels were higher in irreversible pulpitis group than reversible pulpitis and control group.

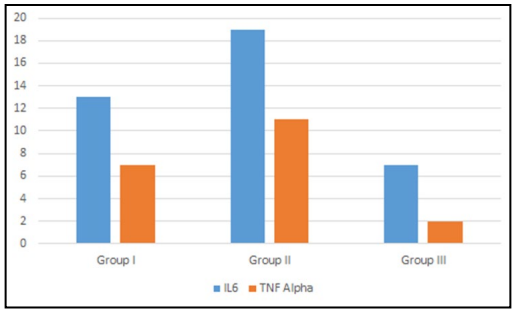

Figure 5. Showing Levels of IL8 in Group I (Reversible pulpitis), Group II (Irreversible pulpitis), Group III (Control group). IL8 levels were found to be higher in irreversible pulpitis group than control and reversible pulpitis group.

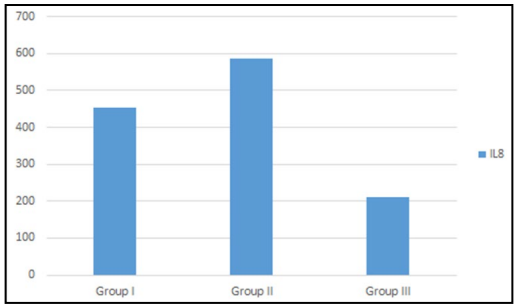

Table 1. Cytokine levels of Interleukin 6, Interleukin 8 and Tumor Necrosis Factors Alpha in Reversible pulpitis group.

Group I	TNF alpha (pg/ml)	IL 6 (pg/ml)	IL 8 (pg/ml)
Sample 1	8.4	7.34	523.7
Sample 2	7.6	6.11	504.8
Sample 3	8.9	8.4	558.1
Sample 4	13.5	24,7	688.7
Sample 5	14.1	27.8	663.8
Sample 6	4.13	14.2	283.6
Sample 7	3.54	11.5	286.1
Sample 8	4.78	12.8	284.7
Sample 9	4.98	12.3	293.4

Table 2. Cytokine levels of Interleukin 6, Interleukin 8 and Tumor Necrosis Factors Alpha in Irreversible pulpitis group.

Group II	TNF alpha (pg/ml)	IL 6 (pg/ml)	IL 8 (pg/ml)
Sample 1	9.2	10.6	516.3
Sample 2	8.7	5.65	584.7
Sample 3	11.2	26.5	705.8
Sample 4	10.7	31.4	756.9
Sample 5	9.1	6.83	506.7
Sample 6	13.7	22.8	555.5
Sample 7	12.96	26.4	566.6
Sample 8	14.5	23.4	559.9
Sample 9	12.08	20.1	533.3

eased pulps, IL-8 was reported to be produced by pulpal inflammatory cells and endothelial cells in addition to odontoblasts [36] In addition, bacterial lipopolysaccharide stimulated pulp fibroblasts produce higher levels of IL-8 than the unstimulated group [37]. Levels of IL-8 were significantly higher in pulpal tissue from irreversible pulpitis as well as pulpal blood. Also, there is an increase in mRNA level of IL-8 in symptomatic teeth compared with healthy teeth. In the present study, IL-8 was detected in pulps with irreversible pulpitis and caries exposure but was not detectable in normal pulps. Irreversible pulpitis pulps have 4-fold higher

Table 3. Cytokine levels of Interleukin 6, Interleukin 8 and Tumor Necrosis Factors Alpha in Control group.

GROUP III	TNF alpha (pg/ml)	IL 6 (pg/ml)	IL 8 (pg/ml)
Sample 1	2.16	7.43	216.4
Sample 2	1.05	6.89	297.3
Sample 3	1.08	6.63	210
Sample 4	2.2	5.25	198.6
Sample 5	2.53	7.81	163.7
Sample 6	2.61	6.32	183.4
Sample 7	2.91	5.89	200
Sample 8	1.99	6.25	211
Sample 9	2.28	6.08	215.2

median IL-8 levels than asymptomatic caries exposure pulps. This suggests that IL-8 may be a good contender for a marker for the degree of pulpal inflammation and appears to be correlated with pulpal symptoms.

TNF-a is a prominent inflammatory mediator and absolutely central in initiating the cascade of inflammatory reactions of the immune system including induction of cytokine production, activation and expression of adhesion molecules and stimulation of cell proliferation. It coordinates the early host response to injury and thus represents an important point of regulation in inflammatory diseases [38, 39]. Symptomatic irreversible pulpitis pulps showed higher levels of TNF-a compared with asymptomatic irreversible pulpitis and normal pulps .

The present data revealed significantly higher levels of TNF-a in irreversible pulpitis than in normal pulps with no significant difference between caries exposure and irreversible pulpitis. Thus, the overall balance of cytokines or relative cytokine levels may be of greater importance than absolute cytokine levels per se. Also, the ratios have no units which make comparisons easy when different types of samples are used. Based on this rationale, we calculated ratios of inflammatory cytokines to the anti-inflammatory cytokine IL-10. IL-2/IL-10, TNF-a /IL-10 and IFN-c/IL-10 ratios were not significantly different between the different groups. However, IL-6/IL-10 and IL-8/IL-10 ratios were significantly higher in the irreversible pulpitis group compared with both caries exposure and normal pulps with no significant difference between normal and caries exposure pulps. This suggests that these two ratios have the potential of being good markers of irreversible pulpitis. However, the histological appearance of the pulp was not studied, the results revealed only the correlation between cytokine levels and different pulpal conditions diagnosed based on clinical signs and symptoms.

Conclusion

Cytokine measurements may help in knowing the exact status of pulp and thereby predict the long-term prognosis of vital pulp therapies. Interleukin 6, interleukin 8, and tumor necrosis factor can be used as a biomarker for dental pulpitis.

Clinical Significance

Dental pulpitis occurs due to the inflammation of pulpal tissue. Assessment of the exact status of the pulp is possible only with the help of histological examination which requires traumatic extraction of pulp tissue or pulpal blood. This study has proved the correlation of cytokines levels with the inflammatory condition of the pulp. So, salivary cytokines have the potential to be used as a biomarker in dental pulpitis.

Limitations

This study was confined to a smaller sample size and this study could not explain the influence of other inflammatory conditions of oral cavity on the cytokine levels.

Future Scope

Further studies should done be to check the influence of other inflammatory conditions in the oral cavity on these cytokine levels.

Author Contributions

Aishuwariya.T - Writing; Original Draft Preparation; Sindhu Ramesh-Review and Editing;

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