

BIOCHEMICAL AND IMMUNOHISTOCHEMICAL EVALUATION OF TRANSFORMING GROWTH FACTOR- BETA1 AND TUMOR NECROSIS FACTOR- ALPHA IN DENTAL DISEASES

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ABSTRACT

Objective: Oral and dental diseases are widely prevalent in the world. So, this research is aimed to evaluate the biochemical and immunohistochemical analysis of transforming growth factor- β 1 (TGF- β 1) and tumor necrosis factor- α (TNF- α) in the gingival crevicular fluid (GCF) and tooth tissues in patients with chronic irreversible pulpitis (CIP), chronic periapical lesion (CPL) and chronic periodontitis (CP) compared to healthy subjects and to study the correlation between tissue level and the GCF level of these markers. **Subjects and Methods:** The GCF and tooth specimens were obtained from 20 chronic irreversible pulpitis patients, 20 chronic periapical lesion patients, 20 chronic periodontitis patients and 20 healthy periodontium subjects. The specimens were processed for biochemical and immunohistochemical analysis of TNF- α and TGF- β 1. **Results:** Statistical analysis revealed significant elevation of the mean GCF TNF- α in CIP, CPL and CP groups ($P < 0.001$) as compared with control group. High significant increase in the mean GCF TGF- β 1 in CP and CPL group than in CIP group ($p < 0.001$). TNF- α and TGF- β 1 were as effective tests in CPL and in CP with sensitivity and specificity of 100%. Significant increases ($P < 0.001$) in mean TGF- β 1 expression in teeth specimens from patients with CIP and CPL and insignificant rise ($P > 0.05$) in patient group with CP compared with the control. **Conclusions:** High significant elevation ($P < 0.001$) in GCF-TNF α and TGF- β 1 were recorded in all patients groups as compared to normal control. The TGF- β 1 and TNF- α depicted the highest correlation of the biochemical and immunohistological expression for CP group.

Keywords: gingival crevicular fluid, TGF- β 1, TNF- α , dental diseases.

INTRODUCTION

Oral health is an essential as well as a vital component of systemic health throughout life. Oral health is related to diet in many ways, for example, nutritional influences on craniofacial development, oral cancer and oral infectious diseases. Dental diseases impact considerably on the quality of life and are expensive to treat. Dental diseases are not just a toothache it can lead to serious problems which may affect the

facial aesthetics as well as the ability to chew, speak or smile¹. Despite a low mortality rate associated with dental diseases, they have a considerable impact on self-esteem, eating ability, nutrition and health both in childhood and older age. Teeth are important in enabling the consumption of a varied diet and in preparing the food for digestion. In modern society, the most important role of teeth is to enhance appearance; facial appearance is

very important in determining an individual's integration into society^{2,3}. Teeth also play an important role in speech and communication. The second International Collaborative Study of Oral Health Systems (ICSII) revealed that in all countries covered by the survey, substantial numbers of children and adults reported an impaired social functioning due to oral disease, such as avoiding laughing or smiling due to poor perceived appearance of teeth^{4,5}. Oral cavity is a multifaceted environment which may port more than 750 bacterial types. Appropriate oral hygiene is important to keep the balance of microbial society and oral health. The bacteria can assist in the creation of dental plaque and caries, leads to periodontal disease and periapical lesion^{6,7}. The severity of dental diseases varies from just a common cavity of tooth or tiny traumatic ulcer up to lethal oral cancer. The most widespread dental diseases are dental caries, gingivitis, pulpitis, periapical lesion as well as periodontitis^{6,8}. The main change in biochemical constituents of dental connective tissue during those diseases was the loss of collagens due to soft and hard tissue destruction. This destruction is thought to reflect a cascade of events involving release of enzymes, pro-inflammatory or anti-inflammatory cytokines, prostaglandin and matrix metalloproteinases by host and bacterial cells. The products of the inflammatory response, which take place during the disease process, can be found in the GCF. Monitoring of the existence of such components can be of potential value in evaluating the periodontal disease status or outcomes of periodontal therapy⁶. TNF- α 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 diseases⁹. TNF- α is produced by monocytes and macrophages in response to oral bacterial components, such as LPS. Elevated levels of TNF- α may promote the release of collagenase from human gingival fibroblasts, leading to cartilaginous collagen destruction and bone resorption. Therefore, it has been suggested that TNF- α may be a marker of inflammatory¹⁰. TNF- α is present at high concentrations in the GCF of diseased periodontal tissues¹¹, and radicular cysts¹². TGF- β is important immunoregulatory cytokine that is produced in the pulpitis, periapical

lesions and periodontitis¹³. Because TGF- β exhibits both pro-inflammatory and anti-inflammatory properties besides its ability to stimulate synthesis of ECM molecules and to inhibit the breakdown of ECM, it has been intensively evaluated in relation to all types of gingival overgrowth¹⁴. Cytokines such as TNF- α and TGF- β 1 play a role in the pathogenesis of pulp inflamed, periapical lesions and periodontitis and plays a vital role in the inflammatory reaction, alveolar bone resorption and in the failure of connective tissue attachment¹⁵.

This study is aimed to examine the correlation of the biochemical and immunohistochemical markers; tumor necrosis factor- α (TNF- α) and transforming growth factor- β 1 (TGF- β 1) in gingival crevicular fluid (GCF) and teeth specimens of normal subjects and patients with chronic irreversible pulpitis (CIP), chronic periapical lesion (CPL), and chronic periodontitis (CP) in an attempt to study the pattern of expression of these markers in dental cells and to find a cutoff value for each parameter that might help in diagnosis and discrimination between these diseases.

MATERIALS AND METHODS

Sixty patients were enrolled in the present study as referred patients from Periodontics, Orthodontics and Oral Diagnosis Departments to Maxillo-Facial and Oral Surgery Department in Teaching Hospital of Dentistry College/ University of Baghdad during the period from 9th of September 2014 to beginning of February 2015. This study was approved by the Institute Review Board at the College of Medicine, Al Nahrain University. Each participant gave a written consent showing his/her agreement for the participation in this study. The general characteristics of the studied subjects are displayed in table 1.

Each patient was subjected to tooth extraction according to specialist's decision who followed a standard criteria for diagnosis¹⁶. The patient groups include; chronic irreversible pulpitis (20 patients, 9 female and 11 male), chronic periapical lesions (20 patients, 9 female and 11 male) and chronic periodontitis (20 patients, 10 female and 10 male). Twenty healthy periodontium control (10 female and 10 male) subjects were enrolled as controls with an age ranging from 27-50 years attending the Outpatient Clinic at the Department of Surgery after accident, trauma or after the diagnosis of the senior in Orthodontic Department. The GCF and teeth samples were obtained from each participant. Any subjects who have any systemic condition that could affect the host's periodontal status

or that would require antibiotics for monitoring or treatment procedures (e.g. heart conditions and joint replacements, diabetes mellitus, hypertensive); the use of antibiotics and/or anti-inflammatory drugs, or professional cleaning or periodontal treatment within the last four weeks was excluded from the study¹⁷.

Gingival crevicular fluid (GCF) samples collection

Subjects were sitting comfortably in an upright position on the dental chair with adequate illumination; the selected test site was isolated with cotton rolls. Without touching the marginal gingiva, the supragingival plaque was removed with a curette to avoid contamination of the GCF sample and blockage of the microcapillary pipette. The crevicular site was then dried gently with an air syringe. The GCF was collected using the white color-coded 1-5 μ l calibrated volumetric microcapillary pipette (Sigma/USA). The tip of the microcapillary pipette was placed in the gingival groove of the proximal area of the selected extracted tooth (unstimulated) for 5-20 min to collect a standardized volume of 4 μ l GCF according to the calibration scale on the microcapillary pipette. The GCF collected was immediately transferred to a plastic eppendorf tube containing 400 μ l phosphate buffered saline (PH 7.4) and stored at -70°C till the time of the assay by using commercially available ELISA and performed as recommended in leaflet with kits, (Human TNF- α and TGF- β 1 ELISA Kit (Mybiosource-USA)¹⁸.

Tissue preparation and staining

After extraction and washing of the teeth with distilled water, the teeth then immediately fixed in 10% formalin for 72 hours and processed routinely into paraffin blocks. Five μ m-thick semi serial longitudinal sections of the teeth were mounted on clean glass slides for routine haematoxylin and eosin staining (H&E) and histopathologically re-evaluated. The intensity of inflammation was assessed according to Accorinte et al¹⁹ Quantitative assessment of the inflammation cells was conducted on five separate microscopic fields for each studied tooth specimen at 40% magnification. The mean count and the severity of the inflammatory response is recorded and then photographed. Other 5 μ m thickness sections were mounted on positively charged microscopic slides for immunohistochemical detection of the tooth TNF- α and TGF- β 1 (Mybiosource, USA) using specific monoclonal antibodies. The degree of inflammation was evaluated in four stages, based on the the criteria of Commission of

Dental Materials, Instruments, Equipment and Techniques in the following manner:

1. Absent: The width of inflammatory zone is similar to control group, absence of/or only a few inflammatory cells (no more than 5 cells).
2. Mild: The average number of the inflammatory cells is less than 10 cells.
3. Moderate: the average number of the inflammatory cells is 10-25 cells.
4. Severe: the average number of the inflammatory cells is greater than 25 cells.

Statistical analysis

Data were analyzed statistically using mean, SD, two extreme values (upper and lower limits), analysis of variance (ANOVA) test, the receiver operating characteristic (ROC), Contingency coefficient test. The association or difference between the studied markers was considered statistically significant when P-value is ≤ 0.05 .

RESULTS

Statistical analysis revealed significant elevation the mean GCF TNF- α in CIP group, CPL group and CP group ($P < 0.001$) as compared with control group and in CP group in comparison to mean values of both CIP and CPL groups. Table (2) show very high significant differences in the means of GCF-TGF- β 1 levels in CIP, CPL and CP group in comparison with the mean GCF-TGF- β 1 level in the control group ($p < 0.001$).

Group comparison revealed high significant increase in the mean GCF-TGF- β 1 in CP and CPL group as compared with CIP group mean value ($p < 0.001$). Furthermore, the mean GCF-TGF- β 1 were significantly higher in CP group than those of the CPL group ($p < 0.001$).

Table (3) reveals insignificant differences ($P > 0.05$) in the mean of TNF- α and TGF- β 1 between male and female mean values within the control, CIP, CPL and CP groups. Group comparison showed highly significant increase ($P < 0.01$) in the male and/or female mean concentration of GCF of (TNF- α and TGF- β 1) in CPL and CP group as compared with the corresponding gender mean levels of the control group. yet, there were insignificant differences in the means of the GCF of TNF- α and TGF- β 1 in male or female with CIP compared to the respective gender in the control group ($P > 0.05$). The means of (TNF- α and TGF- β 1) were significantly increased ($P < 0.001$) in males and females within the CPL and CP groups as compared with the corresponding gender values of the CIP group.

Moreover, there were significant elevation in the male and female mean GCF level ($P < 0.001$) of TNF- α and TGF- β 1 in the CP group compared with similar gender mean values of the CPL group.

TNF- α and TGF- β 1 were found as effective tests in CPL and in CP with sensitivity and specificity of 100% for these biomarkers at cut off reading of ≥ 9.28 pg/ml for TNF- α , ≥ 19.23 pg/ml for TGF- β 1 (Figure 4,5,7,8). In CIP group, TNF- α was observed an effective test at reading ≥ 28.06 pg/ml (Figure 3) with sensitivity of 85% and specificity equals 80%. The TGF- β 1 recorded sensitivity of 95% with lowest specificity which is 50% at reading ≥ 39.53 pg/ml (Figure 6).

The results of mean expression of the TNF- α in the tooth tissue that are depicted in table (4) revealed high significant increases ($P < 0.001$) in CIP, but there were significant reduction in both CPL ($P < 0.001$) and CP ($P < 0.05$) as compared with the control group. There were significant reduction ($P < 0.001$) in the mean expression of TNF- α in CP and CPL compared to those of the CIP group. Yet, there were insignificant differences in the mean level of expression of TNF- α between CP and CPL group ($P > 0.05$).

Table (4) reflected high significant increases ($P < 0.001$) in mean TGF- β 1 expression in teeth specimens from patients with CIP and CPL and insignificant rise ($P > 0.05$) in patient group with CP compared with the control mean values. Current results of group comparisons showed high significant increases ($P < 0.001$) in the means of TGF- β 1 expression in CP in comparison with those of CIP and CPL group. The mean expression of TGF- β 1 in CPL group was lower than those expressed by CIP group ($P < 0.05$).

The correlation between biochemical and immunohistochemical markers can be evaluated by the median values for these data and the number and percentage of values above and below the median value for each biomarker in the GCF (ELISA results) and tooth specimens (immunohistochemistry) were reported in table 5 using contingency coefficient test.

The highest percentages of TNF- α expression above the median of both biochemical and immunohistochemical tests is recorded in CP (16.7% vs 83.3%; $P = 0.01$). Whereas, the TGF- β 1 biochemical and immunohistochemical findings were at their highest levels in CP (16.7% vs 83.3%; $P = 0.01$).

DISCUSSION

Several cytokines have been detected in GCF and in gingival tissues of patients with periodontitis, reflecting the possibility of

evaluating the contents of GCF as "Indicators" or "Markers" of periodontal disease²⁰. Evaluation of the contents of GCF is a promising, non-invasive method for determining tissue changes in periodontium. TNF- α is a pleiotropic molecule that plays a central role in inflammation, immune system development, apoptosis, and lipid metabolism. TNF- α , a proinflammatory cytokine released by macrophages, is known for its substantial role in periodontitis mediated bone loss²¹. The suitability of using cytokine TNF- α in GCF as a possible indicator of periodontal disease was first assessed by Slattery et al²².

Result of current study was similar to the result of the Pezelj-Ribaric et al²³ who found high significant differences between TNF- α concentration in normal control and patients with CIP ($p < 0.001$), this provides relevant information concerning pathogenesis of pulpal inflammatory diseases. Highest concentrations of TNF- α were detected in irreversible symptomatic pulpitis than in asymptomatic pulpitis and control. As irreversible symptomatic pulpal inflammation progressed, TNF- α concentration decreased. It may be speculated that decrease in TNF- α concentration represents a point at which tissue is in late phase of irreversible inflammation, progressing toward total tissue necrosis. They concluded that the TNF- α may be an objective marker for the extent of pulpal inflammation associated with irreversible pulpitis. Elsalhy et al²⁴ demonstrated higher levels ($P < 0.05$) of TNF- α in irreversible pulpitis than in normal pulps with no significant difference between caries exposure and irreversible pulpitis. Although the result partially contradicts the data of Pezelj-Ribaric et al.²³, it is consistent with TNF- α gene expression in irreversible pulpitis, reversible pulpitis and normal healthy teeth²⁵.

Several biologic molecules have been identified in inflamed pulpal tissues and found present in greater concentrations than those reported for healthy pulpal tissues. These substances are chemotactic for inflammatory cells such as polymorphonuclears and macrophages²⁶. TNF- α protein has a particularly potent effect on neutrophil leukocytes and induces chemotaxis and activation of neutrophils. Under the influence of TNF- α , dilatation and increased permeability of blood vessels occur, causing extravasation of leukocytes from blood into the infected area. Cytokines activate inflammatory cells in intracellular areas, causing increased phagocytosis, release of toxic

modulators/agents, and possible elimination of bacteria²⁷.

Slattery et al.²², Teles et al.²⁸, De Paepe et al.²⁹ demonstrated that the TNF- α may play key roles in the host response to inflammation in periodontal diseases. High significant elevation in the mean of TNF-alpha is recorded in CPL group in comparison with control group. Secretion of the cytokines is initiated with the purpose of activating immunological response to irritants and increasing local concentrations of inflammatory cells in order to prevent further colonization of bacteria within the tissues. Enhanced reaction of the host to various antigens results in bone resorption and formation of granulomatous tissue, which are the typical features of periapical lesion³⁰. It was proved that TNF- α leads to bone resorption through osteoclast activation and stimulation of the secretion of proteolytic enzymes, plasminogen activator (PA), and matrix metalloproteinases (MMP), which operate to destroy extracellular matrix of the bone tissue³¹.

De Paepe et al.²⁹ and Wang et al.³² showed that both TNF- α and IL-1 are secreted in the infected rat pulps and periapical lesions. The cells appeared as soon as two days following the pulp chamber opening and their numbers increased steadily until the day 30. These findings have demonstrated the presence of IL-1 α and TNF- α secreting cell in the pulp and periapical tissues immediately following the pulp exposure, which support the assumption that the above mentioned cytokines play a role in the pathogenesis of pulp and periapical lesions.

A study by Kjeldsen et al.³³ using ELISA technique showed significant high concentration of TNF- α in crevicular fluid of patients with chronic adult periodontitis in comparison to healthy subjects. They reported that chronic periodontal infection may evoke an immune response resulting in the production of higher levels of TNF- α cytokine. Which was suggested be a marker of inflammation. TNF- α is a proinflammatory cytokine that is often over expressed in periodontitis and is responsible for alveolar bone resorption during periodontitis²⁷.

The present study revealed that TNF- α can be used as an effective test in discriminating between chronic periapical lesion and chronic periodontitis with sensitivity and specificity of 100% at cut off reading of ≥ 9.28 pg/ml. similar finding was reported by Krishnan et al.³⁴ who revealed that salivary TNF- α could serve as valuable biomarker for differentiating premalignant disease from oral squamous cell

carcinoma with an area under curve (AUC) of 0.981. On the other hand serum TNF- α showed an AUC of 0.865 at a cut-off value of 175pg/ml, with sensitivity and specificity of 97% and 83%, respectively. They concluded that salivary TNF- α to be a better medium for detecting oral squamous cell carcinoma and identification of such early changes may be useful in selecting patients for early interventional therapies.

The production of cytokines such as TNF- α appears to play a central role in the progressive migration of an inflammatory front toward the alveolar bone. This suggests that the production of cytokines at deeper levels within the gingival connective tissue leads to an inflammatory cascade in this area. Once a "critical level" of proinflammatory cytokine TNF- α production is reached, a physiologic response turned to a pathologic response. If the inflammatory front occurs predominantly in the area of attachment to cementum, the result will be loss of attachment. If it occurs near the alveolar crest, the result would be bone loss of bone. If the inflammatory front has not progressed far from the epithelium, the resulting lesion will be restricted to gingivitis¹¹. TNF- α is considered to be a major cytokine involved in the pathogenesis of periodontal disease, affecting the consequences of the tissue destruction and the erosive reaction in periodontitis³⁵.

TNF- α is produced by monocytes and macrophages in response to oral bacterial components, such as LPS. Elevated levels of TNF- α may promote the release of collagenase from human gingival fibroblasts, leading to cartilaginous collagen destruction and bone resorption. Therefore, it has been suggested that TNF- α may be a marker of inflammatory activity¹⁰. GCF was used for assessment of TGF- β 1 levels since although GCF has been shown to originate from the serum in proximal blood vessels, it is generally considered to reflect the ongoing processes in the surrounding periodontal tissues, including inflammation, turnover of connective tissue and resorption of alveolar bone³⁶. The current study revealed high significant differences in the mean of TGF-beta1 in CIP group. A caries related bacteria invade deeply into dentin and come into close proximity to the pulp. Inflammatory cells infiltrate into pulp area and consequently pulpitis develops. Many types of cytokine and adhesion molecules are responsible for the initiation and the progression of pulpitis³⁷.

In this study, highly significant elevation in the mean of TGF-beta1 was found in CPL in comparison with the level of TGF-beta1 of the

control group. This result was in agreement with those of Danin et al.³⁸ and Popović et al.³⁹ who reported that patients with periapical lesions showed significant elevation in TGF- β 1 as compared to healthy control. Cytokine network plays an important role in specific and non-specific immune responses. Many studies have established the production of cytokines in periapical lesions at the level of gene expression, tissue homogenates or cell cultures, and found that in certain circumstances the balance between proinflammatory and immunoregulatory cytokines is disrupted⁴⁰.

TGF- β and IL-10 are important immunoregulatory cytokines that are produced in the periapical lesions¹³. Because TGF- β 1 exhibits both pro-inflammatory and anti-inflammatory properties besides its ability to stimulate synthesis of ECM molecules and to inhibit the breakdown of ECM, it has been intensively evaluated in relation to all types of gingival overgrowth¹⁴.

The TGF- β 1 also inhibits the formation of osteoclasts. It is a key mediator of immune homeostasis, including responses in the pulp and periapical region⁴¹. Under normal circumstances, proinflammatory mechanisms must be strictly controlled to prevent excessive tissue destruction and prevent autoimmune processes⁴². Coordinated expression of TGF- β 1 in pulp may be important in tooth development and repair⁴³. It has been shown that TGF- β 1 as a pulp-capping medicament, enhances the reparative dentine formation in rat molars⁴⁴, and exerts dentine-specific effects inducing differentiation of odontoblast-like cells and stimulating primary odontoblasts⁴⁵.

The current research revealed significant increases in the mean of TGF-beta1 in group with CP. Similar finding was recorded by Sattari et al.⁴⁶ and Vikram et al.⁴⁷ who measured the concentration of TGF- β 1 in GCF of patients with chronic periodontitis before and after treatment and noticed that the TGF- β 1 level was reduced after surgery.

A study done by Skaleric et al.⁴⁸ revealed that low concentrations of TGF- β 1 at the beginning of the inflammatory process stimulate chemotactic recruitment and activation of neutrophils, monocytes and lymphocytes, whereas in advanced gingival inflammation, the TGF- β 1 stimulation is reversed. They consider this as a feedback control mechanism for the progression of inflammation and explain the changes in GCF TGF- β 1 levels at the various stages of periodontal health and disease. Silva et al.⁴⁹ stated that TGF- β 1 is an immunosuppressive

cytokine that stimulates wound healing and up regulation of TGF- β 1 in inflamed gingiva may counterbalance the destructive gingival inflammatory responses that are simultaneously taking place in patients with chronic marginal periodontitis which could explain the increased levels of TGF- β 1 in chronic periodontitis patients. No sex differences in the means of TGF-beta1 were noted between CP and control group. Article survey revealed no previous studies concerned with the effect of gender on the TGF- β 1 in CP group.

TNF- α as a glycoprotein with a variety of biological activity, which is considered one of the most important medium of inflammation and immune response. Studies suggest that TNF- α is a cytokine produced by monocytes/macrophages which has a proinflammatory effect, the biological effects is only relevant with periodontal gum but also directly or indirectly mediated bone tissue resorption and inhibit bone formation⁵⁰.

In this study, data for analysis of expression of TNF- α by dental cells include positive expression by inflammatory cells, macrophages, endothelial cells, and by progenitors and the formative cells (fibroblast, odontoblast, osteoblast and cementoblast) and that revealed significant elevation as in the following sequence :-

Chronic irreversible pulpitis (CIP) < control < chronic periodontitis (CP) < chronic periapical lesion (CPL).

The immunohistochemical results of Gümüş et al.⁵¹ and Liao and co-workers⁵² showed that there is almost no TNF- α expression in the normal control group, the TNF- α positive expression of periodontal tissues of rats in the periodontitis group was significantly different from that in the normal control group. The results suggest that ILs and TNF- α may be expressed through different pathways in the pathophysiology of periodontitis. These observation disagree with the current study that showed high expression of TNF- α in the control tissue and could be explained on the facts that control tissue possess many progenitor cells that expressed positivity for TNF- α immune reaction, in addition to their differentiation to many types of the dental formative cells including odontoblast (dentine formative cell), endothelial cell, fibroblast form collagen fibers), osteoblast (bone formative cell) that in turn express more significant positive TNF- α expression.

Delima et al.³⁰ revealed that secretion of the cytokines is initiated with the purpose of activating the immunological response to irritants and increasing the local

concentrations of inflammatory cells in order to prevent further colonization of bacteria within the tissues. Enhanced reaction of the host to various antigens results in bone resorption and formation of granulomatous periapical tissue, which are the typical features of periapical lesion. The work by Artese et al.⁵³ showed that there is a small fraction of TNF- α positive cells within the periapical lesion with a macrophage-like morphology. Ultrastructural analyses showed that there are some macrophages which have adjusted the extracellular secretion; therefore, these macrophages might be the main source of cytokines in the tissue. Pezelj-Ribari et al.²³ reported that both TNF- α and IL-1 are secreted in the infected rat pulps and periapical lesions. These results supported our findings which illustrated TNF- α positive cells within the inflamed pulp, periodontal tissue and periapical area.

In 2013, Hernadi and colleagues⁵⁴ revealed highly significant expression of TNF- α in the periapical lesion than in control ($P < 0.001$). This result is in contrast with present study that found significant difference in periapical lesion as compared to control. TGF- β is a proinflammatory cytokine, originating mainly from immune cells, and is of great importance in bone resorption. They stimulate the production and activity of osteoclasts and inhibit the activity of osteoblasts⁵⁵.

TGF- β 1 is a mediator of wound healing, and is one of the cytokines which regulate the local bone remodeling⁵⁶. It is produced not only by macrophages, eosinophils, and fibroblasts, but also by osteoclasts and osteoblasts⁵⁷. The present data of the immunohistochemical expressions of TGF- β 1 show positivity by inflammatory cells and the formative dental cells which include odontoblasts, fibroblasts, osteoblasts, and endothelial cells in teeth specimens of all studied groups and that revealed significant elevation as in this order: *Chronic irreversible pulpitis (CIP) < chronic periapical lesion (CPL) < chronic periodontitis (CP) < control*.

These findings may be attributed to followings:

1. It was known that TGF-beta is multifunctional cytokines with biologic effects that depend upon the type of target cells, local concentration and the interaction with other molecules^{58,59}.
2. It initiates odontoblast cytodifferentiation and a local increase in pre-dentine secretion⁶⁰. Such activities might be important during reparative processes in the dentine-pulp complex after tissue injury⁶¹.

3. TGF-beta1 could be directly involved in the regulation of the cell proliferation, migration, and extracellular matrix production in the human dental pulp and eventually in the repair processes occurring after tooth injury⁶².
4. TGF- β 1 might switch from pro- to anti-inflammatory role in order to regulate immune-inflammatory responses and may cause more destructive state as in periodontitis⁶³.
5. TGF-beta1 accelerates the repair of periapical bone loss, and was detected in periapical granulomas and cysts⁶⁴.

Xiao-ping et al.⁶⁵ concluded that positive expression of TGF- β may play an important role in the pathogenesis of human chronic periapical disease, they found significant highly positive expression of TGF- β in periapical lesions than that in the healthy control ($P < 0.01$), this result was in agreement with the results of present study which demonstrated in table 4.

Results of present study were in line with that of Hernadi et al.⁵⁴ who found that TGF- β 1 expression was five times higher in periapical lesions than in controls ($P < 0.05$). Piattelli et al.⁶⁴ revealed higher expression of TGF-beta 1 in the odontoblastic-subodontoblastic layer of the irreversible pulpitis specimens; this difference was statistically significant ($P = 0.0002$) and they concluded that the higher and statistically significant expression of TGF-beta 1 found in the odontoblastic-subodontoblastic layer of irreversible pulpitis specimens may indicate a role for TGF-beta 1 in the dentinal repair processes after pulp inflammation. This finding is similar to results obtained in current study which is demonstrated in table 4.

An improved understanding of the role of TGF- β 1 in the repair of dental tissues, either via a reparative or reactionary dentinogenesis, may lead to new avenues of approach to the knowledge and treatment of dental disease⁶⁶. Elevated levels of TGF- β 1 expression in advanced chronic periodontitis and gingivitis could suggest that this cytokine is one of the components that contribute to the extent of inflammatory response. TGF- β 1 exerts both anti-inflammatory and proinflammatory effects on host cells during the onset and progression of periodontal disease, It is a critical mediator in resolution of inflammation and indicates ongoing wound healing and chronic inflammation during host response^{62,67}. Any or all of these TGF- β 1 dependent mechanisms could contribute both to the initiation and

regulation of inflammation and connective tissue destruction in periodontal diseases. These results are consistent with our study findings.

Results obtained from immunohistochemical reactions of the present study have shown an increment in TGF- β 1 expression from normal healthy to chronic periodontitis, these findings are in agreement with other studies that showed highly significant elevation in CP group as compared to those of the control group^(66,68). In 2013, Ali and Al-Rubaie⁶⁹ was evaluated gingival tissue TGF- β 1 in patients with chronic periodontitis and healthy subjects and found significantly elevated TGF- β 1 expression in patients with chronic periodontitis compared to healthy control ($P < 0.001$). Other study showed reverse the results as the expression levels of TGF- β 1 and IL-10 were significantly decreased ($P < 0.01$) in the periodontal tissues with inflammatory cell infiltration, and in deep periodontal pockets⁷⁰. The present findings for immune reaction of the above mentioned markers in different dental diseases highlight their importance and focused on their roles in the disease prognosis and that their control or their modification may aid in the treatment design of the dental diseases in future.

CONCLUSION

1. High significant elevation ($P < 0.001$) in tumor necrosis factor- α and transforming growth factor- β were recorded in all patients groups as compared to normal control and the sequence of rise in the aforementioned markers in different dental diseases run in the following manner: Chronic periodontitis > periapical lesion > chronic

irreversible pulpitis > control. These elevations make suggested that markers make concluded these markers play a vital role in the inflammatory process, and tooth resorption in CIP, CPL and CP and they may be involved in the pathogenesis of these dental diseases.

2. Males with CPL and CP have higher concentration of TNF- α and TGF- β in GCF as compared to male of the control group with insignificant gender effect on the level of each of the studied biomarkers.
3. Immunohistochemical expression of TGF- β 1 in tooth specimens showed high significant elevation ($P < 0.001$) in chronic irreversible pulpitis and periapical lesion groups, while insignificant differences were reported for chronic periodontitis group in comparison with control group. The TGF- β 1 and TNF- α depicted the highest correlation of the biochemical and immunohistological expression in only CP group.

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Table 1: The general characteristics of the studied participants

	Controls n=20	Chronic irreversible pulpitis n=20	Chronic Periapical lesion n=20	Chronic periodontitis n=20
Females (No.) %	(10) 50%	(9) 45%	(9) 45%	(10) 50%
Males (No.) %	(10) 50%	(11) 55%	(11) 55%	(10) 50%
Age (year)	39.25 \pm 6.3	39.05 \pm 7.6	38.7 \pm 8.2	39.1 \pm 7.9 ^{NS}
Random blood glucose (RBG)(mg/dl)	109.3 \pm 3.3	121.4 \pm 2.5	118.4 \pm 3.2	117.8 \pm 2.5 ^{NS}
Systolic blood pressure (SBP) (mmHg)/hr	120.8 \pm 0.5	120.6 \pm 0.5	121 \pm 0.6	120.9 \pm 0.5 ^{NS}
Diastolic blood pressure (DBP) (mmHg)/hr	80.8 \pm 0.6	80.5 \pm 0.5	81.5 \pm 0.5	80.4 \pm 0.6 ^{NS}

^{NS} Not significant

Table 2: Differences in GCF concentration of TNF- α and TGF- β 1 between chronic irreversible pulpitis (CIP), chronic periapical lesion (CPL) and chronic periodontitis (CP) group

	Studied groups			
	Controls	CIP	CPL	CP
TNF-α (pg/ml)				
Male (No) mean \pm SEM	(10) 20.99 \pm 2.74	(11) 36.95 \pm 2.1	(11) 740 \pm 37.31 <i>b***, c***</i>	(10) 917.7 \pm 21.35 <i>b***, c***, d***</i>
Female (No) mean \pm SEM	(10) 2.71 \pm 2.07 ^{NS}	(9) 34.66 \pm 2.60 ^{NS}	(9) 801 \pm 27.94 <i>b***, c***</i>	(10) 903 \pm 35.22 <i>b***, c***, d***</i>
TGF-β (pg/ml)				
Male (No) mean \pm SEM	(10) 40.42 \pm 4.86	(11) 97.07 \pm 7.71	(11) 1012 \pm 46.71 <i>b***, c***</i>	(10) 1515 \pm 109.5 <i>b***, c***</i>
Female (No) mean \pm SEM	(10) 45.36 \pm 4.82 ^{NS}	(9) 88.19 \pm 13.76 ^{NS}	(9) 960 \pm 78.09 <i>b***, c***</i>	(10) 1377 \pm 85.90 <i>b***, c***</i>

^aANOVA test: CIP, CPL, and CP vs Control group: ***p<0.001

^bANOVA test: CPL and CP vs CIP: ***p<0.001

^cANOVA test: CP vs CPL: ***p<0.001.

Table 3: Effects of the gender on the mean \pm SEM of TNF- α and TGF- β 1 concentration among chronic irreversible pulpitis (CIP), chronic periapical lesion (CPL) and chronic periodontitis (CP)

Descriptive Statistics	Studied groups			
	Controls	CIP	CPL	CP
Mean \pm SEM TNF- α (pg/ml)	20.5 \pm 1.88	35.9 \pm 1.65 ^{a***}	768 \pm 24 ^{a***, b***}	911 \pm 20 <i>a***, b***, c***</i>
Mean \pm SEM TGF- β 1 (pg/ml)	42.9 \pm 3.38	93.1 \pm 7.36 ^{a***}	989 \pm 42.7 ^{a***, b***}	1446 \pm 69.6 ^{a***, b***, c***}

^aANOVA test: Within Controls, CIP, CPL, and CP male vs. female mean values: NS: not significant.

^bANOVA test: Between the male or female biomarker mean values in the CIP, CPL and CP in comparison with the respective sex values of the controls group: ***p<0.001.

^cANOVA test: Between the male or female mean biomarker values of CPL or CP versus CIP group: ***p<0.001.

^dANOVA test: Between the male or female mean biomarker values of CP vs CPL group: ***p<0.001.

Table 4: Mean (\pm SEM) expression of TNF- α and TGF- β 1 in tooth tissue among studied group.

Table 4:

Descriptive statistics	Studied groups			
	control	CIP	CPL	CP
Mean \pm SEM of all positive cell (cell/mm ²) for TNF- α	13.3 \pm 0.5	18.8 \pm 0.5 <i>a***</i> \blacktriangle	7.8 \pm 0.5 <i>a***</i> \blacktriangledown , <i>b***</i> \blacktriangledown	9.8 \pm 0.7 ^{a*} \blacktriangledown , <i>b***</i> \blacktriangledown
Mean \pm SEM of all positive cell (cell/mm ²) for TGF- β 1	6.20 \pm 0.3	23.2 \pm 1.3 ^{a***} \blacktriangle	15.5 \pm 1.1 <i>a***</i> \blacktriangle , <i>b*</i> \blacktriangledown	9.00 \pm 0.7 ^{a, NS} <i>b***</i> \blacktriangledown , <i>c***</i> \blacktriangledown

^aANOVA test: CIP, CPL, and CP vs Controls: ***P<0.001

^bANOVA test: CP and CPL vs CIP: *P<0.05, ***P<0.001

^cANOVA test: CP vs CPL: ***P<0.001.

Table 5: The distribution of the interrelationship of the immunohistochemical and biochemistry results of the biomarkers in different study groups

Markers	Groups	Technique	Below median	Above median	C.C. P-value
TNF- α	Control	Immunohistochemical	16	2	CC= 0.385 **P=0.010
			61.5%	16.7%	
		Biochemistry	10	10	
			38.5%	83.3%	
	CIP	Immunohistochemical	9	9	CC= 0.000 P=1.000 NS
			47.4%	47.4%	
		Biochemistry	10	10	
			52.6%	52.6%	
	CPL	Immunohistochemical	12	6	CC= 0.166 P=0.299 NS
			54.5%	37.5%	
		Biochemistry	10	10	
			45.5%	62.5%	
CP	Immunohistochemical	16	2	CC= 0.385 **P=0.010	
		61.5%	16.7%		
	Biochemistry	10	10		
		38.5%	83.3%		
TGF-B1	Control	Immunohistochemical	17	1	CC= 0.440 **P=0.002
			63.0%	9.1%	
		Biochemistry	10	10	
			37.0%	90.9%	
	CIP	Immunohistochemical	6	12	CC= 0.166 P=0.299 NS
			37.5%	54.5%	
		Biochemistry	10	10	
			62.5%	45.5%	
	CPL	Immunohistochemical	11	7	CC= 0.111 P=0.492 NS
			52.4%	41.2%	
		Biochemistry	10	10	
			47.6%	58.8%	
CP	Immunohistochemical	16	2	CC= 0.385 *P=0.010	
		61.5%	16.7%		
	Biochemistry	10	10		
		38.5%	83.3%		

Contingency Coefficient (CC) test: ***P < 0.001, **P < 0.01, *P < 0.05, NS: Not significant.

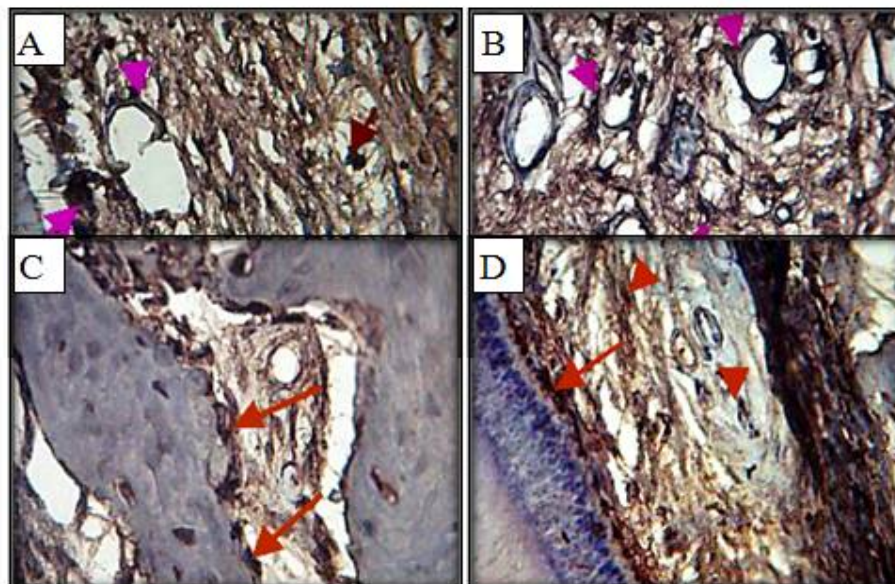


Fig. 1: Immunohistochemical view for TNF- α positive reaction in (A) pulp of healthy sound tooth by odontoblasts (arrow), endothelial cells (arrow head), progenitor cell (red arrows), (B) chronic irreversible pulpitis by fibroblasts (arrows), endothelial cells (arrow heads) in pulp core zone, (C) chronic periapical lesion by osteoblast cells (arrows), (D) chronic periodontitis by cementoblasts (arrow), fibroblasts (arrow heads).DAB stain x40.

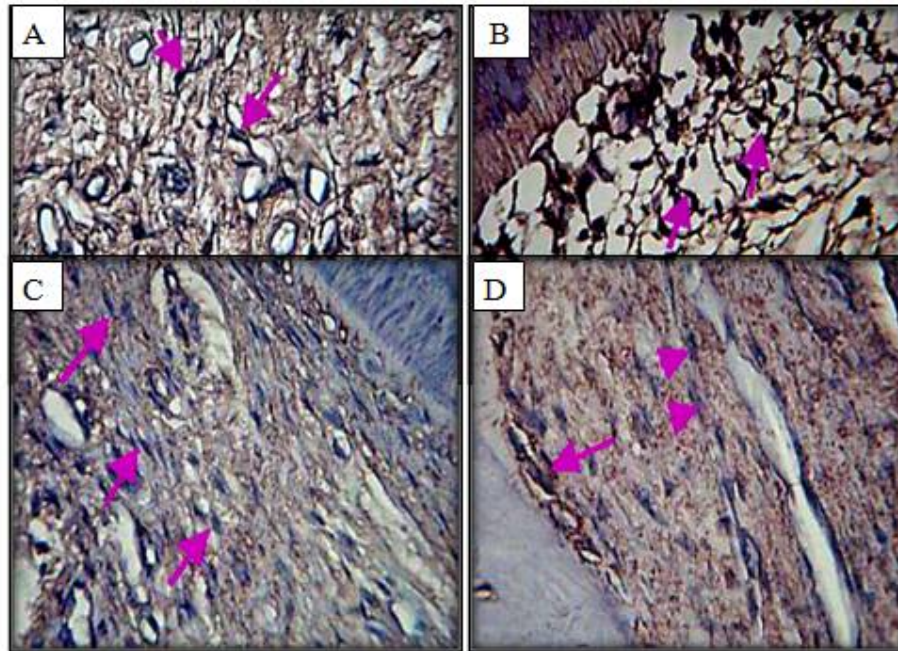
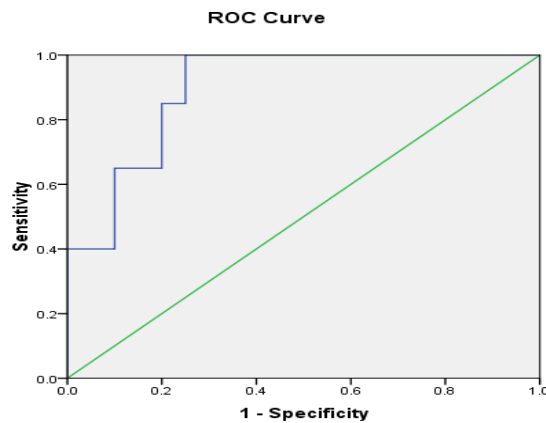
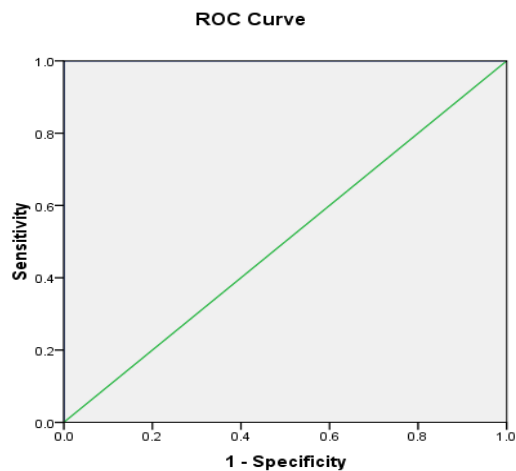


Fig. 2: Immunohistochemical view for TGF-β1 positive reaction in (A) pulp of healthy sound tooth by fibroblasts (arrows), (B) chronic irreversible pulpitis by inflammatory cell (arrow), (C) chronic periapical lesion by fibroblast (arrows), (D) chronic periodontitis by osteoblasts (arrow), fibroblasts (arrow heads).DAB stain x40.



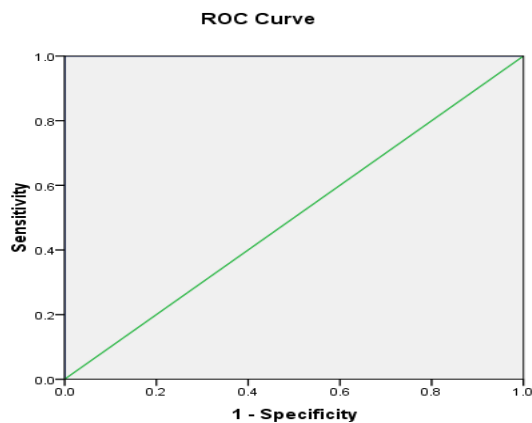
study group	Cut off value	Specificity	Sensitivity	Area under the Curve
TNF-α in CIP	28.06	80%	85%	0.89

Fig. 3: The receiver operating characteristic curve (ROC) for TNF-α in chronic irreversible pulpitis (CIP) showing the cutoff value, sensitivity, specificity, and area under curve



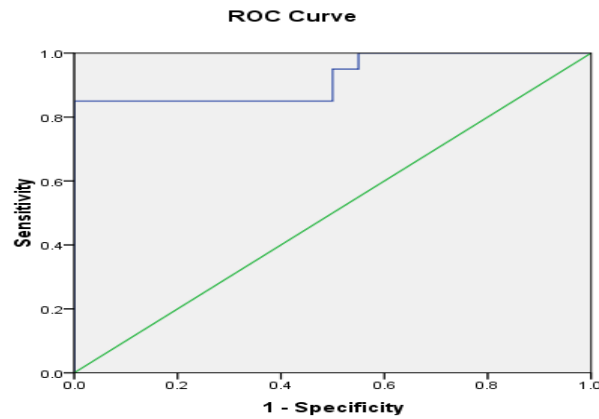
study group	Cut off value	Specificity	Sensitivity	Area under the Curve
TNF- α in CPL	9.28	100%	100%	1

Fig. 4: The receiver operating characteristic curve (ROC) for TNF- α in chronic periapical lesion (CPL) showing the cutoff value, sensitivity, specificity, and area under curve



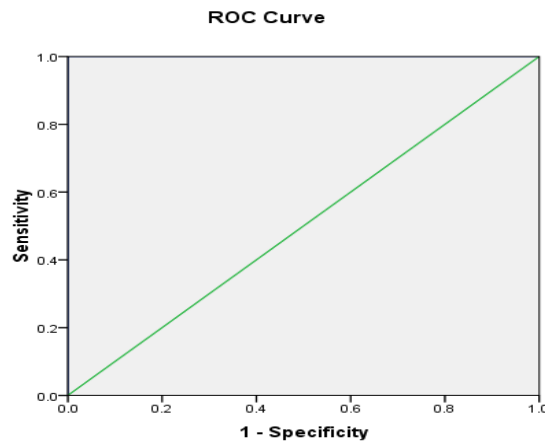
study group	Cut off value	Specificity	Sensitivity	Area under the Curve
TNF- α in CP	9.28	100%	100%	1

Fig. 5: The receiver operating characteristic curve (ROC) for TNF- α in chronic periodontitis (CP) showing the cutoff value, sensitivity, specificity, and area under curve



study group	Cut off value	Specificity	Sensitivity	Area under the Curve
TGF-β1 in CIP	39.53	50%	95%	0.92

Fig. 6: The receiver operating characteristic curve (ROC) for TGF-β1 in chronic irreversible pulpitis (CIP) showing the cutoff value, sensitivity, specificity, and area under curve



study group	Cut off value	Specificity	Sensitivity	Area under the Curve
TGF-β1 in CPL	19.23	100%	100%	1

Fig. 7: The receiver operating characteristic curve (ROC) for TGF-β1 in chronic periapical lesion (CPL) showing the cutoff value, sensitivity, specificity, and area under curve

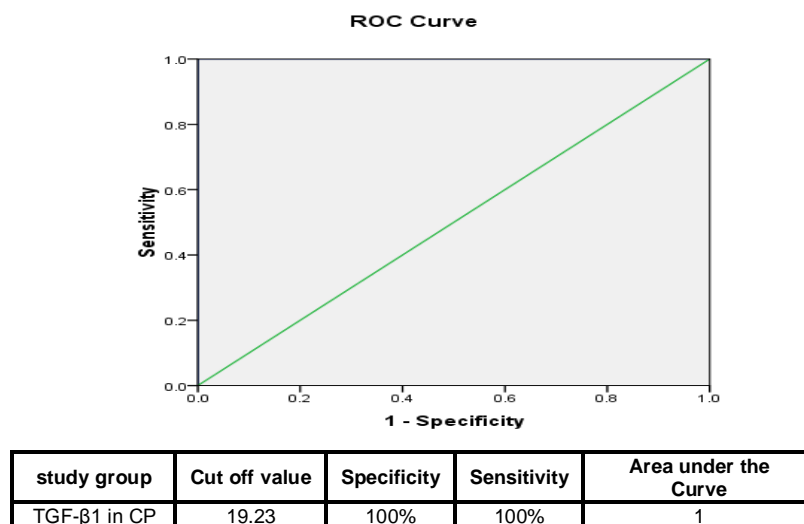


Fig. 8: The receiver operating characteristic curve (ROC) for TGF-β1 in chronic periodontitis (CP) showing the cutoff value, sensitivity, specificity, and area under curve

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