Kuwait University

Immunological Analysis of Dental Pulp Inflammation

Submitted by:

Mohamed Maged Elsalhy

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Supervised by:
Prof. Raj Raghupathy
Dr. Kefah Barrieshi (Co-Supervisor)

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College of Graduate Studies

Signatory Page
(Thesis Examination Committee)

The undersigned certify that they have read, and recommend to the College of Graduate Studies for acceptance, a Master's thesis entitled "Immunological Analysis of Pulpal Inflammation" submitted by Mohamed Maged Elsalhy in partial fulfillment of the requirements for M. Sc degree in Medical Microbiology, Faculty of Medicine.

Signatures of Committee Members                                    Date

_________________________                                                    ___________
Prof. Ziauddin Khan, Professor (Convener)

_________________________                                                    ___________
Prof. Raj Raghupathy, Professor (Supervisor)

_________________________                                                    ___________
Prof. Abu Salim Mustafa, Professor (Member)
Abstract

Assessing the degree of inflammation in the dental pulp poses a diagnostic dilemma as it influences the decision between conservative versus invasive dental treatment. However, there are no objective, quantitative and clinically-practical methods for evaluating pulpal inflammation. Cytokines have been suggested to be markers of pulpal inflammation, but estimation of cytokine levels has been possible only after extraction of inflamed teeth. We set out to develop a method to measure cytokines from the dental pulp prior to decision making.

108 dental pulp blood samples were obtained with cotton pellets from pulp sites exposed on pulpectomy. 25 samples were from normal teeth, 40 from asymptomatic pulps with caries exposure and 43 from symptomatic pulps clinically diagnosed as irreversible pulpitis. The levels of the inflammatory cytokines IL-2, IL-6, IL-8, TNF-α and IFN-γ and the anti-inflammatory cytokine IL-10 were quantified using high-sensitivity ELISA. Levels of cytokines and ratios of inflammatory cytokines to IL-10 were compared using Kruskal–Wallis and Mann–Whitney tests.

Significantly higher levels of IL-6, IL-8, IL-10, TNF-α and IFN-γ were detected in caries-exposed and irreversible pulpitis as compared to normal teeth. IL-2 levels were higher in caries-exposed as compared to normal teeth. IL-2 and IL-10 levels were higher in caries-exposed pulps as compared to irreversible pulpitis, while IL-8 was higher in irreversible pulpitis as compared to caries-exposed teeth. Most interestingly, IL-6/IL-10 and IL-8/IL-10 ratios were significantly higher in irreversible pulpitis compared to both caries-exposed and normal teeth.
IL-8 levels and the IL-8/IL-10 ratio promise to be good indicators of irreversible pulpitis. An important and potentially very useful outcome of this study is the demonstration that cytokine estimation in pulpal blood may help in the diagnosis of pulpal inflammation.
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Chapter 1

Introduction

1.1. Anatomy of the tooth

The tooth can be divided into the crown and the root. The crown is the portion covered by enamel while the root is the portion covered by cementum and lies within the alveolar bone. From inside out, the tooth has a pulp surrounded by dentin. Dentin is covered by enamel in the crown and cementum in the root. Enamel, dentin and cementum are mineralized tissue components of teeth (Berkovitz et al., 2009). Dentin is a hard tissue with dentinal tubules penetrating throughout the entire thickness. The dental pulp is a heterogeneous soft tissue located in the center of teeth, which contains a variety of cell types and extracellular matrix (ECM) molecules. Both dentin and the pulp are derived from neural crest cells (Zhang & Yelick, 2010).

1.2. Caries and its treatment options

Caries is defined as progressive destruction of tooth structure by acids produced by bacteria. Treatment of caries depends on its extension into the tooth structures.

1.2.1. Enamel caries

When caries is only in the enamel, fluoride or fissure sealants can be applied to prevent further progression of caries and it should be closely observed (Griffin et al., 2008).

1.2.2. Dentin caries not reaching the pulp

If caries reaches the dentin, the defective and infected dentin has to be removed and the tooth should be restored with a restorative material (Ricketts & Pitts, 2009).
1.2.3. **Dentin caries reaching the pulp**

When the patient has no symptoms and caries is thought to extend close to, or into the pulp, excavation of the pulpal caries can be stopped at stained but firm dentin. A calcium hydroxide lining is applied over the pulpal dentin followed by glass ionomer restoration. This is classically referred to as the indirect pulp cap. Later caries removal is repeated after 6 to 12 months. The difficulty with this technique is knowing how rapid the carious process has been, how much reactive dentin will be formed and knowing exactly when to stop excavating to avoid pulp exposure. This technique has been evolved to avoid pulp exposure and direct pulp capping (Ricketts, 2001).

A direct pulp cap usually involves the placement of a calcium hydroxide preparation directly in contact with an exposed pulp (Ricketts, 2001). Teeth exposed during caries removal will inevitably have some degree of inflammation although the histological extent of this cannot be accurately predicted from a clinical examination. Direct pulp capping with calcium hydroxide has questionable long term prognosis as the failure rate is high (Barthel et al., 2000; Al-Hiyasat et al., 2006).

In the other hand, when the patient has severe symptoms of pulpal inflammation, pulp is opened and pulpal tissue is removed. The tooth then undergoes root canal treatment and may need an extensive restorative treatment (Torabinejad & Walton, 2009).
1.2.4. Extensively carious teeth

Teeth with extensive caries that affect their restorability have poor prognosis and extraction could be the treatment of choice (Torabinejad & Walton, 2009).

1.3. The Biology and Histology of Dental Pulp

In the healthy adult tooth, odontoblasts and cells of the sub-odontoblastic (Höehl's) layer form a thin border located between the inner margin of the dentin and the outer limit of the pulp. These post-mitotic polarized cells are responsible for the production of dentine. Primary dentine is the dentin formed before tooth eruption while secondary dentine is formed after eruption when the tooth becomes functional. Although decreasing in number and activities, odontoblasts produce dentin extracellular matrix molecules continuously as long the tooth is alive and a gradual thickening of dentin takes place over time (Zhang & Yelick, 2010).

By contrast, in the dental pulp, fibroblasts are renewed constantly (Baume, 1980). In the fully mature and even in aging teeth, the pulp remains a soft tissue. However, aging influences pulp mineralization, and in many cases pulp stones or areas of diffuse mineralization develop gradually. The reduced mineralization potential of dental pulp fibroblasts underlines the fact that they are distinctly different entities from odontoblast/sub-odontoblastic cells, even if they share a common embryological origin (Goldberg et al., 2008).

It is now well established that the normal dental pulp contains heterogeneous cell populations including a majority of fibroblast-like cells, but also inflammatory and
immune cells, and latent or dormant pulp stem cells (progenitors), mostly involved in self-renewal. After damage of the tooth, the progenitors may contribute to pulp repair and mineralization. Nerves, vascular and perivascular cells are also present within the pulp (Goldberg & Smith, 2004).

Immune cells are present both in the normal and pathologic pulp. In the normal pulp, they regulate cell density, and are crucial in the control of cell proliferation and apoptosis (Vermelin et al., 1996; Nishikawa et al., 1999). In the exposed pulp, they may contribute to resolve inflammatory processes. Several immune cell types have been identified, specifically, lymphocytes, macrophages, and neutrophils (Trowbridge, 1990; Stashenko, 1990). CD4\(^+\) T helper, CD8\(^+\) T suppressor/cytotoxic cells and macrophages were detected in normal pulps by Jontell et al (1998). These investigators extracted human teeth with vital normal pulps under local anesthesia, sectioned the pulp tissue and subjected them to indirect immunohistochemistry with monoclonal anti-CD4 and anti-CD8 antibodies as well as to macrophage markers. T helper, T suppressor/cytotoxic cells, macrophages and dendritic cells (DC) were observed; however, no B cells were detected in any of the samples. These immune cells are motile. They migrate independently in contrast to pulp fibroblasts that are closely associated through junctional complexes, and therefore presumably translocate as a whole from the central to the outer part of the pulp, as a syncytial structure (Jontell et al., 1987; Jontell et al., 1998; Nishikawa et al., 1999).

Hahn et al. (1989) demonstrated an increase in the ratio of CD4\(^+\)/CD8\(^+\) lymphocytes in irreversibly inflamed pulps compared to normal pulps. CD8\(^+\) T
lymphocytes were predominant in normal pulps, while the levels of CD4$^+$ T lymphocytes relative to CD8$^+$ T lymphocytes were increased in the irreversible group. Kettering and Torabinejad (1993) detected the presence of natural killer (NK) cells in human chronic periapical lesions.

In response to a slow carious decay, pathological abrasion or to superficial tooth preparation by a dentist, the odontoblasts that are still alive have the capacity to produce a reactionary dentin, more or less similar to the physiological dentin (Lesot et al., 1993). In case of an invasive carious lesion, the odontoblasts are destroyed by bacterial toxins or altered by noxious molecules released by the restorative material, by necrotic cells or by enzymes released by the degradation of the ECM. The cells located in the subodontoblastic layer may in that case ultimately differentiate and replace the wounded cells in producing a layer of reactionary/reparative dentin at the site (Goldberg et al., 2008).

If the pulp is exposed, odontoblasts and Höehl cells can no longer perform repair of the lesion and another process takes place. Stem cells or progenitors located within the pulp get recruited. They proliferate and differentiate into osteoblast-like or odontoblast-like cells (Six et al., 2004) and start to produce an ECM, which will ultimately undergo mineralization. This cascade of events leads to the elaboration of a reparative dentin (Goldberg & Smith, 2004), in the form of a thin dentinal bridge occluding the exposure site, or a bone-like structure (osteodentin) filling the pulp partially or totally (Six et al., 2004).
Autoradiographic data obtained using tritiated thymidine after pulp capping with calcium hydroxide in monkeys have shown that a first replication is observed in the central part of the pulp, and later a second cell division occurs in the outer part of the pulp, near the site of pulp exposure (Fitzgerald et al., 1990). The distribution of labeled cells indicates that cells involved in the reparative process divide at least twice, and that progenitors initially located in the central pulp migrate toward the exposure site. This further suggests that a continuous influx of differentiating cells that contribute to the formation of a dentinal bridge, excluding odontoblasts and sub-odontoblastic cells in the formation of reparative dentin. It seems that these reparative cells originate within the pulp and only a subpopulation of the pulp cells are capable of being committed and to be involved in the repair process. In addition, the identification of stem cells within the dental pulp has led to the hypothesis that the reparative cell progenitors are stem cells, but this has not been clearly demonstrated (Granthos et al., 2002; Goldberg et al., 2008).

Progenitors or adult stem cells exist as dormant or latent cells, and are scarce in the sound pulp (Granthos et al., 2002). During the inflammatory process there is a general increase in the number of pulp cells, but it is not known if this increase is related to fibroblast proliferation, or represent a massive migration of inflammatory cells, or to the proliferation of stem cells (Goldberg et al., 2008).

A link between inflammatory molecules and the regulation of pulp cell population has been suggested previously by studies on the effects of essential fatty acid deficiency (EFAD) (Vermelin et al., 1995). When rats were fed with an EFAD diet, the cell density was increased two-fold and three-fold within the central and outer pulp
respectively, a time-dependent effect related to the period of EFAD diet. In these studies, the increased pulp cell population might result from a decrease or arrest of apoptotic events that normally occur (Vermelin et al., 1996; Nishikawa et al., 1999). In contrast, this diet did not modify either the number or shape of the odontoblasts. As the essential fatty acids are transformed into prostaglandins and leukotrienes, it is possible that these mediators play roles in the balance regulating cell death and cell renewal, as is the case for other cells and tissues.

For years, inflammation in the tooth has been considered mostly as a negative factor leading to pulp destruction by necrosis or apoptosis. However, some data on the reaction to carious lesions and implantation of biomolecules suggest that the inflammatory reaction might be a prerequisite for the burst of progenitors implicated in repair of the pulp (Goldberg et al., 2008).

1.4. Regulation of dental pulp repair by immune cells in carious teeth

The role of odontoblasts and pulp fibroblasts in the regulation of the dental pulp immune and inflammatory responses to cariogenic bacteria suggest that interactions between immune/inflammatory cells and odontoblasts and their precursors influence the process of pulp repair (Goldberg & Smith, 2004).

The progression of bacteria from the outermost enamel to the pulp–dentin interface triggers inflammatory and immune events in the underlying dental pulp through the diffusion of bacterial by-products into dentin tubules. These events may be prevented when reactionary/reparative dentin is formed by odontoblasts at the pulp–
dentin interface, eliminating the bacteria and blocking the route of infection. In the absence of odontoblast reaction, or in case of odontoblast death, bacterial invasion leads to irreversible pulpitis, pulp necrosis, infection of the root canal system and periapical disease (Love & Jenkinson, 2002; Heyeraas & Mjör, 2001).

In the dental pulp, when dentin is being destroyed by caries, immature antigen-presenting dendritic cells (APC) rapidly migrate to the odontoblast layer facing the lesion in a strategic location near the foreign antigens (Yoshiba et al., 1996). Then, a progressive and sequential accumulation of T-lymphocytes, macrophages, neutrophils and B-lymphocytes occurs in the pulp, concomitant with the deepening of the dentin caries lesion, the increase of the bacterial insult and the development of the pulp inflammatory process (Hahn & Liewehr, 2007b; Jontell et al., 1998). So, a close relationship exists between immune cells, especially DCs, that are involved in the initial steps of the immune response in the pulp, and odontoblasts in order to regulate odontoblast dentinogenic activities (Farges et al., 2003).

The rapid accumulation of DCs into the odontoblast layer during the early phase of pulp repair suggests that odontoblast-derived chemotactic molecules might be responsible for the recruitment of DCs that ensure immunosurveillance in the pulp tissue and/or patrolling in pulp blood vessels (Farges et al., 2003). This hypothesis was confirmed by in vitro data that demonstrated the role of odontoblasts in triggering immune/inflammatory events in response to specific bacterial components (Durand et al., 2006; Veerrayutthwilai et al., 2007; Staquet et al., 2008). In addition, odontoblasts
were also found to be more potent attractants of dendritic cells than pulp fibroblasts (Staquet et al., 2008).

### 1.5. Clinical classification of pulpal status

#### 1.5.1. Healthy pulp

Healthy pulp is a vital pulp, without inflammation. It is asymptomatic and reacts to vitality tests such as heat, ice and/or electric pulp tester (EPT). With increasing amount of secondary dentin as the pulp ages, its reaction to thermal test might be decreased (Torabinejad & Walton, 2009). However, a healthy pulp should predictably react to EPT (Fulling & Andreasen, 1976).

#### 1.5.2. Reversible pulpitis

A diagnosis of reversible pulpitis implies that the pulp is vital, but has some local area/s of inflamed tissue that will heal after conservative vital pulp therapy; i.e. removing caries and applying a restoration. Symptoms can be very misleading in this diagnostic category, from none to very intense and sharp sensation associated with thermal stimuli. It is well established that there is a poor correlation between clinical symptomatology and the histopathological state of the pulp (Seltzer et al., 1963; Lundy & Stanley, 1969; Baume, 1970; Dummer et al., 1980). The history of symptoms will most often reveal pain or sensation only on stimulation, such that the tooth will bother the patient only when the tooth is exposed to a stimulus that is hot and/or cold (Asgeir, 2003; Torabinejad & Walton, 2009).
According to the classification, reversible pulpitis should heal once the irritant is removed or, in case of an exposed dentin surface, once the exposed dentin is adequately sealed. However, there is a much higher risk of diagnosing a pulp with mild symptoms as being reversibly inflamed, when in actuality the pulp is irreversibly inflamed. Thus, mistakes in diagnosis of this pulpal category are common and understandable and it is essential to recall and test all patients who have had treatment based on this diagnostic category in order to confirm that the progression of pulpal reaction has gone according to expectation, i.e. that the pulp has healed (Asgeir, 2003).

1.5.3. Irreversible pulpitis

In irreversible pulpitis, the pulp is still vital but is severely inflamed so that healing is an unlikely outcome with conservative pulp therapy. Thus, pulp necrosis and infection is the predicted outcome if vital pulp therapy is attempted. Apical periodontitis will be the final outcome (Asgeir, 2003). In order to avoid pulp necrosis, the pulp is aseptically removed and the entire space filled with a root canal filling material (Torabinejad & Walton, 2009). As with reversible pulpitis, symptoms can be very misleading. It has been well documented that in most cases a pulp that is irreversibly inflamed is asymptomatic. It has been reported that dental pulps can progress from vitality to necrosis without pain in 26–60% of all cases (Bender, 2000).

According to several studies, neither gender nor tooth type appears to matter in case of asymptomatic pulpitis; however, the older the patient is, the less likely is there pain associated with the pulpitis (Hasler & Mitchell, 1970; Michaelson & Holland, 2002).
If the pulp is symptomatic it is most often very sensitive to thermal changes, and the pain sensation has a tendency to linger as a dull ache after the stimulus has been removed. This fact can be used with caution to predict if the pulp is likely to be irreversibly inflamed or not. Therefore, the more dull, throbbing, poorly localized, lingering pain is experienced after the stimulus has been removed, the more severe the inflammation is likely to be and thus the more likely it is to be irreversible in nature (Asgeir, 2003).

It has also been shown that the more severe the pain and the longer it has been symptomatic, the more likely it is to be irreversibly inflamed (Bender, 2000). Perhaps the clearest sign of irreversible inflamed pulp is the history of spontaneous pain, which will ‘hit’ the patient without any thermal stimulation to the teeth, and even wake the patient from sound sleep (Seltzer et al., 1963).

Also included in this category are teeth with carious exposures (Torabinejad & Walton, 2009). Capping of caries-exposed teeth has showed long term failure (Baume & Holz, 1981; Matsuo et al., 1996; Barthel et al., 2000; Al-Hiyasat et al., 2006) and this why it is considered irreversible pulpitis. However, this was not very conclusive. The reluctance to place a direct pulp cap on an exposure in a carious field is based on unpredictable outcomes using traditional materials and treatment protocols. Changes in the materials may change the outcomes.

Moreover, when bacterial byproducts induce pulpal inflammation, compromise immune responses and impede cellular differentiation and recruitment, normal pulpal
repair mechanisms may not function properly. Success rates with direct pulp capping in a carious field vary depending on the technique and materials. Most of the studies with low success rates used calcium hydroxide as pulp-capping materials (Matsuo et al., 1996; Barthel et al., 2000; Al-Hiyasat et al., 2006). Calcium hydroxide does not provide close adaptation to dentin, does not promote consistent odontoblast differentiation and has been shown to be cytotoxic in cell cultures. Also, the resultant reparative dentin is characterized by tunnel defects. Tunnel defects within dentin bridges may provide a pathway for the penetration of microorganisms to activate circulating immune cells, induce pulpal irritation and produce subsequent dystrophic changes (Cox et al., 1996; Andelin et al., 2003).

However, the introduction of mineral trioxide aggregate (MTA) as a capping material is showing promising results. Over an observation period of nine years, Bogen et al. (2008) followed MTA-capped teeth and found that 98% had favorable outcomes on the basis of radiographic appearance, subjective symptoms and cold testing. MTA can be a reliable pulp-capping material on direct carious exposures in permanent teeth. This would change carious-exposed teeth from the irreversible pulpitis category to reversible pulpitis. However, a critical requirement for this is the development of methods that can differentiate between the two categories before capping (Bogen et al., 2008).

Clinically, pulp repair after capping occurs when bacterial contamination is limited and inflammation is mild. However, information gained by examining the
patients before pulp capping is very difficult to assess and does not reflect the actual pulpal status.

1.5.4. Necrotic pulp

The diagnostic category of necrotic pulp implies partial or total necrosis of pulpal tissue. If the pulp is completely necrotic in a tooth with undeveloped root, it is now possible in some cases to disinfect the canal space and stimulate the root to continue formation (Iwaya et al., 2001). In case of a fully formed tooth, root canal therapy is always indicated for both partially and fully necrotic pulp. If the pulpal space is not already infected, it will in most cases become infected in time if left untreated. Prevention of formation of periapical lesion has been shown to have a much more reliable outcome than a treatment on a tooth with a periapical lesion (Sjogren et al., 1990).

1.6. Cytokines and the dental pulp

Cytokines are polypeptides secreted by leukocytes and other cells that act principally on hematopoietic cells, and whose effects include modulation of immune and inflammatory responses. They are pleiotropic in their effects and can have multiple target cells and consequently multiple actions. Structurally dissimilar cytokines can have overlapping but typically non-identical actions, a given effect often being mediated by several different cytokines. Another characteristic of cytokines is that a single cytokine frequently induces or influences the action of other cytokines, and can function synergistically. Cytokines can be classified according to their immune response into Th1 and Th2 cytokines. Th1 cytokines are those involved in cell mediated immunity.
like IFN-γ, IL-2 and TNF-α. TH2 cytokines are those involved in antibody mediated immune response like IL-4, IL-5, IL-6 and IL-10. Another classification is by their inflammatory/anti-inflammatory function. IL-2, IL-6, IL-8, IFN-γ and TNF-α are inflammatory cytokines while IL-4, IL-10 and IL-13 are anti-inflammatory cytokines. As the names indicate, inflammatory cytokines increase inflammation while anti-inflammatory cytokines suppress inflammation. Levels of these cytokines can be used as a reflection of the degree of inflammation.

### 1.6.1. Interleukin-2 (IL-2)

The best known action of IL-2 is to augment the proliferation of T lymphocytes in response to antigenic stimulation, including the generation of both cytotoxic and suppressor T cells (Dooms et al., 2004). Thus, IL-2 controls the 'amplification' phase of the T-cell immune response. IL-2 exerts effects on cellular metabolism and glycolysis that are necessary for long term survival of T cells. It is also important for the differentiation of CD4⁺ T cells into Th1 and Th2 effector subsets (Gaffen & Liu, 2004) and for the development of memory T cells (Williams et al., 2006).

In addition to its function as a T-cell growth and survival factor, IL-2 plays a key role in Fas-mediated activation-induced cell death of CD4⁺ T cells in response to antigen re-stimulation. This is critical in peripheral tolerance for the elimination of auto-reactive T cells (Gaffen & Liu, 2004). IL-2 also promotes the growth and differentiation of mitogen- or antigen-activated B cells in vitro (Mingari et al., 1984).
IL-2 also augments the cytolytic activity of natural killer cells, induces lymphokine activated killer cell activity (Dooms et al., 2004) and increases the proliferation of large granular lymphocytes (London et al., 1986). The actions of IL-2 on monocytes include the stimulation of cytotoxic activity against tumor targets and induction of IL-1 β and IL-6 mRNA (Saraya & Balkwill, 1993; Musso et al., 1995).

These activities emphasize the importance of IL-2 in the immune response particularly in priming the immune response in pulpal inflammation. Significant differences in detectable IL-2 levels in normal and inflamed pulps were reported by Rauschenberger et al. (1997) in their study consisting of 40 human pulp samples; they postulated that presence of elevated levels of IL-2 could be used as a marker to confirm the presence of inflammation in human pulp tissue and be used in clinical diagnostic procedures.

However, in another study by Anderson et al. (2002), no significant difference between the concentrations of IL-2 was observed in any of the 80 pulp samples studied. Thus significant discrepancy exists in the context of IL-2 and further investigation is warranted to determine if a correlation exists between the concentration of IL-2 and the degree of inflammation in the dental pulp.

1.6.2. Interleukin-6 (IL-6)

IL-6 is a multifunctional cytokine with biological activities including regulation of immune response, inflammation, and hematopoiesis (Kishimoto, 2005). IL-6 is responsible for multiple inflammatory manifestations (Kishimoto, 2005; Mima &
Nishimoto, 2009). In vivo treatment with IL-6 induces systemic inflammatory symptoms such as fever, generalized fatigue and anorexia, as well as abnormalities in laboratory test results, including increases in levels of acute-phase proteins, C-reactive protein, serum amyloid A and fibrinogen and decreases in serum concentrations of albumin (Nishimoto, 2010).

In inflammatory tissues, IL-6 induces local infiltration of immunocompetent cells via up-regulation of adhesion molecules. It also induces angiogenesis by augmenting the production of vascular endothelial growth factor. This growth factor increases vascular permeability and leads to inflammatory edema. In the affected joints of rheumatoid arthritis (RA) patients, vascular endothelial growth factor–mediated angiogenesis is necessary for synovial pannus formation, and this causes destruction of the joint. In bone metabolism, IL-6, in the presence of soluble IL-6 receptor, induces osteoclast differentiation, resulting in the characteristic bone resorption and joint destruction seen in RA. This function of IL-6 also explains why osteoporosis is associated not only with chronic inflammation but also with postmenopausal status (Nishimoto, 2010).

In addition, excessive IL-6 signaling induces the production of suppressors of cytokine signaling molecules, intracellular negative feedback factors that inhibit the Janus kinase–signal transducer and activator of the transcription signaling pathway. Given that transduction of the erythropoietin receptor signal requires the same pathway, the action of erythropoietin can be suppressed by suppressors of cytokine signaling. Excessive production of IL-6 therefore contributes to the hypoferremic anemia of
chronic inflammation. IL-6 is a growth factor not only for malignant cells of multiple myeloma and renal cell carcinoma but also for non-tumor cells, including the mesangial cells of the kidney. This suggests that IL-6 overproduction may be involved in the pathogenesis of mesangial proliferative glomerulonephritis (Nishimoto, 2010).

Due to its important role in immune responses and inflammation, IL-6 has been investigated in the dental pulp by Barkhordar et al. (1999). Six inflamed human pulps and six human periapical lesions were compared; pulp samples from impacted third molars were used as controls. The inflamed dental pulp tissue and periapical lesions demonstrated elevated levels of IL-6 compared with normal pulp values. This would suggest that inflamed pulps and periapical tissues have elevated levels of IL-6 which is produced and released locally in inflamed pulpal and periapical lesions, especially in chronically inflamed sites of bone resorption (Barkhordar et al., 1999). This study is consistent with a strong positive correlation of increased expression of IL-6 and IL-8 in gingival fibroblasts isolated from periodontitis patients in vitro (Dongari-Bagtzoglou & Ebersole, 1998).

On the other hand, Nakanishi et al. (1995) detected IL-6 in only 2 of the 18 blood samples from inflamed pulps, and even then were present in extremely small quantities. It was not detected in the normal pulp group.

1.6.3. Interleukin-8 (IL-8)

IL-8 is made by a wide variety of cell types, including virtually all nucleated cells within the body. However, among these cells, monocytes and macrophages
typically represent the principal cellular source. IL-8 is induced by multiple stimuli including lipopolysaccharide (LPS), live bacteria, and other early proinflammatory cytokines such as tumor necrosis factor (TNF-α) and IL-1 (Remick, 2005). IL-8 is actively secreted into the extracellular space following stimulation of cells. IL-8 is relatively unique as it may be produced early in the inflammatory response but will persist for a prolonged period of time, even days and weeks. In this regard, it is in contrast to most of the other inflammatory cytokines, which are typically made and cleared during in vivo situations in a matter of a few hours (DeForge et al., 1993).

IL-8 is a chemokine and induces chemotaxis of inflammatory cells. IL-8 bears principal responsibility for recruitment of neutrophils, the signature cell of the acute inflammatory response (Webb et al., 1993). The cellular recruitment occurs through the development of the chemotactic gradient so that the inflammatory cell moves towards an area of increased chemokine concentration. In vivo, this gradient may be generated by IL-8 binding to basement membrane proteins (Gimbrone et al., 1989). The chemotactic gradient helps to both bring cells toward the local site of inflammation and also to retain them once they have arrived. In addition to recruitment, IL-8 also serves to stimulate the neutrophil to a higher state of activation (Remick, 2005).

IL-8 is important in the regulation of the acute inflammatory response. It is rapidly synthesized at local sites of inflammation where it will fulfill its function to recruit and activate acute inflammatory cells. IL-8 is a small protein that is resistant to heat and proteolysis and relatively resistant to acidic environments. These biochemical characteristics make it an ideal candidate molecule to function at a site of acute
inflammation, where it must withstand suboptimal conditions. An example of a harsh environment is the collection of pus located within an abscess. Another important component of IL-8 is its relative longevity at sites of acute inflammation. As indicated, it may be found for several days during in vivo situations to continue the recruitment of inflammatory cells to combat bacterial infections (Remick, 2005).

Interleukin-8 was induced in vitro when pulpal fibroblasts or human pulp stem cells were challenged by endodontic pathogens, substance P, LPS or TNF (Nagaoka et al., 1996; Yang et al., 2003). However, IL-8 in inflamed pulps is immunohistochemically localized in odontoblasts, lymphocytes, macrophages and endothelial cells, but not in fibroblasts. Hahn & Liewehr (2007a) suggested that IL-8 secreted by fibroblasts may not be relevant to pulpitis.

Huang et al. (1999) studied the expression of IL-8 in inflamed human dental pulp using ELISA and immunohistochemical analysis. Fourteen pulp tissue samples from teeth diagnosed with irreversible pulpitis compared with 15 freshly extracted normal teeth for IL-8 by ELISA. They found that about half of the normal pulps had barely detectable levels of IL-8; the majority of the inflamed pulps had statistically significantly higher levels of IL-8. Immunohistochemical analysis showed that diseased samples were either weakly or moderately stained, whereas normal samples were either negative or weakly stained. In addition, not all diseased samples showed inflammatory cell infiltrates.
Guo et al. (2000) investigated the level of IL-8 from normal and inflamed human dental pulp tissues. Microliters of pulpal blood from 8 normal, 26 acute pulpitis and 22 chronic pulpitis teeth were obtained using filter paper strips and IL-8 levels were measured by ELISA. No IL-8 was detected in the samples from normal pulp, but significant amounts of IL-8 were measured in inflamed pulp tissues, and the level of IL-8 in exudates of acute stage of pulpitis was higher than that of chronic stage. This study demonstrates that IL-8 is produced and accumulated in pulp inflammation and may play a role in the occurrence and development of human pulpitis.

Silva et al. (2009) investigated the location, distribution and concentration of IL-8 in healthy and inflamed dental pulps. Twenty pulps were studied, obtained from healthy third molars and from pulpectomies by immunohistochemistry and ELISA. Immunohistochemically, it was observed that inflamed pulps were strongly stained for IL-8 in inflammatory cells, while healthy pulps were not. ELISA on tissue extracts quantitatively confirmed higher levels of IL-8. It was concluded that inflamed pulps have higher amounts of IL-8 than healthy pulps. Furthermore, pulp fibroblasts stimulated with bacterial LPS produced higher levels of IL-8 than the control group.

An examination of steady-state mRNA expression of various cytokines in symptomatic and clinically-healthy human dental pulps and produced evidence supporting the notion that pulpitis is associated with the up-regulation of IL-6, IL-8 and IL-18 (Zehnder et al., 2003).
1.6.4. **Interleukin-10 (IL-10)**

IL-10 is produced by a number of different cell types, especially inflammatory cells such as T lymphocytes, and is an important inhibitor of cytokine synthesis and macrophage activity. It also inhibits the production of proinflammatory cytokines and extracellular matrix metalloproteinases (Anker & von Haehling, 2004).

IL-10 inhibits the production of several cytokines such as TNF-α, IL-1β and IL-6 in various cell types, and is also self-modulated (Anker & von Haehling, 2004). Furthermore, studies have shown that IL-10 production increases in inflammatory processes such as anemia and rheumatoid arthritis exerting a predominantly immunomodulatory role in such conditions (Yamaoka et al., 1999). It also has an immunoregulatory role in pregnancy (Szereday et al., 1997).

IL-10 has also been described by its protective, i.e. anti-inflammatory, properties in delaying the progression of disease, since high levels of IL-10 are associated with a reduction in apoptosis (Mallat et al., 1999). Also, IL-10 has protective effects in experimental endotoxemia and rescues mice from LPS-induced toxic shock (Marchant et al., 1994).

Hahn et al. (2000) studied the expression of IL-10, IL-4 and IFN-γ mRNA expression in dental pulps. The prevalence of IL-10 mRNA was significantly higher in deep-caries than in shallow-caries.
1.6.5. Tumor Necrosis Factor-α (TNF-α)

TNF-α coordinates the early response to injury and thus represents an important point of regulation in inflammatory diseases. TNF has systemic endotoxic activity leading to fever, hypotension and shock (Malik & Balkwill, 1992). Detailed investigations on its biological activities have confirmed that TNF-α is one of the most prominent inflammatory mediators and absolutely central in starting off the inflammatory reactions of the innate immune system including induction of cytokine production, activation and expression of adhesion molecules and growth stimulation (Clauss et al., 2001; Hehlgans & Pfeffer, 2005).

The blockade of TNF-α with specific antibodies is associated with a reduction in the expression of other proinflammatory cytokines, such as IL-1 and IL-6, both in vitro and in vivo (Pfeffer, 2003). TNF induces the expression of endothelial adhesion molecules and chemokines that attract inflammatory leukocytes to sites of tissue injury and stimulates leukocytes and parenchymal cells to release additional chemokines and inflammatory cytokines such as IL-1, which facilitates further local accumulation and subsequent activation of immunologic effector cells (Hehlgans & Pfeffer, 2005).

Consistent with these functions of TNF-α, the blockade of those cytokines in rheumatoid arthritis patients reduced the TNF-dependent cytokine response and diminished leukocyte recruitment to inflamed tissue (Charles et al., 1999). TNF also has important immunoregulatory properties. It is essential for the development of secondary lymphoid organ structures in lymph nodes, spleen and Peyer’s patches, and its
deficiency results in the absence of germinal centers and follicular dendritic cells (Pfeffer, 2003).

Moreover, TNF-α induces apoptotic cell death in both leukocytes and parenchymal cells. This is important for the immunoregulatory functions of TNF-α, but also may contribute substantially to organ-specific damage, as reported in some acute non-immune nephropathies (Van Herreweghe et al., 2010).

In a study by Nakanishi et al. (1995), TNF-α was detected in 10 of 20 pulpal blood samples in the inflamed pulp group. In the normal pulp group, 2 of the 6 samples contained a detectable amount of TNF-α. However, there was no significant difference between the two groups.

In another study of normal and inflamed human dental pulps, Pezelj-Ribaric et al. (2002) reported high levels of TNF-α in pulpal tissue from teeth clinically diagnosed with irreversible pulpitis, but considerably lower expression of this cytokine in samples from normal teeth.

Kokkas et al. (2007) found a significant increase of TNF-α gene expression associated with irreversible inflammation compared with healthy controls. No such difference was detected in reversibly inflamed pulp in comparison to healthy teeth. These authors concluded that gene expression of TNF-α in inflamed human dental pulp tissue is positively associated with the severity of clinical symptoms.
1.6.6. Interferon-γ (IFN-γ)

IFN-γ is the predominant physiologic macrophage activating factor. It plays a crucial role in promoting non-specific host-defense mechanisms against a number of pathogens. In vitro and in vivo studies have demonstrated that macrophages activated by IFN-γ have the capacity to non-specifically kill a variety of intracellular and extracellular parasites as well as neoplastic cells (Gattoni et al., 2006).

One of the major immunoregulatory roles of IFN-γ is its ability to promote adaptive immune responses by influencing both the number of MHC molecules on the cell surface, as well as the repertoire of peptides presented by MHC molecules. IFN-γ plays an important role in the development of the Th1 response. In vitro, antibody neutralization of IFN-γ greatly reduces the development of Th1 cells and augments the development of Th2 cells (Hsieh et al., 1993).

IFN-γ plays a complex role in regulating humoral immunity. IFN-γ is predominantly responsible for regulating three specialized B-cell functions: B-cell development and proliferation, immunoglobulin (Ig) secretion and Ig-heavy chain switching. Also, IFN-γ plays an important role in the immune-mediated rejection of established tumors (Ikeda et al., 2002).

Hahn et al. (2000) found IFN-γ mRNA in pulp tissue in 67% of shallow-caries teeth and it was about twofold more prevalent than IL-4 and IL-10, supporting Th1 polarization of the response. Furthermore, in 43% of these samples IFN-γ mRNA was the only cytokine message found. In the deep-caries group IFN-γ mRNA remained very
common, but IL-4 and IL-10 were found in similar frequencies and a polarization toward Th1 was no longer apparent.

There are significant gaps in our current understanding of pulpal inflammation and its relation to pulpal immune responses. The quantitative analyses of immunologically-relevant cells and the patterns of production of inflammatory cytokines and chemokines should help elucidate the immunopathologic mechanism of pulpitis. The knowledge gained in these areas can then be applied to immunotherapy of vital pulp in the future. In addition, the understanding of the biochemical and molecular networks and pathways involved in early reversible and later irreversible pulpitis may then be applied clinically to ensure that the dental pulp is vital and healthy.
Chapter 2

Aims and Objectives

1. To determine pulpal levels of IL-2, IL-6, IL-8, IL-10, TNF-α and IFN-γ in irreversible pulpitis, asymptomatic caries exposure and normal pulps using pulpal blood.

>This will help in identifying potential markers to confirm the presence of inflammation in human pulp tissue. Using this marker in clinical diagnosis would decrease the number of caries exposure cases undergoing root canal treatment which would result in reduction in visits to the clinic and treatment costs. It will help in predicting the prognosis of pulp capping.

2. To compare ratios of inflammatory to anti-inflammatory cytokines in human dental pulps in irreversible pulpitis, asymptomatic caries exposure cases and normal pulps.

>This will contribute to our understanding of the pathogenesis of irreversible pulpitis.
Chapter 3

Materials and Methods

3.1. Patient selection

The study was approved by the Joint Committee for Protection of Human Subjects in Research at Kuwait University and all subjects gave their written informed consent. One hundred and eight subjects were involved (57 male and 51 female). The medical histories of all patients in this study were non-contributory. The pulpal status in these patients was categorized as normal, asymptomatic caries-exposed pulp and symptomatic irreversible pulpitis.

The normal pulp group consisted of 25 subjects aged 18-35 years old (12 male and 13 female). Clinical and radiographic examination of these teeth indicated that these teeth had no caries, crown fractures or any restorations. These teeth were planned for extraction for orthodontic reasons.

The asymptomatic caries-exposed pulp group consisted of 40 subjects aged 18-54 years (21 male and 19 female). The teeth in this group had no history of pain to thermal stimuli and were designated for pulpectomy and root-canal treatment due to caries exposure. Clinical and radiographic examination indicated that these teeth had caries and/or restorations and no pulpal necrosis. Pulp sensitivity test to cold yielded sharp instant pain and not prolonged lingering pain.

The irreversible pulpitis group consisted of 43 subjects aged 18-60 years (24 male and 19 female). The teeth in this group had a history of prolonged pain to thermal
stimuli or spontaneous pain and were designated for pulpectomy and root-canal treatment. Clinical and radiographic examination revealed the presence of caries and/or restorations with or without pulp exposure. Pulp sensitivity test on cold elicited immediate prolonged lingering pain reaction.

3.2. Sample collection

After the tooth was isolated with a rubber dam, caries was removed and the pulp was carefully exposed with a spoon excavator and/or a low speed round bar. Blood from the exposed surface of the pulp was collected with a cotton pellet. The pellet was held at the exposed site to allow absorption of the pulpal blood. The pellets were then placed in 1 ml saline in tubes coated with heparin. Samples were stored at -20 °C until they were tested.

3.3. Sample preparation

After thawing, the samples were centrifuged first at 10,000 rpm for 10 min followed by centrifuging at 5,000 rpm for 5 min. The cotton pellet was then removed and the samples were ready for testing.

3.4. Estimation of cytokine levels

IL-2, IL-6, IL-8, IL-10, TNF-α and IFN-γ levels in pulpal blood were determined with commercially available ELISA kits. High sensitivity ELISA kits were used for IL-2, IL-6, IL-10, TNF-α and IFN-γ (Bender Medsystems, Austria) with sensitivities of 0.4, 0.03, 0.05, 0.13 and 0.06 pg/ml, respectively. Regular ELISA kits
were used for IL-8 with a sensitivity of 11.0 pg/ml. All assays were performed in accordance with manufacturer's instructions.

Generally in regular ELISA kits, antibody specific for the cytokine of interest is bound to the walls of plastic microtiter wells. Samples are incubated in the wells, and any cytokine in the sample gets bound to the antibody on the well walls. The wells are then washed, and a second antibody (Horseradish peroxidase (HRP)-conjugated antibody) is added to this one also specific for the cytokine, but recognizing epitopes different from those bound by the first antibody. After incubation, the wells are washed again, removing any unattached antibody. Attached to the second antibody is an enzyme, which, when presented with its substrate (Tetramethylbenzidine (TMB), produces a colored product, the intensity of the color produced being proportional to the amount of bound cytokine. The intensity of the color is then read by a spectrophotometer at 450 nm wave length.

In high sensitivity kits a biotin-conjugated antibody is added first followed by streptavidin-HP antibody after washing. Then, two amplification reagents are added, namely biotinyl-tyramide and streptavidin-HP, separated by a washing step. This is followed by the TMB substrate solution. The intensity of the color is then read by a spectrophotometer at 450 nm. Cytokine concentrations were expressed as picograms per total protein which was measured using the Bio-Rad™ protein assay.
3.5. **Total protein determination**

The Bio-Rad protein assay was used to determine total protein concentration. Dye reagent was prepared by diluting 1 part dye reagent concentrate with 4 parts de-ionized distilled water. Then, the solution was filtered by filter paper to remove particles. Samples were diluted 1: 40 in saline. Ten microliters of each diluted sample solution was added into separate microliters plate wells. Two hundred microliters of diluted dye reagents was added to each well and mixed thoroughly. Samples were incubated at room temperature for 30 minutes. Color intensity was measured at 595 nm wave length. Bovine serum albumin (BSA) was used as control. Five dilutions of BSA were prepared with range between 0.05 mg/ml to 0.5 mg/ml. All solutions were assayed in triplicate.

3.6. **Data Analysis**

Levels of cytokines were estimated and the ratios of cytokines to IL-10 ratio were calculated. Data was tested for data normality by the Shapiro-Wilk test. All concentrations calculated below the sensitivity level of the kits were recorded as the lower sensitivity limit of the kit for the convenience of statistical analysis. Statistical differences between the groups were determined by Kruskal–Wallis and Mann-Whitney U tests for non-parametric comparisons. Differences were considered significant if the $P$ value was equal to or less than 0.05. Data was analyzed using SPSS 17.
Chapter 4

Results

4.1. Testing of normality

Data were tested for normality using the Shapiro–Wilk test. As the data were not normally distributed, levels of cytokines and ratios of inflammatory cytokines to IL-10 were compared using the Kruskal–Wallis and Mann–Whitney tests.

4.2. Cytokine levels

4.2.1. IL-2

IL-2 was detected in 17 out of 25 samples (68 %) in the normal pulp group with levels ranging from 0.005 to 1.373 pg/mg with a median of 0.054 pg/mg (mean +/- standard deviation; 0.173 +/- 0.379 pg/mg). In the caries exposure group, IL-2 was detected in 32 out of 40 samples (80 %) with levels varying from 0.087 to 18.886 pg/mg with a median of 0.875 pg/mg (3.259 +/- 4.811 pg/mg). In the irreversible pulpitis group, IL-2 was detected in 30 out of 43 samples (70 %) with levels from 0.057 to 12.138 pg/mg with a median of 0.261 pg/mg (1.205 +/- 2.109 pg/mg) (Figure 1).

There were significant differences between the groups (p<0.001). IL-2 was significantly higher in the caries exposure group compared to both the normal group (p< 0.01) as well as the irreversible pulpitis group (p< 0.05). However, there was no significant difference between the irreversible pulpitis group and the normal group (p>0.05).
Figure 1. Mean IL-2 levels in different groups.
4.2.2. **IL-6**

IL-6 was not detectable in the normal pulp group. In the caries exposure group, IL-6 was detected in 21 out of 40 samples (50 %) with levels from 0.019 to 1.760 pg/mg with a median of 0.0244 pg/mg (0.233 +/- 0.421 pg/mg). In the irreversible pulpitis group, IL-6 was detected in 30 out of 43 samples (70 %) with levels ranging from 0.086 to 2.309 pg/mg and a median of 0.159 pg/mg (0.459 +/- 0.578 pg/mg) (Figure 2).

There were significant differences between the groups (p<0.001). IL-6 was significantly higher in both the caries exposure group as well as the irreversible pulpitis group as compared to normal pulps (p< 0.001). However, there was no significant difference between irreversible pulpitis and caries exposure groups (p>0.05).

![Figure 2. Mean IL-6 levels in different groups.](image-url)
4.2.3. IL-8

IL-8 was not detectable in the normal pulp group. In the caries exposure group, IL-8 was detected in 16 out of 40 samples (40 %) with levels from 2.294 to 290.957 pg/mg (21.826 +/- 66.091 pg/mg). In the irreversible pulpitis group, IL-8 was detected in 35 out of 43 samples (81 %) with a range from 4.267 to 1107.575 pg/mg and a median of 42.08 pg/mg (154.866 +/- 270.838 pg/mg) (Figure 3).

Significant differences were observed between the groups (p<0.001). IL-8 was significantly higher in both the caries exposure group and the irreversible pulpitis group when compared to normal pulps (p< 0.001). In addition, IL-8 was significantly higher in irreversible pulpitis than in caries exposure (p<0.001).

Figure 3. Mean IL-8 levels in different groups.
4.2.4. **IL-10**

IL-10 was detected in 17 out of 25 samples (68 %) in the normal pulp group with levels ranging from 0.048 to 0.605 pg/mg with a median of 0.266 pg/mg (0.217 +/- 0.183 pg/mg). In the caries exposure group, IL-10 was detected in 39 out of 40 samples (97.5 %) with amount varied from 0.279 to 17.921 pg/mg with a median of 2.303 pg/mg (4.487 +/- 4.894 pg/mg). In the irreversible pulpitis group, IL-10 was detected in 35 out of 43 samples (81 %) with detectable range 0.163 to 29.353 pg/mg with a median of 0.967 pg/mg (3.894 +/- 6.967 pg/mg) (Figure 4).

There were significant differences between the groups (p<0.001). IL-10 was significantly higher in caries exposure and irreversible pulpitis groups than in the normal group (p< 0.001). In addition, IL-10 was significantly higher in caries exposure than in irreversible pulpitis (p<0.01).

![Figure 4. Mean IL-10 levels in different groups.](image-url)
4.2.5. TNF-α

TNF-α was detected in 23 out of 25 samples (92%) in the normal pulp group with levels from 0.038 to 0.212 pg/mg and a median of 0.099 pg/mg (0.105 +/- 0.061 pg/mg). In the caries exposure group, TNF-α was detected in all 40 samples (100%) with levels from 0.089 to 39.447 pg/mg and a median of 1.314 pg/mg (5.802 +/- 9.815 pg/mg). In the irreversible pulpitis group, TNF-α was detected in 41 out of 43 samples (95%) with levels ranging from 0.065 to 8.811 pg/mg and a median of 1.929 pg/mg (1.653 +/- 2.291 pg/mg) (Figure 5).

Significant differences between the groups were evident (p<0.001). TNF-α was significantly higher in caries exposure (p<0.05) and irreversible pulpitis (p<0.001) than in normal pulps. However, no significant difference was observed between irreversible pulpitis and caries exposure (p>0.05).

Figure 5. Mean TNF-α levels in different groups.
4.2.6. **IFN-γ**

IFN-γ was detectable in 8 out of 23 samples (35 %) in the normal pulp group with amounts ranging from 0.001 to 0.019 pg/mg and a median of 0.004 pg/mg (0.006 +/- 0.007 pg/mg). In the caries exposure group, IFN-γ was detected in 19 out of 27 samples (70 %) with levels from 0.007 to 0.699 pg/mg with a median of 0.025 pg/mg (0.141 +/- 0.227 pg/mg). In the irreversible pulpitis group, IFN-γ was detected in 22 out of 28 samples (79 %); levels ranged from 0.01 to 0.621 pg/mg with a median of 0.028 pg/mg (0.131 +/- 0.197 pg/mg) (Figure 6).

There were significant differences between the groups (p<0.001). IFN-γ was significantly higher in caries exposure (p<0.05) and irreversible pulpitis (p< 0.001) as compared to normal. However, there was no significant difference between the irreversible pulpitis and caries exposure groups (p>0.05).

![Figure 6. Mean IFN-γ levels in different groups.](image)
In summary, IL-2, IL-6, IL-8, IL-10, TNF-α, IFN-γ were significantly higher in irreversible pulpitis and caries exposure compared to normal pulps. IL-2 and IL-10 were significantly higher in caries exposure compared to irreversible pulpitis while IL-8 was significantly higher in irreversible pulpitis compared to caries exposure (Figure 7).

Figure 7. Summary of mean cytokines in different groups.
4.3. **Ratio of inflammatory to anti-inflammatory cytokines**

The absolute levels of individual cytokines per se may not be as informative as the ratios of cytokines, i.e. the levels of inflammatory cytokines relative to the level of IL-10, an anti-inflammatory cytokine. Keeping this in mind, the ratios of inflammatory cytokines to IL-10 were calculated.

4.3.1. **IL-2/IL-10 ratio**

There is no significant difference in IL-2/IL-10 ratio between all the groups (p>0.05) (Figure 8).

![Figure 8. Mean IL-2/IL-10 ratio in different groups.](image)
4.3.2. **IL-6/IL-10 ratio**

The IL-6/IL-10 ratio is significantly higher in irreversible pulpitis as compared to both caries exposure and normal pulps (p< 0.05). However, there is no significant difference between normal pulps and caries exposure pulps (p> 0.05) (Figure 9).

![Figure 9. Mean IL-6/IL-10 ratio in different groups.](image-url)
4.3.3. **IL-8/IL-10 ratio**

The IL-8/IL-10 ratio is significantly higher in irreversible pulpitis when compared to both caries exposure pulps (p<0.001) and normal pulps (p< 0.01), but there is no significant difference between normal and caries exposure pulps (p> 0.05) (Figure 10).

![Figure 10. Mean IL-8/IL-10 ratio in different groups.](image-url)
4.3.4. TNF-α/IL-10 ratio

The TNF-α /IL-10 ratio is significantly higher in caries exposure group compared to irreversible pulpitis (p<0.05). However, there is no significant difference between normal and both irreversible pulpitis and caries exposure (p> 0.05) (Figure 11).

![Figure 11. Mean TNF-α /IL-10 ratio in different groups.](image)
4.3.5. IFN-γ/IL-10 ratio

The IFN-γ/IL-10 ratio is significantly higher in normal pulps compared to both caries exposure and irreversible pulpitis (p<0.05). There is no significant difference between irreversible pulpitis and caries exposure (p>0.05) (Figure 12).

![Figure 12. Mean IFN-γ/IL-10 ratio in different groups.](image)
In summary, IL-6/IL-10 and IL-8/IL-10 ratios were significantly higher in irreversible pulpitis compared to both normal and caries exposure pulps (Figure 13).

Figure 13. Summary of cytokine ratios in different groups.
Chapter 5

Discussion

Pulpal diagnosis is indispensable to correct treatment planning. To date, diagnosis and classification of pulpal diseases have been based on symptoms that are not always consistent with histological findings of pulpal pathosis (Seltzer, 1972). In the early stage of pulpal pathosis, the status of the pulp changes from reversible to irreversible pulpitis (Torabinejad & Walton, 2009). Because the dental pulp is enclosed in hard tissues, obtaining direct information on pulpal status is usually difficult unless evaluated microscopically. To determine the histological status of pulp, it is necessary to examine the pulp itself.

However, when the pulp is exposed, it is possible to obtain pulpal blood samples that may contain inflammatory factors that reflect the inflammatory state of the pulp. Analysis of the blood sample would provide valuable information regarding pulpal status. This information could then be used to determine the appropriate treatment.

Due to the necessity of determining pulpal status, Prader (1949) reported objective methods for the diagnosis of pulp disease using pulpal blood. However, he analyzed the white blood cell differential count of the sampled pulpal blood after extirpation or resection of the pulp and that was only qualitatively.

Nakanishi et al. (1995) attempted to take pulpal blood samples rather than the entire pulps. They established a method for collecting pulpal blood samples from exposed pulpal sites. Blood from the exposed surface of the pulp was collected with a
pre-weighed pellet of absorptive material. The pellet was held at the exposed site for 5 seconds to allow absorption of the pulpal blood. Then the quantity of sampled blood was determined by measuring the increased weight of the pellets. Using this method, they quantitatively analyzed the inflammatory factors in these blood samples. However, weighing the pellets accurately is not easy and is difficult to implement in clinical practice. Also, the amount of blood collected was very small to detect many cytokines usually present in minute quantities.

In this study, we tried to make the method of Nakanishi et al. (1995) easier to use clinically. We used cotton pellets which were directly placed in a heparin-coated tube containing 1 ml saline. Later the levels of cytokines were expressed per total amount of protein present in the sample. As the total amount of protein is proportional to the total amount of blood, it will be an accurate reflection of the amount in the sample. The method in this study can be used both for the clinical diagnosis of pulpitis (reversible versus irreversible) and for the elucidation of pulpal pathogenesis.

All previous studies which measured cytokines in the dental pulp focused only on two extremes. They compared normal pulps with irreversible pulpitis. They did not consider the intermediate stage of reversible pulpitis where the pulp still has healing capacity; this intermediate stage is very important in understanding pulpal pathogenesis as well as diagnosis. We measured the levels of different cytokines in normal pulps, asymptomatic caries-exposure pulps and irreversible pulpitis.
5.1. Cytokines and the dental pulp

IL-2, a cytokine secreted by CD4\(^+\) T cells, primarily acts to stimulate T cell proliferation, activates natural killer cells and promotes B cell function through T helper cell activation. Activated T helper cells assist B cells to recognize antigen, produce antibody, and differentiate into plasma cells. These activities emphasize the importance of IL-2 in the immune response. In addition, IL-2 activates transcription of proinflammatory cytokines and increases the cytolytic activity of natural killer cells (Gaffen & Liu, 2004). In the absence of IL-2, acute disease is reduced, and a chronic, inflammatory disorder develops instead (Hoyar et al., 2008).

IL-2 was first measured in the dental pulp is by Rauschenberger et al. (1997). Twenty healthy pulps obtained from soft tissue impacted third molars were compared with twenty inflamed pulpal samples collected from patients diagnosed with irreversible pulpitis. After extraction, teeth were sectioned and half of the pulpal tissue was histologically evaluated while the second half was prepared for measurement of IL-2 levels by ELISA kits prepared in their own laboratory. They detected IL-2 in all vital pulpal samples. Histologically uninflamed pulps exhibited low concentrations of IL-2 irrespective of their clinical classification (normal or irreversibly inflamed). The highest concentrations of IL-2 were detected in mildly inflamed pulps followed by the moderate and severely inflamed tissues. Additionally, they demonstrated that IL-2 concentrations were significantly higher in irreversibly inflamed pulpal tissues as compared to asymptomatic normal samples.
Anderson et al. (2002) tried to repeat the Rauschenberger et al. (1997) study using commercial IL-2 assay kits. They detected IL-2 in only 20 of 72 samples with no significant difference between the experimental groups. Thus, data on IL-2 in inflamed pulps has been somewhat contradictory.

In our study, IL-2 was detected in 17 of 25 normal samples, 32 of 40 caries exposure samples and 30 of 43 in the irreversible pulpitis group. IL-2 was significantly higher in the caries exposure group compared to both the normal group (p< 0.01) as well as the irreversible pulpitis group (p< 0.05).

Our data support the Rauschenberger et al. (1997) study. Both studies found that IL-2 was significantly higher in carious teeth compared to normal. IL-2 was higher in mildly inflamed pulps (i.e. caries-exposed in our study) compared to highly inflamed (i.e. irreversible pulpitis in our study). The failure of Anderson et al. to detect IL-2 in most samples may be explained by the low sensitivity of the assay used which was 7000 pg/ml compared to our study which has a sensitivity of 0.4 pg/ml.

Having higher levels of IL-2 in the caries-exposure group compared to both the normal and irreversible pulpitis may suggest that the pulp is initiating an immunological repair by stimulating the expansion of the helper T-cell population. As pulpal inflammation progressed to irreversible stage, IL-2 concentration decreased. This decrease in IL-2 represents a point where the tissue is in the late phases of irreversible inflammation and is progressing toward total tissue necrosis. Further research
correlating the levels of IL-2 and both histological status and healing capacity is needed to understand its role in pulpal inflammation.

IL-6 is an important cytokine in immune responses. It stimulates the differentiation of mature B lymphocytes to antibody-producing plasma cells, activates T-cells and enhances hematopoiesis. It also 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. IL-6 causes up-regulation of adhesion molecules and induces angiogenesis leading to increase in vascular permeability and leads to inflammatory edema. In addition, it induces osteoclast differentiation and bone resorption (Nishimoto, 2010). In general, pulpal symptoms were always explained by increase in the intra-pulpal pressure due to edema (Bender, 2000). This makes IL-6 a cytokine worth studying in the pulp. Levels of IL-6 can be correlated to the amount of inflammation and edema in the pulp in addition to its role as a mediator of host response following tissue injury and infection.

IL-6 has been studied by Barkhordar et al. (1999) in six inflamed human pulps tissue and six human periapical lesions. The inflamed dental pulp tissue and the periapical lesions demonstrated elevated IL-6 levels compared with the normal pulp.

Nakanishi et al. (1995) studied levels of IL-6 in pulpal blood. IL-6 was detected in only 2 of the 18 blood samples from inflamed subjects and was present in extremely small quantities. It was not detected in the normal pulp.
In our study, IL-6 was not detectable in the normal pulp. It was detected at high incidence in irreversible pulpitis (70 %) than in asymptomatic caries exposure (53 %). IL-6 was significantly higher in both the caries exposure group as well as the irreversible pulpitis group as compared to normal pulps (p< 0.001). However, there was no significant difference between the irreversible pulpitis and caries exposure groups. Our data is consistent with results of Barkhordar et al. (2000) on pulpal tissue.

Although we and Nakanishi et al both used pulpal blood as samples, they could not detect IL-6 in most of the samples. We may attribute our ability to detect IL-6 to the higher sensitivity of ELISA in our study.

IL-8 is one of the most potent chemokines with strong chemoattractive activity for neutrophils. It creates a chemotactic gradient toward the area of increased chemokine concentration. This gradient helps to both bring cells toward the local site of inflammation and also to retain them once they have arrived. In addition to recruitment, IL-8 also serves to stimulate the neutrophil to a higher state of activation. It is rapidly synthesized at local sites of inflammation where it fulfills its function of recruiting and activating acute inflammatory cells (Remick, 2005).

For this reason, it has been studied in order to provide more information on its action in the dental pulp. In diseased pulps, IL-8 was mainly produced by pulpal inflammatory cells and endothelial cells in addition to odontoblasts. It has been demonstrated that odontoblasts are capable of expressing IL-8 messenger RNA in response to lipopolysaccharide stimulation (Levin et al., 1999).
Huang et al. (1999) studied IL-8 in dental pulp tissue by both ELISA and immunohistochemistry. They detected 23-fold higher levels of IL-8 in inflamed pulps as compared to normal pulps. In addition, stronger IL-8 histological staining was observed in inflamed pulps than normal pulps. Guo et al. (2000) used pulpal blood as a sample. They detected higher levels of IL-8 in inflamed pulps while it was undetectable in normal pulps. They also found higher levels in patients with acute symptoms than chronic pulpitis symptoms. In a study by Silva et al. (2008), inflamed pulps showed higher levels of IL-8 by both ELISA and immunohistochemistry as compared to healthy pulps. They also demonstrated that pulp fibroblasts stimulated by bacterial LPS produce higher levels of IL-8 than the control group.

In our study, IL-8 was significantly higher in pulps with irreversible pulpitis and caries exposure than normal pulps. It was not detectable in normal pulps. Irreversible pulpitis pulps have 7-fold higher mean IL-8 levels than asymptomatic caries exposure pulps.

TNF-α is one of the most prominent inflammatory mediators 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 growth stimulation. It coordinates the early response to injury and thus represents an important point of regulation in inflammatory disease (Hehlgans & Pfeffer, 2005).
Nakanishi et al. (1995) reported that TNF-α was detected in 10 out of 20 pulpal blood samples in the inflamed pulp group. In the normal pulp group, 2 out of the 6 samples contained a detectable amount of TNF-α. However, there was no significant difference between the two groups. Pezelj-Ribaric et al. (2002) studied the levels of TNF-α in pulpal tissue from irreversible symptomatic pulpitis, asymptomatic irreversible pulpitis and healthy pulps. They reported high levels of TNF-α in pulpal tissue from teeth clinically diagnosed with irreversible pulpitis, but considerably lower expression of this cytokine in samples from normal teeth. Also, they found significantly higher levels of TNF-α in symptomatic pulpitis patients. Kokkas et al. (2007) found a significant increase in TNF-α gene expression in pulp tissue associated with irreversible inflammation as compared to healthy controls. No such difference was detected in reversibly inflamed pulp in comparison to healthy teeth.

Our study focused on groups similar to the study by Pezelj-Ribaric et al. However, we considered asymptomatic irreversible pulpitis cases as caries exposure cases since irreversibility was still doubtful. Our data showed significantly higher levels of TNF-α in pulpitis cases than in normal subjects with no significant difference between caries exposure and irreversible pulpitis. We used pulpal blood while Pezelj-Ribaric et al (2002) used pulpal tissue which makes comparison is difficult. However, both studies showed that a higher level of TNF-α is present in pulps with inflammation. The low levels in the Nakanishi et al (1995) study may be due to low sensitivity of the assay used.
IL-10 is a very important anti-inflammatory cytokine. It is the main inhibitor of cytokine synthesis and macrophage activity. It also inhibits the production of the pro-inflammatory cytokines like TNF-α, IL-1β and IL-6 in various cell types (Anker & von Haehling, 2004). IL-10 can further inhibit inflammation by increasing the release of IL-1 receptor antagonist by macrophages (Akdis, 2001). It was shown that IL-10 production is increased in inflammatory processes exerting an immunomodulatory role (Yamaoka et al., 1999, Markert, 2003). IL-10 inhibits the differentiation and maturation of DCs (Mosser & Zhang, 2008).

Hahn et al. (2000) found that incidence of IL-10 mRNA was significantly higher in deep-caries than shallow-caries. IL-10 has not been investigated in the dental pulps or been related to pulpal inflammation.

In our study, significantly higher levels of IL-10 were detected in caries exposure and irreversible pulpitis compared to normal pulps. Higher levels of IL-10 were detected in caries-exposed pulps compared to irreversible pulpitis. This suggests that IL-10 may be associated with pulpal symptoms as high levels were present in asymptomatic pulps. Also, having high levels may be an indication that the pulp is attempting to suppress inflammation before it reaches an irreversible stage as in other inflammatory diseases. For example, IL-10 has a critical role in suppressing intestinal inflammation and colitis (Barbara et al., 2000). In addition, Sasaki et al. (2000) found that, IL-10 is an important endogenous suppressor of infection-stimulated periapical bone resorption in vivo.
IFN-γ is one of the predominant cytokines in immune responses. It plays a crucial role in both innate and adaptive immunity as well as in inflammation. IFN-γ activated macrophages produce a variety of toxic substances. It is the main trigger for production and release of reactive oxygen species such as reactive oxygen intermediates and reactive nitrogen intermediates. These powerful oxidants bestow macrophages with cytostatic or cytotoxic activity against bacteria, viruses, fungi, protozoa, helminths and tumor cells (MacMicking, 2009). Inflammatory actions of IFN-γ are closely involved in both rheumatoid synovitis and atherosclerosis. Also, in concert with other pro-inflammatory cytokines, IFN-γ is the most important trigger for the formation and release of reactive oxygen species (Schroecksnadel et al., 2006).

Hahn et al (2000) found IFN-γ mRNA in pulp tissue of both shallow and deep caries teeth. This is the only published study of IFN-γ in the pulp.

In our study, we found significantly higher levels of IFN-γ in caries exposure and irreversible pulpitis compared to normal pulps. There was no significant difference between caries exposure and irreversible pulpitis. In spite of its immunomodulatory effects, IFN-γ may not reflect differences in the degree of inflammation between caries-exposure and irreversible pulpitis.
5.2. Ratios of inflammatory to anti-inflammatory cytokines

Measuring inflammatory to anti-inflammatory cytokines ratios is a good indicator of the cytokine balance in tissues. Also, ratios have no units which make comparisons easy when different types of samples are used. We calculated ratios of inflammatory cytokines to IL-10.

The IL-2/IL-10 ratio showed no significant difference between the different groups. The TNF-α /IL-10 ratio was significantly higher in the caries-exposure group compared to irreversible pulpititis. However, the TNF-α /IL-10 ratio was not significantly different between normal and irreversible pulpititis and between normal and caries-exposure and may not therefore be a good marker of pulpal inflammation.

The IFN-γ/IL-10 ratio was significantly higher in normal pulps compared to both caries-exposure and irreversible pulpititis. However, there is no significant difference between irreversible pulpititis and caries exposure.

The IL-6/IL-10 and IL-8/IL-10 ratios were significantly higher in the irreversible pulpititis group compared to both caries exposure and normal pulps with no significant difference between normal and caries exposure pulps. This makes these two ratios potentially very good markers of irreversible pulpititis.

Pulpal blood is a potentially good sample for pulpal diagnosis in caries exposure cases, as it is easily obtained in clinical setting. Choosing a marker of inflammation and evaluating its levels with the success and failure of pulp capping is key to implement
these cytokine studies in clinical practice. This can help in predicting the long-term prognosis of direct pulp capping. We suggest the use of the IL-8/IL-10 ratio because it gives more information about overall cytokine balance in of the pulp. IL-8 and IL-8/IL-10 are therefore recommended for studies on larger sample sizes, as it may contribute to easy diagnosis of pulpal inflammation in caries-exposure.
References


Mohamed Elsalhy  
Curriculum Vitae

Personal Information
Full Name: Mohamed Maged M M Elsalhy
Birth Date: 21 December 1982
Birth Place: Kuwait
Gender: Male
Nationality: Egyptian

Contact Information
Telephone Number: +965 66857788
E-mail Address: alsalhy1@hotmail.com

Undergraduate Education

Bachelor of Medical Sciences (B.Med.Sc.): Health Sciences Center – Kuwait University (2000 – 2004) / GPA: 3.17

Bachelor of Dental Medicine (BDM): Faculty of Dentistry- Kuwait University (2004 - 2007) GPA: 3.19 – Ranked 4th in a 25 student batch

Postgraduate Education
Membership of the Faculty of Dentistry of the Royal College of Surgeons in Ireland
Diploma (MFDS RCSI)
   Part I: passed April 2008
   Part II: passed June 2010

Master of Science in Medical Microbiology- Faculty of Medicine- Kuwait University (September 2008 – current).
**Work Experience**


**Publications and Abstracts**


ElSalhy M. Short Term Effects of Polyols on *Streptococcus mutans* and Lactobacilli; Presented as a poster in First African and Middle-East IADR Federation Conference 2005, Kuwait.

**Current Research**

Investigating mediators of Inflammation (IL-2, IL-6, IL-8, IL-10, TNF-α, and IFN-γ) in Irreversible pulpitis and asymptomatic caries exposure cases. Elsalhy M. Raghupathy R. Barrieshi-Nusair KM (Master Thesis).

**Interests**

Reading, Swimming, Traveling
الملخص

من المعضلات التشخيصية في طب الأسنان هو تقدير مقدار الإلتهاب في لب الأسنان، إن تقدير مقدار الإلتهاب في اللب مهم جداً في تقرير العلاج التشخيصي أو الجذري للأسنان، لا يوجد طريقة كمية ووضوحية وعملية لتقدير مقدار الإلتهاب حتى الآن، في السابق تم إجراء السينوكون (Cytokines) لتكوين مؤشرات الإلتهاب لerb الأسنان ولكن تقدير كمية السيتوكينات كان فقط ممكنًا بعد خلع الأسنان الملتهبة، في هذه الدراسة قمنا بتنفيذ طريقة لقياس كمية السيتوكينات من اللب بدون خلع الأسنان وقبل اتخاذ القرار.

تم تجميع عينة بلس من 108 لب سنو بواسطة قطعة صغيرة من مكان اللب المكشوف خلال عملية إزالة اللب الجزئية، 25 عينة تم أخذها من أسنان سليمة و 40 عينة من لب أسنان خالصة من الأعراض ولكنها مكشوفة اللب بسبب التسوس و 43 عينة من أسنان مصابة بإلتهاب اللب غير الديد، تم قياس كمية السيتوكينات المحفزة IFN (Interleukin) 2 و (Interleukin) 6 في الإلتهاب وهم إنترولوكين (IFN-γ) والسيتوكين المضاد للالتهاب وهو إنترولوكين 10، بواسطة فحص ELISA (Mann–Whitney و Wallis) تدفقت في الإلتهاب وهم إنترولوكين 10 بواسطة الفحوصات الإحصائية.

وجدنا معدلات مرتفعة من إنترولوكين 6 و 8 و 10 وعامل نخر الورم ألفا (IFN-γ) و إنترولوكين جاما (TNF-α) في لب الأسنان المكشوفة بواسطة التسوس و ذات الالتهاب اللبي غير الديد مقارنة بالأسنان السليمة، كان معدل إنترولوكين 2 أعلى في الأسنان المكشوفة فقط مقارنة بالسليمة، كما كان معدل إنترولوكين 8 و 10 أعلى في الأسنان المكشوفة بالنسبة للأسنان ذات الالتهاب اللبي غير الديد ولكن كان إنترولوكين 8 أعلى في الإلتهاب اللب غير الديد مقارنة بالأسنان ذات الالتهاب اللبي غير الديد مقارنة بالأسنان السليمة، بسبب التسوس و الأسنان السليمة.

نستنتج من هذه الدراسة أن مستوى إنترولوكين 8 و النسبة إنترولوكين 8/إنترولوكين 10 يمكن أن يكونا مشيرات مميزة لالتهاب اللب غير الديد، إن هذه الدراسة توضح أن معدل السيتوكينات موجودة في لب الأسنان يمكن أن تكون وسيلة مفيدة في تشخيص التهاب لب الأسنان.
جامعة الكويت

التحليل المناعي للتهاب لب الأسنان

المقدمة من:
محمد ماجد الصالحي

أطروحة مقدمة لكلية الدراسات العليا لاستيفاء جزء من مطالبات درجة الماجستير في العلوم الطبية (ال mikrobiologية)

بإشراف
أ.د. راج كوبال راجويتاي
أ.د. كفاي محمود باريشي (مشرف مشارك)

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