

## Review Article

# The Bio-Molecular Dynamics of Dental Pulp in Different Clinical Scenarios

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## Abstract

Dental pulp (DP) is a very dynamic tissue both in health and in disease. When exposed to stressors and pathological conditions. It undergoes a complex series of biological reactions whereby alterations affect the pulp tissue at tissue cellular and molecular levels.

The aim of this review is to update the reader on the various bio-molecular alterations in the dental pulp under different clinical conditions: orthodontic treatment (OT), caries, pulpitis and others.

The morphological changes in the composition of the DP range from the reversible remodeling to apoptosis and sometimes necrosis. Many apoptotic factors are involved like Bcl2, Bax and the significant increase in Caspases 9 and 3, as well as, Hsp60, its possible role and its mitochondrial localization.

The inflammatory responses in dental pulp and the role of diffusible and cellular factors as well as DP stem cells were highlighted, in particular, where caries was involved in the pulpitis. Recent data report changes in tissue metabolism and homeostasis inside the DP caused by OT leading to increased levels of iNOS reactivity in the nerve fibers of the pulp.

Moreover, remodeling of the extra cellular matrix (ECM) is an important feature in clinical scenarios like OT and caries whereby alterations in MMP-2 and MMP-9 expression patterns are reported leading to degradation of type IV and V collagens in the ECM.

Furthermore, neurogenic factors are also modified after injuries and OT. Neuropeptides play a significant role not only in pain perception but also in vascular responses. Substance P increases in DP and enhances pain perception and so is the increase in CGRP which is correlated with concomitant gain in bone morphogenetic protein expression resulting in more dentin formation.

The role of stem cells and the possible molecular mechanisms of dentin genesis are presented in this review. They focus on important signaling proteins and the possible role of various scaffolds in this regeneration process.

In conclusion, most alterations in pulp structure are reversible unless the pulp has a history of caries, restorations, trauma or prolonged heavy orthodontic forces. Pulpal symptoms arising from these clinical conditions should be treated appropriately and swiftly. Otherwise, exacerbation of pulpitis and the interplay of the various bio-molecular factors will lead to inhibition of repair and regeneration.

**Keywords:** Dental Pulp; Bio-molecules; Orthodontic treatment; Caries

## Introduction

The dynamics involving cellular and molecular responses of dental pulp (DP) have been approached by considering it as a structure capable of responding actively and dynamically to a variety of stimuli and stressors of different nature. These stimuli and stressors could be related to multiple factors such as changes in oral microflora (influenced by dietary habits and hygiene), orthodontic treatments (OT) and periodontal health [1].

Remodeling changes in dental pulp tissues, periodontal ligament and the alveolar bone do occur as a result of the complex multifaceted biological response elicited by the various stressors and or stimuli.

They produce local alterations in vascularity, as well as cellular and extracellular matrix reorganization, leading to the synthesis and release of various neurotransmitters, cytokines, growth factors, colony-stimulating factors, and metabolites of arachidonic acid among others. Multiple studies, during the past ten years, about the effect of the various stressors and treatment modalities in inducing pulpal alterations were not conclusive. Thus, more investigations are necessary to extend these investigations to a larger number of molecules involved in the physiology and pathology of dental pulp tissue. From this point of view, our review could contribute to the comprehension of mechanisms underlying the histological alterations as well as the biochemical processes involved. For example controversies were reported regarding the effect of orthodontic

treatment on the dental pulp; some authors denied pulpal necrosis [2] while others confirmed it in certain cases [3].

All these considerations point to the need to study thoroughly the morphological and molecular changes in DP in different clinical scenarios. Histologically, DP is a particular type of loose connective tissue characterized by richness in proteoglycans and glycosaminoglycans, and by poverty in collagen fibers. DP has structural and protective functions (synthesis of dentine), a trophic function (provides nutrients through blood and lymphatic vessels) and sensory function (through innervation). The cellular part is characterized by the presence of odontoblasts, fibroblasts (responsible of the production of the components in extra-cellular matrix), mesenchymal cells, endotelocytes and cells of immune system [4]. All these elements are subject to alterations in various dental pulp clinical conditions [5-7].

### Modifications of viability after therapeutic or traumatic stimuli

In the history of odontology, multiple techniques have been established aiming at the repositioning of the teeth to restore proper mandibular closure. The work of archaeologists has allowed us to find evidence of early orthodontic treatment in Greek and Etruscan populations, thus, demonstrating the existence of these practices as early as 1000 years B.C. The advent of new technologies allowed the study of the cell cycle of odontoblasts with or without noxious stimuli. It was proven that the viability of the cells was influenced by a regulated equilibrium between pro and by anti-apoptotic factors. In fact, apoptosis is a key cellular process that can be triggered by both, the external stimuli through the activation of specific receptors and from an intrinsic pathway involving mitochondria. The extrinsic apoptotic pathway involves the activation of Fas receptor and the involvement of caspase 8 as an effector factor. Studies conducted on patients with Class II malocclusion, and treated for 8 months, suggested the involvement of different proteins. Bcl-2, as a factor, is involved in the delicate equilibrium that regulates the initiation of apoptosis, by counteracting the action of the pro-apoptotic factor Bax [8].

In a study reported by Leone and co-workers in 2013 [9], the pulps were extracted and analyzed using both morphological and molecular procedures. Hematoxylin and eosin staining revealed significant structural modifications including vacuolization of the parenchyma, discontinuation of the odontoblasts monolayer and vasodilatation or congestion of blood vessels. Immunohistochemistry and RT-qPCR for Bcl-2 levels showed a strong correlation with the duration of the treatment. In fact, the comparative analysis between controls, 3 months and 8 months treated patients showed a marked lowering of the levels of both in mRNA and protein Bcl-2 levels [9]. The apoptotic process involved the activation of cysteine-aspartic proteases that played essential roles in necrosis and inflammation [10]. Caspase 9 is an initiator caspase activated by many triggering factors, leading to the release of cytochrome c from mitochondria and the activation of other caspases, such as caspases 3 [11].

Another study conducted on patients treated by Straight Wire Technique for different times, analyzed caspase 3 and caspase 9 in correlation to Hsp60. The latter is a 60KDa heat shock protein with mitochondria localization and has a great importance for cell survival

after a series of stresses [12]. The results of immunohistochemical analysis showed a significant increase in the activated forms of caspase 3 and caspase 9 and of Hsp60 in the patients treated for 6 months. These findings led to the analysis of a possible interaction between the effector caspases and Hsp60. Thus, an immunoprecipitation assay showed a high amount of pro-Caspase 3 bound to Hsp60 in patients treated for 3 and 6 months. The same study revealed a high amount of the complexed form of activated caspase 3 with Hsp60 only in the group of 6 months treated patients. In the same study also other viability assays were performed through PCNA and TUNEL reactions. The integration of all these results revealed an increased cell proliferation in treated patients despite the contemporary presence of an increased apoptosis [13].

Further studies conducted on transgenic hBcl-2 mice, without interference with the expression of mBcl-2 and mBax, showed the production of reparative dentine with a significantly higher mineral density, 6 weeks after the creation of artificial cavities. The results showed that Bcl-2 overexpression was able to promote dentine damage repair over the prevention of odontoblast apoptosis. These findings indicate Bcl-2 involvement, both in the maintenance of DP vitality and dentine production under detrimental mechanical stimuli [14].

### Inflammatory responses: diffusible and cellular factors

The triggering and maintenance of the inflammatory status in DP involves secreted factors and cellular responses. In pulpitis there is a massive T cell infiltration acting through the secretion of cytokines and the activation of several membrane receptors which are responsible of different cellular responses and morphological changes in DP. The investigation of an innate immune response during pulpitis focused on the role of dental pulp stem cells (DPSCs). On the other hand, bacteria colonizing the oral surface are responsible of the production of metabolites capable to trigger destruction of tooth enamel and induction of inflammatory responses, leading to pulpitis.

The immunohistochemical study of TLR4 revealed a localization in the odontoblast layer and a co-localization within blood vessels to different levels in healthy teeth and teeth affected by caries. Measurements of TLR4 mRNA, TLR4 protein and mRNA of cytokine showed an increase after stimulations with LPS and extracts from *S. mutans*. The latter, when used to study DPSCs behavior, revealed an inhibition in their proliferation and an increase in their migration. These DPSCs responses were blocked by inhibitory anti-TLR4 antibodies. These evidences suggest a possible involvement of DPSCs in an innate immune response through their TLR-4 mediated chemotactic behavior [15].

The combination of Immunohistochemistry with other experimental procedures, such as in vitro cultures, allows a better investigation of cellular behaviors with a better control of the different variables involved in the pathophysiology of DP through the direct administration of particular compounds and the revelation of cellular responses to very low doses. One example of this approach is represented by the study of cultured human DP cells after activation of pattern recognition receptors (PPRs) and treatment with INF- $\gamma$ . The results of this work revealed a marked increase in CXCL10 and IL-6 production after the treatment with PRRs agonists in combination with INF- $\gamma$ . Furthermore, the detection of IDO by Immuno-blot

and Immunohistochemistry showed an enhanced expression after PPRs activation compared to the exclusive treatment with INF- $\gamma$ . Interestingly, the blockage of IDO through the administration of inhibitors lead to the inhibition in CXCL10 production after stimulation with IFN- $\gamma$ [16]. These findings emanating from all cultures seem to be important in the direct control of different variables involved in the pathophysiology of DP.

### Modifications of nitric oxide synthase expression in DP

The movement of the teeth caused by OT has been shown to change tissue metabolism and homeostasis inside the DP with an initial decrease in blood flow after force application. Subsequently, there is a reactive hyperaemia (30 minutes later) followed by the return to normal blood circulation after 72 hr [17].

Nitric oxide (NO) is a compound with different biological functions produced by many cells of the body including blood cells. First of all, it binds and activates cytosolic guanylate cyclase, increasing intracellular levels of cyclic-guanosine 3',5'-monophosphate and, leading to vasodilation. When nitric oxide is generated by phagocytes (monocytes, macrophages, and neutrophils), it takes an active part in the innate immune response triggered by interferon-gamma (IFN- $\gamma$ ) as a single signal or by tumor necrosis factor (TNF) along with a second signal [18,19].

Many studies investigated a possible relationship between the expression level of Nitric oxide synthase (NOS) and the health status of the DP. NOS is an intracellular constitutive enzyme responsible of synthesis of NO. This enzyme has been well studied and four different isoforms were identified: neuronal NOS and endothelial NOS (found in all cell types); hepatic NOS and macrophageal NOS (macNOS/iNOS) induced by inflammatory processes[20]. iNOS distribution has been studied on DP samples derived from patients who underwent OT for different times durations. One study showed an increased immunohistochemical reactivity of iNOS in nerve fibers after 6 months OT and a peak in odontoblasts after 14 months OT[21].

Another study, conducted on patients subjected to 14 and 24 months OT, showed an increase of iNOS levels only in a particular group. In fact, iNOS was increased only in the subodontoblastic region of 14 months treated patients, while there were no differences in the odontoblasts reactivity considering the other conditions[22].

Characterization of eNOS and iNOS in healthy and inflamed DP showed an elevation in mRNA and protein levels of both enzymes in the pathological samples. Moreover, healthy pulp tissues did not show any iNOS while the increased levels in pathological conditions remained confined to leucocytes[23].

### Matrix remodeling in DP after OT and caries

The study of the effects of orthodontic traction on DP revealed distinct morphological and structural changes such as increase of vacuolization, extra cellular matrix (ECM) and vascular modifications. The latter are represented by an increased vascularization during the early treatment period and a later reduction of vessel diameter[24,25].

Physiological and pathological extracellular matrix (ECM) remodeling are very important features in the oral environment. A group of enzymes capable of degrading almost all ECM proteins are Matrix Metalloproteinases (MMPs). Their expression may be

upregulated in pathological conditions such as inflammation and tumor invasion. The equilibrium between activated MMPs and tissue inhibitors of metalloproteinases (TIMPs) regulates the extent of ECM remodeling. The mineralization process can be controlled by the participation of MMPs organizing the enamel and dentin organic matrix through the regulation proteoglycan turnover. They seem to play an active part in dentinal caries progression through the collagen breakdown in caries and periodontal lesions[26].

Various studies focused on the expression patterns of MMP-2 and MMP-9 in different clinical situations. MMP-2 is a matrix protease able to degrade type IV and V collagens (features shared with MMP-9), denatured collagen and elastin. Immunohistochemistry showed a correlation between the duration of OT and MMPs expression. In fact DPs extracted 6 months after the end of OT showed a significant lowering in MMP-2 and MMP-9 and concomitant alterations in the morphologic features. These findings led the authors to a possible correlation between the decrease in MMPs activity and impediment to the restoration of normal DP structure[22]. In addition, recently, Vidal et al described a correlation between the degradation of dentin matrix components within caries dentin and the activity of MMPs. To substantiate this hypothesis. They assessed the levels of MMPs in caries-affected and in intact dentine. The results showed a high expression of MMP-2 and MMP-9 in caries-affected teeth, indicating that those host-derived enzymes can be intensely involved with caries progression[27].

### Neurogenic factors in DP after injuries and OT

The adaptive response of DP after OT involves alterations in its physiology, resulting in a modified response to sensibility tests. Thus, electrical and thermal tests represent the most important aid to the clinicians to assess and diagnose pulpal pathology [28]. In this context, a study conducted by Alomari and coworkers on 47 patients investigated the changes in pulp sensibility after treatment with fixed orthodontic appliances. The experiments were conducted using electric pulp test (EPT) and thermal test with Endo Ice<sup>®</sup>. The measurements were performed at different times and revealed always a greater failing response of lateral incisors than lateral incisors and canine. The results of EPT showed a relationship between the OT and an increase in the response threshold (maximal after 2 months therapy) that gradually returned to pre-treatment values. To the contrary, thermal tests with Endo Ice<sup>®</sup> displayed a much smaller number of negative responses [29].

Neuropeptides play an important role not only in pain perception but also in vascular responses, in inflammation and in the alveolar bone remodeling. In fact, the released neurogenic factors interfere in the regulation of blood flow to the pulp and the periodontium[30].

Substance P (SP) was the first neuropeptide to be detected in DP and its increase has been linked to an enhancement in pain perception. The latter is probably correlated to the effects of SP on blood vessels in the pulp. This theory was verified through the systemic administration of Somatostatin (antagonist of SP normally present in trigeminal nerve). The results of this study revealed a subsequent reduction in vasodilation in the pulp, followed by inferior alveolar nerve stimulation[31].

Calcitonin gene-related peptide (CGRP), a peptide produced

in both peripheral nerves, and central neurons is involved in vasodilation and transmission of pain[32,33].The cell bodies in the trigeminal ganglion are the main source of CGRP, and its increase in DP is correlated with a concomitant gain in bone morphogenetic protein (BMP) expression that results in more dentin formation[34].

Artificial exposition of DP on rat molars has been used to clarify the behavior of GCRP. These teeth underwent an irreversible pulpitis, with complete necrosis by 3-5 weeks post-injury. The experimental assays performed at various time points post-injury showed a coexistence of vital pulp and peri-apical lesions due to continuous sprouting of CGRP-nerve fibers in the vital portion of DP [33].

### Dental pulp stem cells (DPSCs) potentialities for pulp and dentin regeneration

Damages in tooth enamel lead to the establishment of an infective process. This process caused by caries leads to a lack of collateral blood supply that makes the eradication of the infection by the immune system difficult. Therapeutic procedures such as partial pulpectomy led to unsatisfactory results. Thus, an irreversible pulpitis is diagnosed and the entire pulp should be amputated by pulpectomy. In such a case DPSCs, which show similarities to mesenchymal stem cells, may represent a potential biotherapeutic approach[35].

Other pluripotent cells isolated and characterized from DP are stem cells from exfoliated deciduous teeth (SHED), periodontal ligament stem cells, stem cells from apical papilla (SCAP) and dental follicle progenitor cells[36-39].

In the clinical practice several approaches were used to stimulate the regeneration of DP. In truth, as the volume occupied by DP is very small (~10–100 µl), it is very hard to recreate its microstructure due to the different layers constituted by different cell types, the highly organized dentinal tubes and the complex innervation[40].

This background is complicated when there is a traumatic loss of DP. Endodontic procedures exploit induced hemorrhage to fill the canal space with a blood clot to use as source of growth factors. In truth, these approaches have not yielded satisfactory results because when there is a total loss of pulp tissue due to trauma, the canal space is filled in by peri-apical tissues including bone, periodontal ligament and cementum, but not pulp[41].

Biotechnological procedures using DPSCs loaded in PLG scaffolds put inside emptied canal spaces led to well-vascularized pulp-like tissue and to the deposition of a layer of dentin-like mineral tissue onto the canal's dentin wall after 4 months in mice. Other approaches using collagen as scaffold failed to fill into the deeper part of the canal space, due to contraction phenomenon[40].

OT is responsible of changes in blood supply in human teeth and this phenomenon has been extensively investigated. When laser Doppler flowmetry was used on orthodontically treated patients a reduction in blood flow was registered after 20 minutes, 48 hours and 72 hours, followed by values on day 30[42].

Since a good blood flow is important for the survival of transplanted cells, co-cultures of DPSCs and HUVEC cells were encapsulated in three-dimensional PuraMatrix™. The latter was used to offer a better environment for cell-cell interactions. The results showed pulp-like tissue with patches of osteodentin and more

extracellular matrix, vascularization, and mineralization than the DPSC-monocultures *in vivo*[43].

DPSCs treated with dexamethasone and BMP7 when loaded on nanofibrous PLLA scaffolds underwent odontogenic differentiation with collagen and calcium depositions located at the walls of the scaffold pores, as well as, pulp-like tissue in the lumen of the pores. The extracellular microenvironment offered from this scaffold and the treatment with dexamethasone and BMP7 led to increased mineralization and up-regulation of osteocalcin and DSPP. Morphological studies using vonKossa staining, Masson's trichromatic staining and immunohistochemical staining for dentin sialoprotein revealed an enhancement in odontogenesis. However, the tissue formed did not resemble tubular dentin tissue[44,45].

Several studies have been conducted on the molecular mechanisms involved in the stimulation of dentin genesis focusing on important signaling proteins, such as WNT10A[46,47]. They assessed the molecular stimuli responsible of its expression in DP cells extracted from healthy human premolars. The extracted cells have been transfected with a lentivirus encoding WNT10A and RT-PCR assays have been used to assess the expression of odontoblast-specific genes such as DSPP, ALP, DMP1 and COL1A1. The Immunohistochemistry revealed the expression of WNT10A in the cytoplasm of DP cells (DPCs). The induced overexpression of WNT10A enhanced the proliferation of DPCs beside a down-regulation of ALP activity and of the odontoblast-specific genes studied[47].

### Discussion

It is important to note that different clinical scenarios, stressors and stimuli have different effects on the periodontal tissue leading to a wide spectrum of molecular and biochemical alterations. Dental traumatism, occlusal trauma and orthodontic forces, neither have the same etiology characteristics nor are always of equal intensity and frequency[2]. Although they are caused by forces on the tissues, they are not alike regarding the characteristics of the forces applied as well as their effects on different kinds of soft connective tissue components: cells and extracellular matrix as well as blood vessels and nerves [48].

DP is a highly elastic soft connective tissue, especially when forces are applied gradually, due to the presence of some collagen and elastic fibers in the extracellular matrix. However, sometimes hematomas can occur leading to an inflammatory reaction causing a panel of biological responses at the cellular and molecular levels. In this respect, it is essential to recognize that we are dealing with different clinical entities whereby multiple factors could interfere: The intensity of the stressor, its duration and its main focus and location. Sometimes they are absorbed and dissipated without rupturing vessels by fibrous and elastic connective tissue, and some other times, they induce reorganization of periodontal structures with reabsorption of the periodontal bone surface, cell migration, production of collagen fibers, elastic fibers, twisting of vascular and nerve handles, thus compromising blood and nerve supply to the pulps, or even rupture of vessels causing hematoma [49] and eventually inducing an inflammatory reaction which can be reversible [3]. Such a change in the local environment including vascular changes, recruitment of inflammatory mediators and alveolar socket remodeling might leave, sometimes, pulp side effects: pulpal respiration rate change, internal root absorption and pulpal obliteration [3].

The biological responses in DP after OTs or accidental injuries affect different pathophysiological aspects of the pulp. DP viability is negatively affected by OT based on experimental data showing an increase in effector caspases, a decrease in Bcl-2 and consequently dentin repair [50]. Moreover, stress proteins such as Hsp60 increase after OT and take part in interaction with apoptotic factors. Vascular responses of DP to OT are demonstrated by an increase in NO synthase. In addition, a gain in SP levels after OT correlates with vascular responses and enhancement of pain perception. Moreover, CGRP stimulates dentin formation through influencing more BMP expression, matrix remodeling in DP and more dental formation. It is well documented that matrix remodeling involves factors acting either negatively or positively if teeth are subjected to OT or other injuries. In fact, MMPs show increased levels in correlation with caries progression and decreased levels after OT with concomitant morphological alterations, suggesting an important role of matrix remodeling in the maintenance of the physiological architecture of dentine.

The investigation of the regenerative potential of DP has been approached by endodontic practices in the '70s with unsatisfactory results. Thus, recent research activity focused on the possible isolation and manipulation of stem cells from the teeth. Many experiments employing DPSCs loaded on different types of biomaterials led to the generation of pulp-like and dentin-like tissue. The latter resemble partially to the physiological architecture of the tissues composing the teeth, this might be due to the complexity in the organization of the extracellular matrix and of the different cell types involved. All these findings suggest the need of a deeper study of the molecular dynamics of DP in correlation with stimuli of different nature [44,45].

## Conclusion

Most alterations in pulpal structure that result from orthodontic treatment are reversible, unless the pulp has a history of caries, restorations, trauma (Hamilton and Gutman, 1999) or subjected to heavy and prolonged orthodontic forces. Irreversible pulpal alterations are very rare with orthodontic movement of normal, healthy teeth. Pulpal symptoms that arise during orthodontic treatment or other clinical scenarios should be treated appropriately and quickly, otherwise, the cellular and molecular events described in this review will be induced to reach pathological levels and will exacerbate the inflammation of the pulp leading to an inhibition of the function of dental pulp stem cells' highly needed for repair and regeneration.

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