Evaluation of the Secretion of Nerve Growth Factor by Dental Pulp Fibroblasts that have been Stimulated by Caries Related Bacteria

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Abstract

Introduction: During inflammation of pulp, an elevation in the secretion of various inflammatory biomarkers and cytokines by fibroblasts have been observed. Nerve Growth Factor (NGF) is a protein that has been associated with neuropathic and nociceptive pain. Dental pain involves nociceptive pain mechanism due to the presence of Aδ (myelinated) and C (unmyelinated) fibres. Thus, highlighting the probability of the role of NGF signalling pathway in dental pain. The present study was conducted to evaluate the secretion of NGF by pulp fibroblast cells. **Materials & Methods:** The human fibroblast cell lines were purchased from American Type Culture Collection (ATCC). All these fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM). A pure culture of test strain of *S. mutans* ATCC 29212 was inoculated in sterile nutrient broth. The human dental pulp fibroblasts were stimulated with varying concentrations of S. mutans. Live bacteria were added to the cells at the ratio (bacteria:cells) of 10:1; 50:1 to 100:1. The supernatant collected was subjected to ELISA test to evaluate the levels of NGF. **Results:** An increase in the secretion of nerve growth factor by stimulated dental pulp fibroblasts was observed with increase in the concentration of S. mutans. Highest levels of NGF 68.2 pg/ml were secreted by cells subjected to bacteria in the ratio 100:1. **Conclusion:** The elevation in secretion of NGF by stimulated dental pulp fibroblasts with caries related bacteria suggests the involvement of NGF signalling pathway during pulpal pain which could be used to develop new pharmacological approaches for effective pain management.

Keywords:

Pulpitis; Nerve growth factor; *S. mutans*; Pain; Nociception

Introduction

One of the primary reasons for which a patient seeks dental treatment is to obtain relief from dental pain.

Hence, efficient management of pain to provide relief to the patient is highly important.

A dental practitioner must be well versed with the various techniques of relieving the patient of it before, during and after a dental procedure.

There are various mechanisms involved in dental pain, making it a multidimensional, multifactorial and complex phenomenon.

Various inflammatory biomarkers and proteins are elevated during dentin-pulp complex pathologies. [1] Nerve Growth Factor which belongs to the family of Neurotrophic factors is a protein whose levels have been proven to be elevated during inflammation and peripheral nerve injury. [2]

Growth factors are proteins that play a role in regulating cellular processes like cell proliferations, maturation and differentiation and thus govern the growth of specific tissues. [3] Neurotrophic factors are comprised of three families of growth factors namely; Nerve Growth Factors also known as Neurotrophins, glial cell line derived neurotrophic factor and certain heterogeneous molecules which belong to the family of cytokines. [4] The meurotrophin family includes the Nerve

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Growth Factor, brain derived Neurotrophic factor, NT-3, NT-4 and NT-6. [5,6]

Nerve Growth Factor (NGF) was discovered in 1952 by Levi-Montalcini and was the first member of the neurotrophin family. [7] It plays a role in maintaining the phenotype and the survival of specific peripheral and central neurons during their phase of development and maturation. It has been shown that certain types of sensory neurons involved in nociception require NGF for survival in utero and for their normal development during the initial postnatal phase whereas in adulthood NGF is mainly involved in inflammation and hyperalgesia. [8] Nerve Growth Factor signalling is an active process which is involved in nociceptive and neuropathic pain. Nociceptive pain involves activation of nociceptors by a noxious stimulus. [9] Dental pulp contains the A∂ (myelinated) and C (unmyelinated) fibres which governs the pulpal pain in response to a noxious stimulus. [10]

Previously our team had conducted numerous clinical studies [11-14], case reports [15], in vitro studies [16-19], surveys [20,21] and reviews [22-25] in various aspects of endodontics and conservative dentistry over the past five years which has inspired the idea of the present study.

Thus, the present study was conducted to evaluate the secretion of Nerve Growth Factor by human dental pulp fibroblasts after stimulation with caries related bacteria.

Materials and Methods

Human fibroblast cell lines

The human fibroblast cell lines were purchased from American Type Culture Collection ATCC. All these fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, and subcultured with 1:4 split for several passages when they reached 80–90% confluence. The confluent fibroblasts were lethally treated with mitomycin C at 5 µg/ml for 16 hours and then trypsinized and plated at 2×10^4 cells/cm^2 in 35 mm dishes.

Bacterial strains

A pure culture of test strain of *S. mutans* ATCC 29212 was inoculated in sterile nutrient broth. *S. mutans* were grown in Brain-Heart Infusion broth at 37°C for 8 hours at 37°C for 12 hours. The bacterial concentrations were determined spectrophotometrically with a standard curve. Bacteria grown in liquid media were harvested in the stationary phase by centrifugation at 7000 g for 7 min and washed 3 times; the number of bacteria was then adjusted by dilution with DMEM devoid of antibiotics.

Stimulation procedure

HDPF were seeded in wells of 24-well tissue culture plates and incubated until a confluent monolayer developed (5×10^4 cells/well). The media were then replaced with DMEM containing 1% FBS and antibiotics. After 24 hours, the cells were treated with streptococcus mutans in fresh media for designated times. In the case of bacterial stimulant, live bacteria were added to the cells at the ratio (bacteria:cells) of 10:1; 50:1 to 100:1 in DMEM without antibiotics for 24 hours, respectively.

After incubation, the cell culture supernatants was collected and used to determine NGF concentration.

NGF assay procedure

The NGF ELISA kit was purchased from (Roche, Mannheim, Germany) and assay was performed according to the manufacturer's instruction.

About 100 µL of each standard and samples was added into appropriate wells and incubated for 2.5 hours at room temperature or overnight at 4°C with gentle shaking and the wells were washed by a wash buffer (300 µL).

Then 100 µL of 1X NGF antibody was added to each well. It was incubated for 1 hour at room temperature with gentle shaking. The supernatant was discarded and washed with distilled water. Later, 100 µL of prepared Streptavidin-HRP Solution was added to each well.

It was incubated for 45 minutes at room temperature with gentle shaking. To this, 100 µL of TMB Substrate was added to each well and incubated for 30 minutes at room temperature in the dark with gentle shaking.

The plate was evaluated within 30 minutes of stopping the reaction. The absorbance was measured on an ELISA plate reader set at 450 nm. The optical density observed on the ELISA reader was directly proportional to the levels of nerve growth factor.

Results

With the increase in concentration of *S. mutans*, there was an increase in the optical density as noted in the ELISA reader. Highest optical density was observed when the *S. mutans* were in the ratio of 100:1 (Table 1).

Thus an increase in the secretion of Nerve Growth Factor by stimulated human dental pulp fibroblast cells was observed with an increase in the concentration of *S. mutans*.

Highest level of NGF (68.2 pg/ml) was secreted when *S. mutans* were in the ratio of 100:1 (Figure 1).

Table 1: The NGF absorbance (n=3) of stimulated and unstimulated cells.

		S. mutans/Cell			
Optical Density at 450 nm	n	0	10	50	100
	1	0.126	0.381	0.487	0.581
	2	0.138	0.411	0.472	0.621
	3	0.119	0.457	0.501	0.609

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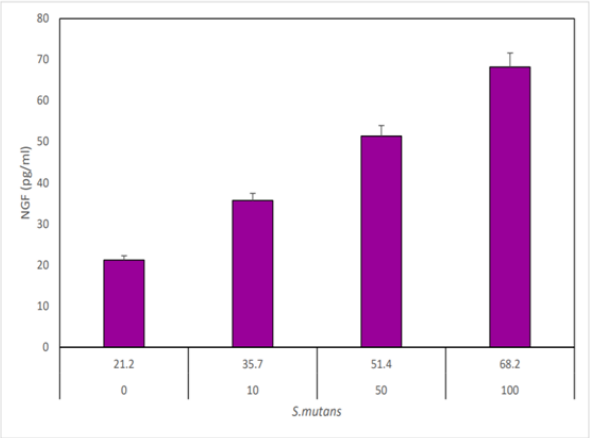

Figure 1: NGF measurement in human fibroblast cell lines after the contamination with *S. mutans.*

Discussion

During a pulpal pathology, various inflammatory mediators and cytokines are secreted by the dental pulp fibroblast cells. [26] According to the results of the present study, the level of NGF secretion by stimulated dental pulp fibroblasts significantly increased with the increase in concentration of *S. mutans*. *S. mutans* were chosen as the bacteria since they are involved in the process of caries initiation and progression which eventually leads to pulpitis.

Nerve Growth Factor acts by binding to two types of surface receptors: Neurotrophin receptors p75 for which it has low affinity and tropomyosin-related kinase A (trkA) receptor for which it has high affinity. [27] The trkA receptor is selectively expressed on the peripheral terminals of A-delta nerve fibres and unmyelinated C-fibers. [27,28] According to the "neurotrophic factor hypothesis" and the classical neurotrophic model, the target tissues synthesize and release and release NGF during embryonic development which promotes the growth, differentiation, and survival of neurons in a dose dependent manner. [29] During embryogenesis, the sensory neurons of the Dorsal Root Ganglia (DRG) shows a high expression of TrkA; however, post natally there is a shift of NGF–trkA signalling from promoting the growth of neuron and its survival to regulating peripheral nervous system's sensitivity to a noxious stimulus. [30]

Nociceptors which are located in peripheral tissues are activated in response to noxious stimuli thereby causing nociceptive pain. Any stimulus (eg, chemical, thermal, or mechanical) that either damages or threatens to cause damage to normal tissues is a noxious stimuli. Following noxious stimuli (eg, injury and inflammation), NGF is produced and released by peripheral tissues secondary to the release of inflammatory cytokines, such as interleukin-1 and tumor necrosis factor alpha. Effects on pain signalling are modulated by the binding of NGF to the trkA receptors on multiple targets. Once the NGF–trkA complex is formed, it gets internalized and is transported retrogradely to DRG cell bodies where it modulates and/or increases the expression of a variety of cell surface receptors involved in nociception. [31] The binding of of NGF to the trkA receptors located on mast cells causes an additional effect on pain processing. This process is proinflammatory and a positive feedback loop is generated by eliciting the release of inflammatory mediators such as histamine, serotonin or 5-hydroxytryptamine (5-HT), protons, as well as NGF itself. [31] Thus, NGF signalling plays two roles, it increase the expression of nociceptive receptors located peripherally and pronociceptive neurotransmitters located centrally, and in response to inflammation, it also sensitizes adjacent nociceptive neurons.

A study conducted Woodnut et.al evaluated the expression of neurotrophin receptors and NGF in nonneuronal cells of normal and injured tooth pulp. The study showed an upregulation of NGF in injured pulp and its accumulation in surviving odontoblast cells. [32]

A study conducted by Mitsiadis et.al showed a weak expression of NGF, p75 NTR and dental pulp fibroblasts and odontoblasts of intact functional teeth, while a strong expression of NGF and p75 NTR molecules was seen in nerve fibres that innervated the dental pulp. An upregulation of NGF and TrkA was seen in carious and injured teeth in odontoblasts surrounding the injury sites. This indicated a correlation between NGF signalling and dental tissue repair events. [33]

Non-Steroidal Anti-Inflammatory (NSAIDs) are routinely used for the management and control of pain. The discovery of Nerve Growth Factor has led to the exploration of new pharmacological approaches targeting the NGF pathway for effective pain management. These approaches mainly aim at sequestration of NGF, prevention of binding of NGF to trkA receptor and inhibition of trkA function. Nerve Growth Factor sequestration involves the use of NGF antibodies. The prevention of binding of the factor to its receptor was done using mouse monoclonal anti-trkA, MNAC13. It was capable of inducing analgesia in models of inflammatory and neuropathic pain. A synergistic was observed when it was used in combination with low-dose opioids. However, analogous species were not introduced into clinical trials due to lack of equivalent humanized antibodies. [34] k252a is a small-molecule protein kinase inhibitor that inhibits the activation of the entire tropomyosin receptor kinase family (trkA, trkB, and trkC). However, due to lack of specificity, no human trials were ever initiated. [35]

Conclusion

The present study demonstrates an elevation of the levels of Nerve Growth Factor secretion by fibroblast cells after stimulation with increasing concentration of *S. mutans* suggesting that it is the stimulation by bacteria which is involved in secretion of high levels of Nerve Growth Factor. As the caries progresses and the concentration of bacteria increases so does the secretion of the factor thus suggestive of its role in dental pain during pulpitis.

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