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Comparison of Gene Expression of Different Isoforms of Osteopontin in Symptomatic Irreversible Pulpitis of Human Dental Pulp

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ABSTRACT

Introduction: Osteopontin (OPN), plays an important role in immune system modulation. OPN can activate osteoclasts, thus causing resorption of bone. In addition, it might have a protective function against polymicrobial endodontic infections. Since different isoforms of OPN might have diverse roles, the aim of the present study was to compare gene expression of different isoforms of osteopontin in symptomatic irreversible pulpitis and normal pulps of the human dental pulp. **Materials and Methods:** Pulps were taken from 20 teeth with symptomatic irreversible pulpitis as the case group and from 20 intact premolars scheduled for extraction as the control group. After RNA extraction and synthesis of complementary DNA (cDNA), quantitative real-time polymerase chain reaction (PCR) was used for the evaluation of gene expression of OPN, OPN2 and OPN3. The Mann-Whitney U, t and Chi-square tests were used to analyze differences between the groups. **Results:** Mean values of OPN, OPN2 and OPN3 in normal pulps were 0.695±0.295, 0.656±0.298 and 0.816±0.422, respectively. Mean values of OPN, OPN2 and OPN3 in symptomatic irreversible pulpitis were 2.52±1.82, 1.99±0.899 and 1.816±0.954, respectively. Unlike OPN and OPN2, OPN3 exhibited significantly higher expression in normal pulps (P<0.05). **Conclusion:** The results of the present case-control study showed that some variants of OPN are upregulated during pulpitis and it might be due to their prominent modulatory roles in dental pulps.

Keywords: Human Dental Pulp; Irreversible Pulpitis; Osteopontin

Introduction

Pulpitis is a condition of the pulp connective tissue, which is induced by microorganisms involved in the caries process, tooth restorative procedures and trauma. There are two distinctive stages in pulpitis, requiring different treatment plans: reversible pulpitis in which the pulp is preserved and irreversible pulpitis in which in most cases, it is not possible to preserve the pulp vitality [1, 2]. Irreversible pulpitis might be symptomatic or asymptomatic, with the symptomatic form being more common and the majority of patients seek treatment only due to pain [3, 4]. In this condition, the pulp loses its reparative capacity and finally undergoes necrosis due to lack of treatment. The treatment consists of pulpectomy and root canal therapy. The pulp disease triggers the host's defensive reactions, the principal aim of which is to limit the inflammation [5]. During these defensive reactions, inflammatory mediators are synthesized, which have the capacity to cause soft and hard tissue destruction [1, 3].

Osteopontin (OPN) is encoded by the secreted phosphoprotein 1 gene. OPN, in the bone marrow, makes up about 2% of non-collagen bone and is predominantly secreted by osteoblasts. It was originally identified as a bone matrix protein but later it became clear that OPN is also a cytokine and is synthesized by different cells, including fibroblasts, preosteoblasts, osteoblasts, osteocytes, odontoblasts, dendritic cells, macrophages, endothelial cells and T cells [6-10]. When

these cells are exposed to proinflammatory cytokines, osteopontin is synthesized. Classic acute inflammatory mediators such as tumor necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β) can result in the expression of this protein [8, 11, 12]. It has recently been shown that osteopontin can play a protective role against polymicrobial endodontic infections. It was also shown that in response to some kind of inflammatory stimuli, there is an increase in OPN in order to participate in tissue repair [13]. In addition, an increase has been shown in osteopontin concentration in reparative dentin and its passive inductive role in the initiation of the pulp regenerative process [14]. On the other hand, the role of osteopontin has been shown in stimulation and activation of osteoclasts [15-17]. It has also been reported that OPN correlates with tumorigenesis, progression and metastasis of different malignancies in experimental and clinical models [18]. OPN also is involved in biological activities such as proliferation, adhesion, and migration of several bone-related cells, such as osteoclasts, osteoblasts and bone marrow mesenchymal stem cells [19]. So, OPN is a multifunctional soluble molecule in extracellular matrix (ECM) [20, 21]. Given the presence of 5 isoforms of osteopontin [22], the differences in the results might be attributed to differences in osteopontin isoforms. It should be pointed out that due to the small size of messenger RNA (mRNA) of isoforms 1 and 4 and 100% similarity to isoform 5, in the pilot study many cross-reactions were observed between primers 1, 4 and 5. In particular, isoform 5 participated in primer dimer reactions, resulting in unreliable responses [22]. Therefore, these three primers were excluded from the study. Since the role of this phosphorylated protein and the expression rates of its different isoforms have not been elucidated in pulpitis and most studies in this field have focused on the reparative effects of osteopontin, the present study was undertaken to compare the expression of its different isoforms (isoforms 2 and 3) in symptomatic irreversible pulpitis and in healthy control teeth, in order to find precise role of OPN in dental pulp health or in pulpitis and determine whether specific isoforms are involved or not. As we know in order to adopt the best treatment strategy, it is necessary to know the mechanism of pathogenesis and the molecules involved in it.

Materials and Methods

Subject selection

This study was ethically conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all of patients. The protocol was approved by Dental Research Center and Ethics Committee of Medical School of Shahid Beheshti University of Medical Sciences (The code of Ethics: IR.SBMU.MSP.REC.1394.121).

The subjects were selected from 14–25-year-old patients referring to the Department of Endodontics, Faculty of Dentistry, Islamic Azad University, Tehran Branch. The sampling method was non-probable and the samples were collected from the available population, based on the prevalence of the disease and Cochran formula (Cochran) [23].

Twenty subjects with symptomatic irreversible pulpitis were selected, with the following symptoms and signs: sustained sensitivity to cold, spontaneous pain, normal or increased response to percussion and absence of any evidence of periapical pathosis on the radiograph of posterior teeth. The subjects had moderate to severe pain for at least 24 h prior to selection and had not taken any analgesics.

After administration of anesthesia the tooth in question was isolated with rubber dam, access cavity was prepared after excavation of caries and the coronal pulpal tissue was removed with a sterile excavator [24].

Twenty normal pulps with a positive response to vitality tests (cold test), without spontaneous pain on percussion or mastication, caries or any symptoms and signs of pathosis in the periapical area on periapical radiographs, were selected in patients referring for extraction of their premolar teeth for orthodontic treatment. After local anesthesia and extraction of teeth, a cylindrical diamond bur was used in a high-speed handpiece to create a vertical cut along the long axis of the tooth without impinging on the root canal. Then a chisel was used to divide the tooth into two halves and the coronal pulpal tissue was gently removed with the use of a spoon excavator [24].

A scalpel blade was used to divide the pulpal samples into small pieces within a Petri dish and transferred into cryotubes containing RNA Later (Catalog number: AM7020; Thermo Fisher Scientific, Cleveland, OH, USA). After 24 h and penetration of the solution into the pulpal tissue, the samples were transferred into a freezer for long-term storage at -20°C [25].

Gene expression analysis

Homogenization of the tissue samples

The pulpal tissue samples were retrieved from the RNA Later solution and homogenized with TissueLyser II (Catalog number: 85300; Qiagen, Hilden, Germany) equipment and prepared for RNA purification.

RNA extraction and cDNA synthesis

After homogenizing the pulpal tissue, RNA was extracted by RNA isolation kit (in the presence of Lysis/Binding buffer, DNase,

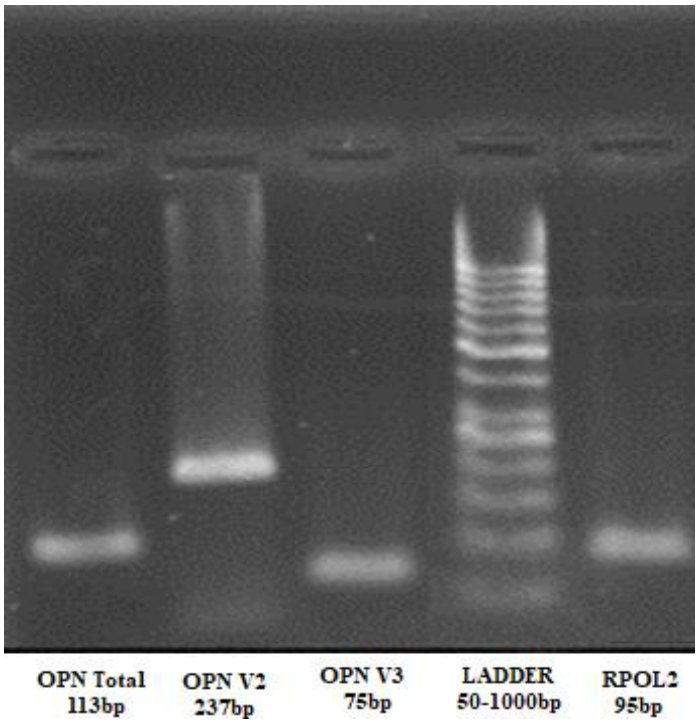

Figure 1. Electrophoresis of real-time PCR products on 2% agarose gel. In relation to OPN total, OPN variant 2, OPN variant 3 and RPOL2 genes, the products have undergone electrophoresis along with a 50-bp marker. The name of each band, along with its size, has been written under the band

washing buffer I,II, and Elution buffer) according to the manufacturer's protocol (High pure RNA Isolation kit, Catalog number: 11828665001; LifeScience, Roche, Mannheim, Germany). The quality of the total RNA was confirmed by the %2 agarose gel electrophoresis, followed by visualization of the bands using ultra violet (UV) transilluminator and Gel Doc and observation 3 bands, 5S, 18S, and 28S RNA. Its concentration and purity were evaluated by measuring the absorbance at 260 nm and the 260/280 nm ratio, respectively, using spectrophotometer, and the Optical density (OD) ratio was >1.8 [26].

Complementary DNA was synthesized, by reverse transcription .01-5µg of total RNA in the presence of Reaction buffer, Oligo (dT), Revert Aid™ MMLV (Moloney Murine Leukemia Virus) Reverse Transcriptase, MgCl2, dNTPs, RiboLock™ RNase Inhibitor, according to the manufacturer's protocol (Reverted First Strand cDNA Synthesis Kit, Catalog number: 00146314; Thermo Scientific Fermentas, Vilnius, Lithuania).

Designing primer

The sequence of the genes were taken from the website at http://www.ncbi.nlm.nih.gov and Beacon Designer software was used to design reverse and forward primers (Table 1).

It should be pointed out that since mRNA of isoforms 1 and 4 was small and due to its 100% similarity with isoform 5, in the pilot study many cross-reactions were observed between primers 1, 4 and 5; in particular, isoform 5 participated in primer dimer reactions, resulting in unreliable responses. Therefore, these three primers were excluded from the study. In order to make it possible to compare the results of the present study with those of other studies in which only the expression of OPN has been evaluated, its primer was used in the present study.

Quantitative real-time PCR (qRT-PCR)

To determine the ideal concentration of primers, different concentrations (0.5, 0.8, 1 and 1.5 µM) were tested and 0.5 mM for each primer was selected as the best.

SYBR Green I real time PCR assay was carried out in final reaction volume of 25 µL with 12.5 µL of SYBR Green I Master mix (SYBR® Premix Ex Taq™ II, Catalog number: RR820L, Takara, Japan), 1 µm of forward and reverse primers, 1.5 µm cDNA and 10 µm DEPC (Diethyl pyrocarbonate) water. Thermal cycling was performed using Rotor gene 6000 machine (Qiagen, Hilden, Germany) under the following cycling condition: Hold at 95°C for 1 min, followed by shuttle heating at 95°C for 6 sec, 54°C for 25 sec and 72°C for 30 sec in 40 cycles.

Calculations to determine the relative level of gene expression were made by reference to the RPOL2 in each sample, using cycle threshold method. The data were analyzed by the system software11. Cycle threshold (Ct) value of reference or housekeeping gene RNA Polymerase II (RPOL2) was subtracted from Ct value of the target gene (IL-22) to obtain ΔCt (Delta ycle threshold). After that ΔΔCt was calculated (ΔCt target0-ΔCt reference).The calculated ΔΔCt was converted to Ratio using the Ratio formula (Ratio=2-ΔΔCt).

Table 1. Designed primers for OPNs and RPOL2

Gene	Sequence
Osteopontin (OPN)	
Forward	AATGATGAGAGCAATGAG
Reverse	GTCTACAACCAGCATATC
Variant 2 or b of OPN	
Forward	ATCTCCTAGCCCCACAGACCC
Reverse	AAACTTCGGTTGCTGGCAGG
Variant 3 or c of OPN	
Forward	AGGCATCACCTGTGCCATAC
Reverse	GAGGACACAGCATTCTGCTTTT
RNA Polymerase II (RPOL2)	
Forward	TAACGCCTGCCTCTTCACGTTGA
Reverse	ATGAGGGACCTTGTAGCCAGCAA

Statistical analysis

All data were analyzed using SPSS 21.0 statistic software program (IBM Corporation, Armonk, NY, USA). Due to the lack of normal distribution of the main data, which was shown by Kolmogorov-Smirnov test, non-parametric test including Mann Whitney U test has been used. $P<0.05$ was considered statistically significant.

Results

The mean ages in the healthy pulp and pulpitis group subjects were 16.95±3.62 and 25.70±4.24 years, respectively, indicating that the two groups were not matched (*t*-test) in relation to their age because healthy samples were collected from subjects who needed orthodontic treatment ($P<0.03$). In relation to gender, the healthy group consisted of 15 females and 5 males and the pulpitis group consisted of 13 females and 7 males. The Chi-squared test did not show any significant difference in gender between the two groups ($P>0.3$).

Kolmogorov-Smirnov test showed that the distribution of the expression of OPN, OPN2 and OPN3 were not normal ($P<0.05$). Therefore, non-parametric tests were used for the analyses of the above mentioned variables.

In Tables 2 and 3 the expression of OPN, OPN2 and OPN3, respectively, was compared between healthy and symptomatic pulpitis groups by the Mann-Whitney U test.

Based on the data presented in Table 3, there was a significant difference in the expression of OPN, OPN2, OPN3 between the healthy subjects and subjects with pulpitis ($P<0.001$). So that there is higher expression of OPN, OPN2, OPN3 in pulpitis.

By comparing different OPNs in healthy pulps, we found higher expression of OPN3 in healthy pulps than the two other OPNs ($P<0.01$) but we could not find any significant difference regarding OPNs expression in symptomatic pulpitis group.

Figure 1 compares the expression of OPN, OPN2 and OPN3 between the healthy and pulpitis groups.

Discussion

Based on the available references, the present study is the first study that evaluate the expression of OPN and its isoforms in human teeth with symptomatic irreversible pulpitis.

The results of the present study showed significant differences in the expression of OPN, OPN2 and OPN3 between healthy pulps and pulps with symptomatic irreversible pulpitis, so that the expressions of OPN, OPN2 and OPN3 in pulpitis were 3.6, 3.04 and 2.34 times more than healthy pulp.

As pointed out previously, osteopontin is the glycoprotein first discovered in osteoblasts but it was later shown that it could be expressed in other tissues, too [6-8] including the dental pulp [27]. A study by Kaneko *et al.* [28] showed a higher expression rate of osteopontin in the apical pulp compared to the coronal pulp, with the highest expression rate of OPN in the periodontal ligament of healthy teeth of rats.

Table 2. Descriptive statistics for OPN, OPN2 and OPN3 gene expression (ΔΔCt) in healthy and symptomatic pulpitis groups

Variables / Parameters	Normal Pulp			Symptomatic Pulpitis		
	OPN	OPN2	OPN3	OPN	OPN2	OPN3
Number	20	20	1.05	20	20	20
Minimum	0.24	0.17	0.20	0.54	0.78	0.90
Maximum	1.05	1.04	1.78	2.52	1.99	1.81
Mean(SD)	0.69 (0.29)	0.65 (0.29)	0.81 (0.42)	2.52 (1.82)	1.99 (0.89)	1.81 (0.95)

Table 3. Comparison of expression of OPN, OPN2 and OPN3 genes between healthy and symptomatic pulpitis groups by Mann Whitney *U* test

OPN				
Group	**Mean rank**	**Total rank**	**Mann-Whitney U**	***P*-value**
Healthy	13.30	266.00	56.000	<0.001
Pulpitis	27.70	554.00		
OPN2				
Group	**Mean rank**	**Total rank**	**Mann-Whitney U**	***P*-value**
Healthy	11.20	224.00	14.000	<0.001
Pulpitis	29.80	596.00		
OPN3				
Group	**Mean rank**	**Total rank**	**Mann-Whitney U**	***P*-value**
Healthy	12.50	250.00	40.000	<0.001
Pulpitis	28.50	570.00		

Consistent with the results of previous studies, indicating that inflammatory mediators result in the expression of this protein [8, 11, 12]. Our study was done on the coronal pulp which has considerably higher number of immune cells than apical pulp [29]; thus, it would be a better region of pulp in order to find inflammatory mediators which are produced by immune cells. In addition, we used Real-time PCR in order to measure gene expression, which is more specific and advantageous to enzyme-linked immune-sorbent assay (ELISA). However, it should be noted that the expression of a gene does not imply the production of a protein or mediator, as RNA may be degraded [30]. In addition, we evaluated the different isoforms of OPNs in the dental pulps. Based on current literatures, there is no similar study up to now.

In the present study, the two groups have a statistically significant difference in age, indicating that they were not matched (*t*-test) in relation to their age. It was because that the healthy samples were collected from subjects who needed orthodontic treatment. On the other hand, there is no source to suggest that OPN gene expression changes with age. Therefore, the difference in gene expression between the two groups does not seem to be due to their age difference. Chatakun *et al.* [31] reported in 2013 that OPN exerts a stimulatory effect on osteoblasts even in early stages. Mori *et al.* [32], also reported in 2010 that OPN functions as an osteoblastic marker and it is even found in stem cells that are differentiated into osteoblasts.

A higher expression of OPN in the pulpitis group and higher expression of OPN3 in healthy pulps, might be attributed to this fact that some isoforms of OPN have a more significant inflammatory roles, while in the case of OPN3 a possible protective role might be considered for it.

Rittling *et al.* [6] reported in 2009 that OPN has a protective role in polymicrobial endodontic infections through enhancing neutrophil function (innate immune response) but it might also enhance bone destruction associated with endodontic infections. In the present study, the relationship between OPN and the expression rate of cytokines involved in bone loss (such as RANKL and IL-1) was not evaluated, although IL-1 is one of the most important inflammatory cytokine and we considered pulpitis as an persistent inflammatory situation; therefore, it cannot be claimed that there is inconsistency between the results of the present study and the above mentioned study. Of course, if periapical lesions are studied, perhaps more relevant results can be obtained.

Kuratate *et al.* [14] reported in 2008 that OPN might have an inductive role in the initiation of pulp regeneration process. Shigetani *et al.* [33] showed that gallium-aluminum-arsenide (GaAlAs) laser irradiation of rat molars induced upregulated dentin matrix protein 1 (DMP1) and osteopontin mRNA expression in the coronal pulp, followed by the formation of reparative dentin. Tomson *et al.* [34] reported that hepatocyte growth factor (HGF) can increase the expression of OPN in dental pulp cells and suggested that along with other factors, it can participate in the repair of the dentine-pulp complex. Similar to the previous case, it might be pointed out that some specific isoforms of OPN, such as OPN3, might have a significant increase in response to environmental factors. It can be concluded from the overall results of the present study that there are differences in the expression of some isoforms of OPN in healthy pulp and during pulpitis. Inflammation results in an increase in the expression of OPNs. However, further studies are needed in this respect, with larger sample sizes to shed more light on the differences between the healthy and inflamed pulps. In the case of OPN2, its expression in dental pulps might be less important than the other isoforms so it might have less important role in inflammation of dental pulp.

Conclusion

The expression of OPN3 was significantly more than other OPNs in the healthy pulp and it might be concluded that OPN3 has a greater role in dental pulp and its higher expression under inflammatory conditions might be a possible mechanism to combat inflammation. OPN, exhibited a higher expression in irreversible pulpitis and it might be advisable to consider it as an inflammatory mediator rather than modulatory factor.

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Conflict of Interest: 'None declared'.

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