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Protease-Activated Receptors 2 and Alpha-Smooth Muscle Actin Expression in the Pulp Tissue of Caries Teeth

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ABSTRACT

Objectives: *The understanding of the molecular events of inflammation in the pulp complex is substantial to facilitate the repair and regeneration occurring in caries teeth. This study aims to assess the protease-activated receptor-2 and alpha-smooth muscle actin (α-SMA) in the pulp tissue of caries teeth and their relations with the caries depth and the presence of pulp tissue exposure.* ***Method:*** *This is a cross-sectional study of 150 extracted teeth with their pulp tissue (108 with caries and 42 without caries, considered as a control group). Samples were collected in Duhok/Iraq from April 2016 to August 2017. The immunohistochemical results for both markers were analyzed.* ***Results:*** *Among the dental caries group, 23.15% (n=25) were shallow caries and 76.85% (n=83) were deep caries. Both markers were significantly higher in caries teeth, but the high percentage was negative for the protease-activated receptor-2 in deep caries (47.2%) and even higher in exposed pulp tissue (51.8%), whereas the α-SMA showed greater positivity in deep caries (59.3) and in exposed pulp tissue (45.8).* ***Conclusion:*** *Detecting high results for both markers in caries teeth, with lower protease-activated receptor 2 results in deep caries, needs more studies, as it may reflect the improper use of anti-inflammatory drugs in our society. The high positive for alpha-smooth muscle actin in deep caries may support this conclusion since it is not affected by this treatment.*

Keywords: Protease-activated receptor-2, Alpha-smooth muscle actin, Pulp tissue, Dental caries

INTRODUCTION

Dental pulp is composed of four layers: the external odontoblastic layer, the cell-free zone, the progenitor cell zone and the internal pulp core of nerves plexus, and blood vessels, which explains the high sensitivity of the pulp [1]. Like other connective tissue, fibrocytes, macrophages, and lymphocytes with a great amount of extracellular matrix are also presented in the pulp tissue. During bacterial infection, the odontoblasts will respond and the core pulpal cells, including fibroblasts, stem cells, endothelial cells, and immune cells will be involved in the activation of the intracellular signaling cascades that help in the repair [2]. Therefore, a better understanding of the molecular and the cellular events occurring in the pulp complex is mandatory to facilitate the repair and regeneration, which may benefit patients with caries teeth in the future.

The protease-activated receptors (PARs) are G protein-coupled receptors that are uniquely activated by proteolysis. The PARs mediate hemostasis, thrombosis, inflammation, embryonic development, and progression of certain malignant cancers [3]. Recognition of bacteria by specific odontoblasts and fibroblast membrane receptors triggers an inflammatory and immune response within the pulp tissue that would also modulate the repair process [4]. The physiological and pathological role(s) of PAR2 is still unclear.

Actins are proteins involved in the cell motility, structure, integrity, cytokinesis, signaling, intracellular transport, and even cell division [5,6]. The α-SMA is commonly used as a marker of myofibroblasts formation typically in wound healing [7,8]. After tissue injury, fibroblasts differentiate into contractile and secretory myofibroblasts that contribute to tissue repair during wound healing [9]. This research study the tissue localization of PAR2 and α-SMA in the pulp of teeth with dental caries by immunohistochemical (IHC) techniques and make a comparison of these results with the

depth of dental caries and the presence of pulp tissue exposure.

PATIENTS AND METHODS

Sampling

This study was approved by the Ethical Committee of Duhok Director of General Health Center. The extracted teeth with their pulps were collected from April 2016 to August 2017 from different private dental clinics and the dental health center of Duhok/ Iraq. From 159 extracted teeth, 150 had sufficient pulp tissue sample and 9 samples were excluded due to problems in processing the small pulp tissue. Dental caries was diagnosed clinically and radiologically in 108 teeth and was considered as "dental caries group", whereas the other 42 were teeth from the control group (without caries) extracted mainly for prosthetic and orthodontic reasons. Samples in the dental caries group were examined grossly and radiologically for the depth of caries, and they were divided into shallow and deep caries according to Schwendicke (Figure 1) [10].

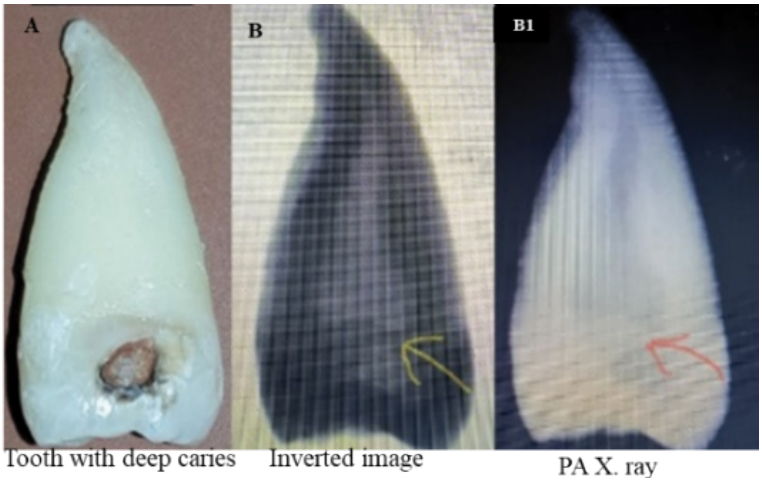

Figure 1 A: posterior tooth with deep caries; B: the inverted image of radiograph using digital processing machine (phosphorus plate) showing the depth of caries with the remaining dentin layer (Yellow arrow); B1: intra-oral periapical radiograph showing remaining of dentin layer (Red arrow)

Histopathological Process

The pulp tissue from all the samples was fixed in 10% formalin, processed into formalin fixed paraffin embedded (FFPE) tissue, they were sectioned and stained by hematoxylin and eosin (H and E) stain. The IHC staining protocol was performed on the FFPE containing the pulp tissue for both PAR2 and α-SMA according to the avidin-biotin complex (ABC) detection system [11]. Sections of 4 microns thickness were placed on positively charged slides. Primary and secondary antibody kits were used, provided by the DAKO Company detected with the EnVision+ system that employs peroxidase-labeled polymer conjugated to anti-mouse immunoglobulin antibodies. Immune complexes were identified by using the peroxidase reaction with DAB+ as chromogen (EnVision+ detection system, K4006, Dako Corp, Carpinteria, CA). The description of the markers used in this study is shown in Table 1.

Table 1 The immunohistochemical markers clone and dilution

Antibody	Clone	Dilution
Monoclonal Mouse Anti-Human PAR2	SAM11	Assay Dependent (75%) PBS, pH7.4
Monoclonal Mouse Anti-Human Actin	HHF35	0.08 mol/EDTA

Statistical Analysis

Data were analyzed using the statistical software for Windows version 17.0 (SPSS), in which the crosstab was applied to analyze the statistical significance of the data when applicable. The critical level of significance was set at $p<0.01$.

RESULTS

The age of all cases ranged from 7 to 69 years, with a mean age of 31.4 years. The youngest patient (7 years old boy)

presented with caries tooth and the oldest patient (69 years old male) with the mobile tooth. The mean age of patients in the dental caries group was 32.4 years (n=108), while that of the control group was 29 years (n=42). Among the dental caries group (n=108), 23.1% (n=25) were found to have shallow caries and 76.9% (n=83) found to have deep caries. Furthermore, deep caries showed exposed pulp tissue in 61.4% of cases (n=51), and the other 38.6% of cases (n-32) showed non-exposed pulp tissue.

The IHC analysis for PAR2 showed positive results in 54 cases, 49 of them (91%) were with dental caries, this represents 32.7% of all cases. Only 5 cases (3.3%) were in the control group. On the other hand, the IHC analysis for α-SMA found positive results in 94 cases, 80 of them (85%) were with dental caries and were representing more than 53% of all cases. Only 14 positive results (9.35%) were in the control group. These results were highly significant at $p<0.001$ level for both PAR2 and α-SMA (Table 2). Positive histological results of teeth pulp tissue to PAR2 and α-SMA marker are shown in Figure 2.

Table 2 IHC Markers results in relation to the presence of the dental caries

Tooth Status	PAR2 Results		Total %	α-SMA Results		Total %
	(-ve)* %	(+ve)** %		(-ve) %	(+ve)%	
Control group	37 (24.7) %	5 (3.3) %	42 (28.0) %	28 (18.65) %	14 (9.35) %	42 (28.00) %
Caries group	59 (39.3) %	49 (32.7) %	108 (72.0) %	28 (18.65) %	80 (53.35) %	108 (72.00) %
Total	96 (64.0) %	54 (36.0) %	150 (100.0) %	56 (37.3) %	94 (62.7) %	150 (100.00)%
P- Value		p<0.001***			p<0.001***	
*Negative (-ve), **Positive (+ve); ***Correlation is highly significant at the p< 0.001; These significant values were seen in positive results but not in negative results						

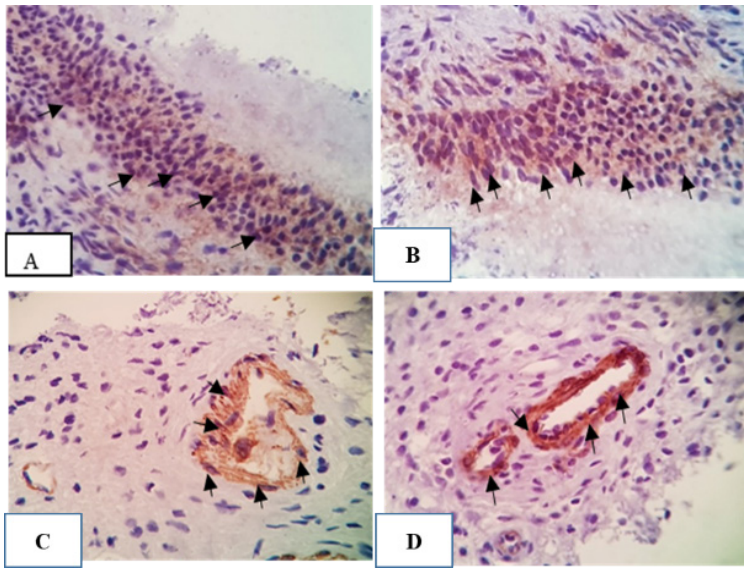

Figure 2 The IHC markers expression in tooth pulp tissue: brawn stain- black arrows; A: odontoblastic zone with PAR2 expression of tooth with shallow caries; B: odontoblastic zone with PAR2 expression of tooth with deep caries; C: perivascular cells with α-SMA expression of tooth with shallow caries; D: perivascular cells with α-SMA expression of tooth with deep caries; pulp cores 10X

The study of the relation between the PAR2 results and the depth of caries in the dental caries group is seen in Table 3. Although the percentage of positive cases of PAR2 were higher in shallow caries (15.7%) than negative cases (7.4%), this result was statistically insignificant. Furthermore, a large percentage of deep caries cases (47.2%) showed negative PAR2 results and only 29.6% of cases were positive. Out of the 32 non-exposed teeth pulp, 24 were positive for PAR2 (75% and representing 28.9% of all teeth caries cases). On the other hand, the exposed teeth pulp showed the higher percentage of negativity for the PAR2 (51.8%).

Table 3 The PAR2 results in relation to the depth of caries and the presence of exposed pulp tissue

PAR2 Marker Results				
Caries Depth	**(-ve)* %**	**(+ve)** %**	**Total %**	**p-value**
Shallow	8 (7.4) %	17 (15.7) %	25 (23.15) %	<0.001***
Deep	51 (47.2) %	32 (29.6) %	83 (76.85) %	
Total	59 (54.6) %	49 (45.4) %	108 (100) %	
Pulp Exposure	**(-ve)* %**	**(+ve)** %**	**Total %**	**p-value**
Non-exposed	8 (9.6) %	24 (28.9) %	32 (38.55) %	<0.001***
Exposed	43 (51.8) %	8 (9.6) %	51 (61.45) %	
Total	51 (61.45) %	32 (38.55) %	83 (100) %	
*Negative (-ve) **Positive (+ve); *** Correlation of dental caries depth and pulp exposure with the PAR2 marker is highly significant at the p< 0.01				

The IHC analysis for the α-SMA marker showed greater expression in deep caries. Out of the 83 deep caries cases, 64 were positive (about 77.1% which represents 59.3 of all caries teeth cases). This result was statistically significant. Unlike the PAR2 results the α-SMA results were higher in both non-exposed and exposed pulp tissue, were 26 out of 32 (81.3%) non exposed pulp tissue showed positive results and 38 out of 51 (74.5%) exposed pulp tissue showed positive results (Table 4).

Table 4 The α-SMA results in relation to the depth of caries and the presence of exposed pulp tissue

Caries Depth	**α-SMA Marker Results**			**p-value**
	(-ve)* %	**(+ve)** %**	**Total %**	
Shallow	9 (8.3) %	16 (14.8) %	25 (23.15) %	<0.001***
Deep	19 (17.6) %	64 (59.3) %	83 (76.85) %	
Total	28 (25.9) %	80 (74) %	108 (100) %	
Pulp Exposure	**(+ve) %**	**(+ve) %**	**Total %**	**p-value**
Non-exposed	6 (7.2) %	26 (31.3) %	32 (38.6) %	<0.001***
Exposed	13 (15.7) %	38 (45.8) %	51 (61.4) %	
Total	19 (22.9) %	64 (77.1) %	83 (100) %	
*Negative (-ve) **Positive (+ve); *** Correlation of dental caries depth and pulp exposure with the α-SMA marker is highly significant at p< 0.001				

DISCUSSION

The highly mineralized enamel and dentine layers of human teeth constitute a protective barrier prevent the pulp soft tissue from different external stimuli and microbial invasion, this protection may be affected by progressive demineralization of the enamel by the acids that are released from certain bacteria when placed in a sugar-rich environment [12]. Therefore, dental caries is considered a chronic infectious disease, which subsequently triggers inflammatory responses in the dental pulp [13]. Clinical and experimental data clearly revealed that dentin barrier formation only occurs when pulp inflammation and infection are minimized [14,15]. For these reasons additional studies, which target the molecular interactions within the dentin-pulp complex, are required as these may identify novel clinical therapies for dental tissue repair.

In this study, a high percentage of the patient presented with caries found to have deep type caries. Moreover, a high percentage of exposed teeth was found in teeth with deep caries. These relatively high percentages may reflect neglecting and the delay in attending dental clinics in our society.

The IHC analysis for both PAR2 and α-SMA found statistically higher positive results in dental caries group than the control group. However, the significant value was seen in positive results but not in negative results, and the negative results were relatively higher for PAR2 than α-SMA. These results were in agreement with several studies that gave different explanations. Bjørndal, et al., proposed that tooth destruction in shallow caries is within the outer half of the

enamel layer and in this depth the external stimuli may be very mild to irritate the pulp or elicit inflammation within the pulp [16]. Others assumed that the mineralized layers that protect the pulp are still enough to protect the pulp and that the dentin-pulp interface may also enable the release of bioactive molecules which subsequently triggers the host protective events including antibacterial, immune, and inflammatory responses. These events may eliminate early stage bacterial infection and block the route of its progression [15].

Unlike other studies, the current study concentrated on the relation of positive results in both PAR2 and α-SMA, with the depth of caries. Unexpectedly the IHC positive results for PAR2 were lower in deep caries than the shallow type and moreover, they were significantly lower in exposed pulp than the non-exposed pulp. This can be partly explained by the frequent use of antibiotics and anti-inflammatory medication, including the painkillers, without medical prescription in our society, which will interfere and inhibit the prostaglandin E2 or other inflammatory molecules [17]. Detection of PARs on peripheral and central neurons suggests that neuronal PARs might be involved in neurogenic inflammation and pain transmission [17]. The intense dental pulp pain is considered to be one of the most frequent reasons that patients attend the emergency dental care to take medication and some of these medications targets or antagonist the PAR2 activation [18].

The PAR2 activation is sufficient to induce neuronal plasticity leading to a chronic pain [19-21]. These effects were found in several *in vitro* and *in vivo* studies, and they clearly suggested that PAR2 plays an important role in periodontal inflammation and mediates reactive host cellular mechanisms which increase the levels of prostaglandin E2, interferon, interleukin-β, interleukin-6 and others [22-24].

Even more, when the caries disease is deep to more than half of the dentine layer, the dentin left a residual dentinal thickness between 0.25-1.0 mm [25]. This layer can prevent the tooth pulp from bacterial invasion but cannot prevent the bacterial toxins to reach the pulp through the dentinal tubules that make the good path to the pulp, elicit the sensory nerves and cause pain. The layer can also stimulate the odontoblasts cells to secrete tertiary dentine [26].

In this study, cases of deep caries with exposed pulp showed a lower PAR2 expression when compared to the non-exposed teeth pulp. It was determined that caries can extend to 100% of the dentin when no residual dentinal layer left to protect the pulp [26]. It is obvious that there is a long time for caries to reach the pulp, and the inflammation becomes a chronic type.

Antibiotics have bactericidal or bacteriostatic properties or both and are used widely to control or eliminate bacteria and their effects. But the mode of action and the extent to which antibiotics have an anti-inflammatory or analgesic effect in irreversible pulpitis remains less clear as observed by Agnihotry, et al., in 2016 [27].

The α-SMA positive expression in this study was significantly higher in dental caries than the control group. Counter to the results of PAR2, they were also significantly higher in deep caries teeth than the shallow and even more in exposed pulp tissue than non-exposed. Because there are little effects of antibiotics and anti-inflammatory medication on the α-SMA when compared to the PAR2, therefore these results may support the previous explanation of the frequent use of antibiotics in our society for the PAR2 but not the α-SMA. An additional explanation is that the process in deep caries and exposed pulp tissue is becoming more chronic and the myofibroblasts continue to regulate the connective tissue remodeling by getting more cytoskeletal property of contractile smooth muscle cells and in creating the high intracellular tension [9]. Since the α-SMA has more relation with the healing and remodeling which continue simultaneously with the injury and inflammation [28], therefore the expression of α-SMA is observed in both the pericytes and smooth muscle cells of blood vessels [29,30]. In the current study, the α-SMA positive expression was frequently seen in the arterioles and capillaries of the dental pulp. More clinical trials dealing with the treatment of deep caries lesion, that elicit more destructive inflammation, to get the best clinical outcome for dental caries patients [16]. This study requires further analysis for more inflammatory molecules to highlight other probable causes of the low PAR2 expression in deep caries and exposed pulp tissue, particularly on the PAR2 antagonists which may lead to a therapeutically new generation of useful antagonists. It might also be beneficial to incorporate more clinical details in the studies as indicators for the regenerative potential of the teeth and dental pulp tissue.

CONCLUSION

The inflammatory process is a fundamental change in dental caries, yet the depth of the carious lesion is seldom studied in researches dealing with the molecular analysis of dental caries. The high percentage of deep dental caries,

the high percentage of exposed pulp tissue and the statistically high positive results for PAR2 and α-SMA in dental caries cases, reflect the importance of this medical problem. The unexpected low positive results for PAR2 in deep caries and the much lower results in exposed teeth can partly be explained by the frequent use of antibiotics in this region, which may affect the PAR2 more than the α-SMA. The fact that the α-SMA was high in both deep caries and exposed pulp teeth may support this conclusion. However, if the research activity in the area combined with the clinical translational approaches this may result in a better therapeutics protocol which enables host defense and repair events.

DECLARATIONS

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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