Protein Activation in Periapical Reaction to Iodoform Containing Root Canal Sealer

Nili Tickotsky*/ Moti Moskovitz **

Objectives: *An association between root canal sealers and periapical lesions in primary dentition has been suggested, yet the chemical-protein interactions that may be involved in it have not been studied. The present study explored root sealer components' effect on periapical tissue proteins using bioinformatics tools.* ***Study design****: For each chemical component of Endoflas F.S. root sealing material we identified the known and predicted target proteins, using STITCH (search tool for interactions of chemicals* http://stitch.embl.de/*). Identified target proteins were grouped into functional categories using the annotation clustering tool from DAVID, the Database for Annotation, Visualization and Integrated Discovery (http://david.abcc.ncifcrf.gov/). STRING Protein-Protein Interaction network database identified associations between the proteins.* ***Results****: Sixteen proteins identified with STITCH served as input to DAVID annotation clustering tool. Only ZnO and Eugenol targeted proteins had statistically significant annotations. Gene Ontology terms of ZnO and Eugenol targeted proteins demonstrated that these proteins respond to mechanical stimulus and to oxidative stress. They highlight these proteins' role in the positive regulation of transcription, gene expression, cell proliferation and apoptosis, and their complementary role in the negative regulation of cell death.* ***Conclusion:*** *When stimulated by Zinc Oxide, Eugenol and Calcium hydroxide, chemical-protein and subsequent protein-protein interactions result in cell proliferation in the periapical area. Our findings indicate that certain root sealers components may cause enlargement of the permanent tooth follicle. Dentists should be aware of this phenomenon and radiographically monitor root canal treated teeth until shedding.*

Key words:*, Endoflas, Protein Interaction, Cytotoxicity, Proliferation, Gene Ontology.*

INTRODUCTION

The biocompatibility of root canal sealing materials is essential for the success of root canal treatments. Toxic materials may provoke inflammatory mediators in periapical tissues to activate various cell proliferation and apoptotic mechanisms[1,2]. In both permanent and primary teeth various root filling materials were shown *in vitro* to be cytotoxic for various kinds of cells [3-5], suppressing growth by apoptosis and/or necrosis [6].

In primary dentition, a number of case reports suggested an association between root canal treatment and periapical lesions[7-10]. An association between intra-canal medicaments and intra-epithelial inclusions found in cyst walls has long been proposed[9,11,12].

In a former study we had found that following root sealing with Endoflas F.S. a root sealing material used in primary teeth, there was a 3.3% incidence of a new radiolucent defect or enlargement of existing periapical radiolucency [13].

Another *in vitro* study established that high concentration of Endoflas F.S. conditioned media reduced cell viability by ~ 80%. However, low concentrations induced a proliferative effect, with cell viability increasing by about 60% compared to the control-media group[14].

Endoflas F.S. (by Sanlor & Cia. S. en C.S. Columbia, South America) comprises a powder of Iodoform (40.6%), zinc oxide (56.5%), calcium hydroxide (1.07%) and barium sulphate (1.63%), with a liquid consisting of eugenol and paramonochlorophenol.

Although the cytotoxicity of ZnO and Eugenol has been established *in vitro* and possible apoptotic mechanisms were suggested [2,4,6], the effect of the other components of Endoflas F.S. on periapical cells was not examined and the chemical-protein and protein-protein interactions involved in root sealer induced biological functions in the periapical region have not been explored yet.

In the present study we explored Endoflas F.S. root sealer components' effect on periapical tissue proteins using bioinformatics tools to identify the proteins affected by each chemical components of the root sealing material. We then examined the role of the identified proteins in human tissues.

Nili Tickotsky DMD, Bar Ilan University, Ramat Gan, Israel.
Moti Moskovitz DMD, PhD, Department of Pediatric Dentistry, Hebrew University, Hadassah School of Dental Medicine, Jerusalem, Israel.

Send all correspondence to:
Moti Moskovitz, Department of Pediatric Dentistry, Hadassah School of Dental Medicine, P.O.Box 12272, Jerusalem 91120, Israel.
Phone: 972-2-6776122
E-mail: motim@md.huji.ac.il

MATERIALS AND METHOD

For each chemical component of Endoflas F.S. root sealing material (i.e. Iodoform, zinc oxide, calcium hydroxide, barium sulphate, eugenol and paramonochlorophenol) we identified the known and predicted target proteins, using STITCH (search tool for interactions of chemicals http://stitch.embl.de/). STITCH links molecular, cellular and phenotypic data related to small molecules. Chemicals are linked to other chemicals and proteins by evidence derived from experiments, databases and the literature[15]. When searching STITCH with a chemical as entry point, the user is presented with a network of related proteins that places the chemical into a biological context [16].

STITCH was queried with both the chemical's name (e.g. 'Calcium hydroxide') and its chemical structure in SMILES (Simplified Molecular-Input Line-Entry System) strings format (e.g. [OH-].[OH-].[Ca+2]) (converted through ChemSPIDER, a chemical structure database http://www.chemspider.com/).

STITCH confidence view that represents the strength of the chemical- protein associations, was set at low confidence of association (score >= 0.15) to identify the proteins associated with each chemical component. Figure 1 illustrates STITCH search results for Eugenol protein targets. Table 1 illustrates the proteins associated with each Endoflas F.S. component.

Selection criteria for further bioinformatics processing was set as STITCH high confidence association (score >= 0.7), as interactions that have higher confidence are more likely to be true positive. Identified target proteins of all root sealing material components with a STITCH high confidence association score were analyzed using DAVID (Database for Annotation, Visualization and Integrated Discovery http://david.abcc.ncifcrf.gov/) functional annotation clustering tool [17] that groups genes according to functional categories to unravel biological processes associated with cellular functions and pathways. Biological functions in DAVID are classified according to the Gene Ontology project (http://geneontology.org/) that provides ontologies of defined terms representing gene products (i.e. proteins) properties.

The functional annotation clustering tool assigns each gene all its related annotations (derived from various databases). Then, Kappa statistics are employed to measure the degree of the common genes between two annotations. The more common genes two or more annotations share, the higher chance they will be grouped together.

To generate fewer functional groups with more tightly associated genes in each group, 'high classification stringency' clustering was employed.

We used STRING Protein-Protein Interaction network to identify interactions between the target proteins. STRING is a database of known and predicted protein interactions (http://string-db.org/). The interactions include direct (physical) and indirect (functional) protein-protein associations [18]. We used the 'actions view' that demonstrates the directionality of the action, if known.

RESULTS

Targeted proteins for each root sealing material component were identified with the STICH tool (Table 1). A total of 33 proteins were identified.

After the exclusion of proteins with STITCH confidence score of less than 0.7, 16 target proteins with STITCH high confidence association score served as input to DAVID Functional Annotation Clustering tool.

Only ZnO and Eugenol targeted proteins were found relevant for clustering by the DAVID tool. The target proteins of the other Endoflas F.S components were not mapped to any of the functional groups.

The main DAVID functional annotation clustering results demonstrating annotations generated for target protein clusters are listed in table 2.

Most ZnO and Eugenol targeted proteins had the following common functions: Response to nutrient levels, response to extracellular stimulus, response to drug, response to oxidative stress, response to hydrogen peroxide, response to mechanical stimulus, regulation of cell proliferation, regulation of transcription and apoptosis and positive regulation of RNA metabolic process.

ZnO targeted proteins (CASP3, RELA, NFKBIA and IGF1) were exclusively assigned the following annotations: Negative regulators of apoptosis of programmed cell death and of cell death.

DISCUSSION

Two Endoflas F.S. components whose targeted proteins were clustered by the DAVID functional annotation tool are ZnO and Eugenol. This means that only ZnO and Eugenol have known target proteins with significant relevance to the biological pathways clustered by DAVID. The other components of the root canal sealer were not mapped to any significant biological pathways according to the DAVID functional annotation clustering results. Possible reasons are that these proteins' genes do not have relationship with any of other genes above similarity threshold, or if they do have a relationship with a few other genes, they do not have enough members to form a functional group based on minimum final cluster members. There is also an option for a false negative result.

The GO (Gene Ontology) terms of the targeted proteins mapped in DAVID demonstrate that these proteins respond to mechanical stimulus and to oxidative stress. They highlight these proteins' role in the positive regulation of transcription, gene expression, cell proliferation and apoptosis, and their complementary role in the negative regulation of cell death; when activated by ZnO and/or Eugenol, these proteins will either promote cell proliferation or apoptosis. Cell synthesis, proliferation and apoptosis were found in human dental periapical lesions in a gene expression study [19].

ZOE (Zinc Oxide Eugenol) overfilled root canal sealings in primary teeth were significantly less successful than those under filled and filled to the apex [20,21]. Root canals filled with ZOE cement showed the presence of inflammatory cells, with severely thickened periodontal ligament in the majority of the roots and cementum and caused bone resorption in a few cases[22].

The factor determining if the sealing material will evoke a negative tissue reaction may be its concentrations: Osteosarcorma cells viability changed in a dose-dependent manner in response to ZnO+eugenol+ FC (Formocreasol): cell survival rate decreased as filling material's concentrations increased [23]. High concentrations of Endoflas F.S. decreased the viability of macrophages and epithelial cells, whereas low concentrations had a proliferative effect[14]. The balance between proliferative and apoptotic pathways in normal periapical tissue may be disrupted by ZnO and eugenol, with high chemical concentrations inducing cell death and low concentrations promoting proliferation.

Table 1: Targeted proteins for each root sealing material component according to STITCH database.

Name of ingredient and its % in Endoflas F.S.	Predicted targets	Details	STITCH Confidence score
Iodoform Formula: CHI3 40.6%	KRI1	KRI1 homolog	0.322
	CHIT1	chitinase 1	0.267
	IARS	isoleucyl-tRNA synthetase)	0.16
	FOS	FBJ murine osteosarcoma viral oncogene homolog	0.150
Zinc oxide Formula: ZnO 56.5%	IGF1	insulin-like growth factor 1	0.800
	RELA	v-rel reticuloendotheliosis viral oncogene homolog A	0.700
	JUN	jun oncogene; Transcription factor that recognizes and binds to the enhancer heptamer motif	0.700
	CASP3	caspase 3, apoptosis-related cysteine peptidase.	0.700
	NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor	0.700
Calcium Hydroxide Formula: CaOH 1.07%	FN1	Fibronectin	0.814
	PIGB	phosphatidylinositol glycan anchor biosynthesis, class B	0.364
	ST5	suppression of tumorigenicity 5	0.272
	ONECUT3	one cut homeobox 3; Transcriptional activator	0.258
	ENGASE	endo-beta-N-acetylglucosaminidase	0.225
	GDF11	growth differentiation factor 11	0.215
	RAB2B	RAB2B, member RAS oncogene family	0.207
	RAB2A	RAB2A, member RAS oncogene family	0.207
	MKI67	antigen identified by monoclonal antibody Ki-67	0.199
	MUTYH	mutY homolog	0.19
Barium sulfate Formula: BaO_4S 1.67%	DNAH11	dynein, axonemal, heavy chain 11	0.639
	SPRY2	sprouty homolog 2	0.519
	SPRY4	sprouty homolog 4	0.448
Eugenol Formula: $C_{10}H_{12}O_2$ liquid	TRPA1	transient receptor potential cation channel, subfamily A, member 1	0.87
	TRPV1	transient receptor potential cation channel, subfamily V, member 1	0.866
	PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	0.842
	UGT2B17	UDP glucuronosyltransferase 2 family, polypeptide B17	0.835
	TRPV3	transient receptor potential cation channel, subfamily V, member 3	0.828
	MAOA	monoamine oxidase A	0.826
	E2F3	E2F transcription factor 3;	0.824
	FIP1L1	FIP1 like 1	0.803
	ALOX5	arachidonate 5-lipoxygenase	0.8
	UGT1A9	UDP glucuronosyltransferase 1 family, polypeptide A9	0.726
Parachlorophenol Formula: C_6H_5ClO liquid	amtR	transcriptional regulator	0.648

Table 2: DAVID annotation clustering results: Enrichment score represents the importance of the gene group among the other groups- higher score means more importance. The proteins responsible for this module are targets of ZnO and Eugenol. Modules composed exclusively of ZnO targets are in italics. The full DAVID annotation clustering results may be found in the supplementary section.

Genes	PValue	Term
Enrichment Score: 3.9063709448593786		
UGT1A9, PTGS2, RELA, JUN, *ALOX5*	5.25E-05	GO:0031667~response to nutrient levels
UGT1A9, PTGS2, RELA, JUN, *ALOX5*	8.07E-05	GO:0009991~response to extracellular stimulus
UGT1A9, PTGS2, RELA, ALOX5	4.51E-04	GO:0007584~response to nutrient
Enrichment Score: 3.3783007792589936		
UGT1A9, PTGS2, RELA, JUN, TRPA1	7.52E-05	GO:0042493~response to drug
PTGS2, RELA, JUN, TRPA1	7.15E-04	GO:0006979~response to oxidative stress
PTGS2, RELA, JUN, TRPA1	0.001364	GO:0010035~response to inorganic substance
Enrichment Score: 3.124345645173428		
E2F3, CASP3, PTGS2, RELA, JUN, NFKBIA, IGF1	1.21E-04	GO:0042127~regulation of cell proliferation
CASP3, PTGS2, RELA, JUN, NFKBIA, IGF1	0.001333	GO:0042981~regulation of apoptosis
CASP3, PTGS2, RELA, JUN, NFKBIA, IGF1	0.001394	GO:0043067~regulation of programmed cell death
CASP3, PTGS2, RELA, JUN, NFKBIA, IGF1	0.001417	GO:0010941~regulation of cell death
Enrichment Score: 2.684229157378862		
RELA, JUN, TRPA1	0.001707	GO:0009612~response to mechanical stimulus
RELA, JUN, TRPA1	0.001707	GO:0042542~response to hydrogen peroxide
RELA, JUN, TRPA1	0.003039	GO:0000302~response to reactive oxygen species
Enrichment Score: 2.1816150636359417		
CASP3, RELA, NFKBIA, IGF1	0.006398	GO:0043066~negative regulation of apoptosis
CASP3, RELA, NFKBIA, IGF1	0.006651	GO:0043069~negative regulation of programmed cell death
CASP3, RELA, NFKBIA, IGF1	0.006703	GO:0060548~negative regulation of cell death

STRING Protein-Protein Interaction network (figure2) demonstrated the central role of PTGS2 protein, activated by eugenol, in the network involved in both proliferation and apoptosis. PTGS2, Prostaglandin-endoperoxide synthase (also known as cyclooxygenase 2- COX2) is regulated by specific stimulatory events, suggesting that it is responsible for the prostaglandin products involved in inflammation and mitogenesis [24]. The products of cyclooxygenase enzymes were shown to have a dual role in the nervous system; some promote the survival of neurons, while others promote apoptosis [25].

The fact that ZnO targeted proteins (CASP3, RELA, NFKBIA, IGF1) were both positive regulators of proliferation and negative regulators of apoptosis, programmed cell death and cell death regulation may indicate that when over- activated by chemicals, these proteins may cause unrestrained cell proliferation.

The findings described here may have a possible additional implication that involves the fact that Endoflas FS has been found to accelerate root resorption in root canal treated primary molars compared to homologous teeth without root canal treatment[26]. In view of the present study's findings, we suggest that high concentrations of ZnO and Eugenol may enhance proliferation of cells that are active in biological root resorption and consequently accelerate it.

The present study provides a broader scope for understanding the possible effects of root filling materials that contain ZnO and/or Eugenol on the periapical tissues, through the chemical-protein and subsequent protein-protein interactions they evoke.

CONCLUSION

Our findings indicate that certain root sealers components may cause enlargement of the permanent tooth follicle. Dentists should be aware of this phenomenon and radiographically monitor root canal treated teeth until their shedding.

Figure 1: STITCH search results for eugenol. The thickness of the lines represents the confidence score of the association; a thick line represents a strong association. Chemical- protein links are colored green, Protein-protein links are colored blue.

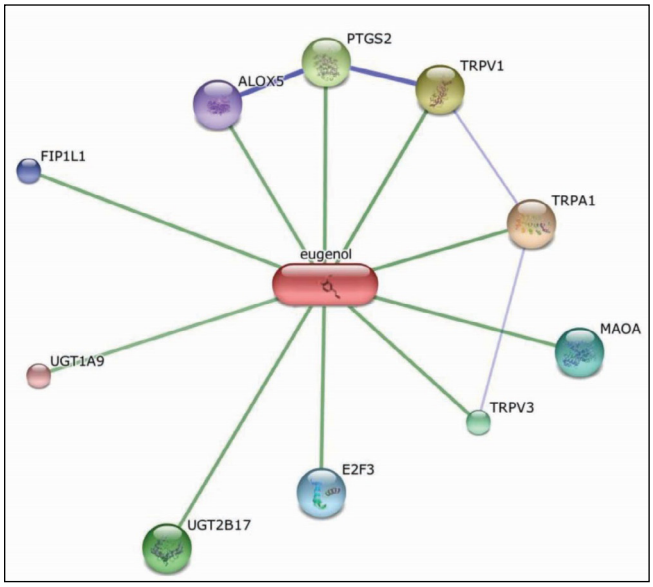


Figure 2: A radicular cyst associated with a lower second primary molar with a root canal treatment. The tooth germ is deflected mesially.

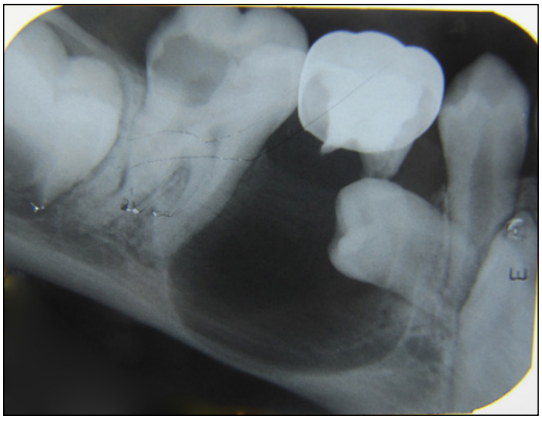
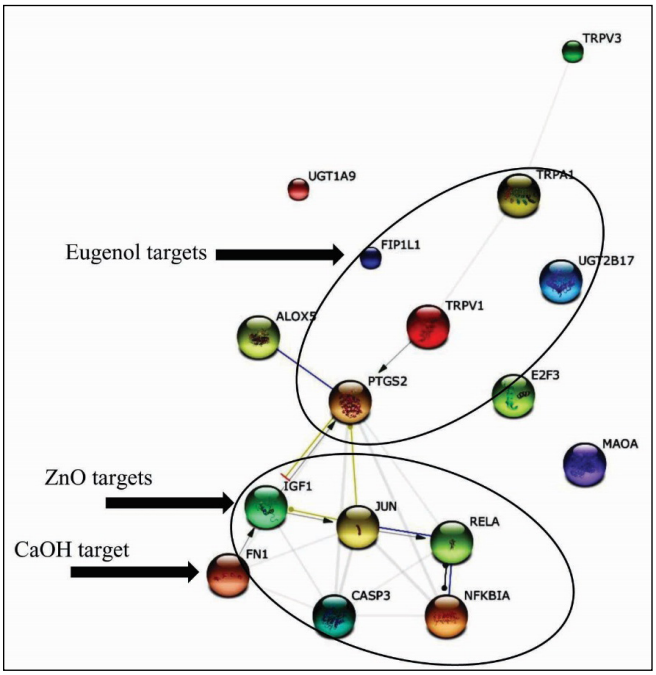


Figure 3: STRING Protein-Protein Interaction network active view demonstrating protein- protein actions for the 16 identified proteins. The color of the circle has no meaning. Circle size represents the amount of data STRING has on the protein. Arrows indicate chemical targets.



REFERENCES

1. Lin LM, Huang GT, Rosenberg PA. Proliferation of epithelial cell rests, formation of apical cysts, and regression of apical cysts after periapical wound healing. Journal of endodontics. 33(8):908-916, 2007.
2. Schwarze T, Leyhausen G, Geurtsen W. Long-term cytocompatibility of various endodontic sealers using a new root canal model. Journal of endodontics. 28(11):749-753, 2002.
3. Geurtsen W, Leinenbach F, Krage T, Leyhausen G. Cytotoxicity of four root canal sealers in permanent 3T3 cells and primary human periodontal ligament fibroblast cultures. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 85(5):592-597, 1998.
4. Guigand M, Pellen-Mussi P, Le Goff A, Vulcain JM, Bonnaure-Mallet M. Evaluation of the cytocompatibility of three endodontic materials. Journal of endodontics. 25(6):419-423, 1999.
5. Huang T-H, Ding S-J, Kao C-T. Biocompatibility of various formula root filling materials for primary teeth. Journal of Biomedical Materials Research Part B: Applied Biomaterials. 80B(2):486-490, 2007.
6. Gorduysus M, Avcu N, Gorduysus O, et al. Cytotoxic effects of four different endodontic materials in human periodontal ligament fibroblasts. Journal of endodontics. 33(12):1450-1454, 2007.
7. Bhat SS, Vidhya M, Sargod S. Radicular cyst associated with endodontically treated deciduous tooth: a case report. Journal of the Indian Society of Pedodontics and Preventive Dentistry. 21(4):139-141, 2003.
8. Rifkin A. The root canal treatment of abscessed primary teeth—a three to four year follow-up. ASDC journal of dentistry for children. 49(6):428-431, 1982.
9. Savage NW, Adkins KF, Weir AV, Grundy GE. An histological study of cystic lesions following pulp therapy in deciduous molars. J Oral Pathol. 15(4):209-212, 1986.
10. Takiguchi M, Fujiwara T, Sobue S, Ooshima T. Radicular cyst associated with a primary molar following pulp therapy: a case report. International journal of paediatric dentistry / the British Paedodontic Society [and] the International Association of Dentistry for Children. 11(6):452-455, 2001.
11. Caldwell RE, Freilich MM, Sandor GK. Two radicular cysts associated with endodontically treated primary teeth: rationale for long-term follow-up. Ont Dent. 76(8):29-33, 1999.
12. Garcia CC, Sempere FV, Diago MP, Bowen EM. The post-endodontic periapical lesion: histologic and etiopathogenic aspects. Med Oral Patol Oral Cir Bucal. 12(8):E585-590, 2007.
13. Moskovitz M, Yahav D, Tickotsky N, Holan G. Long-term follow up of root canal treated primary molars. International journal of paediatric dentistry / the British Paedodontic Society [and] the International Association of Dentistry for Children. 20(3):207-213, 2010.
14. Petel R, Moskovitz M, Tickotsky N, Halabi A, Goldstein J, Houri-Haddad Y. Cytotoxicity and proliferative effects of Iodoform-containing root canal-filling material on RAW 264.7 macrophage and RKO epithelial cell lines. Archives of oral biology. 58(1):75-81, 2013.
15. Kuhn M, Szklarczyk D, Pletscher-Frankild S, et al. STITCH 4: integration of protein-chemical interactions with user data. Nucleic Acids Res. 42(Database issue):D401-407, 2014.
16. Kuhn M, von Mering C, Campillos M, Jensen LJ, Bork P. STITCH: interaction networks of chemicals and proteins. Nucleic Acids Research. 36(suppl 1):D684-D688, 2008.
17. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protocols. 4(1):44-57, 2008.
18. Franceschini A, Szklarczyk D, Frankild S, et al. STRING v9.1: protein-protein interaction networks, with increased coverage and integration. Nucleic Acids Res. 41(Database issue):D808-815, 2013.
19. Takahashi K, MacDonald D, Murayama Y, Kinane D. Cell synthesis, proliferation and apoptosis in human dental periapical lesions analysed by in situ hybridisation and immunohistochemistry. Oral Dis. 5(4):313-320, 1999.
20. Coll JA, Sadrian R. Predicting pulpectomy success and its relationship to exfoliation and succedaneous dentition. Pediatr Dent. 18(1):57-63, 1996.
21. Primosch RE, Ahmadi A, Setzer B, Guelmann M. A retrospective assessment of zinc oxide-eugenol pulpectomies in vital maxillary primary incisors successfully restored with composite resin crowns. Pediatr Dent. 27(6):470-477, 2005.
22. Silva LA, Leonardo MR, Oliveira DS, et al. Histopathological evaluation of root canal filling materials for primary teeth. Braz Dent J. 21(1):38-45, 2010.
23. Huang TH, Ding SJ, Kao CT. Biocompatibility of various formula root filling materials for primary teeth. J Biomed Mater Res B Appl Biomater. 80(2):486-490, 2007.
24. O'Banion MK. Cyclooxygenase-2: molecular biology, pharmacology, and neurobiology. Crit Rev Neurobiol. 13(1):45-82, 1999.
25. Consilvio C, Vincent AM, Feldman EL. Neuroinflammation, COX-2, and ALS—a dual role? Exp Neurol. 187(1):1-10, 2004.
26. Moskovitz M, Tickotsky N, Ashkar H, Holan G. Degree of root resorption after root canal treatment with iodoform-containing filling material in primary molars. Quintessence Int. 43(5):361-368, 2012.