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Validation of Effectiveness of Proteases in Dissolving Dental Pulp Tissue- An in- Vitro Study

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Abstract: *By spot gelatinase assay, minimum inhibitory concentration (MIC) of proteases was determined through pilot study. Then different combinations of proteases solutions (MIC) were used to validate their pulp tissue dissolution capability by following methodology. Pulp tissue was removed from intact, freshly extracted vital premolars and third molars. And same was cut to get an approximately 7mg of tissue for each sample. Total hundred and eight samples of standardized weight (7mg) were taken. These samples were divided into seven groups depending upon different solutions used (Group I & II of three proteases/ Group II & IV of two proteases/ Group V, VI& VII of Collagenase I, NaOCl and Normal Saline respectively) with six subgroups based on different time period i.e. 5, 10, 15, 20, 25, 30 min. Pulp tissue of equal weight (7mg) was placed into each test tube of all groups carrying different solutions of measured volume (1ml each). The solution from each sample was filtered and dried. The residual tissue weight was calculated by filtration method and compared with initial weight to validate tissue dissolution capability. Results showed Group I, Group V and Group VI dissolved the pulp tissue.*

Keywords: *Dental pulp tissue dissolution, Gelatinase assay, Irrigants, Proteases*

I. Introduction

Success in endodontic treatment depends to a great extent upon the practices of mechanical and chemical debridement of the canals. The intricacies of root canal anatomy make it impossible for the instrumentation of the canals to reach all of the fine aspects of its anatomy [1]. Because of anatomical complexities of root canals, organic residues and bacteria located in the dentinal tubules cannot be sufficiently cleaned even after meticulous mechanical procedures [2]. These tissue remnants may provide a source of nutrition for outlived bacteria, consequently, which develops biofilm and ultimately affecting the root canal treatment [3]. Hence, mechanical practices, solitary, unassisted are insufficient in full measure for absolute cleaning of root canal. On account of that, irrigating solutions are expected to support and complement mechanical endodontic preparation. These irrigants expected to flush out dentin debris, dissolve organic tissue, disinfect the canal system and provide lubrication during instrumentation without irritating the surrounding tissues [4]. Some of the irrigants contemporarily used includes sodium hypochlorite (NaOCl), hydrogen peroxide, physiologic saline, water, chlorhexidine. Apart from listed irrigants, Sodium hypochlorite, because of its tissue dissolution ability and antimicrobial action, is 'the irrigant of choice' in endodontics [5] . Though, it shows excellent solvent properties against vital, necrotic and fixed tissues and also an effective antimicrobial agent, its concomitant, universally recognized untoward effects like tissue necrosis, violent tissue response and most importantly inability to remove smear layer cannot be ignored [6].

Owing to the adverse effects of aforesaid 'the irrigant of choice', there is absolute need of the alternative appropriate irrigant which fulfils all the expectations and concurrently without any sort of adverse effect.

This study ab origine, planned to use proteases like collagenase etc for dissolving the vital dental pulp tissue and determine the presumptive effective concentration of proteases in dissolving dental pulp tissue.

II. Materials And Methods

Present study was conducted in the department of conservative dentistry and endodontics of BVDU Dental College and hospital, Pune and in the Interactive Research for Health Affairs (IRSHA) of Bharati VidyapeethDeemed University, Pune.

The protocol of this study was approved and permission was taken from the Research and Ethics Committee. For validation of the presumptive efficacy of proteases i.e. Collagenase I, Dispase and Trypsin and

their combination in dissolving dental pulp tissue we had to determine the effective concentration and combination of proteases and time taken by proteases in dissolving dental pulp tissue.

For validation of proteases in dissolving pulp tissue this study was done in two phases. Gelatinase spot assay [7] to determine minimum inhibitory concentration (MIC) of proteases. Time taken by various combinations of proteases (MIC) in dissolving dental pulp tissue by filtration method was evaluated.

2.1 Gelatinase spot assay for proteases to determine Minimum Inhibitory concentration (MIC) of proteases:

2.1.1 Proteases obtained and solutions prepared:

i) Collagenase I (clostridium hystolyticum) ----Sigma Aldrich
ii) Dispase (Bacillus polymixa) ------- --Sigma Aldrich
iii) Trypsin (Bovine Pancreas) ---- ----Sigma Aldrich

The stock solutions of all proteases were prepared i.e. Collagenase I, Dispase and Trypsin and their minimum concentration was determined by gelatinase spot assay method [7]. Proteases solutions prepared by adding 1 mg of proteases to 1 ml of 50 mM Tris buffer that gives 1mg/ml solution.

2.1.2 Gelatinase spot assay

The gelatinase activities of proteases (Collagenase I, Dispase, Trypsin) were evaluated using x-ray film based assay. Collagenase I, Dispase and Trypsin (50 mM Tris buffer, pH 6.8) were assayed with different concentrations for 5min. This procedure was done on three separate x-ray films, one for each protease. Serial dilutions of each proteases were taken like 1mg, 0.5, 0.25, 0.125....and 2µl spots of each protease were pipette and placed onto x-ray film in duplicate and incubated for 5min. at room temperature. After exposure for 5 min., films were washed to visualize proteases activity as indicated by clear spots of hydrolysed gelatin on x-ray film.

2.2 Evaluation of time taken by various combinations of proteases (with MIC) in dissolving dental pulp tissue by filtration method.

In this study, human dental pulp tissue samples were standardised and divided into groups according to the different combinations of proteases. Sample was placed in each test tube carrying protease solutions of measured volume and kept for specified time interval. The weight loss of sample was calculated by filtration method.

Freshly extracted vital molars and premolars were collected at the department of oral and maxillofacial surgery and freeze at 20°c. until further use. Two longitudinal grooves were prepared on proximal surfaces of the teeth with round bur without reaching the pulp. The teeth were split into two halves with chisel and mallet [8]. The pulp tissue was removed carefully in Toto. The pulp tissue was cut into small pieces with the 15 no. scalpel blade and placed on preweighed filter paper. The weight of the pulp tissue was standardized to 7mg. on analytic balance.

Groups: Hundred and eight samples of standardized weight (7mg) were taken. The samples were divided into different groups of proteases solutions i.e. the MIC of proteases with different combinations and subgroups of different time period i.e. 5, 10, 15, 20, 25, 30 min.

Group I = Collagenase I +Trypsin +Dispase = 1:1:1, Group II = Collagenase I+Trypsin +Dispase = 2:1:0.5, Group III = Collagenase I+Trypsin = 1:1, Group IV = Collagenase I +Dispase = 1:1, Group V = Only Collagenase I , GroupVI = 5 % sodium hypochlorite, GroupVII = Normal saline

Each group had 6 subgroups having 3 test tubes for each time period for which the sample is immersed in the irrigant.

Standardized pulp tissue (7mg) was placed into each test tube that contains cocktail of proteases as well as NaOCl of measured volume (1ml) and mixed by vortexing. The pulp tissues were immersed in the respective solutions of cocktail of proteases as well as NaOCl for specified time, and then it was filtered through preweighed Wattman filter paper No. 1. This was followed by drying at 37 °C in incubator and weight of dried filter paper was measured. The difference in weight of dried filter paper (with residue) and initial filter paper (before filtration) gave us the dry weight of residue left subsequent to filtration which is the sum of weight of pulp tissue residue and solution residue.

Each solution (1ml) was filtered through a preweighed filter paper this was followed by drying of paper at 37° C. Weight of dried filter paper was taken. The difference in the weight of dried filter paper and initial paper gave the dry weight of residue of that solution. This procedure was repeated for all groups.

Weight of residual pulp tissue left on filter paper of each sample was calculated by subtracting the weight of residue of respective solution from weight of total residue on filter paper, measured earlier. This procedure was repeated for all the samples for three times. Weight of wattman filter paper was measured on an analytic balance. Amount of pulp dissolved = Wt. of pulp tissue before immersion – wt. of residual pulp

Thus by filtration method, amount of pulp dissolved by various solutions used at different time interval was measured quantitatively. Wt. Loss % = initial wt. – final wt. / initial wt. × 100 [9]

III Results

3.1 Gelatinase assay results

In this assay, protease activity as indicated by clear spots of hydrolyzed gelatin on x-ray film [7]. Minimum concentration which gave maximum proteases activity was taken as minimum inhibitory concentration for pulp dissolution study. From this assay we got the minimum inhibitory concentrations (MIC) of proteases i.e. Collagenase – 0.125, Dispase – 0 .125, Trypsin - 0 .0078. Results shown in Fig.1 A, B, C

Figure 1 (A) Collagenase I activity showing clear spot on x-ray film

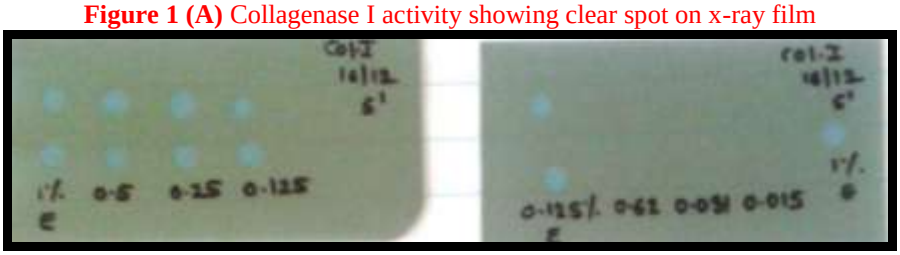

Figure 1 (B)Trypsin activity showing clear spot on x-ray film

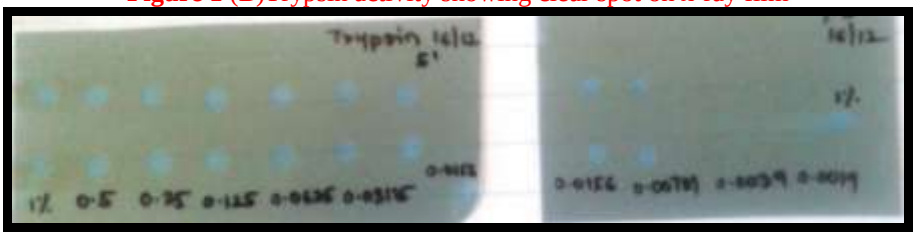

Figure 1 (C) Dispase activity showing clear spot on x-ray film

3.2 Pulp Tissue Dissolution By Filtration Method

Filtration method for pulp dissolution showed that only groups Group I (Collagenase I +Trypsin +Dispase = 1:1:1), Group V (Only Collagenase I) and GroupVI (5 % sodium hypochlorite) were capable of tissue dissolution. No other groups showed tissue dissolution. Group I and Group V showed increased pulp tissue dissolution with passage of time and maximum pulp tissue dissolution activity at 20min. 54.75% and 41.25% respectively, but their pulp dissolution capacity decreased at 25 and 30 min. No dissolution of pulp tissue by saline.

Results are presented in Table 1, 2and 3.

Table 1: Average weight loss of pulp tissue in Group I (Collagenase I +Trypsin +Dispase = 1:1:1)

	Group I (Collagenase I +Trypsin +Dispase = 1:1:1) % weight loss of pulp tissue			
Minutes	I	II	III	Avg % wt. loss
5	9.37	15.6	13.9	12.96
10	15	17.08	23.26	18.44
15	26.45	43.33	32.15	34
20	52.7	47.99	63.56	54.75
25	17.5	33.01	36	28.83
30	6.25	13.3	21.02	13.52

Table 2: Average weight loss of pulp tissue in Group V (Collagenase I)

	Group V (Collagenase I) % weight loss of pulp tissue			
Minutes	I	II	III	Avg % wt. loss
5	28.02	23.90	24.08	25.33
10	33.21	30.01	31.03	31.41
15	36.52	32.88	32.7	34.04
20	41.95	38.62	43.20	41.25
25	22.96	18.54	18.75	20.08
30	0	0	0	0

Table 3: Comparison of average weight loss of pulp tissue in Group I and Group V

	Comparison of average weight loss of pulp tissue in Group I and Group V	
	Group I (Combination of proteases)	Group V (Only collagenase I)
Minutes	Avg % wt. loss	Avg % wt. loss
5	12.96	25.33
10	18.44	31.41
15	34	34.04
20	54.75	41.25
25	28.83	20.08
30	13.52	0

IV. Discussion

The success in endodontic treatment depends on the chemo-mechanical debridement of canals [10]. Anatomical complexities of root canals make it impossible for mechanical procedures alone to remove remnants of pulp tissue so complete debridement and disinfection of root canal with irrigant is very important [11]. Callahan and Grossman demonstrated the importance of solvent ability of endodontic irrigants and emphasized the elimination of pulp tissue from root canal [12].

In the studies assessing the tissue dissolving ability of sodium hypochlorite and other irrigants , the tissues from number of different sources have been used. Bovine muscle tissue [13] porcine muscle tissue [14] rabbit liver [15], rat connective tissue [16], pig palatal mucosa[17], bovine pulp [18], human umbilical cord [19], have been used to determine tissue dissolution ability of different irrigants. Very few studies have been done on direct solubilizing action of irrigants on human dental pulp tissue. Because of the use of different tissues and other variables other than dental pulp tissue, results of these studies cannot be accurately compared or related directly to the clinical conditions [20] so we used vital human dental pulp tissue and its dissolution by various solutions.

Components of human dental pulp tissue are cells, those are fibroblasts, odontoblasts, undifferentiated mesenchymal cells, defense cells, ground substances, and neurovascular structure embedded in the matrix. This matrix composed of collagen fibers which forms a reticular network, support the body of the pulp and other structural element of the pulp thus forms the integrity of pulp organ [21]. Collagen has great tensile strength which makes firm foundation to all contents of pulp; it is the integral part of maintenance of whole structure. If we break or dissolve this collagen complete structure will collapse and destroy this foundation i.e. dissolution of pulp tissue so we thought of using solution that dissolve the collagen.

Various in-vitro studies [22, 23, and 24] have used proteases for isolation of pulp fibroblast cells. This process involves dissolution of extracellular collagen matrix before the cells can be isolated. Various methods have been proposed for obtaining pulpal cells for culture. These methods comprise two major categories these are (1) explants (outgrowth) and (2) enzymatic digestion (dissociation) method.

Enzymatic digestion method (i.e. dissolution of strong collagen by using collagenase, dispase and trypsin etc.) is a common method to obtain single cell suspension from primary tissues and consist of exposing the tissue to enzymes for minimum period of time in order to preserve maximum cell viability [25]. Hence, in this study we had decided and planned to use proteases like collagenase, dispase and trypsin for dissolving dental pulp tissue.

Before using proteases for dissolution of pulp tissue it was necessary to determine their minimum inhibitory concentrations. The gelatinase activity of proteases (viz. Collagenase I, Dispase and Trypsin) were measured by spot gelatinase assay, as gelatin is a substrate for many proteases. Spot gelatinase assay is a validated x-ray film based assay used to measure gelatinase activity of proteases thus minimum inhibitory concentration (MIC) of proteases was determined by assay through pilot study.

Then different combinations of proteases solutions (with MIC) were used to validate their pulp tissue dissolution capability by filtration method. Equal amount of pulp tissue was obtained for standardization and was immersed in test tube containing specific solutions for respective time periods. The solutions from all sample test tubes were filtered through preweighed filter paper and dried to obtain residue left after filtration. In pilot study we found that solutions itself leaves considerable residue, which needs to be reduced from the total residue obtained in order to get accurate reading of the residual pulp. In our study readings were taken and weight of residual pulp was obtained after subtracting weight of residue of irrigant from total residue. We used analytical balance in our study for measurement of weight because of its high degree of precision.

Results of our study showed that only groups, Group I (Collagenase I +Trypsin +Dispase = 1:1:1), Group V (Only Collagenase I) and GroupVI (5 % sodium hypochlorite) were capable of tissue dissolution. No other groups showed tissue dissolution. Group I and Group V showed increased pulp tissue dissolution with passage of time and maximum pulp tissue dissolution activity at 20min. 54.75% and 41.25% respectively. Group I and Group V showed the tissue dissolution as they might be stable at that combination and particular time and showed more activity on collagen fibers of pulp. But their pulp dissolution capacity decreased at 25 and 30 min., this might be because proteases are not stable and their activity reduced after longer period of time.

Sodium hypochlorite showed complete pulp tissue dissolution at all the time period. This is because ionization of NaOCl to liberate hypochlorous acid (HOCL) and hydroxyl ions. When hydroxyl ion levels decreases as a result of saponification and amino acid neutralization reactions, the pH also decreases thereby favoring the formation of HOCL molecules. The chloramination reaction initiated which results in degradation and hydrolysis of amino acids [26]. This result is in agreement with Rosenfield [27, 28] who reported 5% NaOCl as an effective solvent of human pulp tissue.

Results of our study showed no dissolution of pulp tissue by saline as it is ineffective solvent of vital tooth pulp. Further clinical studies are needed to substantiate the findings of this study.

V. Conclusions

1) Group I (Collagenase I +Trypsin +Dispase = 1:1:1), Group V (Only Collagenase I) and GroupVI (5% sodium hypochlorite) were capable of tissue dissolution. No other groups showed tissue dissolution.
2) Sodium hypochlorite showed complete pulp tissue dissolution at all the time period.
3) Results of our study showed no dissolution of pulp tissue by saline as it is ineffective solvent of vital tooth pulp.
4) Group I and Group V showed increased pulp tissue dissolution with passage of time and maximum pulp tissue dissolution activity at 20min., but pulp dissolution capacity decreased at 25 and 30 min.
5) Further clinical studies and research are needed to substantiate the findings of this study.

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