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Effect Of Alprogen As Compared To Subantimicrobial Dose Of Doxycycline On Salivary Collagenases

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ABSTRACT
Periodontitis is treated by sub antimicrobial drugs like Chlorhexidine, Tetracycline and doxycycline. Patients suffer in taking drugs. Periodontitis is the bone destruction in the periodontal cavity along with gingivitis. There is also the presence of matrix metalloproteinases especially salivary collagenases and gelatinases in periodontal disease. Recently, periodontitists are focusing metalloproteinases for treating the bone destruction with the gingival inflammation. By using the drugs, the effect of MMPs was reduced to an extent. Alprogen ,a single component in aloe vera plant has remarkable effect on Periodontal disease .An attempt was made to study in Periodontal disease comparing with the regular drug of choice.

1. INTRODUCTION

The periodontal diseases causes destruction in the susceptible host.[1] The host reacts defensive mechanisms to eliminate the pathogens.[2] Matrix Metallo-Proteinases (MMPs) acts in tissue destruction and degrades denatured interstitial collagens. Periodontal treatment was by removal of infectious agents. Host modulatory therapy with periodontal therapies like SRP and smoking cessation therapy is a comprehensive treatment strategy for periodontitis. Till date, Subantimicrobial Dose Doxycycline (SDD) is used for systemic therapy is a host response modifier. Other antibiotics like tetracyclines and chlorhexidine mouth rinse are used. When MMPs are concerned, the inhibitors are less in number. [2]Keeping in mind the facts, the study was designed to evaluate the anti-inflammatory effect of alprogen from Aloe vera on MMP-2,1,8 and MMP-9 activities and compared with SDD, chlorhexidine and tetracycline.

2. MATERIALS AND METHODS

Fresh aloe vera leaves (10 kg) for purification were crushed in blender (LG Electric Co., Chennai, Tamil Nadu) with 1/3 volumes of extraction buffers (50 mM sodium phosphate, pH 8.0,1.44 mM 2-mercaptoethanol, 1% polyvinylpyrolidine, and 1 mMEDTA). The slurries were taken and filtered through cheese cloth and centrifuged at 10,000g for 30min at 4°C. The supernatant of the extracts was precipitated with 25 to 80% ammonium sulfate saturation. The precipitate was dissolved in 20 mMTris-HCl (pH 8.0) buffer and desalted by Sephadex G-25 column (8.0 3 80 cm) equilibrated with the same buffer. The desalted extracts were applied to a DEAE Sephacel column (3.4 3 30 cm) equilibrated with 20 mMTris-HCl(pH 8.0) buffer. The column was washed .[3] Proteins bounds were eluted with 2 M NaCl were pooled, dialyzed, and adjusted to 0.5 M NaCl in 20 mMTris-HCl (pH 7.4).Then they were chromatographyed through

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column of concanavalin A-Sepharose (3.4 3 26 cm) equilibrated and washed with 20 mMTris-HCl (pH 7.4) and 0.5 M NaCl. Theeluates (negative charged proteins in the pH 8.0 precipitate) were recovered in the pass-through fraction and concentrated by Ultra-filtration and dialyzed by 20 mMTris-HCl (pH 7.4) buffer. Bound glycoproteins were eluted with a 0.5 M methyl-a-D-mannopyranoside and dialyzed against 20 mMTris-HCl (pH 7.4) buffer, and applied to a POROS 50 HQ anion exchange column equilibrated with the same buffer.Then the proteins were eluted at the flow rate of 4 ml/min with a 400-ml linear gradient of 0.0 to 0.5 NaCl. Fractions with antihistamine activity were concentrated to 1.5 ml using AmiconCentriplus 10 concentrator (Amicon, Beverly, MA). [3] It was then filtered through Superdex75 column with 20 mMTris-HCl (pH 7.4) buffer containing 50 mMNaCl with flow rate 3.0 ml/min. Fractions with antihistamine activity were applied to a phenyl Sepharose CL-4B hydrophobic interaction chromatography column (2 ml of total volume) equilibrated with 20 mMTris-HCl (pH 7.4) buffer containing0.5M (NH4)2SO4. After washing proteins were detected, the column was eluted with the flow rate of 0.4 ml/min with 40 ml decreasing linear gradient of 0.5 to 0 M(NH4)2SO4 in the buffer. Fractions with antihistamine activity was collected, and Alprogen was isolated. [3]

ZYMOGRAPHY (isolation of salivary collagenases) Salivary collagenases extracted from saliva sample was stored in PBS buffer solution in -80 degree. The saliva was centrifuged in 10000 rpm.The precipitate was washed in PBS buffer and stored at -7 degree and RIPA (radioimmuno precipitation assay) buffer was added and spun at 1000rpm.The supernatant obtained was stored at -4 degree. [4]

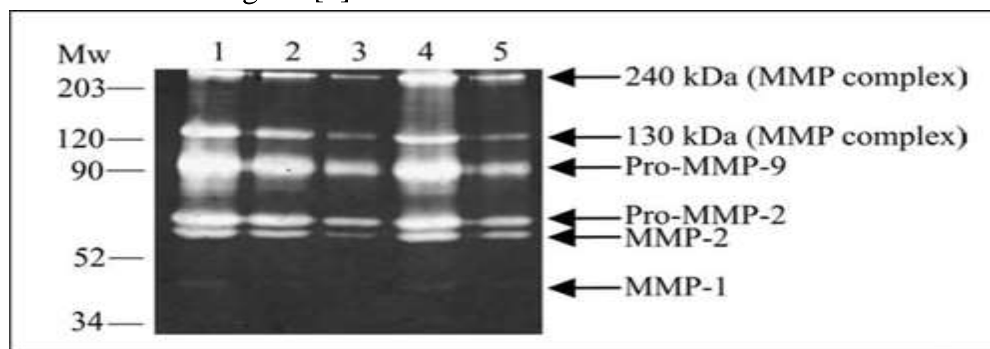

Fig – 1 **Gel Preparation**

The standard method was followed from Marta Toth and Rafael Fridman guidelines (PMCID: PMC3845455). The separating gel was prepared and poured between the electrophoresis plates up to 2 cm from the top and Overlayed with watersaturated n-butanol to polymerize for 30 min at room temperature. [4] The Stacking Gel n-butanol was removed from the gel and gently rinsed with water. The stacking gel was placed on top of the separating gel with comb inserted. Stacking gel was allowed to polymerize approximately for 20 min at room temperature. [5]

2.1.Loading and Electrophoresis

The well comb was removed from the gel and gently rinsed with 1X running buffer. The zymography gel in the electrophoresis setup was installed. The inside chamber was filled with

1X running buffer and the outside chamber with 500 mL 1X running buffer. Then 10 µL of 2X sample buffer was placed on a Parafilm membrane and 10 µL of sample was added. Using gel loading pipette tips and with protein molecular marker it was loaded one well per gel. The gel was made to run at 125 V for 90 min until the dye reaches the bottom. [5] Proteolytic activity was examined by the standardized protocol of zymography. After electrophoresis the gels were cut into strips and was incubated in Tris- $CaCl_2$ containing the natural extract for 16 hours at 37ºC. after that it was washed in triton – X. Quantification was checked. [5] Mmp Activity Assay Levels of the active form of MMP-2 and MMP-9 were measured by an activity assay (Biotrak MMP-2 activity assay and Biotrak MMP-9 activity assay, GE Healthcare) according to the manufacturer's protocol.Supernatants of treated cells were added to the anti-MMP-2 (respectively anti-MMP-9) precoated microplate wells and incubated overnight at 4 °C. The following day, the plates were rinsed 4 times with washing buffer and incubated with the assay buffer in addition to the substrate and detection reagent. The plates were analyzed immediately after the addition of the detection reagent (a dection enzyme that is activated by captured active MMP-2 (respectively MMP-9) through a single proteolytic event, which can be measured using a specific chromogenic peptide substrate) for the baseline measurement and again after 3-6 h for signal recording in a spectrophotometer (ThermoFisher Nanodrop). Standard curves were obtained for each activity assay. [6,7]

3. RESULTS

Alprogen purified showed as a single band by SDS-PAGE and the molecular mass was estimated to be approximately 10.0 kDa on SDS-PAGE. The isoelectric point (pI) value for alprogen is 6.0 by using ampholine polyacrylamide gels. Further characterization is essential[1,8,9,10] Zymography Gel zymogram depicting differences in (pro-)MMP-2 and (pro-)MMP-9 content among discrete samples of saliva. After activation, the gel was stained with Coomassie blue. [9,10] The samples in the individual lanes are: pro – MMP9, pro – MMP2, MMP-2 and MMP-1 (Fig -1)

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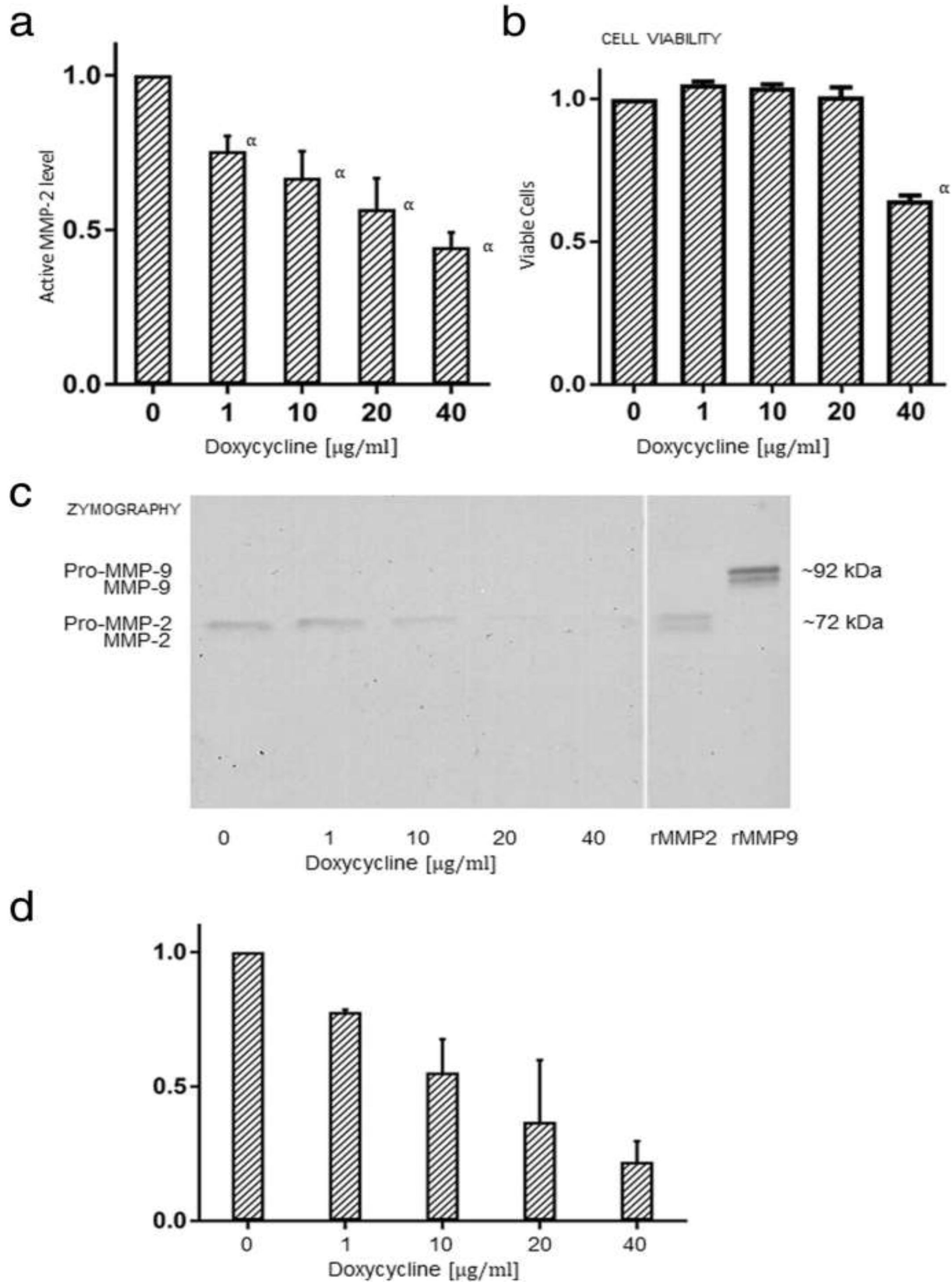

PARTICULARS	DOXYCYCLINE	ALPROGEN
RANGE	78-85	0-65
MEDIAN	82.1	20.9
STANDARD DEVIATION	2.08	16.5

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Range, mean value and standard deviations for doxycycline and alprogen for inhibition of MMP-2 ,MMP-1, MMP-9 percentage

4. DISCUSSION

The doxycycline therapy is used in the treatment of the diseases periodontitis,. Subantimicrobial-dose doxycycline (Periostat® 20 mg) used in periodontitis MMP-2 and MMP-9 (gelatinases) are distributed and identified in fibroblasts, keratinocytes, endothelial cells, monocytes/macrophages, osteoblasts and chondrocytes. Various studies exhibited the relationship between MMPs and periodontal diseases. MMP-2 and MMP-9 could participate in tissue destruction in periodontitis. The activity of MMPs is regulated by inhibitors like tissue inhibitors of metalloproteinases, tetracyclines, doxycycline. Tetracycline's is an adjunct in the treatment of periodontal diseases. Its inhibitory effect on collagenase involves the drug's ability to bind metal ions like Ca^{2+} and Zn^{2+} are required by the enzyme to maintain conformation and hydrolytic activity. This was supported by the observation that Ca2+ can overcome the inhibition of the enzyme by the tetracycline. In recent report, anti-proteolytic effects are by both direct inhibition of MMPs and inhibition of expression. Direct inhibition appears to be mediated by an interaction between the tetracycline molecule and metal ions within the MMP; the mechanism of inhibition is dependent on chelation of structural metals rather than chelation of the active site Zn2+. Doxycycline has a high affinity toward Zn2+ when compared with tetracycline. Hence, doxycycline is more potent than tetracycline's. Today , a shift is towards alternative therapies. Ayurvedic drugs such as Neem, Triphala, Bakul, have been used therapeutically in ancient times to treat diseases, including periodontal diseases. As these drugs have minimal side-effects and cost-effectiveness, their use has increased in recent times. In an in vitro study the ability of a blackcurrant extract and its major anthocyanins to inhibit the activity of MMPs was investigated. MMP-1 and MMP-9 were significantly inhibited by pure anthocyanins. This suggests that such natural compounds may represent promising agents for use in adjunctive treatments for periodontitis as well. The current study also evaluated the inhibitory effect of Alprogen on MMP-2 and MMP-9. Aloe vera, which has aloin as its active component, has anti-inflammatory, antimicrobial, immunostimulatory properties and is also an antioxidant. Barrantes and Guinea showed that aloe gels and aloins are effective inhibitors of stimulated granulocyte MMPs. Aloe vera has various therapeutic uses. The anti-inflammatory activities of Alprogen in periodontal therapy requires attention. In a recent study, Bhat et al.evaluated the effect of Aloe vera in periodontal conditions. The results showed improvement in plaque index and probing depth. The results of our trial, show mean inhibition of MMP-2 by doxycycline as 82.1% and of Alprogen as 20.9%. When the mean difference of inhibition of MMP2 by doxycycline and Alprogen were compared by using Student's paired t-test.

5. CONCLUSION

This was a pioneering in vitro study to evaluate the efficacy of alprogen on MMP-2 and MMP-9 in periodontitis. The results of this study is promising and has established a fact that alprogen

have inhibiting effects on MMP-2 and MMP-9. Having established the efficacy of these agents in vitro, it would only be fair in the interest of science, to further research their efficacy in clinical settings.

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REFERENCES

1. Birkedal-Hansen H, Moore WG, Bodden MK, Windsor LJ, Birkedal-Hansen B, DeCarlo A, Engler JA. Matrix metalloproteinases: a review. Crit Rev Oral Biol Med., 1993; 4: 197–250. [PubMed] [Google Scholar]

2. Liotta LA, Steeg PS, Stetler-Stevenson WG. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. Cell., 1991; 64: 327– 336. [PubMed] [Google Scholar]

3. Inhibitory mechanism of aloe single component (alprogen) on mediator release in guinea pig lung mast cells activated with specific antigen-antibody reactions,j y ro 1 , b c lee, j y kim, y j chung, m h chung, s k lee, t h jo, k h kim, y i park, pmid: 10604937.

4. Nagase H. Activation mechanisms of matrix metalloproteinases. Biol Chem., 1997; 378: 151–160. [PubMed] [Google Scholar]

5. Murphy G, Willenbrock F. Tissue inhibitors of matrix metalloendopeptidases. Methods Enzymol, 1995; 248: 496–510. [PubMed] [Google Scholar]

6. Collier IE, Wilhelm SM, Eisen AZ, Marmer BL, Grant GA, Seltzer JL, et al. H-ras oncogene-transformed human bronchial epithelial cells (TBE-1) secrete a single metalloprotease capable of degrading basement membrane collagen. J Biol Chem., 1988; 263: 6579–6587. [PubMed] [Google Scholar]

7. The inhibitory mechanism of rebamipide on the mediator release in the guinea pig lung mast cells activated with specific antigen-antibody reactions. Ro JY, Kim JY, Kim KH. Pharmacology, 2001; 63(3): 175-84. doi: 10.1159/000056130.PMID: 11598424

8. The effects of cromakalim on the mediator releases from guinea pig lung mast cell activated by specific antigen-antibody reactions.Ro JY, Yim YN, Kim KH.Yonsei Med J., Oct, 1996; 37(5): 325-38. doi: 10.3349/ymj.1996.37.5.325.PMID: 8997165

9. Eupatilin blocks mediator release via tyrosine kinase inhibition in activated guinea pig lung mast cells. Kim JY, Kwon EY, Lee YS, Kim WB, Ro JY.J Toxicol Environ Health A., Dec 10, 2005; 68(23-24): 2063-80. doi: 10.1080/15287390500177024.PMID: 16326424 10. Stetler-Stevenson WG. Progelatinase A activation during tumor cell invasion. Invas Metastas, 1994; 14: 259–268. [PubMed] [Google Scholar]

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11. Himelstein BP, Canete-Soler R, Bernhard EJ, Dilks DW, Muschel RJ. Metalloproteinases in tumor progression: the contribution of MMP-9. Invas Metastas, 1994; 14: 246– 258. [PubMed] [Google Scholar]
12. Murphy G, Crabbe T. Gelatinases A and B. Methods Enzymol, 1995; 248: 470– 484. [PubMed] [Google Scholar]
13. Heussen C, Dowdle EB. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. Anal Biochem, 1980; 102: 196–202. [PubMed] [Google Scholar]
14. Woessner JF., Jr Quantification of matrix metalloproteinases in tissue samples. Methods Enzymol, 1995; 248: 510–528. [PubMed] [Google Scholar]
15. Azzam HS, Arand G, Lippman ME, Thompson EW. Association of MMP-2 activation potential with metastatic progression in human breast cancer cell lines independent of MMP-2 production. J Natl Cancer Inst., 1993; 85: 1758–1764. [PubMed] [Google Scholar]
16. Lewalle JM, Munaut C, Pichot B, Cataldo D, Baramova E, Foidart JM. Plasma membrane-dependent activation of gelatinase A in human vascular endothelial cells. J Cell Physiol., 1995; 165: 475–483. [PubMed] [Google Scholar]
17. Fridman R, Fuerst TR, Bird RE, Hoyhtya M, Oelkuct M, Kraus S, et al. Domain structure of human 72-kDa gelatinase/type IV collagenase. Characterization of proteolytic activity and identification of the tissue inhibitor of metalloproteinase-2 (TIMP-2) binding regions. J Biol Chem., 1992; 267: 15398–15405. [PubMed] [Google Scholar]
18. O'Connell JP, Willenbrock F, Docherty AJ, Eaton D, Murphy G. Analysis of the role of the COOH-terminal domain in the activation, proteolytic activity, and tissue inhibitor of metalloproteinase interactions of gelatinase B. J Biol Chem., 1994; 269: 14967– 14973. [PubMed] [Google Scholar]
19. Toth M, Gervasi DC, Fridman R. Phorbol ester-induced cell surface association of matrix metalloproteinase-9 in human MCF10A breast epithelial cells. Cancer Res., 1997; 57: 3159–3167. [PubMed] [Google Scholar]