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The Role of Proanthocyanidins on Neutrophils in Health and Chronic Periodontitis

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Abstract

Background: Free radicals produced by inflammatory cells like neutrophils in response to pathogens play a major role in tissue destruction observed during periodontitis. Proanthocyanidins from *Vitis vinefera* or Grape seed extract (GSE) have been reported as a potent antioxidant (AO) against oxidative stress. Their AO activity has been tested using blood neutrophils but not salivary neutrophils from chronic periodontitis (CP) patients, making this the first study of its kind. This study aimed to evaluate and compare the AO effect of proanthocyanidins on respiratory burst of human neutrophils in periodontally healthy versus CP patients.

Methods: A case control in-vitro study was conducted from 2008 to 2009 among patients visiting the dental college. Saliva rinse samples were obtained and neutrophils isolated from 60 individuals divided into periodontally healthy (Group A) and CP patients (Group B). Samples were incubated with equal concentrations of nitroblue tetrazolium (NBT), without GSE (control) and with 5µg/mL, 10µg/mL and 15µg/mL of GSE. Colour change was read spectrophotometrically at 560 nm. Student's unpaired t- test was used for analysis with statistical significance set at $p<0.05$.

Results: Inhibition of NBT reduction increased with increasing concentrations of GSE. Effective concentrations were 10µg/ml and 15µg/ml GSE in healthy and 15µg/ml GSE in chronic periodontitis. Chronic periodontitis group showed greater NBT reduction implying greater oxidative stress.

Conclusion: Proanthocyanidins from GSE provided a dose-dependent inhibitory effect on the respiratory burst of salivary neutrophils. Higher concentrations of GSE were needed in CP patients

Keywords: Antioxidants; Chronic periodontitis; Nitroblue tetrazolium assay, Proanthocyanidins; *Vitis vinifera*

INTRODUCTION

Chronic periodontitis (CP) causes progressive destruction of the tooth supporting structures, resulting from complex interactions between host's immune response and pathogenic microbes.[1,2] Bacterial stimulation causes neutrophils, in the gingival sulcular tissues and saliva, to produce free radicals (FR) and reactive oxygen species like superoxide via respiratory burst creating heightened oxidative damage to periodontal tissues.[4, 5]

Imbalance in the body's antioxidant (AO) mechanisms leads to FR-induced oxidative stress (OS) requiring externally administered AO. Higher costs and numerous side effects of commercially available AO are shifting the paradigm towards natural products.[6] *Vitis vinifera* (grapes) seeds are one such natural source of AO. Grape seed oligomeric proanthocyanidins (OPCs) are the most potent natural AO, several folds more active than dietary AO like vitamins C and E.[9-11] OPC can neutralize FR by

inhibiting the activity of xanthine oxidase as well as hyaluronidase, elastase, collagenase and β-glucuronidase involved in CP pathogenesis.[10-12]

This warrants evaluation of the AO property of OPCs. Their direct superoxide-scavenging activity was assessed using nitroblue tetrazolium (NBT) reduction assay.[13-16] NBT offers the advantage of high sensitivity, ready availability, ease of performance and low cost.[13-16] For greater ease, objectivity and to minimize observer bias, spectrophotometry was used.[18] Mitigation of NBT reduction by GSE has been tested using blood neutrophils but not salivary neutrophils from CP patients, making this the first study of its kind.[12]

Thus, this study aimed to evaluate and compare the effect of proanthocyanidins from *Vitis vinifera* seeds on the respiratory burst of human neutrophils in the saliva of healthy and CP patients.

METHODS:

This case control in-vitro study was conducted at KLE V.K Institute of Dental Sciences, Belgaum, India after obtaining ethical clearance from the Institutional Review Board.

The study enrolled 60 individuals aged 25-45 years, of either gender after obtaining written informed consent. Among them 30 individuals were periodontally healthy (Group A) (Figure 1) and 30 were diagnosed with Chronic Periodontitis (Group B) (Figure 2) as per Ramfjord's Periodontal Disease Index (PDI).[19] Individuals with systemic diseases in the past 3 months, pregnant women, those with regular use of antibiotics and/or anti- inflammatory drugs, as well as those who used smoke/smokeless tobacco and betel nut were excluded. The study was carried out by a single observer who was blinded to the study groups.

Subjects were asked to rinse their mouth with 20ml of tap water for 5 seconds and expectorate. Each patient was then asked to rinse with 15ml of Hank's balanced salt solution (HIMEDIA LABS), swish the solution for 30s and expectorated into a polypropylene vessel. This sequence was repeated without interruption for three consecutive times. The samples were then sent for microbiological analysis and were processed within 24hrs under the supervision of a qualified microbiologist.

The collected solution was stirred for 10 min and then centrifuged for 10 min at 200rpm. The sediment was washed once by re- suspension in Hank's balanced salt solution and re- centrifuged. The resulting pellet consisted of PMNs, epithelial cells and debris. This pellet was passed through 20µm nylon mesh to separate PMNs from epithelial cells and debris.

The resultant pellet was divided into four equal parts (1 ml each) and placed in four test tubes. NBT (0.1%) (Journal of Andrology 2003) and different concentrations of GSE were added to the test tubes as follows

Control test tube (TC): 1ml pellet + 1 ml DMSO (0.5%) + 1 ml NBT (0.1%)

Test tube 1 (T1): 1 ml pellet + 5µg/ ml GSE + 1 ml NBT

Test tube 2 (T2): 1 ml pellet + 10µg/ ml GSE + 1 ml NBT

Test tube 3 (T3): 1 ml pellet + 15µg/ ml GSE + 1 ml NBT

The test tubes were incubated at 37^0 C for 30 minutes. (Journal of Andrology 2003)

The colour change from yellow nitroblue tetrazolium to blue black formazan (Figure 3) was read at 560 nm in a spectrophotometer (Shimadzu UV-1700 UV Pharmaspec Spectrophotometer). (Figure 4).

Hank's Balanced Salt Solution (HBSS), GSE solution and NBT solution were prepared according to study instructions.

For HBSS, 9.8g of Hank's balanced salt (HiMedia Laboratories, Mumbai, India) was suspended in 900 mL tissue culture grade water, with constant gentle stirring until the powder was completely dissolved. To this, 0.35g of sodium bicarbonate powder was added and stirred until dissolved. The pH was adjusted to 0.2-0.3 pH units below the desired pH using 1N hydrogen chloride or 1N sodium hydroxide since the pH tends to rise during filtration. The final volume was made up to 1000 mL with tissue grade water. The solution was filtered through a sterile membrane filter with a porosity of 0.22 micron or less. The desired amount was aseptically dispensed into sterile containers and stored in dark at 2ºC - 8ºC until use.

For Grape Seed Extract solution, a standardized GSE containing 95% OPC fraction was commercially

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obtained. 10mg of this GSE was dissolved in 10 mL of 0.5% dimethyl sulfoxide (DMSO) solution (containing 1mL DMSO in 200 mL water). 0.5 mL, 1 mL and 1.5 mL of this solution were dissolved in 0.5% DMSO separately to make the final volume up to 100 mL each, such that three different GSE solutions were obtained with GSE concentrations of 5 µg/mL, 10 µg/mL and 15µg/mL respectively. Concentrations above 15µg/mL showed cytotoxic effects on the cells.[10]

NBT solution (0.1%) was prepared by adding 10 mg (1 tablet) of NBT (Sigma Laboratories) to 100 mL of phosphate buffer saline (pH 7.2) and stirred at room temperature for 1 hour. NBT solution was filtered with a 0.2 mm filter.

The color change from yellow NBT to blue black formazan was read at 560 nm in a spectrophotometer.

Percent NBT reduction was calculated by the following formula: NBT reduction % = (OD of test – OD of control) / OD of control.[17]

Data was compiled & analyzed using statistical software R software version 3.6.1 and Microsoft Excel. Categorical variables were represented as frequency table. Continuous variables were recorded as mean ± standard deviation (SD). Inter-group comparisons and changes in NBT assay levels were analyzed using student's unpaired t- test. p-value <0.05 was considered statistically significant.

RESULTS:

The mean NBT assay values for both groups are presented in Table-1. With increasing GSE concentrations, a decrease in the mean NBT assay values (signifying mean NBT reduction) was noted in both groups. These NBT assay values for different GSE concentrations as well as the control were found to be significantly greater in Group B – Chronic Periodontitis compared to Group A – Periodontally healthy (p<0.0001) (Table-1).

Table-2 summarizes the intra-group comparison of NBT assay values between different GSE concentrations. No significant difference in these values was noted between T1 (5 µg/mL GSE) and TC (control) in both the groups (p>0.05). Within Group A, the mean NBT reduction was significantly lower in T2 (10 µg/mL GSE) and T3 (15 µg/mL GSE) when each was compared to TC (p=0.0372 and p=0.0197, respectively) and T1 (p=0.0204 and p=0.0055, respectively), while it showed no significant difference between T2 and T3 (p=0.0917). Within Group B, the mean NBT reduction was significantly lower in T2 and T3 when each was compared to TC (p<0.0001 for both) and T1 (p<0.0001 for both), while it was significantly lower in T3 compared to T2 (p<0.0001).

Table-3 presents the intra-group comparison of percent NBT reduction by different concentrations of GSE with respect to the control. With an increase in GSE concentration, there was a decrease in the mean % NBT reduction in both the groups. Hence, the maximum decrease in % NBT reduction was seen with 15 µg/mL GSE concentration in both the groups.

DISCUSSION:

Inflammatory conditions like chronic periodontitis are associated with increased oxidative stress generated from neutrophils and bacterial interactions during phagocytosis. This contributes to disease pathogenesis and can be mitigated by antioxidants.[4-6] With the body's own inherent AO defense system falling short and the commercial AO products posing limitations, the focus has shifted to harnessing the benefits of natural AO (phytochemicals).[8-11] Hence, this study was conducted to evaluate and compare the AO effect of GSE-derived OPC on the respiratory burst of human neutrophils isolated from the saliva of healthy individuals versus CP patients.

Salivary PMNs were employed since their functional activity was found to be comparable with that of blood and crevicular PMNs.[6] Moreover, studies indicated that salivary PMNs could function even after leaving the gingival crevices and their functions could be correlated with the disease state.[21]

The pathogenesis underlying CP involves the production of FR-like superoxide by PMNs.[4-6] This superoxide converts yellow NBT to blue formazan.[13-16] Anti-oxidants such as GSE-derived OPC inhibit these FR, thus inhibiting NBT reduction.[13-16] This was reflected in the present study by the decrease in the mean NBT assay values with increasing GSE concentrations, implying a decrease in the respiratory burst of neutrophils. The dose-dependent inhibition of NBT reduction, and hence, superoxide generation, by OPC was in concordance with the works of Carini et al, who studied the inhibitory properties of

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proanthocyanidins from *Vitis vinifera* seeds on the respiratory burst of activated human blood neutrophils.[12] This concentration-dependent increase in AO activity was also in line with the studies conducted by Houde et al as well as Bagchi et al.[10,11]

In the current research, NBT reduction was observed to be greater in CP patients compared to periodontally healthy individuals, implying an increased production of oxygen FR (ROS) in CP, thus requiring a higher concentration (15µg/mL) of AO (GSE) to neutralize the same. These findings are in accordance with the works of Chapple et al who concluded that increased oxidative stress arising from host-microbial interactions lies at the heart of periodontal tissue damage.[5] GSE provides useful AO effects in such cases due to their FR-scavenging and metal-chelating properties.[22] Another in vitro study by Houde et al found that GSE-treated macrophages, when stimulated with lipopolysaccharide of periodontal pathogens like *Actinobacillus actinomycetemcomitans* or *Fusobacterium nucleatum*, demonstrated decreased nitric oxide and ROS production than non-GSE-treated macrophages.[10]

Hence, the present study is the first to establish that GSE-derived OPC provided a dose-dependent inhibitory effect on the respiratory burst of human salivary neutrophils in CP as well as in health. Higher concentrations (15µg/ml) of this AO are needed in CP patients compared to healthy individuals due to greater oxidative stress in CP. This paves the way for the exploration of GSE/OPC preparations in various forms as preventive and/or therapeutic adjuncts to conventional periodontal therapy.[22]

However, this research has its limitations in being a single-center, in-vitro study with limited sample size. These can be overcome by multicentric, long-term, prospective, interventional clinical studies with a larger sample size.

CONCLUSION:

Proanthocyanidins derived from *Vitis vinifera* extract have provided a dose-dependent inhibitory effect on the respiratory burst of human salivary neutrophils in CP as well as in health. Higher concentrations of this AO are needed in CP patients compared to healthy individuals due to greater oxidative stress in CP.

The findings of the study are significant and prove that grape seed proanthocyanidins can be applied clinically as preventive and/ or therapeutic adjuncts to conventional periodontal therapy. Further clinical studies are required in order to unveil the mechanism of this novel and highly potent antioxidant.

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FIGURES:

LEGENDS –

Figure 1 – Healthy periodontium

Figure 2 – Chronic periodontitis

Figure 3 – Colour change

Figure 4 – Spectrophotometer

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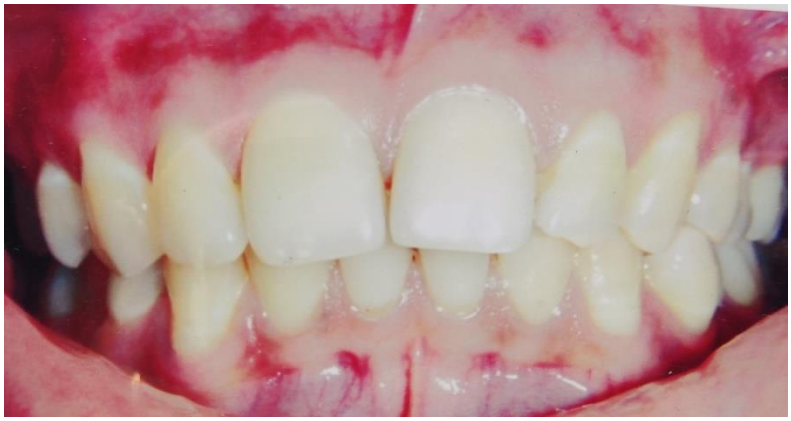

Figure 1: Healthy periodontium

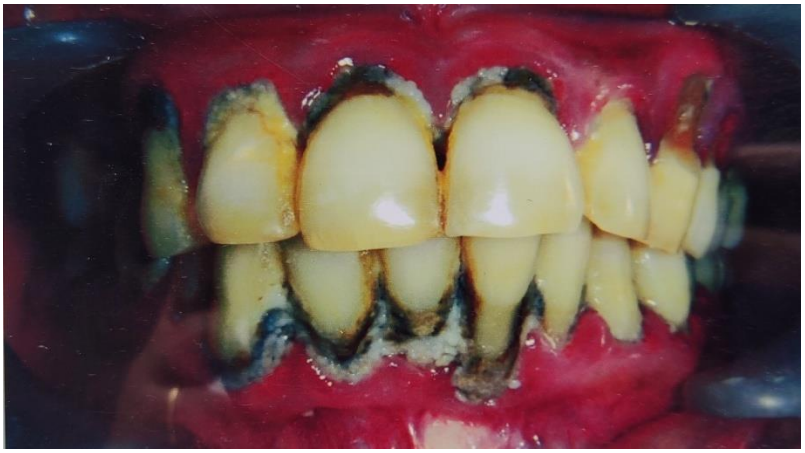

Figure 2: Chronic periodontitis

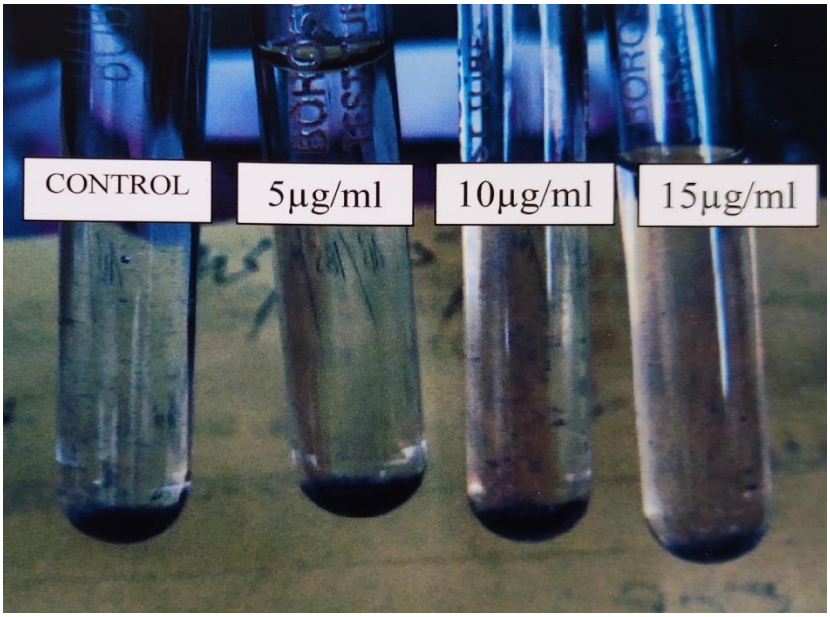

Figure 3: Colour change

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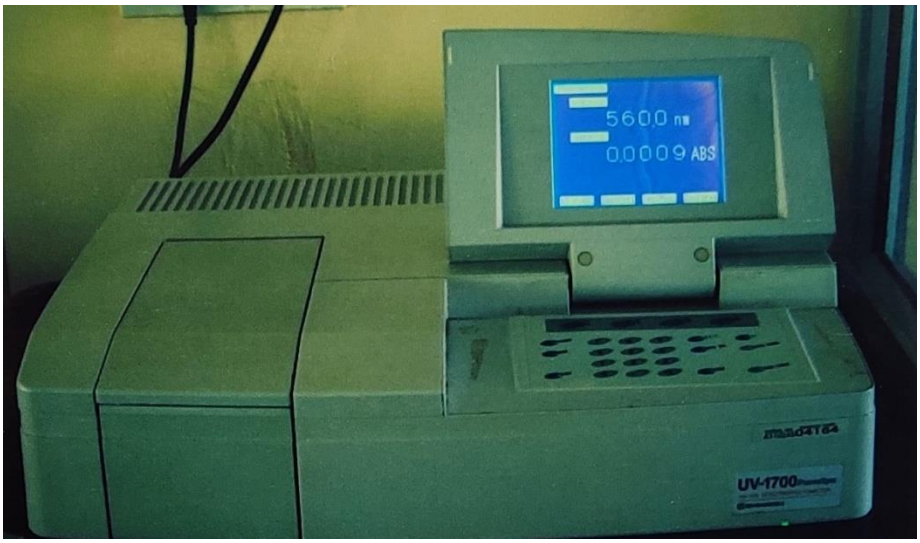

Figure 4: Spectrophotometer

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