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In vitro studies on inhibitory effect of proanthocyanidins in modulation of neutrophils and macrophages

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The role of proanthocyanidins (PC), a novel flavonoid extracted from grape seeds was studied *in vitro* in the modulation of neutrophil and macrophage function. We attempted to assess the levels of non-enzymatic and enzymatic mediators in the presence or absence of PC in 4-phorbol-12-β-myristate-13-acetate (PMA)-stimulated neutrophils isolated from humans and rats, *E. coli* endotoxin-stimulated macrophages and macrophages isolated from *E. coli* endotoxin-induced experimental periodontitis in rats. Addition of PC at a concentration of 50 µg/ml effectively blocked the release of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and exhibited a marked inhibition of myeloperoxidase (MPO) and lysosomal enzymes (p<0.001), as compared to PMA-stimulated neutrophils (human and rats) and neutrophils isolated from experimental periodontitis in rats. The levels of ROS, RNS and lysosomal enzymes were found to be elevated (p<0.001) and addition of PC significantly (p<0.001) reduced these levels as compared to those from *E. coli* endotoxin-stimulatedmacrophages from rats and macrophages isolated from experimental periodontitis in rats (p<0.001). Thus, the study demonstrated that PC decreased the levels of ROS and RNS and also inhibited the MPO and lysosomal enzymes activities in experimental periodontitis in rats. In addition, this study clearly indicated that PC could be developed as an effective antiinflammatory agent.

Keywords: Lysosomal enzymes, Macrophages, Neutrophils, Proanthocyanidins, Reactive nitrogen species, Reactive oxygen species.

Periodontal disease is the consequence of complex interactions between a diverse oral bacterial community and host tissues. In acute inflammation, a large number of neutrophils are recruited to the sites of tissue injury, where they die by undergoing apoptosis[1]. The removal of neutrophils and their toxic contents from the inflamed site is a pre-requisite for resolution of tissue injury and engulfment of intact, senescent neutrophils by macrophages represents an important neutrophil disposal process[2]. Monocytes and macrophages, which are found in higher numbers in active periodontal lesions than in inactive sites[3] play an important role in the host inflammatory response to periodontopathogens[4].

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Abbreviations: ACP, acid phosphatase; EP, experimental periodontitis; MPO, myeloperoxidase; PC, proanthocyanidins; PMA, 4-phorbol-12-β-myristate-13-acetate; RNS, reactive nitrogen species; ROS, reactive oxygen species.

The primary function of neutrophils is the phagocytosis as well as destruction of microorganisms and the release of myeloperoxidase (MPO) and hydrogen peroxide (H_2O_2) into the phagosome generally leads to a rapid microbicidal effect[5]. During phagocytosis, neutrophils undergo a burst of respiration in which oxygen is reduced to superoxide anion ($O_2^{\bullet-}$), which dismutates to form H_2O_2[6]. MPO, which is released from cytoplasmic granules of neutrophils and monocytes by a degranulation process reacts with the H_2O_2 formed by the respiratory burst to form a complex that can oxidize a large variety of substances[7]. The liberation of MPO as a potent injurious oxidative enzyme from the azurophilic granules of neutrophils into the extracellular space gives rise to highly reactive oxygen species (ROS)[8]. Proteolytic enzymes, such as cathepsins B, D and L are released from lysosomes to the site of destruction of periodontal tissues. In addition to the defensive role, phagocytic cells may also be responsible for damage to periodontal tissue as a collateral effect of their phagocytic function, thus worsening the periodontal lesion[9].

Microbial components, especially lipopolysaccharide (LPS) have the capacity to activate macrophages to synthesize and secrete a wide array of molecules, including the cytokines viz., interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), prostaglandins, especially PGE_2, and hydrolytic enzymes. These cytokines manifest potent pro-inflammatory and catabolic activities and they play key roles in periodontal tissue breakdown[10]. Understanding of these mechanisms is critical in designing new therapeutic interventions capable of reducing the putative deleterious effects of neutrophils and macrophages.

Several drugs have been developed which can curtail the release of these effectors and of recent interest is the role of proanthocyanidins (PC), a highly purified polyphenolic bioflavanoid extract from grape seeds, having a wide range of biological properties against oxidative stress[11]. Several *in vitro* studies on cancer and tumour cells have been carried out on the biological, pharmacological and chemoprotective properties of PC against oxygen free radicals[12]. This study is the first attempt to investigate the effects of PC on the production of ROS, reactive nitrogen species (RNS) and lysosomal enzymes release by neutrophils stimulated with 4-phorbol-12-β-myristate-13-acetate (PMA) and macrophages stimulated with *E. coli* endotoxin. Preliminary experiments have been performed on human neutrophils and experiments have also been carried out on neutrophils isolated from experimental periodontitis rats, as well as macrophages isolated from *E. coli* endotoxin-stimulated rats and experimental periodontitis in rats.

Materials and Methods

Materials

4-Phorbol-12-β-myristate-13-acetate (PMA), horse radish peroxidase, dextran, superoxide dismutase (SOD), histopaque, phenol red, *o*-dianisidine hydrochloride, hexadecyl trimethyl ammonium bromide, haemoglobin, Medium 199 (M-3274), foetal calf serum (FCS), and nitroblue tetrazolium (NBT) salt and dimethylsulfoxide (DMSO) were purchased from M/s Sigma Chemical Co., USA. MEM (Modified Eagle's Medium) and HBSS (Hank's balanced salt solution) were obtained from M/s Hi Media, Bombay, India. All other reagents used were of analytical grade.

Animals

Male Wistar rats weighing approximately 250 g were housed in solid-bottomed polypropylene cages under strict veterinary supervision and maintained in control rooms with 12 h light/dark cycle. The animals received commercial rat diet and water *ad libitum*. This study conformed to the guiding principles of Institutional Animal Ethical, Committee (IAEC) Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the Guide for the care and use of laboratory animals.

Proanthocyanidins (PC)

PC (50 µg/ml) from grape seeds[13], purchased from M/s "Terravita" Brampton, Ontario, Canada was dissolved in 0.1% DMSO, filtered through 0.22 µm filter (Millipore) and stored as aliquots at -20°C. Prior to use, it was gently vortexed and added to the culture.

Induction of experimental periodontitis (EP)

EP was induced by injecting *E. coli* endotoxin as described by Ramamurthy *et al*[14]. Briefly, under anesthesia (ketamine 50 mg/kg body weight, ip), 1 mg of endotoxin dissolved in 1 ml of saline was injected into the labial and palatal aspects of maxillary anterior gingivae, buccal and palatal aspects of maxillary molars to induce periodontitis.

Isolation of neutrophils

Neutrophils were isolated according to the method described by Neuman *et al*[15], with slight modifications. Heparinised whole blood (1:2) was overlayered on 12 ml of 6% dextran and mixed adequately. After the setting of blood, the yellowish supernatant was carefully aspirated and was centrifuged at 1500 *g* for 12 min at 4°C. The pellet was resuspended in 12 ml of ice-cold distilled water and dispersed repeatedly to break the pellet. Then, 2.0 ml of 0.6 N KCl was added and mixed. The solution was layered on ficoll and histopaque and centrifuged at 1500 *g* for 30 min at 4°C. After centrifugation, the supernatant was discarded and the pellet was resuspended in 2 ml HBSS. Cell concentration was determined using haemocytometer.

Stimulation and assays

Neutrophils (1×10^6 cells/well) were left for adherence for 1 h and stimulated by the addition of PMA (100 ng/ml) for 1 h. In case of unstimulated cells and experimental periodontitis rat neutrophils, after 1 h adherence, PC was added. The cultures were terminated after 1 h of PC treatment and H_2O_2[16], superoxide ($O_2^{\bullet-}$)[17] and nitrite[18] were assayed immediately.

For H_2O_2 assay, the medium was removed and to the adherent neutrophils, 100 µl of PMA (1 µg/ml) was added, followed by the addition of 1 ml of HBSS containing horse radish peroxidase (19 units/ml) and phenol red (0.02%). After 1 h of incubation, 100 µl of 1 M NaOH was added and the colour developed was read at 605 nm.For assay of $O_2^{\bullet-}$, the medium was removed and the adherent neutrophils were incubated for 1 h in HBSS containing 80 µM cytochrome *c* and 10 µM PMA. The color developed was read at 550 nm. The amount of $O_2^{\bullet-}$ released was measured by the amount of cytochrome *c* reduced which would be inhibited by SOD and quantitated using a molar extinction coefficient of 21 x 10^3 M^{-1} cm^{-1}. For nitrite assay, 100 µl of cell-free medium from well plates was mixed with equal volume of Griess reagent (5 ml of equal volumes of 1% sulphanilamide and 0.1% naphthyl ethylenediamine dihydrochloride in 5% H_3PO_4). The color developed was read at 550 nm. $NaNO_2$ (10-100 nmoles) served as standard.

For the enzyme assays, after removing the cell-free medium, cells were lysed by adding 0.5 ml of ice-cold 0.1% Triton-X-100 in 0.25 M sucrose, subjected to repeated freezing and thawing and assayed for lysosomal enzymes viz., cathepsin D[19] and acid phosphatase (ACP)[20]. The level of myeloperoxidase (MPO) was assayed in the cell lysate after extracting the enzyme in phosphate buffer (pH 6.0) containing hexadecyl trimethylammonium bromide[19].

Isolation and culture of peritoneal macrophages

Peritoneal macrophages were recovered by starch elicitation by the method of Mc Millan[21] with slight modification. Briefly, 10 ml of 2% sterile starch solution was injected into rats intraperitoneally. After 96 h, 20 ml of ice-cold MEM was injected into the peritoneal cavity of the rats and without removing the needle, the abdomen was gently massaged and the lavage was collected. The contents were immediately transferred to a sterile heparinised container (10 IU heparin/ml) and centrifuged at 1800 rpm for 15 min. The pellet was suspended in M199 medium containing 5% heat-inactivated FCS. The percentage of macrophages was ascertained by non-specific esterase staining and the viability was assessed by trypan blue exclusion method.

The cells were plated as follows: for cover slip preparations, 0.5 × 10^6 cells/well in 12-well Costar plates; for enzyme assays and superoxide anion release, 1 × 10^6 cells/well in 12-well Costar plates; for nitrite and H_2O_2 release assays, 0.1 × 10^6 cells/well in 12-wells in 96-well plates. The plates were left overnight for adherence at 37˚C in a humidified atmosphere with 5% CO_2.

Infection and assays

Peritoneal macrophages were preincubated with phenol extracted *E. coli* endotoxin for the indicated periods and washed with HBSS twice. Macrophages were infected with 10 ng/ml endotoxin. 1 h after infection, the unphagocytosed organisms were removed by gentle washing and infected macrophages were cultured in M199 containing 5% FCS.

In order to find out the effect of PC on the biochemical changes in the infected macrophage cultures, PC was added at a final concentration of 0.5 µg. The culture was terminated at various time points viz. 1, 12, 24, 48 and 72 h.

Statistical analyses

Results were reported as mean ± standard error (SE). Statistical analysis was performed using one-way-analysis-of-variance (ANOVA) and further comparisons among groups were made according to Post-Hoc Tukey's test.

Results

Neutrophils

The stimulation of the respiratory burst by PMA in neutrophils isolated from humans resulted in an increase (p<0.001) in the levels of ROS and nitrite, which were significantly reduced (p<0.001) after the addition of PC. Nitrite levels in the culture medium, which are direct indicators of the amount of nitric oxide produced, were found to be elevated under stress and addition of PC was shown to decrease the nitrite levels (Fig. 1A). A significant increase in MPO activity was observed (Fig. 1A) in PMA stimulated human neutrophils (p<0.001) along with the increase in the levels of H_2O_2 and superoxide anion. Addition of PC significantly inhibited MPO activity (p<0.001) as well as the levels of H_2O_2 and superoxide anion, as compared to PMA-stimulated cells. PC was also shown to reduce the levels of cathepsin D and ACP, as compared to PMA-stimulated cells (Fig. 1B).

The results of the studies on PMA-stimulated human neutrophils showed the beneficial effect of PC on the radical production and lysosomal enzymes release. Subsequently, experiments were performed on rat neutrophils isolated from control rats, neutrophils stimulated with PMA and neutrophils

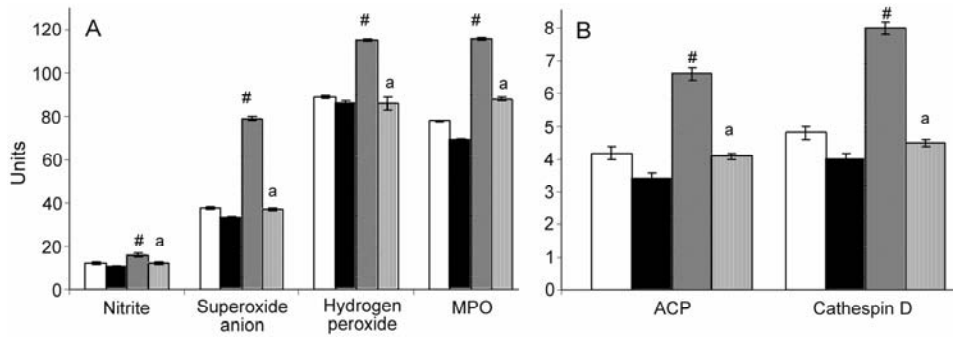

Fig. 1—(A): Effect of PC on human neutrophil ROS generation. Nitrite level expressed as nmoles of nitrite/0.5 x 10^6 cells. H_2O_2 expressed as µ moles H_2O_2 liberated/0.5 x 10^6 cells. Superoxide anion expressed as nmoles of superoxide anion liberated/0.5 x 10^6 cells. MPO activity expressed as µ moles H_2O_2 utilized/min/mg protein; and (B): Effect of PC on human neutrophils lysosomal enzymes. ACP activity expressed as µ moles of *p*-nitrophenol liberated/h/mg protein. Cathepsin D activity expressed as µ moles of *p*-nitrophenol liberated/h/mg protein. All values were mean ± SE (n = 6). $^{\#}p< 0.001$ as compared to control. $^{a}p< 0.001$ as compared to PMA-stimulated cells. (□) Control neutrophils; (■) PMA-treated neutrophils; (▨) Control neutrophils + PC; (▥) PMA-treated neutrophils + PC

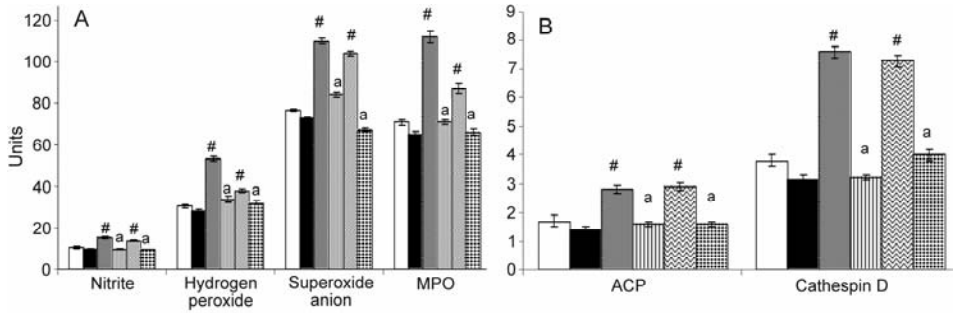

Fig. 2—(A): Effect of PC on rat neutrophil ROS generation and MPO release; and (B): Effect of PC rat neutrophils lysosomal enzymes. Other details are as same as in Fig 1. (□) Control neutrophils; (■) PMA-treated neutrophils; (▨) Control neutrophils + PC; (▥) PMA treated neutrophils + PC; (▩) Experimental periodontitis neutrophils; (◪) Experimental periodontiits + PC

isolated from experimental periodontitis rats. The levels of ROS and nitrite were observed to be significantly elevated ($p<0.001$) both in PMA-stimulated and also in neutrophils isolated from experimental periodontitis rats and addition of PC effectively scavenged the radicals species production (Fig. 2A).

The effect of PC on lysosomal enzymes was more marked on experimental periodontitis rat neutrophils (Fig. 2B). While PMA stimulation resulted in a significant increase in the activity of cathepsin D and ACP ($p<0.001$), the increased activity of these enzymes in the experimental periodontitis rats was reduced upon treatment with PC ($p<0.001$).

Macrophages

Assay of nitrite, H_2O_2 and superoxide anion levels

Nitrite levels in the culture medium were found to be elevated and the values reached peak levels at 48 h. Addition of PC significantly ($p<0.001$) reduced the nitrite levels (Fig. 3A). The $O_2^{\bullet-}$ production at all time points studied was found to be high and PC significantly ($p<0.001$) inhibited $O_2^{\bullet-}$ production at all time points (Fig. 3B). The H_2O_2 levels were increased from 1 h of infection and the increase was found to sustain throughout the period of study. The inhibitory effects of PC on H_2O_2 production was significant ($p<0.001$) at all time points in the culture (Fig. 3C).

Lysosomal enzymes

Significant elevation in the activities of ACP and cathepsin D was observed in *E. coli* endotoxin infected rat macrophages. ACP activity increased from 1 h onwards, with the maximum observed at 72 h and addition of PC significantly ($p<0.001$) reduced the ACP activity (Fig. 4A). Cathepsin D activity also showed an increase from 1 h onwards with a peak value reached at 48 h and addition of PC significantly reduced the cathepsin D activity (Fig. 4B).

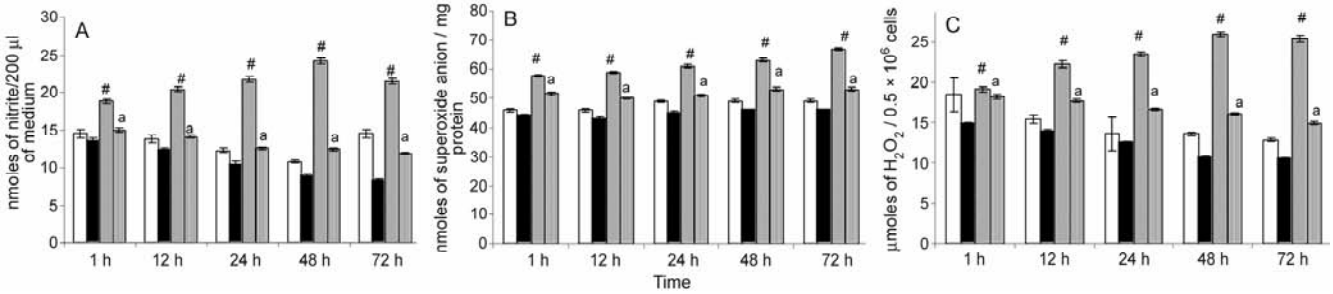

Fig. 3—(A): Effect of PC on rat macrophage nitrite generation when challenged *in vitro* with *E. coli* endotoxin; (B): Effect of PC on rat macrophage superoxide anion level when challenged *in vitro* with E. coli endotoxin; and (C): Effect of PC on rat macrophage H_2O_2level when challenged *in vitro* with *E. coli* endotoxin. Other details are as same as in Fig. 1. (□) Control macrophages; (■) Control macrophages + PCl; (▨) *E. coli* endotoxin stimulates mactophages; (▥) *E. coli* endotoxin stimulates macrophages + PC

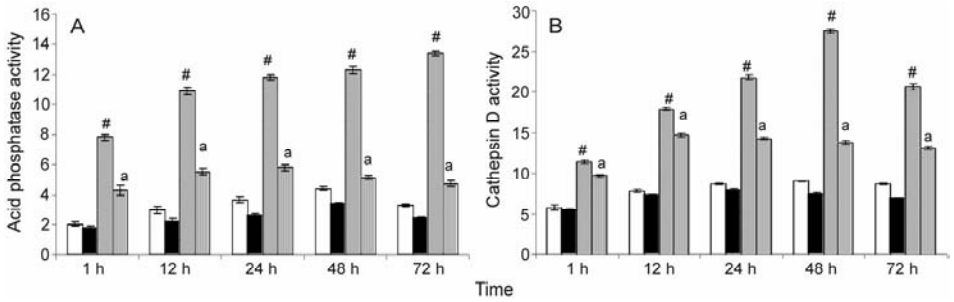

Fig. 4—(A): Effect of PC on rat macrophage acid phosphatase release when challenged *in vitro* with E.coli endotoxin; and (B): Effect of PC on rat macrophage cathepsin D release when challenged *in vitro* with E. coli endotoxin. Other details are as same as in Fig. 1. (□) Control macrophages; (■) Control macrophages + PC; (▨) E. coli endotoxin stimulated marophages; (▥) E. coli endotoxin stimulated mactophages + PC

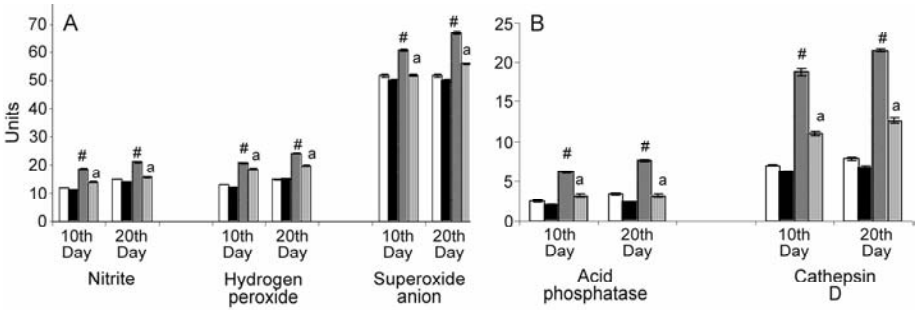

Fig. 5—(A): Effect of PC on rat neutrophil ROS generation and MPO release; and (B): Effect of PC on rat macrophage lysosomal enzymes release when challenged *in vitro* with *E. coli* endotoxin. Other details are as same as in Fig. 1. (□) Control macrophages; (■) Control macrophages + PC; (▨) *E. coli* endotoxin stimulated marophages; (▥) Experimental periodontitis macrophages + PC

Nitrite, H_2O_2 and superoxide levels of peritoneal macrophages isolated from control and experimental periodontitis rats

Nitrite level was found to be high in acute (10^{th} day), as well as chronic phases (20^{th} day) of the disease. Incubation of the resident macrophages with PC resulted in a decrease in the nitrite level comparable to control. The trend in the increase of superoxide and H_2O_2 levels was similar to that of nitrite and addition of PC significantly (p<0.001) reduced these levels (Fig. 5A).

Lysosomal enzymes of peritoneal macrophages isolated from control and experimental periodontitis rats

ACP as well as cathepsin D (Fig. 5 B) activities were significantly increased in macrophages isolated from experimental periodontitis rats in both acute (10^{th} day) and chronic phases (20^{th} day)and addition of PC to the macrophages significantly (p<0.001) reduced the activities of both the enzymes.

Discussion

Since isolation of human neutrophils was easier, preliminary experiments were carried out using human neutrophils. Once the protective effect of PC in inhibiting the generation of ROS, nitrite and lysosomal enzymes release was established, this study was extended later to neutrophils isolated from control rats and experimental periodontitis rats.

The components of microbial plaque have the capacity to induce the initial infiltrate of inflammatory cells including lymphocytes, macrophages, and PMNs. The role of PMN is primarily a protective one and host tissue damage can result indirectly from over exuberant PMN and monocyte responses as well as directly from the colonizing pathogens themselves. Several studies have shown that the role of ROS and RNS produced by inflammatory and immune cells[22] have specific role in tissue destruction associated with inflammatory periodontal diseases[23]. Activated macrophages are a rich source of nitric oxide (NO) production and NO may either potentiate or inhibit neutrophil-mediated cytotoxicity[24].

The finding of this study showing a significant increase in $O_2^{\bullet-}$, H_2O_2 and NO_2levels both in neutrophilsand macrophages isolated from experimental periodontitis rats was in accordance with the above report[24]. In an earlier report, it was conclusively shown that PC from grape seeds significantly decreased the levels of reactive oxygen species and markedly lowered the activity of nitric oxide synthase as well as the content of NO in carrageenan-induced paw edema in rats and the observations of this study concurred with this finding[25]. This finding demonstrated that inhibition of lipid peroxidation and NO formation was an anti-inflammatory mechanism of PC.

Lysosomal acid hydrolases are released from viable cells over a prolonged period of time by various agents known to cause, or associated with chronic inflammation[26]. Cathepsins K, B, S, L, and D are shown to be secreted simultaneously by cultured monocyte-derived macrophages[27]. Axline and Cohn[28] found that the phagocytosis of digestible particles induces the production of ACP and to a lesser extent cathepsin D and β-glucuronidase in mouse peritoneal macrophages. The therapeutic interventions affecting macrophage function included: i) neutralization of monokines, ii) blockade of monocytes recruitment or antigen presentation, and iii) blockade of effector's function viz. neutralization of macrophage products[29] and PC could fall in the last category as the present studies indicated. Active compounds endowed with a capacity to modulate the host inflammatory response are now receiving considerable attention, since they may prove to be potential new therapeutic agents for the treatment of periodontal diseases[30].

A great body of evidence has demonstrated that the human neutrophil is both a target and a source of various proinflammatory cytokines, chemokines, and growth factors, and therefore it exerts its proinflammatory functions through an auto-regulatory pathway[31]. Neutrophils are exquisite targets of proinflammatory cytokines viz., IL-1 and TNF-α, chemokines such as IL-8, and growth factors such as granulocyte/monocyte colony stimulating factor. Indeed, these cytokines are shown to amplify several functions of neutrophils, including their capacity to adhere to endothelial cells and to produce ROS[32].

In this study, rat peritoneal macrophages were challenged *in vitro* with *E. coli* endotoxin. After 1 h of phagocytosis, PC was added to the culture. The ability of *E. coli* endotoxin to induce the macrophages to synthesize lysosomal enzymes such as ACP and cathepsin D, nitric oxide and ROS was measured both in the presence and absence of PC. The implication of oxidative stress in the etiology of several chronic and degenerative diseases suggests that antioxidant therapy represents a promising avenue for treatment. PC as a potent antioxidant attenuated oxidative stress by neutralizing the free radicals.

In conclusion, this study demonstrated that PC was shown to decrease the levels of ROS, RNS, myeloperoxidase and lysosomal enzymes in experimental periodontitis in rats. The study also showed that PC, possessing potent radical scavenging, antioxidant and antiinflammatory properties could be safely developed as an anti-inflammatory drug for periodontitis.

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