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

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Received 03 December 2009; revised 27 April 2010

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-stimulated macrophages 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 apoptosis¹. 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 process². Monocytes and macrophages, which are found in higher numbers in active periodontal lesions than in inactive sites³ play an important role in the host inflammatory response to periodontopathogens⁴.

The primary function of neutrophils is the phagocytosis as well as destruction of microorganisms and the release of myeloperoxidase (MPO) and hydrogen peroxide (H₂O₂) into the phagosome generally leads to a rapid microbicidal effect⁵. During phagocytosis, neutrophils undergo a burst of respiration in which oxygen is reduced to superoxide anion (O₂⁻), which dismutates to form H₂O₂⁶. MPO, which is released from cytoplasmic granules of neutrophils and monocytes by a degranulation process reacts with the H₂O₂ formed by the respiratory burst to form a complex that can oxidize a large variety of substances⁷. 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 lesion⁹.

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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.
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 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) for the care and use of laboratory animals.

Proanthocyanidins (PC)

PC (50 µg/ml) from grape seeds, 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. 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., with slight modifications. Heparinised whole blood (1:2) was overlaid 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 × 10⁶ 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 H₂O₂, superoxide (O₂⁻) and nitrite were assayed immediately.
For \( \text{H}_2\text{O}_2 \) 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 \( \text{O}_2^- \), the medium 
was removed and the adherent neutrophils were incubated 
for 1 h in HBSS containing 80 µM cytochrome \( \text{c} \) and 
10 µM PMA. The color developed was read at 
550 nm. The amount of \( \text{O}_2^- \) released was measured 
by the amount of cytochrome \( \text{c} \) reduced which would 
be inhibited by SOD and quantitated using a molar 
extinction coefficient of 21 x 10\(^2\) 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% 
\( \text{H}_3\text{PO}_4 \)). The color developed was read at 550 nm. 
\( \text{NaNO}_2 \) (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 
asayed 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, \( 0.5 \times 10^6 \) cells/well in 12-well Costar 
plates; for enzyme assays and superoxide anion 
release, \( 1 \times 10^6 \) cells/well in 12-well Costar plates; for 
nitrite and \( \text{H}_2\text{O}_2 \) release assays, \( 0.1 \times 10^6 \) cells/well in 
12-wells in 96-well plates. The plates were left 
overnight for adherence at 37°C in a humidified 
atomosphere with 5% \( \text{CO}_2 \).

**Infection and assays**

Peritoneal macrophages were preincubated with 
phenol extracted \( \text{E. coli} \) 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 \( \text{H}_2\text{O}_2 \) and superoxide anion. Addition 
of PC significantly inhibited MPO activity \( (p<0.001) \) 
as well as the levels of \( \text{H}_2\text{O}_2 \) 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
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 scavenge 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 \((p<0.001)\).

**Macroophages**

**Assay of nitrite, \(\text{H}_2\text{O}_2\) 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 \(\text{O}_2^-\) production at all time points studied was found to be high and PC significantly \((p<0.001)\) inhibited \(\text{O}_2^-\) production at all time points (Fig. 3B). The \(\text{H}_2\text{O}_2\) 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 \(\text{H}_2\text{O}_2\) 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).
Nitrite, H$_2$O$_2$ and superoxide levels of peritoneal macrophages isolated from control and experimental periodontitis rats

Nitrite level was found to be high in acute (10\textsuperscript{th} day), as well as chronic phases (20\textsuperscript{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$_2$O$_2$ 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\textsuperscript{th} day) and chronic phases (20\textsuperscript{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 have specific role in tissue destruction associated with inflammatory periodontal diseases. Activated macrophages are a rich source of nitric oxide (NO) production and NO may either potentiate or inhibit neutrophil-mediated cytotoxicity.

The finding of this study showing a significant increase in $O_2^-$, $H_2O_2$ and $NO_2$ levels both in neutrophils and macrophages isolated from experimental periodontitis rats was in accordance with the above report. 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. 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. Cathepsins K, B, S, L, and D are shown to be secreted simultaneously by cultured monocyte-derived macrophages. Axline and Cohn 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 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.

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. 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.

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.

Acknowledgements

The authors thank the Principal, Meenakshi Ammal Dental College, Dean, Meenakshi Medical College and Research Institute and the Research Committee members for their encouragement and support. The authors also thank Dr. A B Mandal, Director, CLRI, Chennai for his support. The award of CSIR Emeritus Scientist project to Dr. R Puvanakrishnan is gratefully acknowledged.

References

GOVINDARAJ et al: INHIBITORY EFFECT OF PROANTHOCYANIDINS