ISSN 2348-6201

Cranberry Extract Suppressive Effect on Secretion of Inflammatory Cytokines by the Monocytic Cells

Jegdish P. Babu[1,3]*, Vreiti Sangha [1,2] and David A. Tipton[1,3]
[1]College of Dentistry, [2]Department of Periodontics, and [3]Department of Bioscience Research, University of Tennessee Health Science Center, Memphis, TN USA.
email: jbabu@uthsc.edu

Key Words: Cranberry; LPS; Cytokines; Periodontitis; Monocytic cells.

ABSTRACT:

The proanthocyanidin rich fraction of cranberry (NDM) has long been speculated to possess the capacity to suppress the production of inflammatory mediators by macrophages. The **objective** of this investigation was to examine the effect of NDM on lipopolysaccharide (LPS)-mediated stimulation of the cultured monocytic cells (THP-1) to secrete two inflammatory cytokines; IL-1β and TNF-α. The LPS used in the study was isolated from periodontal pathogen, *Porphyromonas gingivalis*. **Methods**: Cultured monocytic cells were incubated with bacterial LPS in the presence or absence of NDM, and the culture supernatants were assayed for secreted TNF-□ and IL-□ by ELISA. Cells incubated with NDM secreted significantly lesser amounts of the two cytokines than the cells without. **Conclusion**: The study demonstrated the ability of NDM to suppress production of inflammatory cytokines by the human monocytic cells when stimulated with LPS. The findings of this study provides promising perspectives for the development of novel host-modulating therapies for adjunctive treatments of periodontitis or other inflammatory diseases by the NDM isolated from cranberries.

Council for Innovative Research
Peer Review Research Publishing System
Journal: JOURNAL OF ADVANCES IN BIOTECHNOLOGY
Vol. 4, No. 3.
www.cirjbt.com, jbteditor@gmail.com

ISSN 2348-6201

Introduction:

Periodontal disease is a chronic microbial and inflammatory process characterized by the presence of sulcular pathogenic bacteria, exacerbated host immune response and destruction of the connective tissue attachment. Periodontitis is associated with a complex microflora, in which Gram-negative anaerobic rods and spirochetes dominate (1). Components of the microbial dental plaque have the capacity to activate the local host response by inducing the initial infiltrate of inflammatory cells including lymphocytes, macrophages, and polymorphonuclear leukocytes (2). It has been well established that under the stimulation of bacterial products, that monocytes/macrophages, lymphocytes, and local resident cells such as fibroblasts and vascular endothelial cells synthesize a broad spectrum of cytokines many of which exert pro-inflammatory effects and are related to periodontal tissue destruction (3). LPS and other substances gain access to the gingival tissues, initiate and perpetuate production of high levels of pro-inflammatory cytokines. These induce production of matrix metalloproteinases which destroy the connective tissues of the gingiva and periodontal ligament, and prostaglandins which mediate alveolar bone destruction (3).

The Cranberry (*Vaccinium macrocarpon*) is a native North American fruit that has recently received considerable attention in health research, particularly in the field of infectious diseases. Within the past 25 years, the body of scientific evidence to support its use has grown as investigations have shown that cranberry juice can prevent adherence of *Escherichia coli* bacteria to uroepithelial and other eukaryotic cells (4). The biological activities of cranberry can be attributed to a diverse group of phytochemicals, including flavonoids, hydroxycinnamic acid derivatives, organic acids, and isoprenoids including ursolic acid and lutein. The flavonoids fall primarily into three classes: anthocyanins, flavonols, and proanthocyanidins (PACs) or polyflavan-3-ols. In the area of dental research, it has been reported that a high molecular weight fraction prepared from cranberry juice inhibits the co-aggregation of many oral bacteria (5) and affects dental biofilm formation (6,7). The purpose of the study was to investigate the effect of NDM on the secretion of inflammatory cytokines by the cultured monocytic cells in response to bacterial LPS.

Materials and Methods

Cranberry NDM Preparation:

NDM was prepared from concentrated cranberry juice made from the American cranberry, *Vaccinium macrocarpon*, and provided by Ocean Spray Cranberries, Inc. (Lakeville-Middleboro, MA) as described previously (8). Briefly, the cranberry concentrate was dialyzed and the contents of the dialysis bag containing the high molecular weight (> 12,000 daltons) material was lyophilized. The lyophilized cranberry component was designated NDM, and was found to be soluble in water up to 4 mg/ml. It exhibited tannin like properties, suggesting that it is rich in phenolic compounds such as proanthocyanidins (9). Chemical analysis revealed that this fraction is devoid of sugars, acids, and nitrogen; but contained 0.35% anthocyanins (0.055% cyanidin-3-galactoside,0.003% cyanidin-3-glucoside, 0.069% cyanidin-3-arabinoside, 0.116% peonidin-3-galactoside, 0.016% peonidin-3-glucoside and 0.086% peonidin-3-arabinoside), and 65.1% proanthocyanidins, similar to previously isolated components from cranberry (8).

Bacterial lipopolysaccharide (LPS) Preparation:

Porphyromonas gingivalis (*Pg)* 33287, obtained from ATCC (Manassas, VA) was grown in liter batches of thioglycollate broth for 72 hours in an anaerobic jar. The bacteria were harvested by centrifugation and the cell pellets were washed with de-ionized water and centrifuged at (10,000xg, for 20 minutes). The bacterial cell pellets were lyophilized and the LPS was prepared by the hot phenol-water extraction method as described by Westphal and Jann (10). The purity and protein contamination of the isolated LPS was confirmed by gel electrophoresis. LPS samples were sonicated gently prior to electrophoresis, and then the gel was stained with silver nitrate stain. The endotoxin activity of isolated LPS was measured by the *Limulus* assay (11).

Treatment of THP-1 Cells with LPS:

The human myelogenous leukemia cell line THP-1 (22) was obtained from ATCC (Manassas, VA). These non-adherent cells were maintained in continuous culture in RPMI-164 (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco) and 0.05 mM 2-mercaptoethanol (Gibco) as described by Shim and Babu (12). One milliliter aliquots of THP-1 cells ($1x10^6$ cells) were cultured in a 48-well culture plates. These cells were treated with $1x10^{-7}$ M phorbol 12-myristate 13-acetate (PMA; Calbiochem Co., La Jolla, CA, USA) for 2 hours to induce maturation of the monocytes and become macrophage-like. The differentiated macrophages were identified by morphological features and their ability to adhere to plastic surfaces. Throughout the study, the cell viability was monitored by Trypan Blue dye-exclusion assay before and after treatment with test reagents. These cells were incubated with *P. gingivalis* LPS (1-10 ng/ml) for 24 hours, and the secreted cytokines were measured by ELISA. In order to study the effect of NDM on THP-1 cells, transformed cells were first incubated with NDM (10-20 µg/ml) for 60 minutes and then challenged with the isolated LPS (10 ng/ml), incubated for 24 hours and the culture supernatants were collected for cytokine assays. To investigate if NDM alters the activity of LPS, the bacteria were grown as described earlier in the media supplemented with NDM (10-20 µg/ml), and then the LPS was isolated as described before and added to the THP-1 cells.

Cytokine Assays:

The collected supernatant fluids were assayed for secreted cytokines, IL-1□ and TNF-□ using ELISA kits obtained from R & D Systems, Inc. (Minneapolis, MN, USA) according to the manufacturer's suggested protocol. The

ELISA plates were read using BMG Lab tech Spectro Star ELISA reader at 490nm. Results were expressed as picograms (pg) of cytokines per milliliter of supernatant fluid calculated from the standard curve.

Data Analysis:

Each experiment was repeated a minimum of three times, with triplicate determinations (of viable bacteria) at each data point. Mean values ± standard error of mean (SEM) from triplicate samples of all assays were calculated and the significance of differences was assessed using ANOVA followed by Scheffe's *f*-test. Differences in results with a *p*-value < 0.05 were considered significant.

Results

Secretion of TNF-α and IL-1β by THP-1 cells in response to *Pg* LPS:

Activated THP-1 cells were incubated with 1-10 ng/ml of *Pg* LPS for 24 hours. After incubation, culture supernatants were assayed for the secreted cytokines by ELISA. The results (Figure 1) show that THP-1 cell's ability to secrete the two cytokines was found to be dependent upon the amount of LPS in the reaction mixture. Cells stimulated with 1 ng/ml of LPS secreted 110 ± 28, and 49 ± 6 pg/ml of TNF-α and IL-1β , respectively. Upon stimulation of cells with 10 ng/ml of LPS a six-fold increase (P <0.029) was seen in the quantity of cytokines secreted, when compared to 1 ng/ml of LPS. In the absence of LPS, THP-1 cells secreted significantly less (P < 0.039) 36 ± 5 of TNF-α and 18 ± 3 pg/ml of IL-1β (data not shown).

Effect of NDM on secretion of cytokines by the THP-1 cells:

To study the effect of NDM on secretion of inflammatory cytokines by monocytic cells, THP-1 cells were incubated with both NDM (10-20 µg/ml) and LPS (10 ng/ml) and assayed for the two cytokines in the culture supernatants. In the presence of NDM, the amount of the two cytokines secreted was inhibited significantly. When NDM was added to the cells, the cells secreted significantly less (P <0.028) TNF-α and IL-1β . The cells secreted 197 ± 31 and 73 ± 12 pg/ml of TNF-α in the presence of 10 and 20 µg/ml of NDM, respectively, compared to 619 ± 26 pg/ml, in its absence (Figure 2). IL-1 β secretion was reduced from 173 ± 15 to 68 ± 10 and 42 ± 8 pg/ml in the presence of 10 and 20 µg/ml NDM. The results (Figure 2) suggest strongly that NDM has the ability to suppress the secretion of both the inflammatory cytokines by the THP-1 cells.

Secretion of TNF-α and IL-1β by THP-1 cells stimulated with LPS of *Pg* grown in presence or absence of NDM:

In order to investigate the possibility that NDM may be altering the functional feature of LPS, we grew *Pg* in the culture media supplemented with NDM (10 and 20 µg/ml). After bacterial growth, LPS was isolated as described earlier. The immunogenic property of LPS isolated from NDM-grown bacteria was found to be similar to control bacterial LPS, when tested by *Limulus* assay (data not shown). However, the LPS of NDM-grown bacteria was less effective (P < 0.021) in stimulating THP-1 cells to secrete TNF-α and IL-1β than the control LPS (Figure 3). About 80-90% reduction in cytokine secretion by THP-1 cells was observed when they were stimulated with NDM-grown bacterial LPS when compared to the amount of cytokines secreted in response to control LPS. The present study demonstrated the effects of cranberry NDM on LPS-mediated interactions with cultured monocytic cells. NDM appeared to suppress the secretion of inflammatory cytokines by the monocytes in response to isolated LPS from *Pg*, a periodontal pathogen. The results also suggest that NDM is capable of inhibiting the effect of LPS to stimulate secretion of cytokines without altering its endotoxin activity.

DISCUSSION

The first line of defense against any infection consists of the recruitment of monocytes and neutrophils. These cells are the most abundant leukocytes in circulating blood, representing greater than fifty percent of the white blood cell count. In this study cultured monocytes (THP-1 cells) were exposed to *Pg* LPS and the amount of secreted cytokines, TNF-α and IL-1β was measured The results (Figure 1) showed that the LPS induced the monocytes to secrete significant quantities of both cytokines. This finding may be relevant to the previous study implicating the systemic inflammatory response as a major factor in severity of periodontitis, atherosclerosis and cardiovascular disease. Excessive secretion of inflammatory cytokines leads to irreversible destruction of tooth-supporting tissues, with tooth loss as a common end point (1). It is very likely that periodontal pathogens cause oral pathogenesis by their ability to stimulate inflammatory cytokines with LPS thus contributing to the severity of the disease.

Cytokines have been recognized as potential markers of the progression and severity of periodontitis as well as indicators of an appropriate response to treatment (13). Moreover, local inhibition of both IL-1 and TNF production in periodontal tissues significantly inhibits the inflammatory response and bone loss in ligature-induced periodontitis in monkeys (14). This suggests that local inhibition of cytokines may be a successful approach for inhibiting bone resorption in periodontitis. In this study, we found that treating macrophages with the cranberry's non-dialyzable material resulted in significant reduction in the secretion of both IL-1β and TNF-α cytokines. Our finding appears to agree with the previous study of Yang et al. (15), who demonstrated that green tea polyphenol-rich epigallocatechin gallate blocked mouse macrophage production of TNF-α .

The growth and viability of *P. gingivalis* was found to be unaffected by cranberry NDM. Ahuja et al.,(16) reported that cranberry juice concentrate had no antibacterial activity on *E. coli*. A recent study by Babu et al. (17) demonstrated cranberry NDM did not affect viability of oral plaque bacterium, *Streptococcus gordonii* but inhibited bacterial metabolic activity. Taken together the results of our study and the existing literature attribute various beneficial roles of the Cranberry NDM. It appears that NDM has the ability to inhibit the production of cytokines by the monocytic cells when stimulated with LPS.

The LPS isolated from bacteria grown in the presence of NDM led to a significant reduction in secretion of the cytokines. In this scenario it may be due to alteration of LPS structure or that NDM may render the lipid-A region of the LPS ineffective thus altering the biological function of LPS. The exact underlying mechanism by which the NDM suppresses the biological function of LPS is far from clear. It would be beneficial to undertake further studies to delineate the mechanisms by which NDM suppresses LPS-induced secretion of cytokines by the monocytic cells. Nevertheless, it is important to note that cranberry NDM can alter the function of LPS in such a way that lesser quantities of cytokines are being secreted, which in turn may reduce the severity of periodontal disease.

Acknowledgements: The authors thank the University of Tennessee College of Dentistry Alumni Endowment and the Tennessee Dental Association Foundation for their financial support of this study.

REFERENCES

1. Darveau, R.P., A. Tanner, and R.C. Page, The microbial challenge in periodontitis. Periodontol 2000, 1997. 14: 12-32.
2. Kornman, K.S., R.C. Page, and M.S. Tonetti, The host response to the microbial challenge in periodontitis: assembling the players. Periodontol 2000, 1997. 14: 33-53.
3. Gemmell, E., R.I. Marshall, and G.J. Seymour, Cytokines and prostaglandins in immune homeostasis and tissue destruction in periodontal disease. Periodontol 2000, 1997. 14: 112-43.
4. Sobota, A.E., Inhibition of bacterial adherence by cranberry juice: potential use for the treatment of urinary tract infections. J Urol, 1984. 131(5): 1013-6.
5. Weiss, E.I., et al., Inhibiting interspecies coaggregation of plaque bacteria with a cranberry juice constituent [published erratam appear in J Am Dent Assoc 1999 Jan;130(1):36 and 1999 Mar;130(3):332]. J Am Dent Assoc, 1998. 129(12): 1719-23.
6. Steinberg, D., et al., Effect of a high-molecular-weight component of cranberry on constituents of dental biofilm. J Antimicrob Chemother, 2004. 54(1): 86-9.
7. Yamanaka, A., et al., Inhibitory effects of cranberry juice on attachment of oral streptococci and biofilm formation. Oral Microbiol Immunol, 2004. 19(3): 150-4.
8. Bodet, C., F. Chandad, and D. Grenier, Anti-inflammatory activity of a high-molecular-weight cranberry fraction on macrophages stimulated by lipopolysaccharides from periodontopathogens. J Dent Res, 2006. 85(3): 235-9.
9. Ofek, I., J. Goldhar, and N. Sharon, Anti-Escherichia coli adhesin activity of cranberry and blueberry juices. Adv Exp Med Biol, 1996. 408: 179-83.
10. Westphal, O., and K. Jann. 1965. Bacterial lipopolysaccharide. Extraction with phenol-water and further applications of the procedure. Methods Carbohydr. Chem. 5: 83-91.
11. Obayashi, T., et al., Development of a new chromogenic Limulus test specific to endotoxin. Rinsho Byori, 1985. 33(6): 639-44.
12. ~~Shim, E., and Babu J.P. Glycated albumin produced in diabetic hyperglycemia promotes monocyte secretion of inflammatory cytokines and bacterial adherence to epithelial cells. J Periodont Res 2014; doi: 10.1111/jre.12194~~ .
13. Hou, L.T., C.M. Liu, and E.F. Rossomando, Crevicular interleukin-1 beta in moderate and severe periodontitis patients and the effect of phase I periodontal treatment. J Clin Periodontol, 1995. 22(2): 162-7.
14. Assuma, R., et al., IL-1 and TNF antagonists inhibit the inflammatory response and bone loss in experimental periodontitis. J Immunol, 1998. 160(1): 403-9.
15. Yang, F., et al., Green tea polyphenols block endotoxin-induced tumor necrosis factor-production and lethality in a murine model. J Nutr, 1998. 128(12): 2334-40.
16. Ahuja, S., B. Kaack, and J. Roberts, Loss of fimbrial adhesion with the addition of Vaccinum macrocarpon to the growth medium of P-fimbriated Escherichia coli. J Urol, 1998. 159(2): 559-62.
17. Babu, J.P., et al., Inhibition of *Streptococcus gordonii* metabolic activity in biofilm by cranberry juice high-molecular-weight component. J Biomed Biotechnol, 2012: ~~590384~~.

ISSN 2348-6201

Figure Legends:

***Figure 1*:** Secretion of cytokines by the monocytic cells in response to *P. gingivalis* LPS. Cultured and *pmsf*-activated THP-1 cells ($1x10^6$ cells) were incubated with LPS (1 to 10 ng/ml) for 24 hours in a 48-well culture plate. Supernatants were collected and assayed for the cytokines by ELISA. Data are the mean ± SEM of triplicate determinations.

***Figure 2*:** Effect of NDM on secretion of TNF-□ and IL-1□ by THP-1 cells in response to LPS of *P. gingivalis*. THP-1 cells ($1x10^6$ cells) incubated with bacterial LPS (10ng/ml) and NDM (10-20 µg/ml) for 24 hours. The released cytokines by the cells into the culture media was measured by ELISA. Data are the mean ± SEM of triplicate determinants. The experiment was repeated three times under similar conditions.

***Figure 3*:** Influence of LPS of *P. gingivalis* grown in the presence of ± NDM on THP-1 cells ability to secrete the cytokines. Cultured monocytic cells ($1x10^6$ cells) were incubated with LPS for 24 hours. Culture supernatants were collected and assayed for TNF-□ and IL-1□ by ELISA. Data are the mean ± SEM of triplicate determinations.

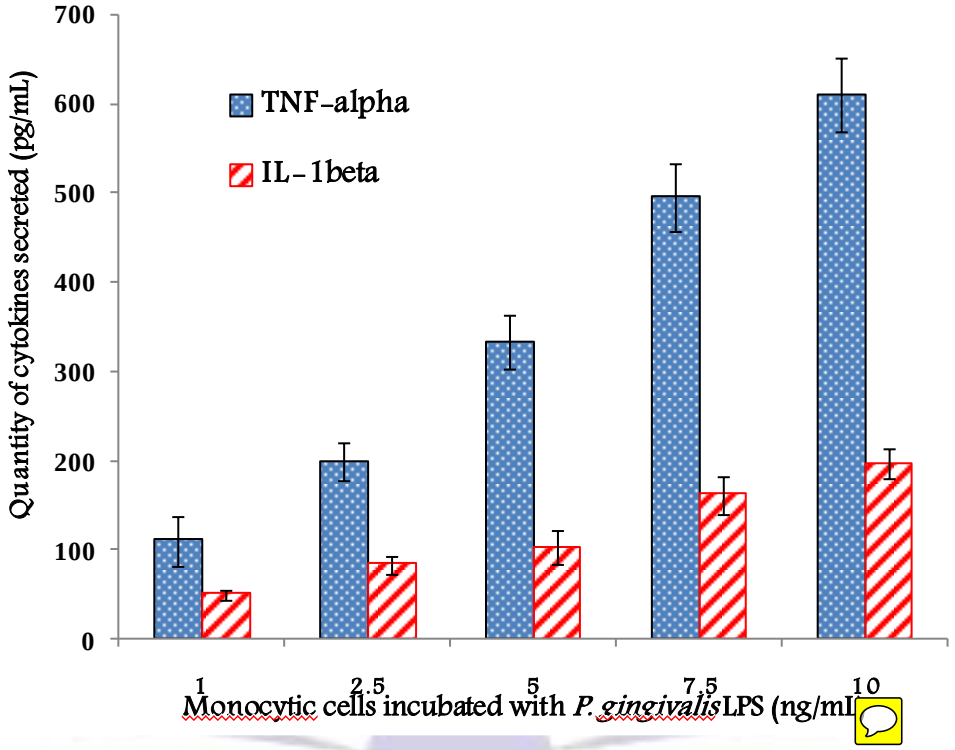

***Figure 1*:** Secretion of cytokines by the monocytic cells in response to *P. gingivalis* LPS. Cultured and *pmsf*-activated THP-1 cells ($1x10^6$ cells) were incubated with LPS (1 to 10 ng/ml) for 24 hours in a 48-well culture plate. Supernatants were collected and assayed for the cytokines by ELISA. The results are given as mean and standard error of triplicate measurements, and are expressed as pg/mL of media from culture supernatants, after subtracting the absorbance values obtained from controls without THP-1 cells. The monocytic cells were found to be viable using trypan blue exclusion asay after incubation with LPS. All values are significantly different from each other (p <0.05).

ISSN 2348-6201

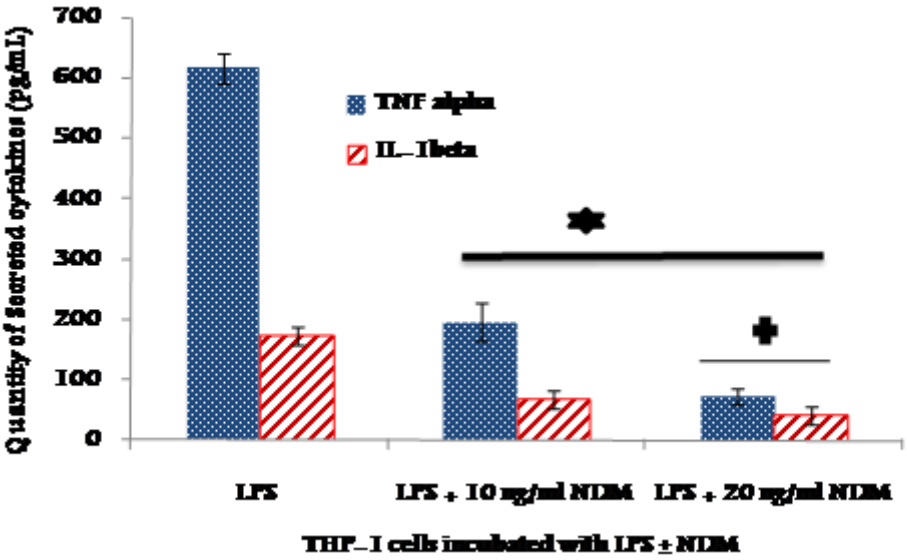

Figure 2: Effect of NDM on secretion of TNF-□ and IL1□ by THP1 cells in response to LPS of P. gingivalis. THP-1 cells (1x106 cells) incubated with bacterial LPS (10ng/ml) and NDM (10-20 µg/ml) for 24 hours. The released cytokines by the cells into the culture media was measured by ELISA. The results are given as mean and standard error of triplicate measurements, and are expressed as pg/mL of media from culture supernatants. The experiment was repeated three times under similar conditions. The horizontal line with an asterisk (*) represents a significant difference (p <0.05) in the secretion of cytokines between cells incubated with LPS and LPS+NDM. Cells incubated with LPS+20 µg/mL (—) secreted significantly less cytokines than those incubated with LPS+10 µg/mL.

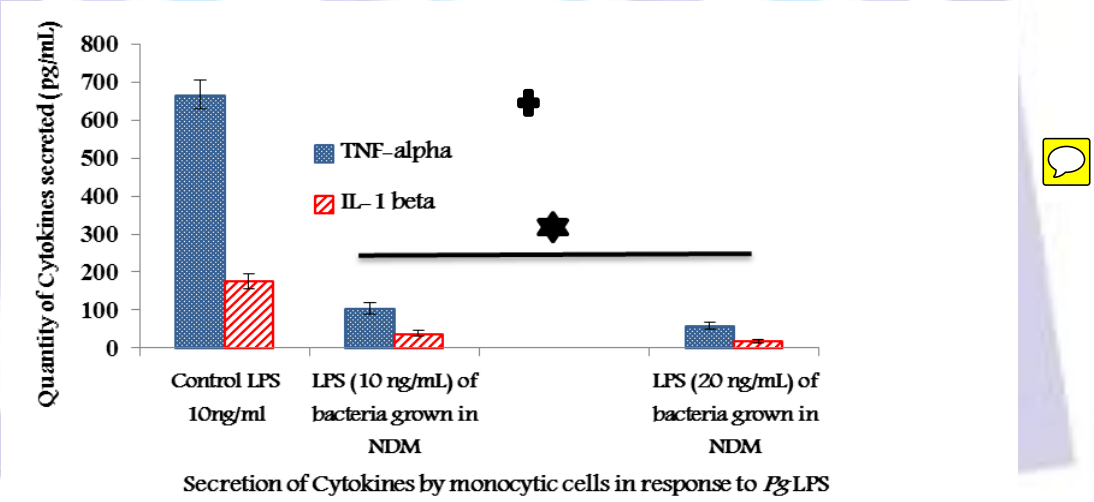

Figure 3: Influence of LPS of *P. gingivalis* grown in the presence of ± NDM on THP-1 cells ability to secrete the cytokines. Cultured monocytic cells (1x10 cells) were incubated with LPS for 24 hours. Culture supernatants were collected and assayed for TNF-□ and IL-□ by ELISA. The results are given as mean and standard error of triplicate measurements, and are expressed as pg/mL of media from culture supernatants. The horizontal line with an asterisk (*) represents a significant difference (p <0.05) in the secretion of cytokines by THP-1 cells in response to control LPS and LPS of bacteria grown in the presence of NDM.