CHAPTER IV

RESULTS

4.1 mRNA Expression of TLRs on HGECs.

TLRs are predominantly expressed on many cells and known as key pattern recognition-receptors of the innate immune system. To obtain additional information on the expression of TLRs in HGECs, total RNA from HGECs was analyzed by RT-PCR using of a panel of specific primers of TLRs 1-10. We found the mRNA expression of TLRs 1, 2, 3, 4, 5, 6, 9, and 10 on HGECs but not TLRs 7 and 8 (Figure 1A). However, TLR4 mRNA expression was very minimal. The results were reproducible in all 4 HGEC lines (Appendix: figure 7A, 7B, and 7C). Human PBMC were used as a positive control and shown to express all mRNA of TLRs 1-10 (Figure 1B). GAPDH expression was used to confirm the quality of all RNA preparation for RT-PCR.

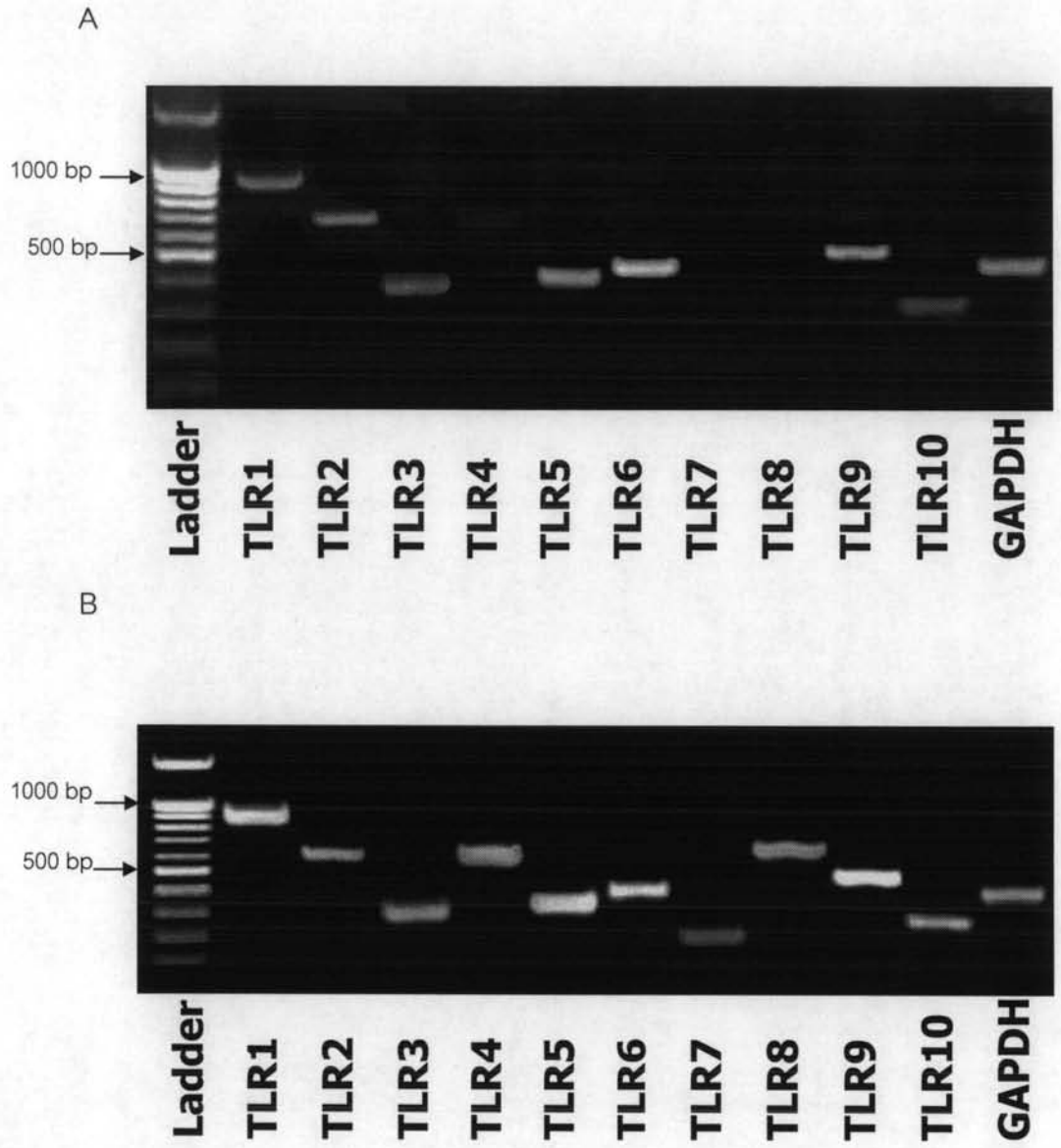


Figure 1. TLR expression in HGECs. TLRs1-10 mRNA was determined in cultured HGECs by RT-PCR (A). PBMC mRNA was used as positive control (B). GAPDH mRNA was used as an internal control. Data are representative of 4 separate HGEC lines.

4.2 TLR ligands stimulate expression of HBD-2

To characterize the functional relevance of TLRs in HGECs, expression of anti-microbial peptide HBD-2 was determined after stimulation with a variety of highly purified TLR ligands. Pro-inflammatory cytokine TNF-α is known to induce epithelial HBD-2 expression (Krisanaprakornkit et al., 2000), therefore we used TNF-α-treated HGECs as a positive control. As depicted in Figure 2A, un-stimulated HGECs constitutively expressed negligible levels of HBD-2 mRNA. HGEC production of HBD-2 was induced by stimulation with *P. gingivalis* LPS, poly I:C, and *S. typhimurium* flagellin , respective ligands for TLRs 2, 3, and 5 , which coincided with their mRNA expression. The mean ratio of HBD-2:GAPDH ± SEM in stimulated HGEC cultures was significantly up-regulated as compared to un-stimulated control (*, $p < 0.05$; Figure 2B). As expected, we did not detect HBD-2 expression in *E. coli* LPS-stimulated HGECs since the culture media used in this study did not contain FCS, the source of LPS-binding protein. Even though the cells expressed TLR9 mRNA, no HBD-2 expression was observed in HGECs stimulated with CpG ODN 2006, (Figure 2A). The results of all 4 HGEC lines are presented in Appendix: figure 8A, 8B, 8C and 8D.

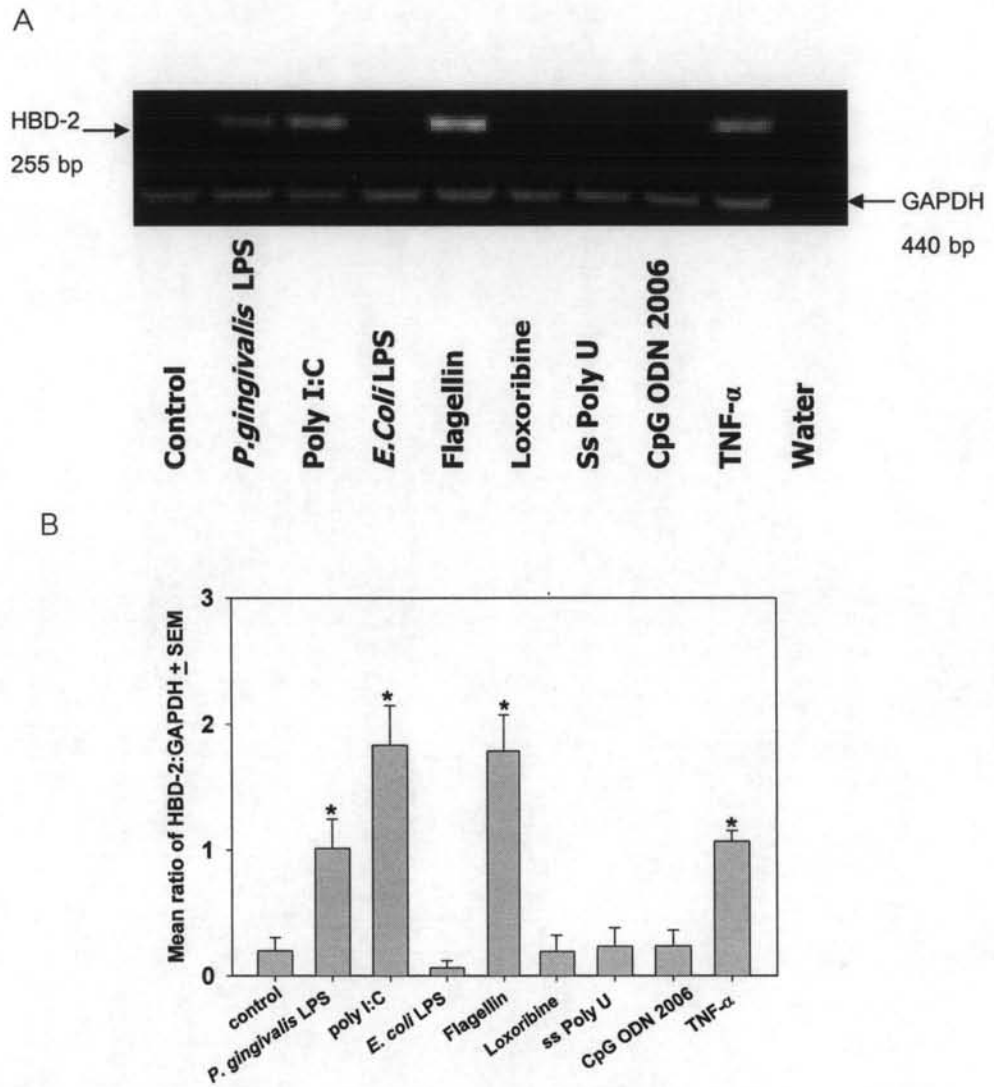


Figure 2. Expression of HBD-2 in HGECs after stimulation with various TLR ligands. HGECs were cultured in 48-well plates, and stimulated with the following ligands: *P. gingivalis* LPS (TLR2 ligand), poly I:C (TLR3 ligand); *E. coli* LPS (TLR4 ligand); *S. typhimurium* (TLR5 ligand); Loxoribine (TLR7 ligand); polyU (TLR8 ligand); CpG ODN 2006 (TLR9 ligand). Culture medium was used as a control. After 24 h incubation, stimulated cells were harvested and mRNA expression of HBD-2 was analyzed by RT-PCR. TNF-α-stimulated HGEC was used as positive control. GAPDH mRNA was used as an internal control. Data are representative of 4 separate experiments (A). Semiquantitative analysis of HBD-2 expression is shown as mean ratio of HBD-2:GAPDH ± SEM from 4 separate experiments (*, $p < 0.05$, compared with un-stimulated control) (B).

4.3 Combination of *P. gingivalis* LPS and TNF-α stimulates expression of epithelial HBD-2

TNF-α, a pro-inflammatory cytokine, has been consistently detected in periodontitis lesions and associated with severity of disease (Seymour, 1991; Page et al., 1997). We next investigated HBD-2 expression in HGECs when stimulated with the combination of this cytokine and LPS from *P. gingivalis*, a product of a key periodontal pathogen. Cells were treated with *P. gingivalis* LPS (50 μg/ml), TNF-α (30 ηg/ml), or a combination of *P. gingivalis* LPS (50 μg/ml) and TNF-α (30 ηg/ml) for 24 h, and the expression of HBD-2 was analyzed by RT-PCR. HGECs stimulated with the combination of *P. gingivalis* LPS and TNF-α led to markedly enhancement of HBD-2 mRNA expression (Figure 3A). The mean ratio of HBD-2:GAPDH ± SEM in the combined-stimulation HGEC cultures was significantly up-regulated when compared to a single-stimulation culture (*, $p < 0.05$; Figure 3B). The results of all 4 HGEC lines are presented in Appendix: figure 9A, 9B, 9C and 9D.

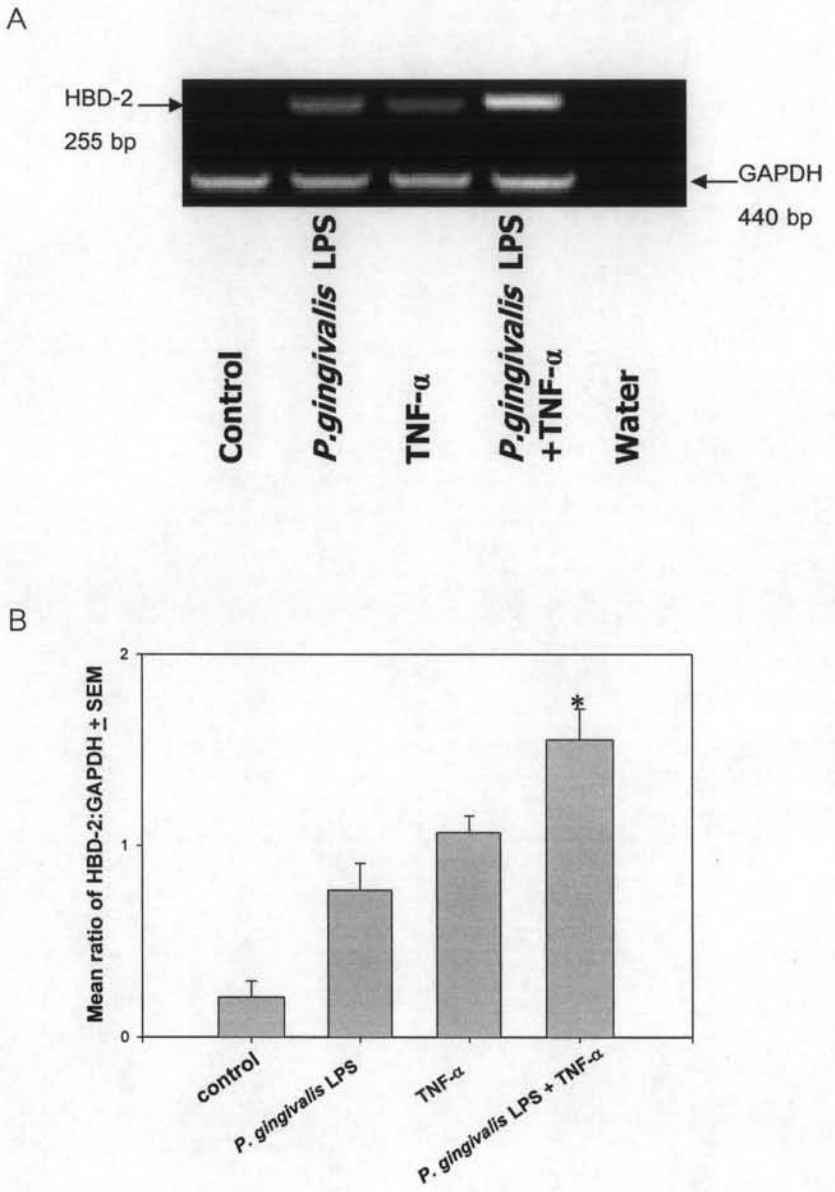


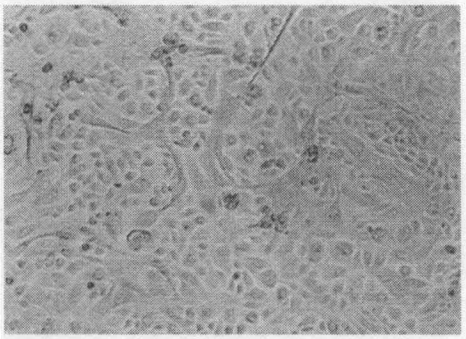
Figure 3. Expression of HBD-2 in HGECs after stimulation with *P. gingivalis* LPS and TNF-α combination. HGECs were cultured in 48-well plates and stimulated with *P. gingivalis* LPS, TNF-α, or *P. gingivalis* LPS and TNF-α. Culture medium was used as a control. GAPDH mRNA was used as an internal control. Data are representative of 4 separate experiments (A). Semiquantitative analysis of HBD-2 expression is shown as mean ratio of HBD-2:GAPDH ± SEM from 4 separate experiments. (*, $p < 0.05$, compared with single-stimulation culture) (B).

4.4 Non-toxic doses of nicotine

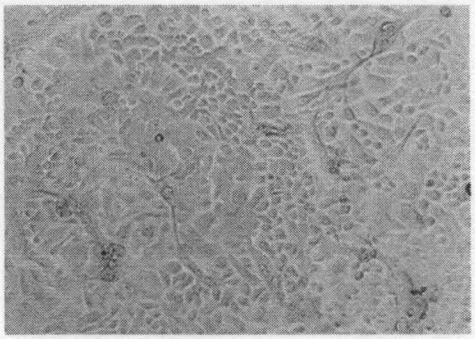
Nicotine, a major component of cigarette smoke, is well recognized for its immune suppression, both in innate and adaptive immunity (King et al., 1988; Martin, 1997; Ferson et al., 1979; Holt, 1987). So far, there have been no studies on the effect of nicotine on the innate immune response of human gingival epithelium. In order to investigate the effects of nicotine on HGEC function, non-toxic doses of nicotine in HGEC culture were determined. Two HGEC lines were treated with different concentrations of nicotine (0.1, 0.3, 1, and 10 mM) for 24 h. Cell viability was then evaluated by the MTT assay. Mean ± SEM of % cell viability in nicotine-treated HGEC culture is presented in table 4. At 10 mM nicotine, about 97% of the HGECs died. In contrast, at 0.1, 0.3, and 1 mM nicotine, the cultured HGECs were more than 100% viable. The observation of the treated cells under microscope confirmed the MTT results (Figure 4). Therefore, the non-toxic doses of 0.1, 0.3, and 1 mM nicotine were selected for HGEC treatment.

Table 4: Cell viability of nicotine-treated HGECs by MTT

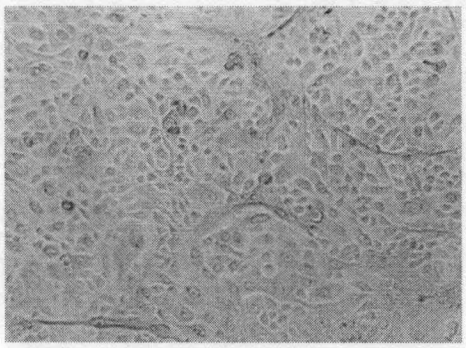
Concentrations of nicotine	% cell viability (mean ± SEM)
Control (untreated HGECs)	100
0.1 mM	109.84 ± 1
0.3 mM	103.76 ± 0.27
1 mM	104.22 ± 0.61
10 mM	3.56 ± 0.09



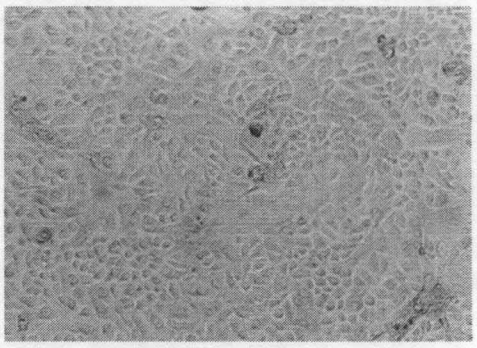
Control (Untreated HGECs)



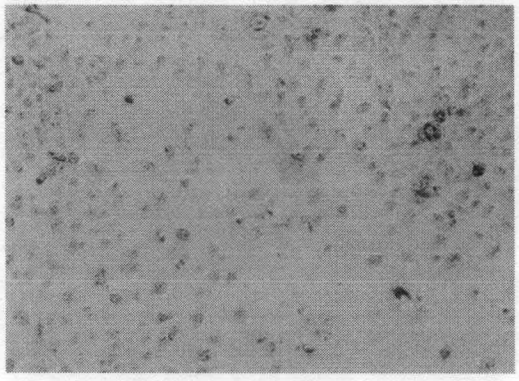
Nicotine 0.1 mM



Nicotine 0.3 mM



Nicotine 1 mM



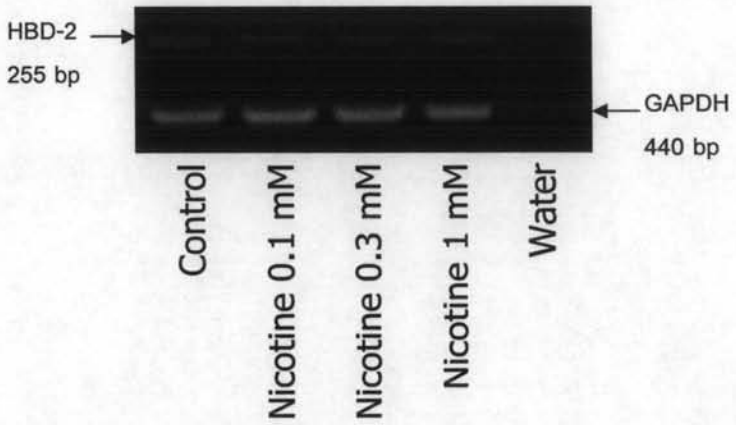
Nicotine 10 mM

Figure 4. Toxicity test of nicotine on HGEC culture. Nicotine at 0.1, 0.3, 1, or 10 mM was added to HGEC culture. The cell condition was observed under microscope (4x).

4.5 The effect of nicotine on HBD-2 expression in unstimulated HGECs

We evaluated the effect of nicotine on HBD-2 expression in unstimulated HGECs. Figure 5A is a representative of 5 HGECs lines which were treated with 0.1, 0.3, and 1 mM of nicotine for 24 h and HBD-2 expression was determined by RT-PCR. The results of treatment by nicotine alone showed a trend of HBD-2 suppression (Figure 5B), however the significant suppression of the mean ratio of HBD-2:GAPDH in nicotine-treated HGECs could not be observed as compared to that of untreated HGECs. The results of all 5 HGEC lines are presented in Appendix: figure 10A, 10B, 10C, 10D and 10E.

A.



B.

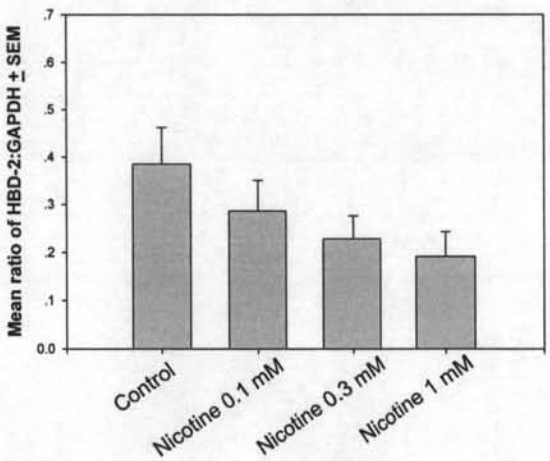
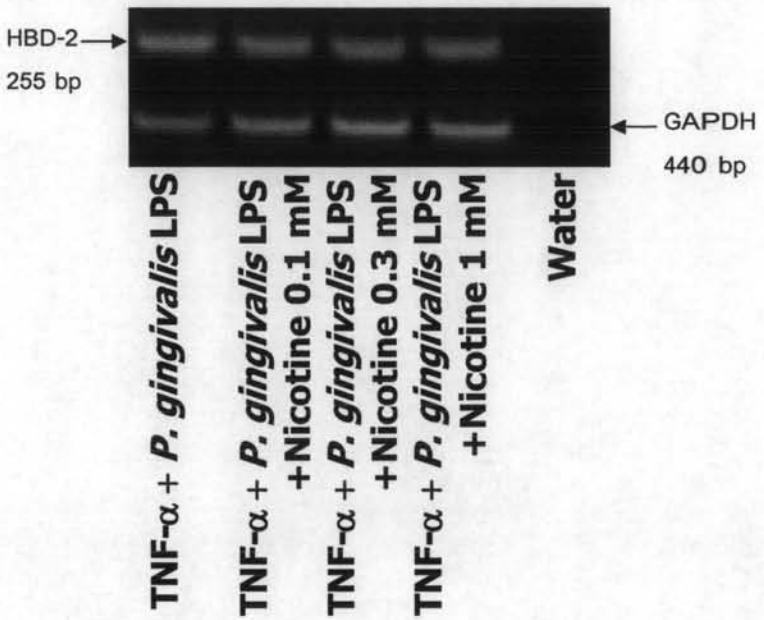


Figure 5. The effect of nicotine on epithelial HBD-2 expression in unstimulated HGECs. HGECs (1.2 x 10^5 cells/ml) were treated with different concentrations of nicotine (0.1, 0.3, and 1 mM), Culture medium was used as a control. After 24 h incubation, treated cells were harvested and mRNA expression of HBD-2 was analyzed by RT-PCR. GAPDH mRNA was used as an internal control. Data are representative of 5 separate experiments (A). For semiquantitative analysis of HBD-2 expression, data shown are mean ratio of HBD-2:GAPDH ± SEM from 5 separate experiments (B).

4.6 The effect of nicotine on HBD-2 expression in stimulated HGECs

Next we studied the effect of nicotine on HBD-2 expression in stimulated HGECs. Figure 6A is a representative of nicotine-treated HGECs which were stimulated with *P.gingivalis* LPS and TNF-α combination, and HBD-2 expression was determined by RT-PCR. The trend of suppressive effect on HBD-2 by nicotine could be observed (Figure 6B) and nicotine at high doses (0.3 and 1 mM) demonstrated a significant HBD-2 suppression (*, $p < 0.05$, compared with cultures stimulated with *P.gingivalis* LPS and TNF-α combination, in the absence of nicotine) (Figure 6B). The results of all 5 HGEC lines are presented in Appendix: figure 11A, 11B, 11C, 11D and 11E.

A.



B.

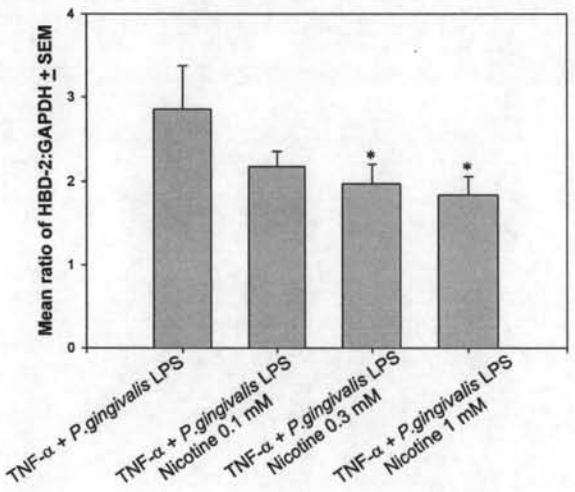


Figure 6. The effect of nicotine on epithelial HBD-2 expression in stimulated HGECs. Nicotine (0, 0.1, 0.3, and 1 mM) was added into HGECs (1.2×10^5 cells/ml) which were stimulated with *P.gingivalis* LPS and TNF-α combination. After 24 h incubation, stimulated cells were harvested and mRNA expression of HBD-2 was analyzed by RT-PCR. GAPDH mRNA was used as an internal control. Data are representative of 5 separate experiments (A). For semiquantitative analysis of HBD-2 expression, data shown are mean ratio of HBD-2:GAPDH ± SEM from 5 separate experiments (B). (*, $p < 0.05$, compared with cultures stimulated with the combination of *P.gingivalis* LPS and TNF-α in the absence of nicotine).