Effect of epigallocatechin-3-gallate on tumor necrosis factor-alpha production by human gingival fibroblasts stimulated with bacterial lipopolysaccharide: An in vitro study

Elahe Karami, Zeinab Rezaei Esfahrood, Reza Mansouri, Ahmad Haerian, Amir Abdian-Asl

Abstract:
Background: Evidence shows that epigallocatechin-3-gallate (EGCG) in green tea has anti-inflammatory effects. Aim: This study assessed the effect of EGCG on the production of tumor necrosis factor-alpha (TNF-α) as an inflammatory cytokine in periodontitis, which produced by human gingival fibroblasts (HGFs) stimulated with lipopolysaccharide (LPS) of Porphyromonas gingivalis. Materials and Methods: In this study, HGFs were cultured and subjected to LPS and EGCG. Cell viability of different concentrations of EGCG (10, 25, 50, 75, and 100 µM) and LPS (1, 10, 20, and 50 µg/mL) was assessed using methyl-thiazole-tetrazolium (MTT) assay. Then, the best concentrations of EGCG and P. gingivalis LPS were used simultaneously and separately to assess the production of TNF-α by HGFs using the enzyme-linked immunosorbent assay (ELISA). Assessments were done at 1, 3, and 5 days. Results: LPS at 1, 10, and 20 and EGCG at 10.25 and 50 µg/mL showed the least cytotoxicity in MTT assay. ELISA showed EGCG alone decreased the production of TNF-α in all days, except 10 µM on day 1. 1, 10, and 20 µg/mL LPS increased the output of TNF-α on days 1 and 3 while reducing it on day 5. The combination of EGCG and LPS showed a decrease of TNF-α in all days except on day 5 that revealed an increase in the production of TNF-α at 25 and 50 µM EGCG. Conclusion: In the combination use of EGCG and LPS, EGCG shows anti-inflammatory effects by decreasing the production of TNF-α by HGFs stimulated with P. gingivalis. Key words: Epigallocatechin-3-gallate, fibroblasts, Porphyromonas gingivalis, tumor necrosis factor-alpha

INTRODUCTION

Periodontitis is a chronic inflammatory disease that leads to connective tissue destruction. It results from the production of inflammatory cytokines such as interleukins (IL-6 and IL-1) and the tumor necrosis factor-alpha (TNF-α) by host immune cells in interaction with gram-negative bacteria such as Porphyromonas gingivalis. Human gingival fibroblasts (HGFs) have been shown to actively take part in inflammatory processes of periodontitis by producing TNF-α, so the modulation of its activity can be an effective way to reduce the severity of the periodontal disease. Due to the harmful side effects of chemical drugs, more attention has been paid to medicinal plants with antimicrobial and anti-inflammatory properties to regulate host responses in inflammatory diseases in recent years. Green tea has been considered as one of the most effective herbal remedies in treating periodontitis, and the polyphenols have inhibitory effects against the production of inflammatory factors by HGFs exposed to bacterial lipopolysaccharide (LPS). Among green tea polyphenols, epigallocatechin-3-gallate (EGCG) has been suggested as one of the significant therapeutic components with anti-inflammatory and antibacterial effects. However, studies on the anti-inflammatory effects of EGCG,

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particularly on HGFs stimulated by *P. gingivalis* LPS, are limited. Most of them are related to assessing the production of other types of ILs by stimulated cells,\(^{14}\) not specifically TNF-\(\alpha\) or related to using of other types of cell.\(^{15}\) In this study, an attempt has been made to examine the effects of EGCG on TNF-\(\alpha\) expression on HGFs stimulated by *P. gingivalis* LPS while HGFs were exposed to EGCG and LPS simultaneously, unlike previous research that first affected the cells to LPS, then after time and the production of some cytokines, the effect of EGCG was investigated.\(^{16}\) We used the MTT method to select the best concentration of LPS and EGCG to affect on HGF, and also applied the enzyme-linked immunosorbent assay (ELISA) kit to determine LPS and EGCG effects on TNF production by smulated HGFs.

### Materials and Methods

This *in vitro* experimental study was approved in the ethics committee of Yazd University of Medical Sciences (IR.SSU.REC.1394.74).

**Culture of human gingival fibroblasts**

HGFs (C165 NCBI code) were obtained from the Pasture Institute of Iran and placed in 25 cm\(^2\) flasks containing Dulbecco’s modified Eagle’s medium (Gibco) containing 1.16 g/L L-glutamine supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 pg/mL streptomycin and incubated at 5% CO\(_2\), and 95% moisture at 37°C (Memmert, Germany). Cells were passaged five times. During culture, the cells were controlled daily and the culture medium was refreshed based on cell density. After reaching 80%-85% confluence, they were transferred to new flasks containing complete culture medium and 10% FBS in a 1:3 ratio. For cell counting, 0.08% trypan blue was used. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to assess cell viability and proliferation.

**Preparation of epigallocatechin-3-gallate and lipopolysaccharide**

The EGCG extract and the standard LPS were purchased in pure from Sigma Aldrich and Invivo company, respectively.

**Preparation of MTT solution**

One milliter of sterile phosphate-buffered saline was added to a vial containing 5 mg of 10X stock solution of MTT and vortexed. Unsolved particles were filtered (0.45 \(\mu\)m) or eliminated with centrifugation; 20 \(\mu\)L of stock solution was added to a 96-well plate (SPL, Korea).

**Assessment of viability and proliferation of human gingival fibroblasts**

For assessment of the LPS and EGCG cytotoxicity, first, 5000 cells in 150 \(\mu\)L of culture medium containing 10% FBS and 15 mM/L D-glucose were added to each well of a 96-well plate and incubated for 24 h. Next, 10, 25, 50, 75, and 100 \(\mu\)M concentrations of EGCG and 1,10, 20, and 50 \(\mu\)g/mL concentrations of *P. gingivalis* LPS (InvivoGen, San Diego, CA, USA) were sterilized and added to cells and incubated for 1, 3, and 5 days. Each test was performed in triplicate for each concentration. The study groups were as follows: (1) EGCG alone, (2) LPS alone, (3) EGCG + *P. gingivalis* LPS, and (4) no treatment (control). After incubation, each well’s supernatant was discarded, then replaced with 100 \(\mu\)L of ready to use-RPIM1640 according to the manufacturer’s instructions. Next, 10 \(\mu\)L of MTT solution was added to each well and the plates were incubated again at 37°C for 4 h. After the completion of incubation time, the culture medium in each well was removed and 100 \(\mu\)L of dimethyl sulfoxide was added to each well to dissolve the formazan crystals. The optical density (OD) of each well was read by the ELISA reader (Anthos, USA) at 570 and 620 nm wavelengths. The mean percentage of cytoxicity and cell proliferation was calculated, and the results were compared with those of the control group.

Quantification of produced TNF-a using ELISA: the TNF-\(\alpha\) ELISA kit (Crystal Day, China) was used. According to the instructions provided in the kit, standard solution and blank wells were prepared. The blank well contained only A and B chromogenic substrates and was considered to have OD = 0. Each standard well contained a certain volume of TNF-\(\alpha\) with a specific OD read by the ELISA reader. The standard solution contained 960 ng/L TNF-\(\alpha\) attached to the antibody and the first standard well containing 50 \(\mu\)L of this solution and 50 \(\mu\)L of conjugated streptavidin-horseradish peroxidase was prepared. The sample wells were prepared to contain 40 ng/L of the respective sample, 10 ng/L of TNF-\(\alpha\) antibody and 50 ng/L of a conjugated enzyme in a 96-well plate. The sample wells contained different concentrations of EGCG (10, 25,50 \(\mu\)M) and LPS (1, 10, and 20 \(\mu\)g/mL). After final incubation and rinse, 50 ng/L of A and 50 ng/L of B chromogenic substrates was added to the wells, and they were incubated in the dark for 10 min. The wells contained blue-colored solutions, which turned yellow a few minutes after adding the stop solution. The ELISA Reader read the OD of the yellow solution in 450 nm wavelength and the obtained OD indicated the concentration of cytokine in each sample.

**Data analysis**

Data were analyzed using the SPSS software version 22 (SPSS Inc., IL, USA). Two-way ANOVA was applied to compare the mean OD values in the test and control (100% viability) groups. \(P < 0.05\) was considered statistically significant.

### RESULTS

**Assessment of viability and proliferation of human gingival fibroblasts by MTT**

Figures 1 and 2 show the cytotoxic effects of EGCG and *P. gingivalis* LPS on HGFs at 1, 3 and 5 days. Significant differences were noted among different concentrations of EGCG and LPS at 1, 3, and 5 days in terms of the mean and standard deviation (SD) of OD (indicative of the percentage of cell viability) (all \(P < 0.001\)). Cell viability on days 1 and 3 was higher than 77% in the presence of different concentrations of EGCG and with an increase in EGCG concentration, cell viability, and proliferation significantly decreased \((P = 0.00)\). Although a reduction in cell activity was noted on day 5, cell viability was acceptably high compared to the control group \((P = 0.00)\). Cell viability in all 3 days and the presence of different concentrations of LPS (except for 50 \(\mu\)g/mL on day 1) indicated increased viability and cell proliferation compared to the control group, which was proportional to the increase.
in LPS concentration and meaningfully significant ($P = 0.00$). Nonetheless, the best levels of EGCG (10, 25, 50 µM) and *P. gingivalis* LPS (1, 10, and 20 µg/mL) with the highest cell viability were chosen for ELISA.

**Assessment of tumor necrosis factor-alpha-a production by sandwich enzyme-linked immunosorbent assay**

Figures 3 and 4 show the effects of different concentrations of EGCG and LPS on the production of TNF-α by HGFs at 1, 3 and 5 days. The mean and SD of ODs indicate the level of produced TNF-α by HGFs, which showed significant differences on days 1, 3 and 5 ($P < 0.001$). ELISA showed that EGCG decreased the production of TNF-α by HGFs in all days and was proportionate to the increase in the concentration of EGCG except for 10 µM on day 1, which caused a slight increase in the production of TNF-α. In contrast, for 1, 10, and 20 µg/mL concentrations of LPS on days 1 and 3, an increase in the production of TNF-α was noted, while a reduction was reported on day 5.

Figures 5-7 show the effect of different concentrations of EGCG on the production of TNF-α by HGFs stimulated with 1, 10, and 20 µg/mL concentrations of LPS at 1, 3, and 5 days. In a combination of EGCG and LPS, 10 µM concentration of EGCG caused a reduction in the production of TNF-α in fibroblasts stimulated with all levels of LPS at 1, 3, and 5 days. A reducing effect was noted with 25 and 50 µM concentrations of EGCG on days 1 and 3 in all the concentrations of LPS. However, on day 5, no decreasing effect on TNF-α production was noted; instead, a combination of EGCG and LPS increased the output of TNF-α compared to unstimulated cells.

**DISCUSSION**

Although inflammation is part of the whole host’s defensive responses against pathogenic bacteria in periodontal diseases, extensive secretion of pro-inflammatory factors by immune cells due to constant bacterial irritation can lead to periodontal damages.[1,15,16] Therefore, regulation of the host response is known as a logical supplemental therapy to decelerate the periodontal collapses.[17,18] For this purpose, to avoid the side effects of chemical drugs,[19] various types of natural products such as plant extracts and polyphenols have been suggested in periodontal treatment.[19,20] An animal study has represented the modulation effects of green tea and the anti-inflammatory effect of epigallocatechin-3-gallate as the most bioactive green tea polyphenol in cytokine expression in the periodontium.[21] Since the LPS component of bacteria can stimulate the expression of inflammatory cytokines such as IL6, IL8 and TNF-α in HGF as a significant cell component of gingival mesenchymal tissues,[22] and the studies on the anti-inflammatory effects of EGCG on HGFs in TNF-α production, while *P. gingivalis* LPS stimulates HGFs are limited. Hence, this study assessed the impact of EGCG on the production of TNF-α by HGFs stimulated with LPS of *P. gingivalis*.

Firstly, the MTT assay determined the cytotoxic concentration of LPS and EGCG and showed a reduction in HGFs.
al.: Inhibitory effect of EGCG on TNF-α production by HGF stimulated with LPS

Figure 5: Effect of different concentrations of epigallocatechin-3-gallate (EGCG) on production of tumor necrosis factor-alpha (TNF-α) by human gingival fibroblasts stimulated with 1 µg/mL concentration of lipopolysaccharide at 1, 3, and 5 days; lipopolysaccharide (LPS)

Figure 6: Effect of different concentrations of epigallocatechin-3-gallate (EGCG) on production of tumor necrosis factor-alpha (TNF-α) by human gingival fibroblasts stimulated with 10 µg/mL concentration of lipopolysaccharide at 1, 3, and 5 days

Figure 7: Effect of different concentrations of epigallocatechin-3-gallate (EGCG) on production of tumor necrosis factor-alpha (TNF-α) by human gingival fibroblasts stimulated with 20 µg/mL concentration of lipopolysaccharide at 1, 3, and 5 days

proliferation to the increase in dosage of EGCG and an enhancement in HGF proliferation to the increase in dosage of LPS, similar to the results of the study by Jung et al.,[35] which was applied to periodontal ligament fibroblasts (PDLF). The low concentrations of LPS promoted the proliferation of PDLF, while high levels of LPS inhibited the proliferation of PDLF in the long term.[33,34] Weisburg et al.[35] also showed that EGCG caused a reduction in the proliferation of cancerous HGFs. The study results by Wu et al.[36] indicate the inhibitory effect of EGCG on Human dermal fibroblast proliferation which tended to decline by raising EGCG concentration to more than 50 µM and this effect was enhanced after 72 h. This inhibitory effect of EGCG on a variety of cell types such as HGF, in our study can be related to its cytostatic nature or amphiaphilic properties, as EGCG binds to cellular proteins and creates interference in the process of transcription and nuclear conversion.[29,30] Therefore, the applied dose of epigallocatechin-3-gallate is crucial in therapeutic effects, whereas the dose-dependent effect of EGCG has been shown in our study. It has also been reported that a concentration of more than 100 µM of EGCG leads to oxidative DNA damage and increment of H2O2 production.[38]

Secondly, the ELISA assay showed that LPS increased the production of TNF-α by HGFs, which was in line with the results of Imatani’s study on HGF and other previous studies on other types of cell.[23,31,32] In fact, the oxidative stress (OS) and production variety species of free radicals in cells following stimulation by bacterial toxin are responsible for the excessive expression of TNF-α.[29,30] However, some free radicals such as reactive oxygen species and reactive nitrogen species have vital roles in different physiological processes, but in the presence of an inefficient anti-oxidant system, they can cause tissue and cell damages to result in enhancing periodontal destruction.[29,30] Since the secretion of TNF-α is managed by nuclear factor kappa B (NF-κB) signalling,[13,14,31] inflammatory diseases are caused by an improper adjustment of NF-κB due to LPS irritation.[14,29] In our study, the expression of TNF-α induced by LPS was reversed on the 5th day and turned downward. This may be related to advances in OS leading to damage to fibroblast cells and induce cell apoptosis.[30] Furthermore, our ELISA assay showed that cytokine production decreased proportionately to the increase in the dose of EGCG, which can be related to the effect of EGCG on cell proliferation due to its cytostatic property.[26,27] Generally, previous research has proven that the optimal effects of EGCG as an anti-oxidant and its anti-inflammatory effects on periodontal diseases[13,14,30] is related to inhibiting NF-κB activation.[13,14]

In ELISA assay, we also confirmed the inhibitory effects of EGCG on the production of TNF-α by stimulated HGFs in combination use of EGCG and LPS. We have shown that 10 µg/mL concentration of EGCG decreased cytokine production in stimulated cells on all 3 days and only on days 1 and 3 for other levels, while on day 5, an increasing trend was noted. This increase in cytokine production may be due to a reduction in viability and proliferation of cells on day 5 after the induction of EGCGT and LPS. However, the reason behind this increase in the production of cytokines on day 5 requires a more accurate assessment at the molecular level. Out results showed EGCG diminishes the secretion of pro-inflammatory cytokines dose-dependently[13,14,31] and time dependently[38] and most clear effects are visible in the early days[31,32] as it has been confirmed in studies about other types stimulated cell or even stimulated HGFs with other different factors. This feature of EGCG could be the cause of unusual results on the 5th day of our study. Moreover, some research has declared that EGCG has significantly stimulated the release of IL-10,[39] which is known as an anti-inflammatory cytokine.
and suppresses the expression of pro-inflammatory cytokines in inflammatory processes.[37] Thus, the production of IL-10 could be an interference in the results of our study.

It should be considered that the difference in the required concentration of polyphenols for inhibitory effects in different studies depends on the method of drug and bacterial toxin application, type of bacteria, type of cell and duration of drug and toxin effects on cells.[39]

CONCLUSION

In an in vitro model of Porphyromonas LPS-induced inflammation in HGF cells, we confirmed that EGCG could exert anti-inflammatory effects via the down-regulation of TNF-α expression, time-dependently and dose-dependently. Therefore, EGCG can be useful in treating periodontal disease caused by P. gingivalis by reducing the inflammatory activity of stimulated HGF. However, the study had several limitations and further research is needed to manage the release of EGCG and other anti-inflammatory factors.

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Conflicts of interest
There are no conflicts of interest.

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