Original Article

Quercetin Decreases Th17 Production by Down-Regulation of MAPK- TLR4 Signaling Pathway on T Cells in Dental Pulpitis

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ABSTRACT

Statement of the Problem: Quercetin is a pharmacological flavonoid that can inhibit high mobility group box1 (HMGB1) protein, a non-histone nuclear protein that is implicated in inflammation. Th17 cells are important cells in the pathogenesis of inflammation. Pulpitis is the inflammation of dental pulp, which usually is accompanied by pain. Quercetin may alleviate this inflammation.

Purpose: The current study aimed to compare blocking of HMGB1 function and stimulation of HMGB1 function with quercetin and investigate the effects of the blockage on T helper 17 (Th17) cells and mitogen-activated protein kinase Toll-like receptor 4 (MAPK-TLR4) signaling pathway.

Materials and Method: T cells isolated from the pulp involved with pulpitis and the normal pulp were cultured. The cells suspensions were plated in 6-wells culture plates and stimulated with 0.5 µg/ml of HMGB1 for 2, 4, 8, and 12 hours. For blocking TLR4, 10 µg/ml rabbit anti-human TLR4 antibody was added 1 hour before treatment with HMGB1.

Results: The level of these cytokines decreased; moreover, western blot data showed that quercetin could decrease MAPK signaling pathway by means of inhibition of HMGB1 on T cells. The results showed the reduction of TLR4 pathway and Th17 cell polarization.

Conclusion: Our results indicated that the levels of IL-17, IL-33, and IL-6 in supernatants from patients' cultured T cells were increased after stimulation with HMGB-1 following employing quercetin. It also could inhibit MAPK signaling pathway, which subsequently could decrease Th17 production and IL-17. Quercetin could decrease pro-inflammatory cytokines and IL-17 production.

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Introduction

Quercetin is a plant polyphenol from the flavonoid glycoside group found in many fruits, vegetables, leaves, and grains. It is contraindicated with some antibiotics like fluoroquinolones since quercetin competitively binds to bacterial DNA gyrase. The effect of quercetin on the fluoroquinolones is not definite yet. [1]

Latest studies identify quercetin as an inhibitor of cytochrome P_{450}2C8 (CYP2C8). CYP2C8 retains epoxygenase activity and metabolizes fatty acids, and

has potential harmful interactions with taxol/paclitaxel, a chemotherapy medication. Since paclitaxel is metabolized primarily by CYP2C8, its bioavailability might be increased unpredictably, which potentially leads to harmful side effects. [2-4]

Moreover, quercetin produces the glycosides quercitrin, which performs like guaijaverin, a plant flavonoid with potential antiplaque activity. CTN-986 is a type of quercetin derivative attained in cottonseeds and cottonseed oil. Miquelianin (C21H18O13) is the quercetin 3-*O*-β-D-glucuronopyranoside that shows an antioxidant effect in human plasma. [5-9]

Toll-like receptors (TLRs) are found on the membranes of pattern recognition receptors and not only play important roles in activating immune responses but are also involved in the pathogenesis of inflammatory disease, injury and cancer. [10] Extracellular high mobility group box1 (HMGB1) acts through multiple receptors, including TLR2, TLR4, and TLR 9. HMGB1 generates a positive feedback loop and in turn, triggers secretion of some proinflammatory cytokines by monocytes; hence, sustaining prolonged inflammation. [11-14] The ability of immune system to recognize molecules that are broadly shared by pathogens is partly because of the presence of TLRs. TLRs are expressed on the membranes of leukocytes including dendritic cells, macrophages, natural killer cells, cells of the adaptive immunity (T and B lymphocytes) and non-immune cells such as epithelial cells, endothelial cells, and fibroblasts. [15]

The binding of ligands to TLRs scripts the key molecular events that ultimately initiate immune responses and subsequently lead to development of antigen-specific acquired immunity. [16]

Upon activation, TLRs recruit adapter proteins within the cytosol of the immune cell in order to propagate the antigen-induced signal transduction pathway. [6] These recruited proteins are then responsible for the subsequent activation of other downstream proteins, including protein kinases (IKKi, IRAK1, IRAK4 and TBK1) that further amplify the signal and ultimately lead to the upregulation or suppression of genes that adjust inflammatory responses and other transcriptional events. Some of these events lead to cytokine production, proliferation, and survival, while others lead to greater adaptive immunity. [6] The pathogen might be phagocytized and digested if the ligand is bacterial factor and its antigens are presented to CD4+T cells. In the case of a viral factor, the infected cell may shut off its protein synthesis and may undergo programmed cell death namely apoptosis. Immune cells that have detected a virus might also release anti-viral factors like interferons. [1, 16]

Recently, some studies have reported that quercetin could play a role as an anti inflammatory agent. [17-18] It must be reasonable to speculate on the inhibitory effect of quercetin on TLR-mediated mitogen-activated protein kinase (MAPK) activation and further inflammatory events. Therefore, the present study was focused on the effect of flavonoid quercetin on the mRNA expression of TLR4 and MAPKs activation with stimulation of HMGB1, cytokine production, activities of different inflammatory enzymes induced during inflammation in humans and evaluating the effect of this flavonoid on dental pulpitis considering the inhibition of inflammation.

Materials and Method

Reagents

TLR4 primer was obtained from Shanghai Sangon Company (Shanghai, China). Recombinant human IL-17, IL-6, and IL-33 ELISA kits were purchased from Bender Med System (Vienna, Austria). Fluorescein isothio cyanate (FITC)-conjugated rabbit anti-human CD14 mAb (Leu M3; Becton Dickinson, Franklin Lakes, NJ, USA), Rabbit anti-human CD4 antibody labelled with PIPE and goat anti-human IL-17 antibody were purchased from Invitrogen Company(Hercules; CA, USA). HMGB1 was obtained from Invitrogen Company, (USA). As a gene recombinant product, its purity was more than 93% as determined by SDS-PAGE, without DNA, and the lipo-polysaccharide (LPS) was less than 1.0 EU per 1 gram of the protein as determined by the limulus amebocyte lysate (LAL) method. LPS (Escherichia coli serotype 055:B5) was purchased from Worthington (Lakewood; NJ, USA).

Patients

Pulpal tissues were obtained from 60 patients at the time of extraction. The inflamed tissues were obtained from extracted carious teeth and normal tissues were obtained from the teeth extracted for orthodontic reasons. Informed consents were obtained from each pa-

tient before surgical extraction, explaining the future analysis on teeth pulp tissues.

Cell culture and HMGB1 stimulation

T cells isolated from the pulpitis and normal tissues were cultured at 1×10^6 cell/ml in RPMI-1640 medium (GIBCO; Invitrogen corporation, UK)supplemented with 20% fetal bovine serum, and 1% streptomycin or penicillin. The cells suspensions were plated in 6-wells culture plates and stimulated with 0.5µg/ml of HMGB1 for 2, 4, 8, and 12 hours. For blocking TLR4, 10µg/ml rabbit anti-human TLR4 antibody was added 1 hour before treatment with HMGB1. In addition, we dissolved 2mg/ml quercetin in ethanol and added 0.5% of total volume of cell culture before treatment with HMGB1.

Reverse transcriptase (RT) - polymerase chain reaction (PCR) analysis for Th-17 and TLR4

Total RNA of the frozen pulp samples was extracted with TRIzol reagent (Invitrogen) and quantified. RT-PCR was performed and analyzed in triplicate assays by SYBR® Green master mix and either the ABI Prism™ 7700 Sequence Detection System or the Step one plus (Applied Biosystems; Darmstadt, Germany) different kinds mRNA in the liver using specific primer pairs (Invitrogen Shanghai Co. Ltd). PCR products were analyzed by electrophoresis on a 1.5% agarose gel. The primers for TLR4 were 5"-ttgtgcaaacttgccgggagga-3" and 5"-acttctccttcagcttggcagc-3" and for Th-17 were 5"-ttgtcccgtgcaaacttgccggggagga-3" and 5"- aagtcccgttattacttgccggttagga-3" respectively. Quantification of gene expression was calculated relative to β-actin.

Western blot analysis

T cells from pulpitis, treated with quercetin, were lysated and then electrophoresed on 12% SDS-PAGE gels and transferred onto polyscreen PVDF transfer membranes (PVDF; PerkinElmer, USA). Membranes were blocked with 5% (w/v) non-fat dry milk, 1% (v/v) Tween 20 in PBS for 1h at room temperature and incubated overnight with commercially available anti-MAPKs antibody (1:1000) (Abcam, USA) at 4^oC. Detection was performed with electrochemiluminescence (ECL) and the blots were quantified by densitometry using the image analysis program (Amercontrol Biosciences, USA).

Flow cytometry analysis

For measuring the T cells count after isolation, the cells were washed and then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-CD14 mAb (Leu M3; Becton Dickinson, Franklin Lakes, NJ). After washing, the cells were re-suspended in 1% FCS/ PBS. Analysis was performed on a flow cytometer (Becton Dickinson, USA), and the cells were specifically analyzed by selective gating based on parameters of forward and sidelight scatter. The T cells were cultured and stimulated with HMGB1, and then mRNA levels of TLR4 were measured by qRT-PCR.

Statistical analysis

All statistical analyses were performed using SPSS17.0 statistical analysis software. Comparisons between paired and unpaired groups were performed using the t-test or one-way ANOVA with Bonferroni correction and *p* Values <0.05 were considered statistically significant.

Results

The mRNA of TLR4 was increased in T cells with HMGB1 stimulation from pulpitis and normal groups, especially when the cells were co-cultured with HMGB1 for eight hours. TLR4 did not increase in quercetin treatments group (Figure 1).

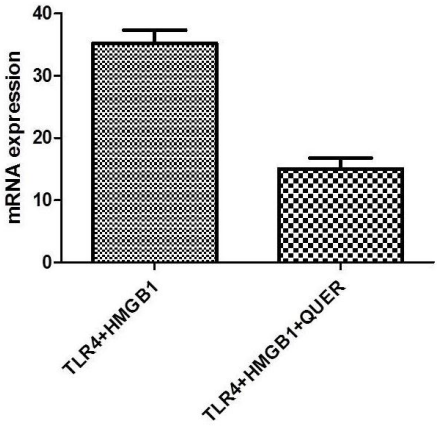

Figure 1: The level of mRNA expression of TLR4 was increased in T cells with HMGB1 stimulation from pulpitis and normal groups, especially when the cells were co-cultured with HMGB1 for 8 hours. TLR4 did not increase in quercetin treatment group

To scrutinize if quercetin could alter MAPK activity, T cells lysates, treated with quercetin, were immuno-blotted with Ab to MAPK and activity was significantly down-regulated following quercetin as compared to sham controls (Figure 2). At 24 and 48 hour of incubation, quercetin significantly down- regulated MAPK gene expression at concentrations of 5 to

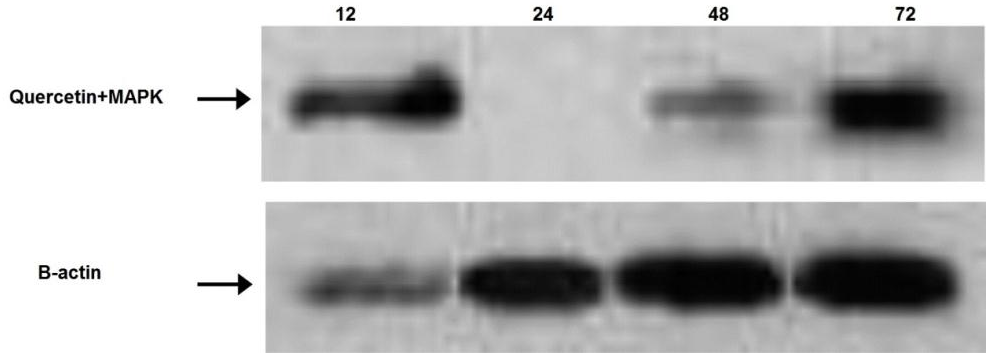

Figure 2: T cells lysates treated with quercetin were immuno-blotted with Ab to MAPK and their activity was down-regulated following employment of quercetin

50 M; and at 72 hour, quercetin significantly down-regulated MAPK gene expression at 10 to 50 M concentrations.

Discussion

T cells are the most important pro-inflammatory cells in the host defense mechanism including normal metabolism, phagocytosis, cytokine generation, and anti-tumor effects. [19-22] We wanted to find a way to activate the cells, and then stop the signaling pathways. Therefore, we stimulated T cells with HMGB1, which is a DNA-binding protein that could also be released extracellularly and function as a late mediator of inflammatory responses. The results indicated that the TLR4 could be a good receptor for HMGB1 on T cells. Some studies discussed that TLR4 could be involved in ST2 and IL-33 activation. [23-26] IL-33 mediates its biological effects by interacting with the receptors ST2 and IL-1 receptor accessory protein, activating intracellular molecules in the NF-κB and MAP kinase signaling pathways that drive production of type 2 cytokines from polarized Th_2 cells. The induction of type 2 cytokines by IL-33 could induce the severe pathological changes observed in mucosal organs following administration of IL-33 *in vivo*. [27] In addition, this signaling pathway could activate MAPK pathway, subsequently cause an increase in the production of pro-inflammatory cytokine IL-6. Our previous study showed how proinflammatory cytokines could active Th17 and increase IL-17 in auto immunity disease and inflammation; however, the main aim of this study was blocking the inflammation in pulpitis. [28] Therefore, we employed quercetin, which is included in a large class of bioflavonoids. Flavonoids represent a group of phytochemicals exhibiting a wide range of biological activities arising mainly from their antioxidant properties and ability to modulate several enzymes or cell receptors. [29] The beneficial effects have been attributed to their antioxidant and anti-inflammatory properties. [30] Quercetin can decrease proinflammatory cytokines such as IL-6 and IL-1β with blocking HMGB1 and inhibition of MAPK signaling pathway. As previously mentioned, MAPK signaling pathway can activate ST2 and TLR4 signaling pathways. Our data showed that quercetin could also inhibit MAPK signaling pathway.

Conclusion

Inhibition of MAPK signaling pathway can decrease Th17 production and decrease IL-17. This study demonstrated that quercetin could decrease pro inflammatory cytokines and IL-17 production.

Conflict of Interest

There was no conflict of interest to declare.

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