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Brief report

Anti-inflammatory Effects of Quercetin and Vitexin on Activated Human Peripheral Blood Neutrophils

- The effects of quercetin and vitexin on human neutrophils -

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Key Words

human neutrophils, myeloperoxidase, nitric oxide, quercetin, tumor necrosis factor-α, vitexin

Abstract

Objectives: Polymorphonuclear neutrophils (PMNs) constitute the first line of defense against invading microbial pathogens. Early events in inflammation involve the recruitment of neutrophils to the site of injury or damage where changes in intracellular calcium can cause the activation of pro-inflammatory mediators from neutrophils including superoxide generation, degranulation and release of myeloperoxidase (MPO), productions of interleukin (IL)-8 and tumor necrosis factor α (TNF-α), and adhesion to the vascular endothelium. To address the anti-inflammatory role of flavonoids, in the present study, we investigated the effects of the flavonoids quercetin and vitexin on the stimulus-induced nitric oxide (NO), TNF-α, and MPO productions in human neutrophils.

Methods: Human peripheral blood neutrophils were isolated, and their viabilities were determined by using the Trypan Blue exclusion test. The polymorphonuclear leukocyte (PMNL) preparations contained more than 98% neutrophils as determined by morphological examination with Giemsa staining. The viabilities of cultured neutrophils with various concentrations of quercetin and vitexin (1 - 100 μM) were studied using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays. Neutrophils were cultured in complete Roswell Park Memorial Institute (RPMI) medium, pre-incubated with or without quercetin and vitexin (25 μM) for 45 min, and stimulated with phorbol 12-myristate 13-acetate (PMA) (10^{-7} M). NO production was carried out through nitrite determination by using the Griess method. Also, the TNF-α and the MPO productions were measured using enzyme-linked immunosorbent assay (ELISA) kits and MPO assay kits.

Results: Neutrophil viability was not affected up to a concentration of 100 μM of quercetin or vitexin. Both quercetin and vitexin significantly inhibited TNF-α, NO, and MPO productions in human neutrophils ($P < 0.001$).

Conclusion: The present study showed that both quercetin and vitexin had significant anti-inflammatory effects. Thus, treatment with either quercetin or vitexin may be considered as a therapeutic strategy for treating patients with neutrophil-mediated inflammatory diseases.

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1. Introduction

Recent studies have evidenced that neutrophils are key regulators of acute and chronic inflammatory responses. Neutrophils play a well-established role in host defense by exterminating pathogens *via* phagocy-

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tosis and succeeding intracellular and extracellular killing mechanisms as a critical part of immunity [1]. Phagocytosis consists of pathogen internalization as phagosomes, as well as subsequent destruction *via* fusing phagosomes with toxic compounds containing granules and generation of reactive oxygen species (ROS) *via* myeloperoxidase (MPO), a pro-oxidant enzyme copiously found in the primary granules of neutrophils [2]. In addition to MPO, both nitric oxide (NO) produced by inducible NO synthase (iNOS) and tumor necrosis factor α (TNF-α) are other significant participants from neutrophils during the inflammatory process of immune response [3]. Due to the important effects of neutrophils throughout inflammation, modulating their functions is an interesting therapeutic strategy to reduce inflammation. In recent years, flavonoids have been thought to exert various beneficial effects, including antioxidant, anti-inflammatory, and anti-allergic effects [4, 5].

Quercetin (3,3′,4′,5,7-penta hydroxyl flavone) and vitexin (apigenin-8-C-β-D-glucopyranoside), which are found in a variety of fruits and vegetables, are among the most widely distributed flavonoids and have been demonstrated to have strong anti-inflammatory, antioxidant, and neuroprotective activities [6-8]. Quercetin has also been shown to attenuate effectively lipopolysaccharide (LPS)-induced acute lung injury by inhibiting the levels of TNF-α and interleukin (IL)-6 secretions and by reducing the MPO activity in animals [9]. Vitexin has also been shown to induce an anti-inflammatory effect by reducing pro-inflammatory cytokines such as IL-1β, IL-6, IL-33, and TNF-α [10]. Thus, the aim of this study was to investigate the anti-inflammatory effects of both quercetin and vitexin against phorbol 12-myristate 13-acetate (PMA)-induced neutrophil stimulation by evaluating their potential roles in modulating NO, TNF-α, and MPO productions in tissue.

2. Material and Methods

The protocol used in this study was approved by the Ethics Committee of Qazvin University of Medical Sciences (IR.QUMS.REC.1394.112) and all participants gave signed informed consent. Human blood from healthy volunteers was collected and placed in heparinized tubes (5 U/mL). The entire procedure was conducted under endotoxin-free conditions. Neutrophils were isolated by using Ficoll-Hypaque gradient centrifugation (at 400 × g for 30 min at room temperature (RT)). The layer consisting of erythrocytes and polymorphonuclear leukocytes (PMNLs) was harvested using a sterile Pasteur pipette and mixed with one volume of dextran/NaCl solution (3% dextran in 0.9% NaCl). The cell suspension was kept at room temperature for 30 min in the dark.

Neutrophils were collected from the upper layer by centrifugation at 200 × g for 10 min at RT. The residual erythrocytes were hemolyzed. The neutrophil-enriched pellet was re-suspended in ice-cold 0.2% NaCl for 30 sec and isotonic osmolarity was re-established by adding one volume of ice-cold 1.6% NaCl. Cells were washed twice with phosphate buffer saline (PBS), re-suspended in culture medium (Roswell Park Memorial Institute (RPMI) medium 1640, 10% fetal bovine serum (FBS), nonessential amino acids, 50 U/mL penicillin, 50 μg/mL streptomycin), and adjusted to 1×10^6 cells/mL. Neutrophils viability was determined by using the Trypan Blue exclusion test, and the viability was 98%. The PMNL preparations contained > 98% neutrophils, as determined by using morphological examinations with Giemsa staining.

The viability of the neutrophils was determined by using the tetrazolium-based colorimetric test. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) experiment relies on the reduction of the yellow MTT to blue formazan crystals by the mitochondrial dehydrogenase enzymes in living cells. In fact, the increase in absorbance is related to an increment in enzyme activity. Neutrophils were cultured in 96-well plates in the presence of different concentrations of both quercetin and vitexin (1-100 μM) at 37°C for 2 h. Dimethylsulfoxide (DMSO) was used to dissolve both flavonoids. After 2 h of treatment, the MTT solution (5 mg/mL) was added, and the cells were incubated for 4 h at 37°C in a 5% CO_2 incubator. Then, DMSO was added to dissolve the MTT formazan crystals. The absorbance was measured at 570 nm by using a microplate reader spectrophotometer. Experiments were carried out in triplicate, and the percentage of MTT reduction compared to untreated control cells was calculated.

NO production was carried out a through nitrite determination by using the procedure described by Ding *et al* [11]. Nitric oxide is rapidly changed into nitrite in aqueous solutions. Therefore, the total nitrite can be used as an indicator of nitric-oxide concentration. The spectrophotometric analysis of the total nitrite content was performed by using the Griess method (1% sulfanilic acid, 0.1% N-1-naphthylethylenediamine dihydrochloride) in the supernatant of the neutrophil culture. Neutrophils (5×10^5/well) were cultured with and without 25 μM of quercetin or 25 μM of vitexin for 45 min and were then PMA-stimulated (10^{-7} M) for 4 h in RPMI-1640 medium supplemented with 10% fetal bovine serum. Then, the same volume of Griess was added to the cell culture supernatant, and after 15 min, the absorbance was measured at 550 nm by using a spectrophotometer. The nitrite concentration was determined using sodium nitrite as a standard (0 - 60 μM).

The levels of the TNF-α cytokine in the neutrophil culture supernatant were measured with ELISA kits according to the manufacturer's instructions (eBioscience, USA). Neutrophils (1×10^6 cells/mL) were cultured with vitexin (25 μM) or with quercetin (25 μM) for 45 min and then were stimulated with PMA (10^{-7} M) for 6 h. Afterwards, cells were centrifuged (1000 × g, 4°C, 10 min), and the supernatant was collected and stored at - 80°C until it was used for the cytokine determination. The measurement of MPO enzyme activity was performed using MPO assay kits according to the manufacturer's instructions (Abcam, UK). Neutrophils (2×10^6 cells/well) were exposed for 45 min at 37°C with and without 25 μM of quercetin or vitexin; then, 10^{-7} M PMA was added for 2 h.

Statistical analyses of the data were performed using GraphPad Prism software. Results were expressed as means ± standard errors of the mean (SEMs) and were tested for significance by using the Student's *t*-test. Results with $P < 0.05$ were considered significant.

3. Results

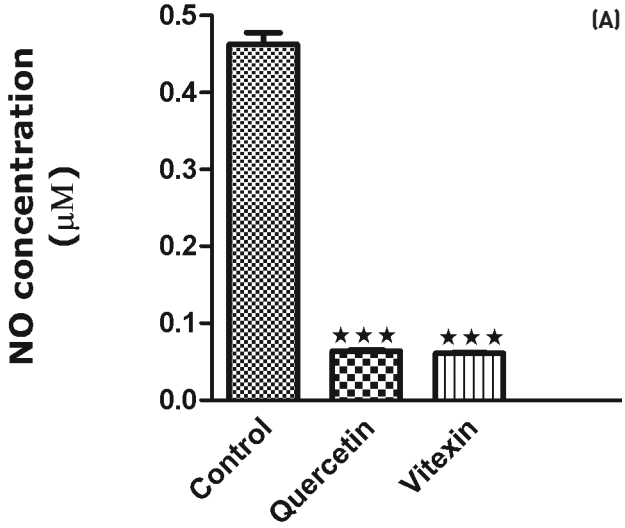
Neither quercetin nor vitexin at concentrations up to 100 μM affected neutrophil viability during a 4-h incubation period. The nitric-oxide production was evaluated in cells treated with quercetin (25 μM) or with vitexin (25 μM) and in control. Decreases of 86.09%, and 86.74% in NO production were observed in the cells treated with quercetin and with vitexin groups, respectively, when compared with the control (Fig. 1A). Human neutrophils responded to PMA stimulation by secreting TNF-α while cells pre-treated with quercetin and with vitexin did not produce this cytokine. Quercetin (25 μM) and vitexin (25 μM) groups significantly inhibited TNF-α production by 79.31%, and 80.94%, respectively (Fig. 1B). The MPO activity in neutrophils was evaluated after induction of neutrophil degranulation by the addition of PMA for 2 h. As compared with the control, the MPO activities were reduced by 83.7% and 87.3% in the cells treated with quercetin and with vitexin, respectively (Fig. 1C).

4. Discussion

Inflammation is the most common appearance of tissue pathology and is involved in the pathogenesis of numerous diseases, such as diabetes, cancer, and neurodegenerative diseases [12-14]. Reviews of the literature on the effects of flavonoids on various inflammatory processes and immune functions have shown that they may inhibit several factors that are activated under inflammatory conditions [6]. In the present study, the effects of administering two flavonoids, quercetin and vitexin, on activated human neutrophils were investigated.

Results showed that activated isolated human peripheral blood neutrophils exhibited significantly increased NO, TNF-α, and MPO productions while pre-incubation of neutrophils with quercetin or vitexin significantly reduced the productions of these factors. The results of this study were similar to those of a previous study on the administration of rutin to activated human neutrophils [5]. During the inflammatory process, NO is produced by inducible iNOS and plays an important role in the early inflammatory response [15].

Quercetin pretreatment inhibits LPS-induced iNOS gene expression and NO release in LPS-activated macrophages



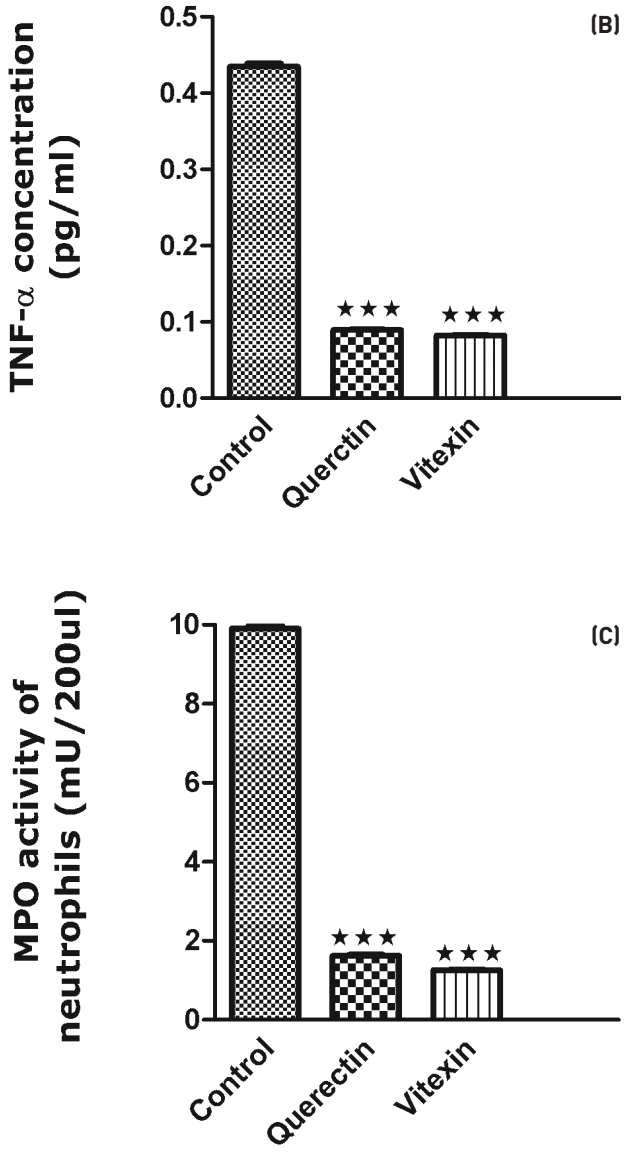


Figure 1 Anti-inflammatory effects of quercetin and vitexin against PMA-induced neutrophils stimulation of (A) NO, (B) TNF-α, and (C) MPO productions. Values are expressed as means ± SEMs (n = 6 with 3 repeated experiments). The *t*-test was used for analyses, and $^{***}P < 0.001$ compared to the control.

PMA, phorbol 12-myristate 13-acetate; NO, nitric oxide; TNF-α, tumor necrosis factor α; MPO, myeloperoxidase; SEMs, standard errors of the mean.

[16]. Similar effects of quercetin on macrophages were also demonstrated in another study [17]. Moreover, vitexin has been reported to reduce NO production *via* neutrophils [18]. These results are in good agreement with the results of this study as the administration of either quercetin or vitexin significantly reduced *in-vitro* NO production by neutrophils. The underlying mechanism in neutrophils may be similar to that in the aforementioned studies on macrophages. TNF-α is important for the induction of inflammatory responses, and quercetin administration has been shown to reduce the TNF-α level significantly and to protect against radiation-induced pulmonary injury in mice [19].

Similarly, another study reported the inhibitory effects of quercetin on LPS-induced NO, iNOS and TNF-α mRNA and protein expressions while it promoted *in-vitro* heme-oxygenase-1 induction in a dose- and time-dependent manner [17]. Quercetin has also been reported to inhibit in mice the productions of pro-inflammatory cytokines, such as IL-1b, IL-6, and TNF-α, from macrophages in LPS-induced inflammation [20]. In line with the above study, the inhibitory effects of quercetin have been demonstrated in patients with sarcoidosis, and those effects have been shown to be due to the reductions of inflammatory markers, such as TNF-α and IL-8 [21]. Furthermore, some studies have reported similar effects of vitexin on TNF-α. Vitexin has been shown to have an inhibitory effect in mice on the productions of different pro-inflammatory cytokines (TNF-α, IL-1B, IL-6, and IL-33) induced by carrageenan [10]. The protective effects of vitexin against myocardial ischemia/reperfusion injury in rats have also been shown to be due to an inhibition of the activation of the transcription factor $NF_{\kappa}B$ and the subsequent generation of TNF-α [22, 23].

Similarly, our results showed that administration of either quercetin or vitexin could remarkably decrease TNF-α production by neutrophils. This reduction of TNF-α may be caused by a mechanism similar to that in the previously mentioned studies. MPO is an enzyme that is mainly found in the primary granules of neutrophils, and its principal duty is to destroy microorganisms; however, under certain conditions, it generates an excess of oxidants, which causes tissue damage [24]. Quercetin has been demonstrated to down regulate significantly the MPO activity in hypercholesterolemic rats, which is an indication of decreased neutrophil infiltration and thereby reduced generation of ROS [25]. Also, the incubation of human neutrophils stimulated with quercetin has been shown to be able to reduce the productions of ROS and MPO [26]. Furthermore, another study revealed that quercetin decreased MPO release in the injured tissue after a spinal cord injury in rats [27]. Vitexin has also been shown to cause a significant reduction of neutrophil infiltration and an attenuation of MPO activity in a model of rats with acute doxorubicin cardiotoxicity [28]. The results of the present study support the finding of previous studies as both quercetin and vitexin significantly inhibited the release of MPO from PMA-activated human neutrophils.

5. Conculsion

This study demonstrated that PMA-activated human peripheral blood neutrophils and the significant ensuing productions of NO, TNF-α, and MPO could be inhibited *via* pretreatment of neutrophils with quercetin or with vitexin. These results show that these flavonoids may be promising agents for the prevention and the treatment of patients with various inflammatory diseases. However, further studies are required in order to elucidate the underlying cellular and molecular mechanisms of therapy with the two mentioned flavonoids.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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