

Hydrolyzed Olive Vegetation Water in Mice Has Anti-Inflammatory Activity¹

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ABSTRACT Fruit and vegetable simple and polyphenols are potent antioxidants. One of the most effective in terms of free radical scavenging is 3,4-dihydroxyphenyl ethanol or hydroxytyrosol (HT), a simple phenol found predominantly in *Olea europea*, or the olive plant. HT is most abundant in the aqueous fraction of olive pulp with trace amounts in the olive oil fraction and in the leaves. For these experiments, we evaluated the anti-inflammatory activity of olive vegetation water (OVW), which we showed previously to have potent antioxidant activity. Because some simple phenols and polyphenols with antioxidant activity have shown varying anti-inflammatory activities, we tested OVW and HT for their ability to inhibit the production of tumor necrosis factor- α (TNF- α), a pivotal cytokine in inflammation. In lipopolysaccharide (LPS)-treated BALB/c mice, a model system of inflammation, OVW at a dose of 125 mg/mouse (500 mg/kg) reduced serum TNF- α levels by 95%. In the human monocyte cell line, THP-1, OVW reduced LPS-induced TNF- α production by 50% at a concentration of 0.5 g/L (equivalent to ~0.03 g/L simple and polyphenols). OVW had no toxic effects in vitro or in vivo. When OVW was combined with glucosamine, a component of proteoglycans and glycoproteins that was shown to decrease inducible nitric oxide synthase production in cultured macrophage cells, the 2 compounds acted synergistically to reduce serum TNF- α levels in LPS-treated mice. These findings suggest that a combination of OVW and glucosamine may be an effective therapy for a variety of inflammatory processes, including rheumatoid and osteoarthritis. J. Nutr. 135: 1475–1479, 2005.

KEY WORDS: • olive • inflammation • cytokines • antioxidant

The Mediterranean diet, rich in fruit, vegetables, and fish, has been associated with a lowered incidence of disease and an overall improvement in health (1–3). Many of the diseases affected by the Mediterranean diet, including cancer, diabetes, and cardiovascular disease, have a component of inflammation, or the disease is exacerbated by inflammatory mediators. Concomitant with inflammation is the generation of reactive oxygen species (ROS)³ or free radicals, which increase oxidation of proteins and lipids, resulting in signals that trigger more inflammation. The health benefits of the Mediterranean diet have been attributed to high concentrations of free radical-scavenging simple and polyphenols and flavonoids. Olive fruit, a major component of this type of diet, is particularly beneficial to health by virtue of its oil composition (predominantly oleic acid) and antioxidant phenolics.

Olive phenolics possess the highest antioxidant activities of all known natural antioxidants. Olive phenolics were shown to inhibit LDL oxidation and platelet aggregation, scavenge superoxide and other ROS, scavenge hypochlorous acid, inhibit neutrophil respiratory burst, and increase plasma antioxidant capacity (4). Hydroxytyrosol (HT), an olive phenolic structurally related to caffeic acid, was shown to inhibit free

radical generation, scavenge reactive oxygen and nitrogen, inhibit smoking-induced oxidative stress in rats, and increase plasma antioxidant capacity (5–10).

In addition to their activities as antioxidants, some simple and polyphenolics were shown to possess anti-inflammatory activity. For example, polyphenolic compounds extracted from red wine and from black tea modulate cyclooxygenase 2 (COX-2) activity and COX-2 gene expression in cells (11). Epigallocatechin gallate (EGCG), the major green tea polyphenol, dose dependently decreased lipopolysaccharide (LPS)-induced tumor necrosis factor- α (TNF- α) production in a human macrophage cell line (12). In vivo, LPS-induced serum TNF- α was reduced by 80% after administration of green tea polyphenols to BALB/c mice (12).

In this study, the effects of olive vegetation water (OVW) on LPS-treated human THP-1 cells and in LPS-treated BALB/c mice were investigated. The effect of glucosamine, an essential component of glycoproteins and proteoglycans that had efficacy in human trials of arthritis, was also evaluated. Finally, the combined effect of OVW and glucosamine on TNF- α production was measured to determine whether these compounds act synergistically to attenuate inflammation.

MATERIALS AND METHODS

Reagents. RPMI and fetal bovine serum (FBS) were purchased from GIBCO. The TNF- α ELISA kit was purchased from R&D Systems; 3,4-dihydroxyphenyl ethanol (HT) was purchased from Cayman Chemical. Oleuropein was purchased from Indofine Chemical and glucosamine from Protein Research Labs. Trolox, 1,1-diphenyl-2-picrylhydrazyl, dexamethasone (DEX), LPS, and Folin reagent

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³ Abbreviations used: AUC, area under the curve; COX-2, cyclooxygenase-2; DEX, dexamethasone; ED, effective dose; EGCG, epigallocatechin gallate; FBS, fetal bovine serum; HT, hydroxytyrosol; NF- κ B, nuclear factor- κ B; ORAC, oxygen radical absorbance capacity; OVW, olive vegetation water; RA, rheumatoid arthritis; ROS, reactive oxygen species; TNF- α , tumor necrosis factor- α .

were purchased from Sigma. Standards including caffeic acid, gallic acid, tyrosol, catechol, and protocatechuic acid, as well as all other reagents, were purchased from Sigma.

Sample preparation. Manzanilla olive fruit, collected in the 2000–2001 crop year, was first depitted, and then the pitless pulp was mechanically pressed to yield a liquid phase mixture including olive oil, vegetation water, and solids. Solids were removed from the liquid phase mixture by filtration and centrifugation at $8000 \times g$ for 40 min. The oil and aqueous fractions were gravity-separated, and the aqueous phase was decanted. The OVW was treated with citric acid (1%) for 6 mo before analytical HPLC. For *in vitro* and *in vivo* studies, the OVW was freeze-dried, yielding a light brown crystalline product containing at least 6% polyphenol (OVW refers to the freeze-dried form of the OVW).

Analysis of phenolic composition. The freeze-dried olive extract was resuspended in water at a concentration of 100 g/L, and a sample was evaluated by HPLC on a Beckman-Coulter 125 NM series system consisting of a 125 NM series pump, a 166 NMP series detector, and an analytical Ultrasphere reverse-phase column (C-18; 150×4.6 mm i.d.). Separation was achieved by elution gradient essentially as described by Romani et al. (13). Data were collected and analyzed using Beckman 32 Karat Software. Compound identification was confirmed by analyzing retention times and absorption of standards at 5 different wavelengths (220, 240, 280, 320, and 340 nm). The concentration of HT in this OVW was determined to be 17 mg/g by comparison of HT in OVW with HPLC analysis of various concentrations of pure HT.

Oxygen radical absorbance capacity (ORAC). ORAC was employed to evaluate the antioxidant activity of OVW, freeze-dried OVW, olive oil, olive leaf, and pure HT. ORAC was performed essentially as described by Cao et al. (14) by Brunswick Laboratories. Final ORAC values were calculated using the regression equation between Trolox concentration and area under the curve (AUC), and expressed as $\mu\text{mol Trolox equivalence/L}$ for liquid samples or $\mu\text{equivalence/g}$ for solid samples.

TNF- α measurement in THP cells. The human monocytic cell line, THP-1, was obtained from ATCC. OVW or HT was added to THP-1 cells in 96-well microtiter plates for 1 h before stimulation with LPS ($10 \mu\text{g/L}$) for 3 h at 37°C . After LPS stimulation, supernatants were collected by centrifugation at $200 \times g$ for 20 min and were evaluated for levels of TNF- α by ELISA. DEX (100 nmol/L), an inhibitor of TNF- α release, was used as a positive control.

TNF- α level *in vivo*. Treatment of the mice (Charles River Laboratories) adhered to regulations outlined in the USDA Animal Welfare Act and the conditions specified in the NRC guidelines. All protocols were approved by IACUC. The mice were observed at least daily for signs of illness or distress. The mice used in this study were nulliparous nonpregnant BALB/c mice weighing 20–25 g. The mice were housed in a dedicated room in a stand-alone facility after receipt and 3 d of quarantine. Primary enclosures were as specified in the USDA Animal Welfare Act (9 CFR, Parts 1, 2 and 3) and as described in the NRC guidelines. Mice were housed 4/cage. The rooms were ventilated (>12 air changes/h) with 100% fresh air (no air recirculation). A 12-h light:dark photoperiod was maintained, except when room lights were turned on during the dark cycle to accommodate blood sampling or other study procedures. Room temperature was maintained between 18 and 26°C . The mice were allowed free access to food and water before testing. Standard rodent diet (Prolab[®] RMH 2500; PMI Nutritional International) consisted of 24% protein, 10.5% fat, 49% carbohydrates, minerals, and vitamins. The day before the experiment, the mice were food deprived for 24 h. At the end of the food-deprivation period, OVW or glucosamine or OVW + glucosamine at different doses (“test article”) was administered to the mice by oral gavage, and the mice were allowed to rest for 12 h. Control mice were administered water by oral gavage. During the food-deprivation and post-treatment periods, water was consumed *ad libitum*. OVW was comprised of ~ 6 – 10% polyphenols ($\sim 3\%$ HT), 65% carbohydrates, 20% fat, and 6% protein. After 12 h, a second dose of test article was given to the mice by oral gavage. At 1 h later, they were administered LPS ($50 \mu\text{g}$ in water) by i.p. injection. At 2 h after LPS treatment, the mice were killed by cervical dislocation while anesthetized with isoflurane in

accordance with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association; blood was collected by retro-orbital bleeding. Blood was allowed to clot at 4°C for 12 h. After clotting, the blood was centrifuged at $1000 \times g$ for 10 min, and serum was collected for measurement of TNF- α levels by ELISA.

Determination of synergy. To determine whether OVW and glucosamine have a synergistic effect on LPS-induced inflammation *in vivo*, we performed an isobolographic analysis as described previously (15). For this analysis, 2 series of dosage curves were obtained. First, using a fixed concentration of OVW, inhibition curves were generated; then, using a fixed concentration of glucosamine, another set of inhibition curves was generated. The 85% effective dose (ED_{85}) values of each of the compounds was determined and used to plot the isobologram. The combination of OVW and glucosamine yielded an effect that fell below the line of additivity, suggesting a synergistic effect.

Statistical analysis. The results are expressed as means \pm SEM of at least 10 independent measures. Data were analyzed by 1-way ANOVA to determine significant differences among the groups, followed by Dunnett’s post test to compare each of the test conditions with LPS-treated cells. Differences were considered significant when $P < 0.05$.

RESULTS

Composition of vegetative water from depitted organic olives. HPLC of hydrolyzed OVW showed that the major retention peaks at 4.7, 9, 12, and 29 min corresponded to HT, tyrosol, caffeic acid, and oleuropein, respectively (Fig. 1), with no detectable levels of catechol, protocatechuic acid, or homovanillic acid, sometimes seen in “waste water,” or the water obtained from the crushing of olives containing pits (16,17). At the higher wavelengths, complex molecules such as oleuropein, verbascoside, and cinnamic acid derivatives were evident (data not shown); however, the concentration of these molecules in the OVW was $<10\%$ of HT levels; thus, the most abundant detected phenol was HT.

Comparison of antioxidant activities and phenolic content. Aqueous and freeze-dried OVW possess far more total phenolics and a concomitantly better oxygen radical absorbance capacity than olive oil (Table 1). Freeze-drying the aqueous olive fraction (comprised of $\sim 11\%$ solids) resulted in a >10 -fold increase in the percentage of total phenolics and a concomitant increase in ORAC, which suggested that freeze-drying does not compromise the activity of the fraction. Moreover, it was shown recently that freeze-drying is the method of choice for retaining the phenolic integrity of foods (18). Olive oil had undetectable levels of HT and oleuropein, whereas aqueous and freeze-dried OVW had relative concen-

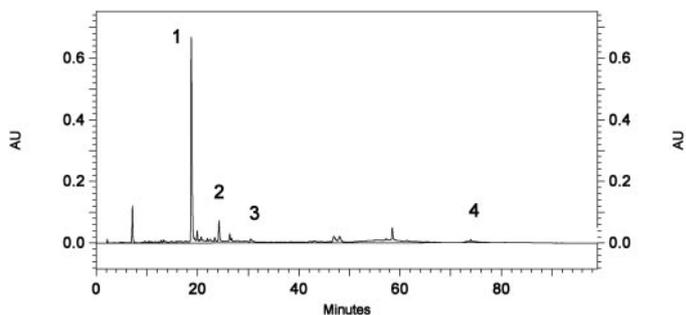


FIGURE 1 Analytical HPLC chromatogram of phenolics from hydrolyzed OVW identified by comparison with commercial standards. Signal was recorded at 240, 280, 320, and 340 nm. Shown below is the recording at OD_{280} . Peak assignments are: 1, HT; 2, tyrosol; 3, caffeic acid; and 4, oleuropein.

TABLE 1

ORAC and total phenolic concentrations for olive oil, OVW, and freeze-dried OVW^{1,2}

| Compound name | ORAC | Phenolics | HT | ORAC | ORAC |
|------------------|----------------------|---------------|------|--------------------------------|-------------------------|
| | $\mu\text{mol TE/g}$ | mg/g | | $\mu\text{mol TE/g phenolics}$ | $\mu\text{mol TE/g HT}$ |
| OVW | 140 | 5.0 | 2.5 | 28,000 | 56,000 |
| Freeze-dried OVW | 2011 | 69 | 35 | 29,145 | 57,460 |
| Olive oil | 0 | <1 | <0.2 | 0 | 0 |
| Olive leaf | 1079 | 51 | 0 | 21,157 | n/a |
| HT (pure) | 42,560 | 1000 | 1000 | 42,560 | 42,560 |

¹ For OVW and freeze-dried OVW, the values are those recorded for the samples used in this study and are representative of the mean for ORAC, total phenolics, and HT.

² TE, Trolox equivalence; n/a, not applicable.

trations of HT and oleuropein of 50 and 11%, respectively (Table 1). It was of interest whether OVW, highly enriched in HT, possessed other activities in addition to its antioxidant activity, and whether HT specifically contributed.

OVW decreases TNF- α in THP-1 cells. To examine the effects of OVW on cytokine expression associated with inflammation, we first evaluated the effects on TNF- α production in the human monocyte cell line, THP-1. OVW significantly reduced expression of TNF- α relative to the control (only LPS-treated; Fig. 2). HT did not affect expression.

OVW decreases TNF- α production in mice. To determine whether the above findings were applicable to an in vivo model, we evaluated the effect of OVW in a murine model of inflammation. OVW significantly decreased LPS-induced TNF- α production at a dose of 35 mg OVW/mouse (1.4 g/kg; Fig. 3). At 125 mg OVW/mouse (5 g/kg), TNF- α production was decreased >90% with no overt toxicity observed. The potent inhibitory effect of OVW on TNF- α production in vivo suggests that OVW may be effective in inflammatory disorders.

Glucosamine decreases TNF- α production in mice. Female BALB/c mice were dosed by oral gavage with various concentrations of glucosamine at 12 h and then again at 1 h before treatment with LPS. Glucosamine significantly inhibited LPS-induced TNF- α production at 6.2 mg/mouse (248 mg/kg) with complete inhibition at 50 mg glucosamine/mouse (Fig. 4).

OVW and glucosamine show synergy in protecting mice from LPS-induced TNF- α production. OVW and glu-

cosamine were either given alone or in combination at concentrations predicted to give a positive response. All combinations of OVW and glucosamine suppressed LPS-induced TNF- α production in vivo (Fig. 5). The effect of combining OVW with glucosamine was additive, as seen when we compared 35 mg OVW alone and 12.5 mg glucosamine alone with a 35-mg OVW + 12.5-mg glucosamine combination. When OVW was combined with glucosamine, the percentage of TNF inhibition was much greater than that predicted by doubling the concentration of one of the components; thus, the combination of olive extract and glucosamine was more synergistic than additive (Fig. 6). Moreover, an isobolographic analysis was performed to evaluate synergy (Fig. 7). Possible synergistic action of OVW and glucosamine was examined at the ED₈₅ of each of the 2 compounds. The straight line drawn between the points of data acquired for the ED₈₅ of OVW and the ED₈₅ of glucosamine alone predicted the inhibition of TNF- α if the combination of the 2 compounds resulted in an additive effect. The combination of OVW and glucosamine fell below the line, suggesting a synergistic interaction between the compounds.

DISCUSSION

In the present work, we described the composition and activity of hydrolyzed OVW from depitted olives. OVW was evaluated for its ability to inhibit LPS-induced TNF- α pro-

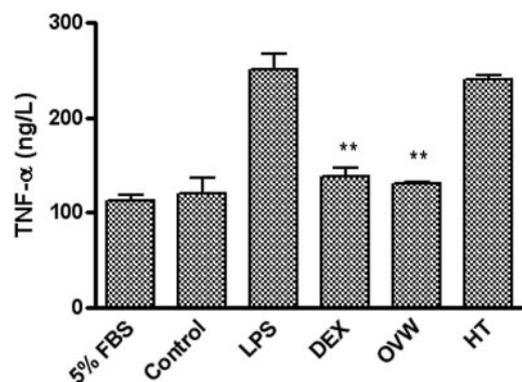


FIGURE 2 Effects of OVW on LPS-induced TNF- α production in human THP-1 cells. THP-1 cells were cultured in the absence of LPS with 5% FBS or with serum ("Control"), in the presence of LPS alone, or with DEX (positive control), OVW, or HT. Values are means \pm SEM, $n = 10$ independent experiments. Asterisks indicate different from LPS-treated cells: ** $P < 0.01$.

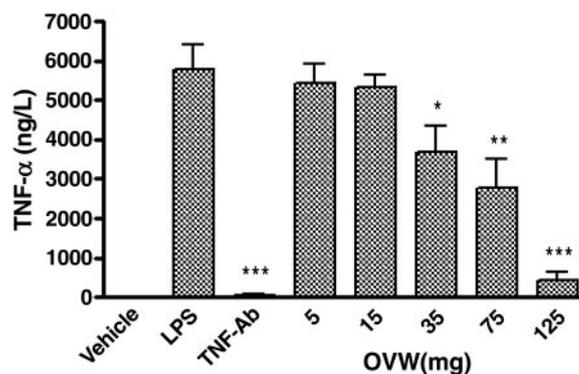


FIGURE 3 Effects of OVW on LPS-induced TNF- α production in BALB/c mice. Female BALB/c mice were treated with the indicated doses of OVW for 12 h followed by LPS treatment for 90 min. Control mice were treated with vehicle only, LPS only, or LPS + anti-TNF- α antibodies (positive control). Values are means \pm SEM, $n = 10$ independent experiments. Asterisks indicate different from LPS-treated cells: *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

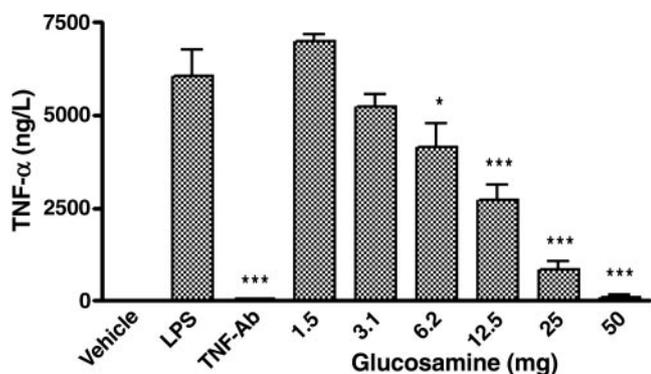


FIGURE 4 Effects of glucosamine on LPS-induced TNF- α production in BALB/c mice. Female BALB/c mice were treated with the indicated doses of glucosamine by oral gavage for 12 h followed by LPS treatment for 90 min. Control mice were treated with vehicle only, LPS only, or LPS + anti-TNF- α antibodies (positive control). Values are means \pm SEM, $n = 10$ independent experiments. Asterisks indicate different from LPS-treated cells: *** $P < 0.001$, * $P < 0.05$.

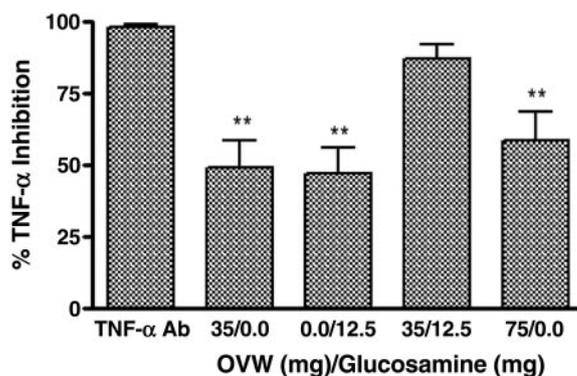


FIGURE 6 Synergistic effects of OVW and glucosamine on the inhibition of TNF- α production following LPS-treatment in BALB/c mice. Female BALB/c mice were treated with the indicated doses of OVW + glucosamine by oral gavage for 12 h followed by 90 min of LPS treatment. Control mice were treated with vehicle only, LPS only, or LPS + anti-TNF- α antibodies (positive control). Values are means \pm SEM, $n = 10$ independent experiments. Asterisks indicate different from LPS-treated cells: ** $P < 0.01$.

duction in a mouse model of inflammation. We found that the aqueous olive fraction, produced in this way, has a unique profile of simple and polyphenolics distinct from olive oil and olive mill waste water, with HT as the principal phenolic moiety.

Hydroxytyrosol was shown to be effective in vitro and in vivo as an antioxidant, antithrombotic, and antimicrobial agent. For example, HT was shown to inhibit free radical generation, scavenge reactive oxygen and nitrogen, inhibit smoking-induced oxidative stress in rats, and increase plasma antioxidant capacity (5–10). HT also possesses cardioprotective activities because it was shown to inhibit LDL oxidation, a prerequisite for atherosclerosis (19,20); inhibit the platelet aggregation that occurs in thrombosis (21); protect cells from cell death induced by reactive oxygen intermediates, as occurs during ischemia and reperfusion injury (22); and inhibit a variety of pathogenic gram-negative and gram-positive bacteria (23). Finally, in human trials, consumption of olive oil with increasing polyphenol content (specifically, HT and tyrosol)

resulted in a dose-dependent decrease in oxidized LDL, a decrease in 8-oxo-7,8-dihydro-2'-deoxyguanosine in mitochondrial DNA, and a decrease in urine malondialdehyde levels (24,25).

In this study, we showed that OVW significantly decreased production of TNF- α after LPS treatment in THP-1 cells, a model of joint inflammation. TNF- α is the primary cytokine induced in this system and the cytokine responsible for the perpetuation of the inflammatory response in monocytes. Interestingly, HT, the major phenol present in OVW, was ineffective at attenuating TNF- α production in this cell system. We evaluated the effectiveness of pure HT in other anti-inflammatory cell models and found that it was also ineffective in those models (unpublished results). Thus, although the major phenolic component of hydrolyzed olive water is HT, the anti-inflammatory activity may be attributable to another component of the water that is as yet unidentified.

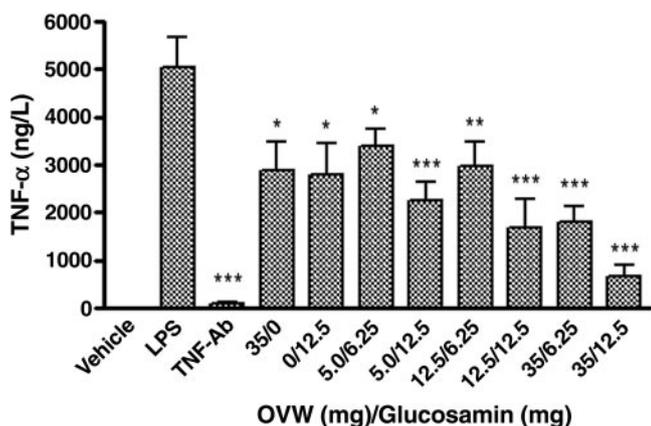


FIGURE 5 Effects of OVW with glucosamine on LPS-induced TNF- α production in BALB/c mice. Female BALB/c mice were treated with the indicated doses of OVW + glucosamine for 12 h followed by LPS treatment for 90 min. Control mice were treated with vehicle only, LPS only, or LPS + anti-TNF- α antibodies (positive control). Values are means \pm SEM, $n = 10$ independent experiments. Asterisks indicate different from LPS-treated cells: *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

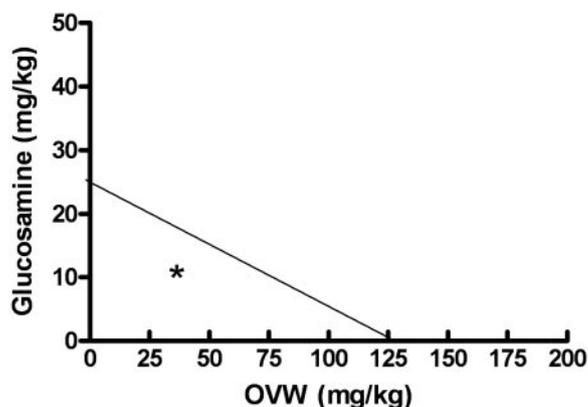


FIGURE 7 Isobolographic analysis of OVW and glucosamine on the inhibition of TNF- α production after LPS-treatment in BALB/c mice. An isobologram was constructed to determine the synergistic inhibition of TNF- α production between OVW and glucosamine. Plotted are the actual doses of glucosamine or OVW that resulted in an 85% inhibition of TNF- α production. A straight line was drawn between the ED₈₅ of glucosamine in the absence of OVW and the ED₈₅ of OVW in the absence of glucosamine. The combined compounds (*) resulted in an inhibition of TNF- α that fell below the line, suggesting a synergistic effect.

To determine the effectiveness of OVW, we tested its activity in an *in vivo* model of inflammation, LPS-treated BALB/c mice. Previous studies showed that other simple and polyphenols are capable of attenuating or inhibiting inflammation caused by a variety of inducers. For example, black tea extract was shown to attenuate endotoxin-induced interleukin (IL)-6 production *in vivo* (26). EGCG, the major polyphenol in green tea extract, was shown to inhibit LPS-induced TNF- α production *in vitro* in mouse peritoneal macrophages and *in vivo* (12). EGCG inhibited LPS-induced TNF- α mRNA expression and nuclear factor- κ B (NF- κ B) activity *in vitro* (12,27), suggesting a possible mechanism of action. Additionally, resveratrol was shown to suppress TNF-induced NF- κ B activation (28), to significantly attenuate LPS- and phorbol-12-myristate-13-acetate-induced COX-2 (29), and to suppress NF- κ B activation in mouse skin and macrophages (30).

We showed that OVW given to mice by oral gavage decreased the production of TNF- α after LPS treatment. The decrease in TNF- α production was dose dependent with an ED₅₀ of ~75 mg OVW/mouse (3 g/kg). Because TNF- α is also a major contributor to inflammation associated with rheumatoid arthritis (RA), we wanted to determine whether OVW can work in conjunction with glucosamine, which was shown to promote joint health, to decrease destruction, and to increase rebuilding of tissue in RA.

Glucosamine, thought to protect joints from inflammation and physical contact-induced destruction by contributing to joint matrix, was also shown to have some anti-inflammatory activity. *N*-Acetyl glucosamine and, to a lesser extent, glucosamine, were shown to inhibit IL-1 β -induced NO production in cultured human articular chondrocytes (31). The resultant inhibition was the consequence of inhibition of inducible nitric oxide synthase mRNA and protein expression. *N*-Acetyl glucosamine, but not glucosamine, also suppressed production of IL-1 β -induced COX-2 and IL-6.

To begin to address the question of whether OVW and glucosamine have synergistic or additive effects as anti-inflammatory agents, we evaluated the effects of glucosamine on LPS-induced TNF- α production *in vivo*. Glucosamine was effective at inhibiting TNF- α production, with an ED₅₀ of ~8 mg/kg. When we tested a combination of OVW with glucosamine, the effect was synergistic.

This is the first demonstration of both OVW and glucosamine as effective inhibitors of LPS-induced TNF- α production, and of the synergistic effects of OVW with glucosamine to inhibit LPS-induced cytokine production. The advantages of combination therapy include an ability to increase safety by decreasing the dose of glucosamine necessary for efficacy, thereby decreasing the potential for negative side effects. OVW was shown to be free of side effects even at very high doses (32). Thus, OVW could prove to be an effective dose-sparing compound not only for glucosamine but for other anti-inflammatory compounds and drugs.

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