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The effect of citrus-derived oil on bovine blood neutrophil function and gene expression in vitro

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ABSTRACT

Research on the use of natural products to treat or prevent microbial invasion as alternatives to antibiotic use is growing. Polymorphonuclear leukocytes (PMNL) play a vital role with regard to the innate immune response that affects severity or duration of mastitis. To our knowledge, effect of cold-pressed terpeneless Valencia orange oil (TCO) on bovine PMNL function has not been elucidated. Therefore, the objective of this study was to investigate the effect of TCO on bovine blood PMNL chemotaxis and phagocytosis capabilities and the expression of genes involved in inflammatory response in vitro. Polymorphonuclear leukocytes were isolated from jugular blood of 12 Holstein cows in mid-lactation and were incubated with 0.0 or 0.01% TCO for 120 min at 37°C and 5% CO_2, and phagocytosis (2×10^6 PMNL) and chemotaxis (6×10^6 PMNL) assays were then performed in vitro. For gene expression, RNA was extracted from incubated PMNL (6×10^6 PMNL), and gene expression was analyzed using quantitative PCR. The supernatant was stored at −80°C for analysis of tumor necrosis factor-α. Data were analyzed using a general linear mixed model with cow and treatment (i.e., control or TCO) in the model statement. In vitro supplementation of 0.01% of TCO increased the chemotactic ability to IL-8 by 47%; however, migration of PMNL to complement 5a was not altered. Treatment did not affect the production of tumor necrosis factor-α by PMNL. Expression of proinflammatory genes (i.e., *SELL*, *TLR4*, *IRAK1*, *TRAF6*, and *LYZ*) coding for proteins was not altered by incubation of PMNL with TCO. However, downregulation of *TLR2* [fold change (FC = treatment/control) = −2.14], *NFKBIA* (FC = 1.82), *IL1B* (FC = −2.16), *TNFA* (FC = −9.43), and *SOD2* (FC = −1.57) was observed for PMNL incubated with TCO when compared with controls. Interestingly, expression of *IL10*, a well-known antiinflammatory cytokine, was also downregulated (FC = −3.78), whereas expression of *IL8* (FC = 1.93), a gene coding for the cytokine IL-8 known for its chemotactic function, tended to be upregulated in PMNL incubated with TCO. Incubation of PMNL with TCO enhanced PMNL chemotaxis in vitro. The expression of genes involved in the inflammatory response was primarily downregulated. Results showed that 0.01% TCO did not impair the function of PMNL in vitro. Future studies investigating the use of TCO as an alternative therapy for treatment of mastitis, including dose and duration, for cows during lactation are warranted.

Key words: citrus oil, Holstein cow, bovine neutrophil

INTRODUCTION

The economic losses due to mastitis in the United States exceed $2 billion annually (Nickerson and Oliver, 2014). Mastitis, an inflammation of the mammary gland, is usually associated with the presence of a pathogen such as *Escherichia coli*, *Klebsiella* spp., *Streptococcus* spp., and *Staphylococcus* spp. (Kehrli and Shuster, 1994). During mastitis, circulating neutrophils, also known as PMNL, are of key importance in controlling the severity and duration of mastitis (Burvenich et al., 2007). During inflammation, PMNL are the most predominant cell type in the mammary gland, accounting for approximately 95% of somatic cell population (Kehrli and Shuster, 1994), and are, therefore, of critical importance for resolution of mastitis. Antibiotics to treat mastitis have been extensively used in the past 60 yr (Plastridge, 1958). However, growing consumer concerns regarding antibiotic use have led to the examination of alternative strategies for controlling mastitis while reducing the use of antibiotics on-farm (Bannantine et al., 2013).

Citrus oils, or fractions thereof, have been traditionally used as flavoring agents in foods, and it has frequently been noted that many possess antimicrobial properties (Smith-Palmer et al., 1998; Alzoreky and Nakahara, 2003; Muthaiyan et al., 2012a,b). Citrus essential oils have been part of the human diet for hundreds

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of years. Therapeutic use of a variety of essential oils as either individual compounds or mixtures has been satisfactorily evaluated to inhibit the activity (growth inhibition) of *Staphylococcus* strains and *E. coli* (Fratini et al., 2014). Citrus essential oils are complex mixtures of volatile and nonvolatile compounds. The volatile compounds are a mixture of monoterpene and sesquiterpene hydrocarbons and their oxygenated derivatives (Fisher and Phillips, 2008). Removal of the terpene fraction from essential oils, which are readily oxidized when exposed to air, is performed by fractional distillation, by extraction of the oxygenated compounds with diluted alcohol or other solvents, or by combination of these methods resulting in significant reduction of the limonene fraction (Kirchner and Miller, 1952). Studies evaluating the therapeutic use of citrus essential oils in ruminants are very limited and are more focused on its antibacterial effect in reducing pathogen population in feces (Jacob et al., 2009; Callaway et al., 2011).

Cold-pressed terpeneless Valencia orange oil (**TCO**) contains limited amounts of the essential-oil limonene (0.3%) and is rich in linalool (20.2%), decanal (18%), and geranial (9.1%; Nannapaneni et al., 2009). This product is more stable and contains most of the odor and flavor of the original oil (Kirchner and Miller, 1952). Several in vitro studies, using different concentrations of TCO, reported that TCO inhibits the growth of a wide range of microbes such as *Mycobacterium tuberculosis* and *Mycobacterium bovis* (Crandall et al., 2012), *E. coli* O157:H7 (Nannapaneni et al., 2008), *Campylobacter jejuni* and *Campylobacter coli* (Nannapaneni et al., 2009), *Staphylococcus aureus* (Muthaiyan et al., 2012b), and different *Listeria* strains (Shannon et al., 2011; Muthaiyan et al., 2012b). Other studies have evaluated the antimicrobial effect of TCO on growth reduction of *C. jejuni* and *C. coli* in retail chicken legs and thighs (Nannapaneni et al., 2009), *Samonella* ssp. and *E. coli* O157:H7 in beef meat (Pittman et al., 2011), and *Staph. aureus* in keratinocytes (Muthaiyan et al., 2012a). To our knowledge, no study has evaluated the effect of TCO as an alternative therapy to control mastitis in dairy cows. Because of their vital role in controlling mastitis, examining the effect of TCO on PMNL function will identify the role of TCO on the host response that may affect the severity and duration of mastitis. Therefore, the objective of this study was to investigate the effect of TCO on bovine blood PMNL chemotaxis and phagocytosis capabilities in vitro and the expression of genes involved in the inflammatory response of PMNL. Results will identify the effect of TCO on PMNL inflammatory response and advance our knowledge regarding its potential use as an alternative therapy for mastitis in dairy cows.

MATERIALS AND METHODS

Animals

All procedures involving the use of live animals were approved in accordance with the regulations and guidelines set forth by the USDA Beltsville Animal Care and Use Committee. Twelve Holstein cows in mid-lactation (129 ± 22 DIM; 7 primiparous and 5 multiparous) were used for this study. All cows were free of clinical signs of disease before the study. Cows were housed and fed in freestalls, had free access to water, and were milked twice daily at 0800 and 1800 h. Cows were fed a TMR to provide 100% of NRC requirements daily at 1100 h.

Jugular blood (~150 mL) was collected from each cow after the morning milking and before the morning feeding. Blood was collected into evacuated tubes containing acid-citrate dextrose (Fisherbrand, Thermo Fisher Scientific Inc., Pittsburgh, PA), inverted to mix, and placed on ice. Blood samples were processed within 1 h of collection.

Isolation of PMNL

Polymorphonuclear leukocytes were isolated according to procedures described by Moyes et al. (2009), with minor modifications. Blood was transferred to 15-mL conical tubes (Fisherbrand, Thermo Fisher Scientific Inc.) and centrifuged (model 5810R, Eppendorf, Hauppauge, NY) for 20 min at 1,000 × *g* at 4°C. After centrifugation, the plasma, buffy coat, and one-third of the red blood cells were discarded. The remaining cells were transferred to a 50-mL conical tube (Fisherbrand, Thermo Fisher Scientific Inc.), and the cell suspension was lysed with 18 mL of ice-cold deionized water. The cell suspension was inverted gently for no longer than 45 s, and isotonicity was restored by addition of 2 mL of 10× PBS (pH 7.4; Sigma-Aldrich, St. Louis, MO). The solution was then centrifuged for 10 min (200 × *g* at 4°C) and the supernatant decanted. The pellet was washed with 20 mL of endotoxin-free, calcium- and magnesium-free Hanks' balanced salt solution (**CMF-HBSS**, Sigma-Aldrich) and centrifuged for 5 min (850 × *g* at 4°C). If needed, cells were lysed for a second time by adding 1.8 mL of ice-cold deionized water followed by 200 μL of 10× PBS and 20 mL of CMF-HBSS and centrifuged for 5 min (850 × *g* at 4°C). Cells were washed twice with 20 mL of CMF-HBSS and centrifuged for 5 min (850 × *g* at 4°C). After the final wash, the pellet was resuspended in 1 mL of CMF-HBSS, and PMNL concentrations were measured using a TC-20 automated cell counter (Bio-Rad Laboratories Inc., Hercules, CA). Using the trypan blue (0.1%, Bio-Rad Laboratories Inc.) exclusion method (Freshney, 1987),

the average viability of PMNL was 92.3%. Cell differentials were determined microscopically on cytospin preparations using a commercially available hematology staining kit (Hema-Fast 3-Step Hematology Staining Kit; Fisherbrand, Thermo Fisher Scientific Inc.). Results indicated that 88.5% of isolated cells were PMNL.

Citrus Essential-Oil Product and Cytotoxicity

Commercially available TCO was obtained from the Firmenich Citrus Center (Safety Harbor, FL). Citrus essential oils are complex mixtures of volatile (85–99%) and nonvolatile compounds (1–15%). The volatile compounds are a mixture of monoterpene and sesquiterpene hydrocarbons and their oxygenated derivatives including aldehydes, ketones, acids, alcohols, and esters (Fisher and Phillips, 2008). Moufida and Marzouk (2003) reported that limonene (~88%) is the major component of the sweet orange oil, whereas linalool is found in minimal proportions (0.02%). However, removal of the terpene fraction drastically reduces the proportion of limonene (Kirchner and Miller, 1952). Nannapaneni et al. (2009) listed linalool (20.2%), decanal (18%), and geranial (9.1%) as the main compounds for TCO grown in Florida, whereas limonene is only present at 0.3%.

Cytotoxicity. The cytotoxicity of TCO on PMNL was determined in vitro. Polymorphonuclear leukocytes (~4×10^4 cells in 200 μL of CMF-HBSS), isolated from 9 cows (134 DIM), were added to a sterile, round-bottom, 96-well plate (Fisherbrand, Thermo Fisher Scientific Inc.) according to methods previously described (Muthaiyan et al., 2012a) with modifications for PMNL cytotoxicity. Briefly, plates were centrifuged (model 5810R, Eppendorf) at 23°C at 1,000 × *g* for 10 min and supernatant was discarded. The pellet was resuspended with 200 μL of CMF-HBSS containing a fixed amount (0.1%) of dimethyl sulfoxide (**DMSO**, BDH chemicals, VWR International, Philadelphia, PA) and either 0, 0.005, 0.01, or 0.1% of TCO. Cells were incubated (Binder Inc., Bohemia, NY) at 37°C and 5% CO_2 for 2 h. Cells were centrifuged at room temperature at 1,000 × *g* for 10 min, and supernatant was discarded after centrifugation. Cells were reconstituted again with 200 μL of CMF-HBSS and centrifuged at 23°C at 1,000 × *g* for 10 min. After centrifugation, cells were resuspended in 50 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg/mL) reconstituted in 1× PBS. Cells treated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were incubated for 2 h at 37°C and 5% CO_2. After incubation, plates were centrifuged at 1,000 × *g* for 10 min, 150 μL of DMSO was added to each well, and formed crystals were dissolved by vigorously pipetting up and down. Finally, plates were incubated at room temperature at a constant rotation for 15 min, and absorbance was measured at 570 nm. Relative cytotoxicity was calculated as 1 − (absorbance of treated PMNL/absorbance of control PMNL). Supplemental Figure S1 (http://dx.doi.org/10.3168/jds.2014-8450) depicts the relative expression of the different doses of TCO compared with the control (0% TCO and 0.1% DMSO). The assay demonstrated that 0.01% of TCO or less in 0.1% DMSO was not toxic ($P > 0.10$) to the surveillance of PMNL, whereas a dose of 0.1% TCO was toxic ($P < 0.01$). Therefore, the dose of 0.01% of TCO in 0.1% DMSO was selected for further analysis.

Treatment Preparation. Stock of TCO (commercial product) was diluted at 10% concentration with DMSO. A further dilution was performed using CMF-HBSS to reach a working concentration of 0.1% TCO. Incubations were performed in 50-mL conical tubes (Fisherbrand, Thermo Fisher Scientific Inc.). Cell suspensions containing 2×10^6 PMNL/mL (phagocytosis assay; final volume of 4.0 mL), 6×10^6 PMNL/mL (chemotaxis assay; final volume of 1.0 mL), or 6×10^6 PMNL/mL [quantitative PCR (**qPCR**); final volume of 1.0 mL] were added using RPMI/5% heat-inactivated fetal bovine serum (**FBS**; Sigma-Aldrich). For all assays, working solutions of either control (0.0% TCO) or TCO (0.01% TCO) were added to each tube. All PMNL were incubated for 120 min at 37°C, 95% relative humidity, and 5% CO_2 using a constant rotator.

After incubation, PMNL chemotaxis and phagocytosis capabilities were assessed as described below. For qPCR analysis, 1.0 mL of cell suspensions was transferred into 2.0-mL RNase-free tubes (Fisherbrand, Thermo Fisher Scientific Inc.) and centrifuged (10,000 × *g* at 4°C for 2 min). The spent media were collected and stored at −80°C for analysis of tumor necrosis factor-α (**TNF-α**), whereas the cell pellet was washed twice with 1.0 mL of 1× PBS and 1.0 mL of TRIzol Reagent (Invitrogen, Carlsbad, CA) with 1 μL of linear acrylamide (Ambion Inc., Austin, TX) added. Polymorphonuclear leukocytes were homogenized (VDI 25 Homogenizer, VWR International) and stored at −80°C until further analysis.

PMNL Chemotaxis

Chemoattractants used in this experiment included human IL-8 (Sigma-Aldrich) and recombinant human complement 5a (**C5a**; Sigma-Aldrich). Chemoattractants were adjusted to a final concentration of 100 ng/mL and 10^{-9} *M* for IL-8 and C5a, respectively, using 1× PBS. In triplicate, 600 μL of each chemoattractant was added to the bottom wells of a 24-flat bottom well plate

(Fisherbrand, Thermo Fisher Scientific Inc.). Filters were added (pore size of 5.0 μm; 6.5-mm diameter; 0.3-cm^2 growth area; Fisherbrand, Thermo Fisher Scientific Inc.) to the top of each well. A total of 100 μL of cell suspension adjusted to 6×10^6 PMNL/mL with RPMI-1640 (Sigma-Aldrich Co.) containing 5% FBS (Sigma-Aldrich Co.), and previously incubated with treatments (control or TCO), was added to the top of the filter. Plates were incubated for 90 min at 37°C, 5.0% CO_2, and 95% relative humidity. Following incubation, the filter was washed vigorously with 200 μL of CMF-HBSS twice, and the filter was removed. The total number of PMNL migrated was counted using a TC-20 automated cell counter (Bio-Rad Laboratories Inc.).

PMNL Phagocytosis

Phagocytosis was performed according to procedures described by Moyes et al. (2009). Briefly, before incubation fluorescent beads (Fluoresbrite yellow-green Carboxylate 1.75-μm Microspheres; PolySciences, Warrington, PA) were first precoated with heat-inactivated FBS (250 μL of FBS per 1 μL of beads; Sigma-Aldrich Co.), and the mixture was protected from the light and incubated at room temperature for 45 min. After TCO incubation above, 1.0 mL of PMNL (2×10^6) was transferred into four 5-mL polystyrene round-bottomed tubes (Becton Dickinson Labware, Franklin Lakes, NJ). To each tube, 100 μL of the FBS-coated beads was added to obtain a 10:1 ratio (beads:PMNL). Samples were run in duplicate and incubated for 2 h at 37°C, 5% CO_2, and 95% relative humidity and then centrifuged for 5 min (1,000 × *g* at 4°C). The pellets were washed in 1 mL of CMF-HBSS and fixed with 400 μL of 4% paraformaldehyde (Sigma-Aldrich Co.). Samples were stored at 4°C and protected from light until analysis (~1–2 d) for percentage phagocytosis using an XL Flow Cytometer (Beckman Coulter, Fullerton, CA). The PMNL populations were gated by forward scatter and side scatter. Data were collected on 20,000 events per sample in the gate set for bovine PMNL, and percentage fluorescence was used as the quantitative index of PMNL response. Phagocytosis by PMNL was expressed as the total percentage of PMNL engulfing one or more beads.

TNF-α Assay

The spent media collected after 2 h incubation of PMNL with treatments was analyzed for concentration of TNF-α using a bovine-TNF-α commercial kit (Vet-Set Elisa Development Kit; Kingfisher Biotech Inc., St. Paul, MN) following the manufacturer's protocol. Standards were prepared with 4% bovine serum albumin in PBS, and samples were diluted to 90% concentration with the same standard diluents (4% bovine serum albumin in PBS). Samples were run in duplicate. The intraassay coefficient of variation was 2.1%.

RNA Isolation, cDNA Synthesis, and Quantitative Reverse-Transcription PCR (qPCR)

A subset of 6 cows (4 primiparous, 2 multiparous) were randomly selected for gene-expression analysis. Specific details for total RNA isolation and quality evaluation, cDNA synthesis, primer selection and efficiency evaluation, and qPCR are found in the Supplemental Materials (http://dx.doi.org/10.3168/jds.2014-8450). Purity of extracted RNA was calculated by absorbance ratio of 260/280 measured using a NanoVue Plus (GE Healthcare, Piscataway, NJ) and averaged 1.8. Integrity of RNA was measured by the RNA quality indicator number using a Experion bioanalyzer (Bio-Rad Laboratories Inc.) and averaged 7.6. Primer sequences and accession number are presented in Supplemental Table S1 (http://dx.doi.org/10.3168/jds.2014-8450). Primer and qPCR validation detailing the slope, intercept, and R^2 of the dilution curve as well as efficiency are found in Supplemental Table S2 (http://dx.doi.org/10.3168/jds.2014-8450). The quantification-cycle (**Cq**) values from target genes were normalized by the geometric mean of 3 (*GAPDH*, *GOLGA5*, and *OSBPL*) out of 4 candidate target genes that were previously verified as suitable reference genes in bovine tissue (Janovick-Guretzky et al., 2007; Kadegowda et al., 2009; Moyes et al., 2010). These 3 reference genes were selected based in their lower M value as recommended by the genorm analysis (M <0.74) using the Biogazelle q^+ software (Biogazelle NV, Zwijnaarde, Belgium) and based on the work of Vandesompele et al. (2002).

The qPCR results, given as Cq values for each transcript, were analyzed using the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001). Briefly, the Cq of each target gene was calculated and normalized by the geometric mean of the 3 selected reference genes to generate delta ΔCq values. The ΔCq values of control samples (n = 6) were averaged and subtracted from each ΔCq value of the TCO samples (n = 6) to generate the ΔΔCq. The ΔΔCq values were linearized, to give the fold change (**FC**) value, using the formula $2^{-\Delta\Delta Cq}$. Then, a positive and a negative FC value indicate an upregulation and downregulation in the mRNA expression of a given gene by TCO with respect to control, respectively. General description of the main biological function of the analyzed genes can be found in Supplemental Table S3 (http://dx.doi.org/10.3168/jds.2014-8450).

Statistical Analysis

Results for the phagocytosis, chemotaxis, and gene expression ($2^{-\Delta\Delta C_q}$) assays were $\log_{10}$ transformed for statistical analysis. The PROC MIXED procedure of SAS (SAS/STAT version 9.2; SAS Institute Inc., Cary, NC) was used for statistical analysis. Separation of least squares means, for significant effects, was accomplished using the Tukey's option within the MIXED procedure of SAS. The main effect of parity and the interaction of parity × treatment were not significant ($P > 0.05$) for all variables; therefore, parity was not included in the final model. For analysis of cytotoxicity, the class variables included cow and treatment (i.e., control, 0.05, 0.01, or 0.1% of TCO). For chemotaxis, the class variables included cow, treatment (i.e., control or 0.01% TCO), and chemoattractant (i.e., IL-8 or C5a), and the model was run by chemoattractant. For phagocytosis, TNF-α, and gene expression, the class variables included cow and treatment (i.e., control or TCO). The models were used to determine the effect of TCO on bovine blood PMNL response in vitro, using the following statistical model: $Y_{ij} = \mu + C_i + T_j + \varepsilon_{ij}$, where Y_{ij} is the dependent variable for the ith cow for the jth treatment, μ is overall mean, C_i is the fixed effect of cow (cow = 1, 2, …, n), T_j is the fixed effect of treatment, and ε_{ij} is the residual error. For all models, the degrees of freedom were estimated with the Kenward-Roger specification in the model statement. The effect of cow was removed from the model if not significant ($P > 0.25$). Statistical differences were declared as significant and highly significant at $P < 0.05$ and $P < 0.01$, respectively. Trends toward significance are discussed at $0.05 < P < 0.10$. The $\log_{10}$ least squares means were back-transformed, and data are presented as least squares means and confidence interval for presentation in tables and figures.

RESULTS AND DISCUSSION

Neutrophils are the most abundant nonspecific leukocytes in blood and are actively recruited to the site of infection, such as mammary gland during mastitis (Sordillo, 2005). In the healthy mammary gland, the most abundant somatic cells are macrophages and lymphocytes, with very few PMNL (Kehrli and Shuster, 1994; Bannantine et al., 2013). However, during mastitis, PMNL migrate from the blood to the mammary gland and become the predominant somatic cells in milk (>95%, Kehrli and Shuster, 1994).

Phagocytosis is one of the hallmarks of PMNL function, and incubation of PMNL with TCO did not affect the ability of PMNL to phagocytose in vitro (14.2 vs. 15.9% for control and TCO, respectively, Figure 1). These results indicate that TCO does not impair PMNL function. Recruitment of PMNL to the mammary gland initiates a multistep adhesion cascade involving L-selectin coded by the gene *SELL* in PMNL (Williams et al., 2011; Mayadas et al., 2014). The migration process of PMNL is aided by the orchestrated action of chemoattractants, such as IL-8 and C5a, released by resident macrophages and mammary epithelial cells (Burvenich et al., 2003; Paape et al., 2003). This migration process, also known as chemotaxis, is still not fully understood, even though different hypotheses and mechanisms have been postulated. Among the most accepted mechanism is the hierarchical multistep migration capacity postulated by Foxman et al. (1997) indicating the preference of PMNL for end-target chemoattractants such as C5a instead of intermediate chemokines such as IL-8. This mechanism was complemented recently by Byrne et al. (2014) postulating that PMNL can migrate back and forth in an oscillatory manner when confronted with different chemoattractant signals to finally migrate to the sites of infection. Therefore, L-selectin expression is influenced by IL-8 and C5a for adherence of PMNL to the endothelial wall (Paape et al., 2003). In the current study, chemotactic ability of PMNL by IL-8 in vitro was improved ($P = 0.03$) by 47% when PMNL were incubated with TCO (Figure 2A), whereas chemotactic activity due to C5a did not differ because of treatment (Figure 2B). However, expression of *SELL* was not affected by TCO (FC = −1.20, Table 1). Interestingly, expression of *IL8* tended ($P = 0.08$) to be upregulated by TCO (FC = 1.93), which may indicate that greater expression of *IL8* increased the synthesis of IL-8, and this enhanced the observed increased chemotaxis ability of PMNL incubated with TCO. However, most studies evaluating the use of different citrus essential oils (Hirota et al., 2010; Kummer et al., 2013) reported reduction of migration ability of rodent PMNL in response to different chemokines including IL-8. This response may be dose dependent where lower concentrations do not alter migration of PMNL (Caldefie-Chézet et al., 2006; Kacem and Meraihi, 2009). For the current study, incubation of PMNL with 0.01% TCO did not impair PMNL ability to migrate. Regardless, increased expression of *IL8* was unexpected based on the downregulation of other proinflammatory genes (see below) and results from others (Hart et al., 2000; Juergens et al., 2004).

Bovine TLR-2 and TLR-4 generally mediate responses to gram-positive (i.e., *Streptococcus* spp.) and gram-negative (i.e., *E. coli*) bacteria, respectively (Prince et al., 2011). These receptors recognize pathogen-associated molecular patterns present on invading

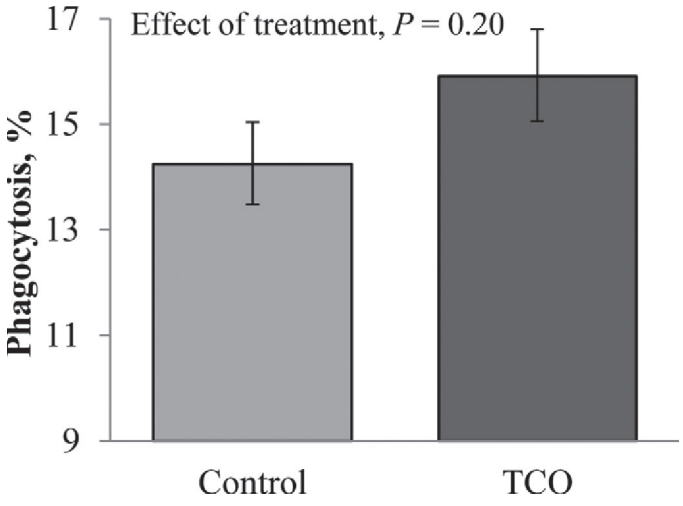

Figure 1. Percent phagocytosis of PMNL incubated with 0 (control) or 0.01% of cold-pressed terpeneless orange oil (TCO) in vitro.

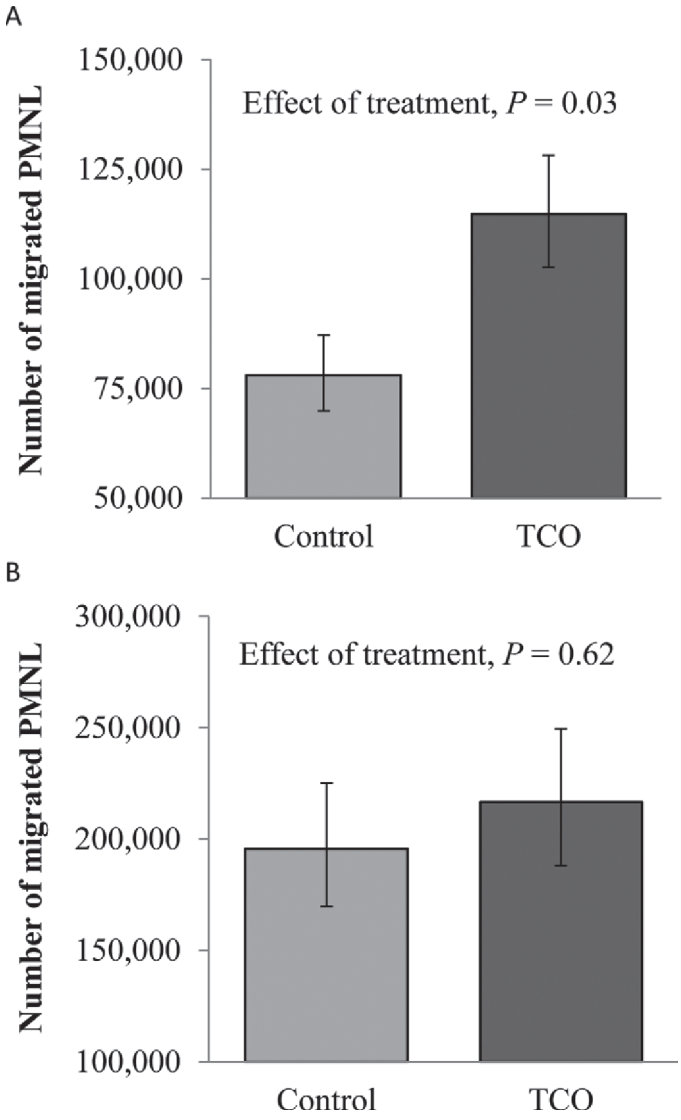

Figure 2. Number of PMNL migrating in response to chemokine IL-8 (A) and C5a (B) after incubation with either 0 (control) or 0.01% of cold-pressed terpeneless orange oil (TCO) in vitro.

microorganisms and can generate a wide range of effects on PMNL including migration, activation of signaling factors, cytokine production, reactive-oxygen-species generation, priming, receptor expression, phagocytosis, degranulation, and programmed cell death (Prince et al., 2011; Thomas and Schroder, 2013). In the current study, expression of *TLR4* was not affected by treatment (FC = 1.13), but *TLR2* was downregulated in PMNL incubated with TCO (FC = −2.14, $P = 0.03$). Smith-Palmer et al. (1998) observed that gram-negative bacteria were less susceptible to the microbial effect of plant essential oils and hypothesized that the outer membrane of gram-negative bacteria is a strong permeability barrier restricting the entry of hydrophobic compounds. Studies at the University of Arkansas using the same product as in the current study (TCO) and from the same provider reported that 0.1% TCO reduced the growth of *Staph. aureus* (gram positive, Muthaiyan et al., 2012b) but failed to inhibit the growth of 11 serotypes and strains of *Salmonella* (gram negative) when used in 4 serial dilutions starting with 1% TCO (O'Bryan et al., 2008). Therefore, TCO may have a more potent antimicrobial effect against gram-positive bacteria via the downregulation of *TLR2* rather than to gram-negative bacteria but warrants further investigation.

The recognition of pathogen-associated molecular patterns by TLR-4 present on the surface of PMNL elicits a cascade of signaling events that enhance the synthesis of proinflammatory cytokines and proinflammatory mediators (Prince et al., 2011; Thomas and Schroder, 2013). Toll-like receptors activate the NF-κB proinflammatory signaling cascade that encompasses the activation of MyD88, TRAF-6, IRAK-1, and the IKK complex that ultimately leads to secretion of proinflammatory cytokines TNF-α, IL-6, and IL-1β (Akira et al., 2001). In the current study the expression of *IRAK1* and *TRAF6* was not altered by TCO. To the contrary, *NFKBIA*, the gene coding for the IκBα protein, was downregulated by TCO (FC = −1.82, $P = 0.05$). This downregulation would be expected considering that NF-κB binds to the IκB promoter to activate its transcription; therefore, a decrease in *NFKBIA* expression should be associated with a decrease in NF-κB activation, resulting in an overall decrease in expression of proinflammatory genes (Camargo et al.,

Table 1. Expression of genes in PMNL incubated with 0 (control) or 0.01% of cold-pressed terpeneless citrus oil (TCO) in vitro

Symbol	Name	Fold-change[1]	*P*-value	CI
Adhesion molecule and pattern recognition receptor				
TLR2	Toll-like receptor 2	−2.14	0.03	−1.79 to −2.55
TLR4	Toll-like receptor 4	1.13	0.57	−0.98 to 1.30
SELL	L-Selectin	−1.20	0.50	−1.00 to −1.44
Signaling factors				
IRAK1	IL-1 receptor-associated kinase 1	1.15	0.70	−0.90 to 1.46
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, α	−1.82	0.05	−1.50 to −2.20
TRAF6	Tumor-necrosis-factor receptor-associated factor 6	−1.11	0.72	−0.92 to −1.33
Cytokine production				
IL1B	IL-1, β	−2.16	0.02	−1.82 to −2.55
IL8	IL-8	1.93	0.08	1.56 to 2.39
IL10	IL-10	−3.78	<0.01	−3.13 to −4.58
TNFA	Tumor necrosis factor α	−9.43	<0.01	−7.28 to −12.22
Killing of pathogens				
LYZ	Lysozyme	1.12	0.76	−0.88 to 1.42
SOD2	Superoxide dismutase 2 mitochondrial	−1.57	0.08	−1.33 to −1.88

[1]Correspond to the fold change ($2^{-\Delta\Delta Cq}$) of TCO versus control. Positive value is upregulation and negative is downregulation of a given gene in PMNL incubated with 0.01% TCO compared with control PMNL. Cq = quantification cycle.

2010). Furthermore, our results showed a downregulation of *IL1B* (FC = −2.16, $P = 0.02$) and *TNFA* (FC = −9.43, $P < 0.01$), encoding for the proinflammatory cytokines IL1-β and TNF-α, respectively, and TCO did not alter TNF-α production in the spent media of PMNL (29.6 vs. 35.3 ± 4.73 pg/μL, TCO and control, respectively, $P = 0.42$). However, these results conflict with the downregulation of *IL10* (FC = −3.78, $P < 0.01$), a known antiinflammatory cytokine. The overall downregulation of proinflammatory genes in the current study indicates that TCO may have an antiinflammatory effect on PMNL transcripts and that TCO does not mount a proinflammatory response.

To kill invading microorganisms, PMNL secrete several potentially harmful substances during the inflammatory response. Among these, the protein lysozyme, coded by the gene *LYZ*, has a strong antimicrobial effect via degrading the bacteria cell wall (Amulic et al., 2012). In the current study, expression of *LYZ* was not affected by the incubation of PMNL with TCO. During phagocytosis, the reactive oxygen cascade results in the production of reactive oxygen species such as superoxide anion and H_2O_2 that aid in the killing of engulfed microorganisms (Paape et al., 2003). Superoxide dismutase 2 is an enzyme encoded by *SOD2* that converts superoxide anion to H_2O_2 in the mitochondria. In the current study, expression of *SOD2* tended ($P = 0.08$) to be downregulated in PMNL incubated with TCO (FC = −1.57), whereas respiratory burst was not measured. Results indicate that TCO does not significantly alter transcripts involved in phagocytosis and respiratory burst of nonstimulated bovine blood PMNL in vitro.

CONCLUSIONS

Incubation of PMNL with TCO increased chemotactic capability in vitro, whereas PMNL phagocytosis was unchanged. The expression of genes involved in the proinflammatory response was primarily downregulated; however, TNF-α production was not altered by TCO when compared with controls. Results show that 0.01% TCO does not impair the function of PMNL in vitro, indicating that TCO inhibits bacterial growth without negatively altering PMNL function. Future studies investigating the use of TCO as an alternative therapy for treatment of mastitis, including dose and duration, for cows during lactation are warranted.

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