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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 method (0.1%, Bio-Rad Laboratories Inc.) exclusion method (Fresh-