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Effects of polyphenols including curcuminoids, resveratrol, quercetin, pterostilbene, and hydroxypterostilbene on lymphocyte pro-inflammatory cytokine production of senior horses *in vitro*

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ABSTRACT

Senior horses (aged ≥20 years) exhibit increased chronic, low-grade inflammation systemically, termed inflamm-aging. Inflammation is associated with many afflictions common to the horse, including laminitis and osteoarthritis, which are commonly treated with the non-steroidal anti-inflammatory drugs (NSAIDs) flunixin meglumine and phenylbutazone. Although these NSAIDs are effective in treating acute inflammatory problems, long-term treatment with NSAIDs can result in negative side effects. Thus, bioactive polyphenols including curcuminoids, resveratrol, quercetin, pterostilbene, and hydroxypterostilbene were investigated to determine their effectiveness as anti-inflammatory agents *in vitro*. Heparinized blood was collected *via* jugular venipuncture from senior horses (n = 6; mean age = 26 ± 2 years), and peripheral blood mononuclear cells (PBMC) were isolated using a Ficoll density gradient. PBMC were then incubated 22 h at 37 °C, 5% CO_2 with multiple concentrations (320, 160, 80, 40, 20, 10 μM) of all five polyphenols (curcuminoids, resveratrol, quercetin, pterostilbene, and hydroxypterostilbene), dissolved in DMSO to achieve the aforementioned concentrations. PBMC were stimulated the last 4 h of the incubation period with phorbol 12-myristate 13-acetate (PMA)/ionomycin and Brefeldin A (BFA). A Vicell-XR counter evaluated cell viability following incubation. PBMC were stained intracellularly for interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) and analyzed *via* flow cytometry. Data was analyzed by one-way analysis of variance (ANOVA). Viability of PBMC incubated with various compound concentrations were compared with PBMC incubated with DMSO alone (positive control) to determine at what concentration each compound caused cytotoxicity. The highest concentration at which cell viability did not significantly differ from the positive control was: 20 μM for curcuminoids, 40 μM for hydroxypterostilbene, 80 μM for pterostilbene, and 160 μM for quercetin and resveratrol. Flunixin meglumine and phenylbutazone were then evaluated within this range of optimal concentrations for the polyphenol compounds (160, 80, 40, 20 μM) to compare the polyphenols to NSAIDs at equivalent concentrations. The highest concentration at which viability did not significantly differ from the positive control was: 40 μM for flunixin meglumine and 160 μM for phenylbutazone. All five polyphenols and flunixin meglumine significantly decreased lymphocyte production of IFN-γ, while only hydroxypterostilbene, pterostilbene, quercetin, and resveratrol significantly reduced lymphocyte production of TNF-α compared to the positive control ($p < 0.05$). Polyphenols performed similarly to or more effectively than common NSAIDs in reducing lymphocyte production of inflammatory cytokines of the senior horse *in vitro*. This study therefore supports the further investigation of polyphenols to determine whether they may be effective anti-inflammatory treatments for chronic inflammation in the horse.

1. Introduction

The senior horse population is growing globally, with horses 20 years or older comprising an estimated 7.6% of the population in the United States (USDA, 2006) and horses ≥15 years old comprising 29% of the population in the United Kingdom (Ireland et al., 2011). In addition to increasing lifespan, healthspan must be

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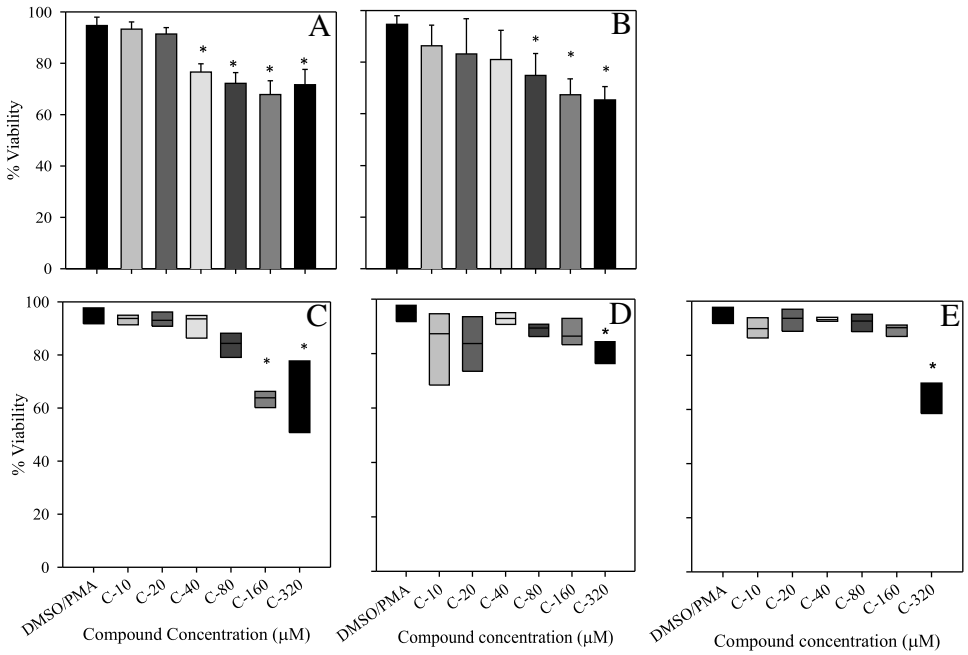

Fig. 1. Effect of compound concentrations (ranging from 10 to 320 μM in two-fold dilutions; denoted as C-10–C-320) on % viability of PBMC of n = 6 senior horses following incubation with DMSO or compounds and PMA/ionomycin. Compounds include (A) curcuminoids, (B) hydroxypterostilbene, (C) pterostilbene, (D) quercetin, and (E) resveratrol. Bar graphs show mean ± SD, while box plots show the median, 25th, and 75th percentile. Bars denoted with an asterisk (*) signify a significant difference ($p < 0.05$) in that compound concentration relative to DMSO/PMA, the positive control.

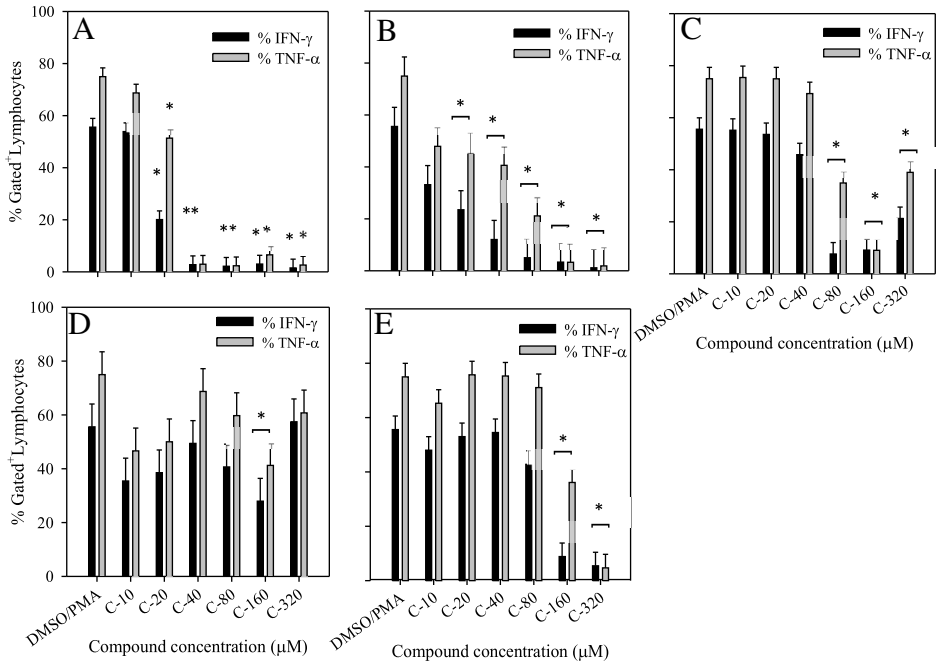

Fig. 2. Effect of compound concentration on lymphocyte production of pro-inflammatory cytokines interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) using an *in vitro* model with cells from n = 6 senior horses. Compounds include (A) curcuminoids, (B) hydroxypterostilbene, (C) pterostilbene, (D) quercetin, and (E) resveratrol. Compound concentrations ranged from 10 to 320 μM in two-fold dilutions (denoted C-10–C-320), with DMSO/PMA serving as a positive control for inflammation. Bars denoted with an asterisk (*) signify a significant difference ($p < 0.05$) in that compound concentration relative to DMSO/PMA, the positive control. Bars denoted with an asterisk $\overset{*}{\rule{1em}{0.4pt}}$ indicate no interaction between the cytokines, but an overall significant difference ($p < 0.05$) of inflammatory cytokine production when comparing the compound concentrations to the positive control.

taken into account. With increased age, an increase in inflammation at both the gene expression [interleukin (IL)-1b, IL-15, IL-18 and TNF-α] and protein levels [tumor necrosis factor-alpha (TNF-α)] has been characterized in the horse (Adams et al., 2008; Katepalli et al., 2008). This increased inflammation with aging indicates that horses, like many other species, exhibit inflamm-aging or low-grade, chronic inflammation that occurs systemically with aging (Franceschi et al., 2007). Although clearly established clinical conditions of the horse associated with inflamm-aging have not yet been discovered, human studies have shown that inflammation is