Evaluation of the *in vivo* effects of resveratrol on innate immune function in dogs

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Introduction

- Resveratrol, a naturally-occurring phytophenol found in various fruits, vegetables, and nuts, has demonstrated potential to serve as a dose-dependent immunomodulator, bolstering immune function at low doses and suppressing immune function at high doses.
- There is a great need, in both human and veterinary fields, for a therapeutic agent that could modulate the immune system in a state of dysfunction. Resveratrol has been found to reverse immunosenescence, bolster immune cell activity against neoplastic cells, and counteract harmful inflammation in human and murine studies.
- Use of a canine model to test the *in vivo* effects of resveratrol will enable us to learn more about its direct effects on innate immune function and its potential to serve as a future therapeutic agent. In this study, our objective was to evaluate the effects of resveratrol on the phagocytic and oxidative burst function of monocytes and neutrophils and cytokine production.

Hypothesis

- We hypothesize that dogs receiving resveratrol will have more effective phagocytosis, reduced oxidative burst function, and decreased leukocyte pro-inflammatory cytokine production in comparison to control dogs.

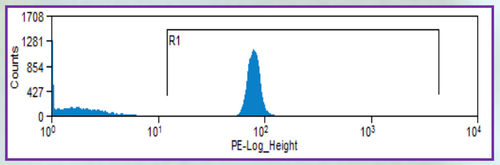

Figure 1. Gating for DNA positive cells. Propidium iodide (PI) DNA stain positive cells were isolated for further analysis based upon level of fluorescence.

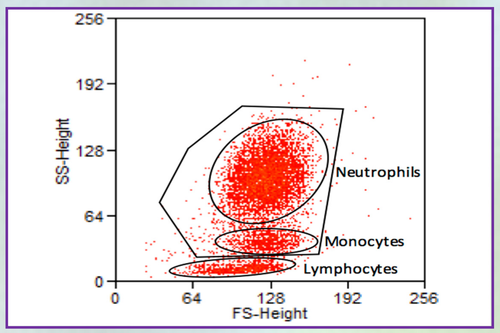

Figure 2. Gating for phagocytosis and oxidative burst. PI DNA stain positive cells were projected into a scatter plot based upon size (FS-Height) and granularity (SS-Height). Neutrophils and monocytes were isolated to determine percent cells performing phagocytosis and oxidative burst.

Materials and Methods

- **Population:** Twelve adult, client-owned dogs volunteered by owners were determined as "healthy" via physical exam, complete blood count, and chemistry panels performed by a veterinarian. In an open label trial, dogs received either the resveratrol (200mg/kg/day, PO x 3 days) or control group (no supplementation).
- **Blood collection:** Whole blood was collected on day 0 and day 3 from both the resveratrol and control group. Blood was collected from the resveratrol group four hours post-administration (PO) on day 3 to achieve peak plasma concentrations of resveratrol in blood.
- **Phagocytic and oxidative burst function:** Granulocyte phagocytic and respiratory burst function tests were determined using commercially available test kits (Orpegen Pharma, Heidelberg, Germany) previously validated for dogs (LeBlanc et al., 2010). For phagocytic function tests, blood was incubated with opsonized, FITC-labeled *E. coli* for 10 min, and extracellular fluorescence was removed via quenching solution. Fixing solution was added for red blood cell lysis and cell fixation. Granulocytes with phagocytized *E. coli* were measured by flow cytometry after DNA staining using propidium iodide. For the measurement of oxidative burst, samples were incubated with either opsonized *E. coli* or phorbol myristate acetate (PMA) for 10 min. Formation of reactive oxidants during oxidative burst was monitored by the addition and oxidation of dihydrorhodamine 123 to Rhodamine 123. The reaction was stopped by addition of fixing and RBC lysing solution. Propidium iodide was then added and oxidative burst capacity determined using flow cytometry. Flow cytometry was performed at the University of Missouri Cell and Immunology Core Facility using the CyAn ADP machine and summit software. DNA positive cells were identified on an FL2 histogram plot and applied to a forward vs side scatter plot. Then granulocytes were identified and applied to an FL1 histogram (Figure 1). Percentage of FITC positive granulocytes having performed phagocytosis was recorded for assessment of phagocytic function. The percentage of Rhodamine 123 positive granulocytes having produced reactive oxygen metabolites was recorded for assessment of oxidative burst function. A minimum of 10,000 events were recorded for each sample.
- **Cytokine production:** Leukocyte cytokine production was performed as previously described (DeClue et al., 2008; Deitschel et al., 2010; Fowler et al., 2011). Two mls of whole blood was diluted 1:2 with complete **Roswell Park Memorial Institute medium** containing 200U/mL of penicillin, 200 mg/mL of streptomycin (Gibco®, Invitrogen, Grand Island NY), placed in 12 well plates, and stimulated with one pathogen associated molecular pattern motif: lipopolysaccharide (LPS) from *Escherichia coli* (*E. coli*) O127:B8 (Sigma-Aldrich, St. Louis, MO), lipotechoic acid (LTA) from *Streptococcus faecalis* (Sigma-Aldrich, St. Louis, MO), peptidoglycan (PG) from *Staphylococcus aureus* (Sigma-Aldrich, St. Louis, MO), or control phosphate buffered saline (PBS). Wells were gently mixed on a plate rocker for 5 minutes, then incubated for 24 hours at 37ºC and 5% CO_2. The supernatant was then collected and frozen at −80°C until analysis. Tumor necrosis factor (TNF), interleukin (IL)-6, and IL-10 will be measured in the supernatant using a canine specific multiplex bead based assay (Millipore, Billerica, MA) (Karlsson et al., 2012).
- **Statistical analysis:** Statistical analysis was performed using commercially available software (SigmaStat, Systat Software Inc.). Data distribution properties were tested using histogram plots. Differences between treatment groups over time were compared using two way repeated measures ANOVA and Fisher LSD method. A *P* -value of < 0.05 was considered statistically significant.

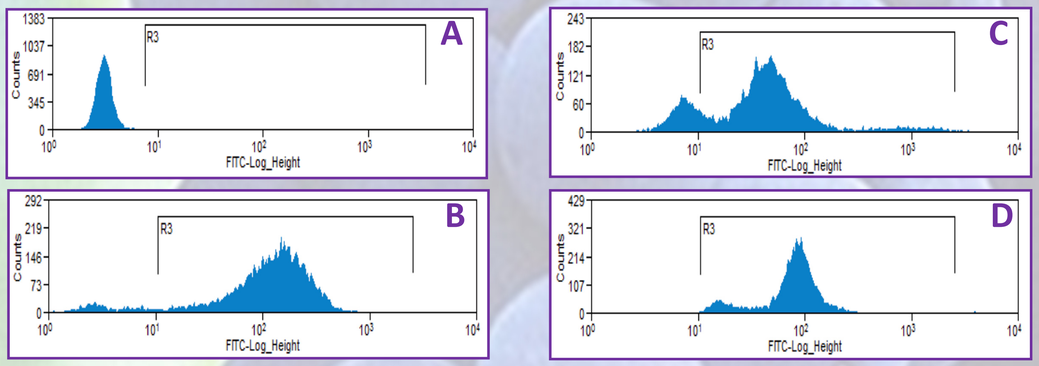

Figure 3. Gating for neutrophils and monocytes performing phagocytosis and oxidative burst. **A**) Control samples incubated with washing solution enabled the exclusion of neutrophils and monocytes negative for performance of phagocytosis and oxidative burst. **B**) Neutrophils and monocytes positive for FITC were performing phagocytosis and fluoresced within the preset gate. **C, D**) Neutrophils and monocytes positive for DHR were performing oxidative burst due to stimulation by either C) *E. Coli* or D) PMA, and fluoresced within the preset gate.

Acknowledgements

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Results

•**Phagocytosis.** The mean fluorescence intensity (MFI) of FITC-labeled *E. Coli* cells performing phagocytosis increased significantly from the Day 0 and Day 3 dogs in the resveratrol group (Figure 4-B). This increase in MFI indicates an increased phagocytic capability, or an increase in the number of bacteria ingested per cell for the cells performing phagocytosis. There was no significant variability in the percent cells performing phagocytosis between Day 0 and Day 3 for the control group or between Day 0 and Day 3 for the resveratrol group. There was also no significant variability between the control group and resveratrol group (Figure 4-A).
•**Oxidative Burst.** The MFI of cells experiencing PMA-induced oxidative burst decreased significantly in the Day 0 and Day 3 dogs in the resveratrol group (Figure 4-F). This decrease in MFI indicates a reduced robustness of the oxidative burst performed within the cell. There was no significant variability in the MFI of *E. Coli*-induced oxidative burst cells (Figure 4-D). The percent cells performing oxidative burst of *E. Coli* (Figure 4-C) and PMA (Figure 4-E) did not experience significant change between Day 0 and Day 3 for the control group or between Day 0 and Day 3 for the resveratrol group, nor did these groups experience significant change between the control group and the resveratrol group.

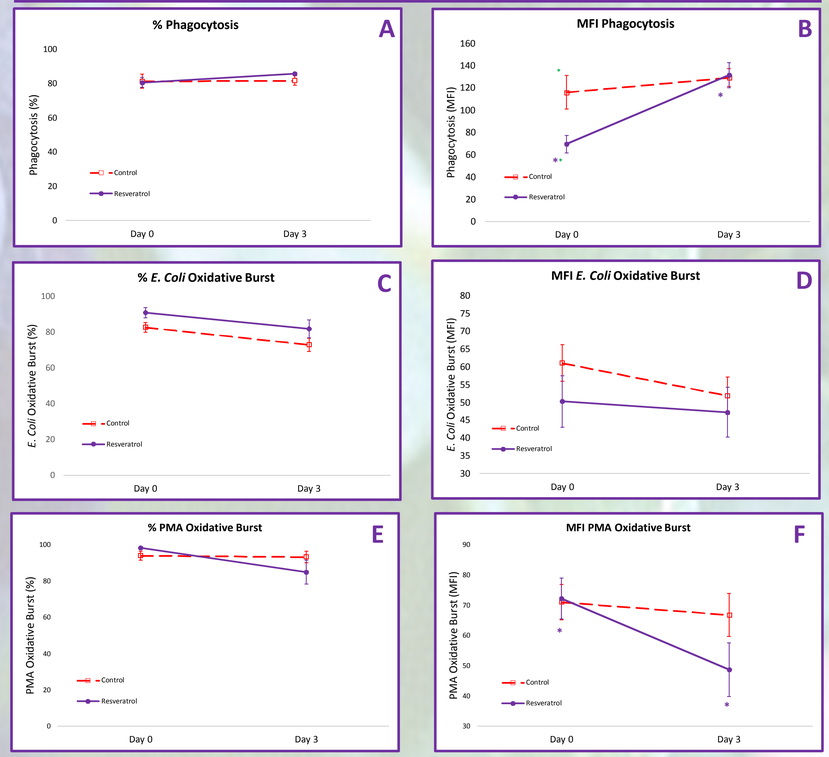

Figure 4. Means of Control vs. Resveratrol. **A)** Percent cells performing phagocytosis of FITC-labeled *E. Coli*. **B)** Mean fluorescent intensity (MFI) of cells performing phagocytosis of FITC-labeled *E. Coli*. **C & E)** Percent cells undergoing *E. Coli*-induced oxidative burst (C) and PMA-induce oxidative burst (E). **D & F)** MFI of *E. Coli*-induced oxidative burst (D) and PMA-induced oxidative burst (F).
* Indicates significance ($P < 0.05$) between Day 0 and Day 3 in the resveratrol group.
◦ Indicates significance ($P < 0.05$) between Day 0 in the control group and Day 0 in the resveratrol group.

Conclusions

•Resveratrol use *in vivo* in canines demonstrated a direct effect on active, phagocytic cells by increasing the number of bacteria engulfed during phagocytosis. In support of our hypothesis, the oxidative burst performed by cells induced by PMA was reduced in intensity; however, *E. Coli*-induced cells experienced no significant change. Cytokine profiles are yet to be determined in this study. With the current data, use of resveratrol as a therapeutic agent holds potential to serve as an immunomodulator. Further study is warranted to determine the *in vivo* effects of resveratrol at various doses and if its associated effects are consistent within a larger population.