Evaluation of the *in vitro* dose-dependent effects of resveratrol on innate immune function in dogs

Rowena Woode, Sandra Bechtel, Yan Zhang, Juliana Amorim, Kaoru Tsuruta, Amy DeClue

Comparative Oncology & Internal Medicine Laboratories, College of Veterinary Medicine, University of Missouri, Columbia, MO

Introduction

- Resveratrol, a compound found in the skin of grapes, has immunomodulatory activity, stimulating immune function at low concentrations and inhibiting it at high concentrations in human and murine immune cells *in vitro*, as well as counteracting inflammation and improving immune function *in vivo*.
- Resveratrol has potential use as a therapeutic agent in humans and animals, helping to bolster immune function in patients with suppressed immunity, and to suppress immunity in patients with immune dysfunction.
- Companion dogs are ideal to evaluate resveratrol in this capacity since humans and dogs share many of the same environmental influences and develop similar diseases spontaneously.

Objectives/Hypothesis

- The objective of our study was to evaluate the *in vitro* effects of resveratrol on canine leukocyte phagocytic function, oxidative burst, leukocyte cytokine production capacity, and natural killer cell function.
- We hypothesized that resveratrol would demonstrate a dose-dependent effect on immune cell function in dogs *in vitro* with low concentrations being stimulatory and high concentrations being inhibitory.

Materials and Methods

- Dogs: Whole blood samples from 6 healthy, adult, client-owned dogs were used for each assay.
- Cell viability: Blood was incubated with 120ug/mL (20x concentration) of resveratrol, ethanol or PBS for 4 hours at 37ºC, followed by 24 hour incubation with phosphate buffered saline, lipopolysaccharide, lipoteichoic acid and peptidoglycan. Percent live versus dead cells were assessed in each group.
- Incubation: Blood was incubated with high (6ug/mL), intermediate (3ug/mL) and low (1ug/mL) concentrations of resveratrol and a control solution for 4 hours at 37ºC.
- Leukocyte phagocytosis: Samples were incubated with FITC-labeled *Escherichia coli* or a negative control solution for 10 minutes at 37ºC. Phagocytic activity was measured via flow cytometry.
- Leukocyte oxidative burst: Samples were incubated with unlabeled opsonized *E. coli* bacteria, phorbol 12-myristate 13-acetate (PMA) or a negative control solution for 10 minutes at 37ºC. Dihydrorhodamine was added as a fluorogenic substrate. Samples were analyzed via flow cytometry.
- Cytokine Production: Whole blood treated with resveratrol was incubated with phosphate buffered saline, lipopolysaccharide, lipoteichoic acid and peptidoglycan) for 24hrs at 37ºC. Supernatant was collected and cytokine production will be measured using a canine-specific multiplex bead assay.
- Natural Killer (NK) Cell Cytotoxicity: Whole blood will be treated with resveratrol or control solution, peripheral blood mononuclear cells separated, and then incubated with canine thyroid adenocarcinoma cells (CTAC) for 24 hours at 37ºC at NK:CTAC ratios of 1:1, 10:1, 25:1 and 50:1. NK cell cytotoxicity was measured via flow cytometry.

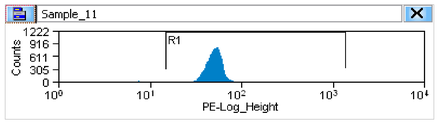


Figure 1. Gating for DNA positive cells. Cells staining positive for propidium iodide (PI) DNA stain were isolated based on fluorescence within the first and third decades (R1).

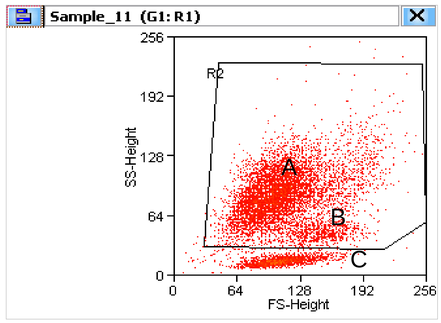


Figure 2. Gating for DNA positive cells. Cell populations were separated based on size (FS - forward scatter) and complexity (SS – side scatter), isolating cells into neutrophil (A), monocyte (B) and lymphocyte (C) cell populations. Neutrophils and monocytes were gated in our analysis to evaluate phagocytosis and oxidative burst.

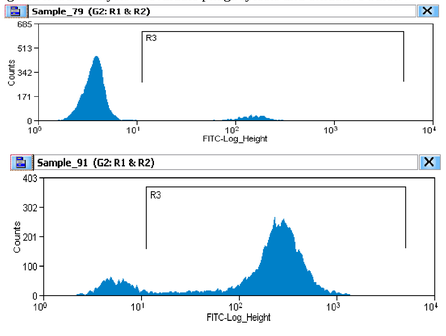
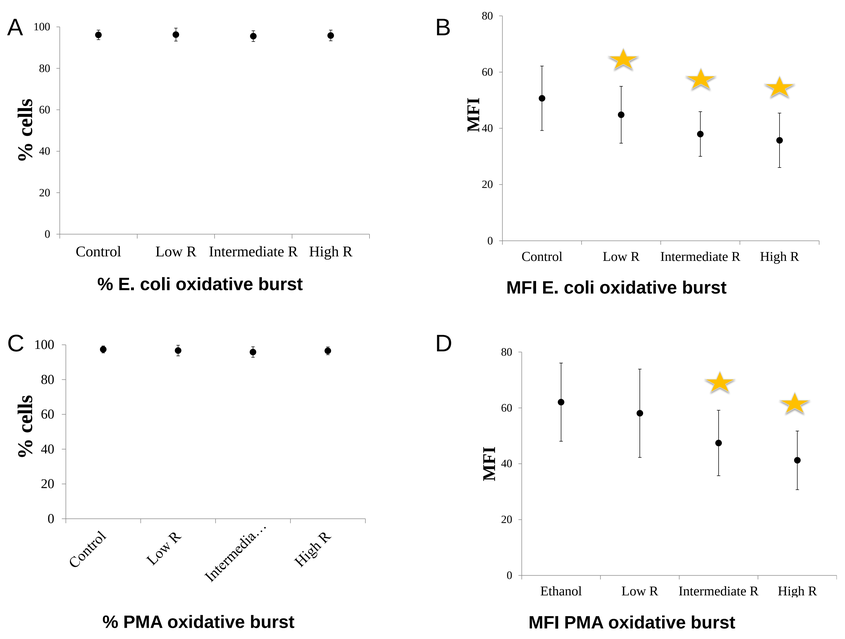


Figure 3. Gating for FITC positive cells. Monocyte and neutrophil populations were determined to be either FITC negative (top) or FITC positive (bottom) depending upon whether or not they fluoresced within the first and third decades (R3).

•Statistical analysis for phagocytosis and respiratory burst: Shapiro-Wilk test was used to test normality assumptions. One way repeated measures ANOVA and post hoc-Fisher least significant difference method were used to compare data and a p value of <0.05 was considered significant.

Results

- Cell viability: Cells incubated with 120ug/mL resveratrol showed average cell viability >85% in all groups, indicating no significant adverse effects on cells.
- Phagocytosis: There was no statistically significant difference between ethanol control and resveratrol treated groups in percentage of cells performing phagocytosis and the MFI of cells performing phagocytosis.
- Oxidative Burst: For both *E. coli* and PMA induced oxidative burst, the mean fluorescent intensity was significantly less for cells treated with resveratrol, and this appears to be a concentration dependent response (Figure 4).
  - The MFI for E. coli respiratory burst decreased with increasing concentrations of resveratrol.
  - The MFI for PMA respiratory burst decreased with increasing concentrations of resveratrol.
- Leukocyte cytokine production capacity and natural killer cell assays: These assays are currently being performed.



% Ecoli		
Treatment Name	Mean	Std Dev
Control	96.155	2.328
Low R	96.285	3.116
Intermediate R	95.552	2.6
High R	95.868	2.578

% PMA		
Treatment Name	Mean	Std Dev
Control	97.313	2.017
Low R	96.668	3.039
Intermediate R	95.818	3.036
High R	96.517	2.249

MFI E. coli		
Treatment Name	Mean	Std Dev
Control	50.69	11.469
Low R	44.823	10.115
Intermediate R	37.972	7.947
High R	35.73	9.686

PMA MFI		
Treatment Name	Mean	Std Dev
Ethanol	62.062	14.026
Low R	58.078	15.832
Intermediate R	47.43	11.723
High R	41.233	10.504

Figure 4. The mean percentage of cells in the cell population engaging in oxidative burst activity upon stimulation with E. coli (A) and PMA (C) showed no significant difference between treatment groups. The mean fluorescence intensity (MFI) of cells stimulated with *E. coli* (B) and PMA (D), indicating the degree to which each cell engaged in oxidative burst activity on average, showed statistically significant differences between treatment groups. Star denotes a statistically significant difference compared to control.. Furthermore, this difference shows a dose-dependent effect, with oxidative burst activity decreasing progressively as doses of resveratrol increase..

Conclusion and Future Directions

This data suggests that resveratrol has immunomodulatory effects in healthy dogs *in vitro*, and that these effects are dose dependent in nature. Further study is warranted *in vitro* to further define these changes, and leukocyte cytokine production assays and natural killer cell assays are underway.

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