FINAL STUDY REPORT

STUDY TITLE: **Investigation on the *in vitro* effects of resveratrol on peripheral blood mononuclear cellsharvested from healthy and atopic dogs**

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STUDY OBJECTIVE

Canine peripheral blood mononuclear cells (PBMC) will be cultured and used to assess the cytotoxic and anti-inflammatory effects of resveratrol.

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IDENTITY OF SITE AT WHICH THE STUDY WAS CONDUCTED

College of Veterinary Medicine University of Florida Gainesville, Florida

KEY STUDY DATES

Study Start	Study End
01 May 2021	25 November 2021

DATA RECORDS:

All data sheets or notebook pages will be maintained by the study team performing the study until the final report is filed.

EXPERIMENTAL DESIGN:

Summary:

Canine peripheral blood mononuclear cells (PBMC) harvested from ten healthy and ten atopic dogs were used to assess the cytotoxic and anti-inflammatory effects of resveratrol. The purpose of the present study was to evaluate whether resveratrol is associated with any cytotoxic effect and if it is able to inhibit inflammatory mediators or stimulate anti-inflammatory molecules after 24 hours of incubation.

Experiments:

Five mL of blood were collected, transferred to EDTA tubes and processed within 1–2 h for cell culture. Briefly, 5 mL of undiluted room temperature blood was layered over 12 ml of ice cold Lymphoprep™ (Axis-Shield PoC, Oslo, Norway) in a 15 ml SepMate® polypropylene tube and processed following the manufacturer's recommendations. Previous experiments, by the manufacturer, using flow cytometry determined an average purity of >95% lymphocytes will be isolated using this technique. The collected PBMC were plated in a T25 flask with fresh RPMI medium for five days to allow monocytes to adhere. Then the suspended lymphocytes were harvested and counted for viability using a hemacytometer and assessed for viability (>95%) with trypan blue exclusion staining.

Cell culture set-up

Peripheral blood mononuclear cells were re-suspended in RPMI media to a concentration of $1x10^6$ cells per ml, and then plated into flat-bottomed 24-well Costar plastic microtiter plates (0.8mL/well). Cells were allowed to rest overnight in fresh RPMI media a 37ºC in a 5% CO_2 incubator. The next day, the cells were incubated for 24 hours at 37ºC in a humidified atmosphere with 5% CO_2 under the following conditions:

1. Without addition of phytohemagglutinin (PHA) and resveratrol (negative control),
2. Stimulation by PHA without resveratrol (positive control),
3. Resveratrol alone
4. Stimulation by PHA with resveratrol.

The day after, the cells were centrifuged for 5 min and the supernatant transferred to 1.5 ml Eppendorf tubes and frozen at −80ºC until used in ELISA assays.

Negative control (RPMI)	RPMI alone
Positive control (µg/mL) (PHA)	2
Res (µg/mL)	1.5
	3
	6
	9

Table 1: Concentrations of Res (1.5-9µg/mL) tested in RPMI with and without PHA (2µg/mL), negative and positive controls.

Cell viability assay:
The cell viability was assessed using two different methodologies: lactate dehydrogenase and DNA-histone (apoptosis) assays.

Lactate Dehydrogenase (LDH) Assay: This set of experiment was performed used Pierce LDH Cytotoxicity Assay (ThermoFisher, Waltham, MA, USA) following the manufacturer's instructions. Briefly, 10µl of lysis buffer was added to the positive control well, and 10µl of sterile PBS was added to the negative control wells on each culture plate. The wells were gently tapped and incubated 37°C, 5% CO_2 for 45 mins. Then, 50µl of supernatant was taken from each well and transferred to another sterile 96 well flat bottom plate in which 50µl of the reaction mixture was added and the plates were incubated at room temperature for 30 minutes in the dark. Finally, 50µl of stop solution was added to each sample well and gently tapped. Care was taken to avoid any air bubbles. The wells were then read at absorbance of 490nm and 680 nm using the Cytation-1 Image Reader.

Cell apoptosis assay: This set of experiment was performed using the Cell Death Detection ELISA^{PLUS} assay (Roche Diagnostics GmbH, Mannheim, Germany). This assay measures the DNA-histone complex released by a cell going through apoptosis. This set of experiment was performed following the manufacturer's instructions. Briefly, 20µl of centrifuged cell supernatant was transferred to the coated plate and 80µl of immnoreagent were added. The plate was covered with adhesive cover foil and incubated on a shaker (300rpm) for 2h at 25°C. Then the well content was emptied and washed three times with 300µl of incubation buffer and dried. Finally, 100µl of ABTS substrate solution were added to each well and incubated on a shaker (250rpm) for 20 minutes at 25°C. Finally, 100µl of ABTS stop solution was added to each well. The wells were then read at absorbance of 405nm and 490 nm using the Cytation-1 Image Reader

Antioxidative stress:
The antioxidative stress evaluation was performed on cell lysates using the catalase and superoxide dismutase colorimetric kits (ThermoFisher, Waltham, MA, USA). This set of experiment was performed following the manufacturer's instructions using cell lysates instead of supernatants.

Host defense peptides:
Protein levels of canine β-defensin (cBD)3-like and canine cathelicidin (cCath), present in the supernatant, were measured using a previously validated custom-made competition inhibition ELISA.

Inflammatory markers:
Once the supernatant was collected, one aliquot was saved for the inflammatory markers' evaluation using the Milliplex MAP canine Multiplex® assay (Millipore, Billerica, MA, USA), according to manufacturer's protocol. Multiplex technology allows the simultaneous assessment of multiple cytokines using the Luminex-100 system Version 1.7 (Luminex, Austin, TX). Thirteen canine cytokines (Granulocyte-Monocyte- Colony stimulating factor [GM-CSF], Interferon [IFN]-γ, IL-2, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18, IFN-γ-induced protein 10 [IP-10/CXCL10], keratinocyte chemotactic-like [KC-like], monocyte chemotactic protein-1 [MCP-

1/CCL2], and Tumor necrosis factor [TNF]-α) were assessed in this study. Data analysis was performed using the MasterPlex QT 1.0 system (MiraiBio, Alameda, CA). A five-parameter regression formula was used to calculate the sample concentrations from the standard curves.

Statistical analysis:
Once collected, the data were first tested for normal distribution using the Kolmogorov-Smirnov test (alpha = 0.05). Differences in cytokine, apoptosis index, and antioxidative stress parameters were compared with the baseline (media or media with PHA) using the repeated measurement ANOVA and the Friedman's multiple comparison test (non-normally distributed data). Either the Dunnett's or the Dunn's multiple comparison test was sued as post-hoc analysis. P-values of <0.05 were considered statistically significant. All statistical comparisons were performed using the GraphPad Prism9 Software (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

Cell viability assay:
The LDH cell viability assay (Figure 1) did not show any significant difference between any of the condition tested and the baseline (media only) in the atopic group. However, a significant increase in cell viability was seen in all the tested concentrations of resveratrol, with or without PHA when compared to media alone ($p<0.01$). In addition, compared to atopic PBMC, healthy PBMC has a significantly higher cell viability in all conditions tested ($p<0.002$).

Cell apoptosis assay:
Active apoptosis was not seen at any of the concentration tested (Figure 2), when compared to the baseline (media only) in either group. However, when atopic PBMC were exposed to PHA and 6µg/mL of resveratrol there was a significantly lower absorbance when compared to PHA alone ($p=0.028$). Finally, the atopic PBMC had a significantly higher absorbance than the healthy PBMC for controls and testing solutions ($p\leq0.001$).

Antioxidative stress:
Catalase (Figure 3): A significant increase un catalase activity was seen in healthy PBMC after exposure to 1.5 ($p=0.012$) and 3µg/mL ($p=0.03$) of resveratrol when compared to baseline (media alone). However, no changes were seen in the atopic PBMC exposes to resveratrol with or without PHA. No effects were also seen in the healthy PBMC exposed to PHA and resveratrol.

Superoxide dismutase: There was no detectable superoxide dismutase activity in the cell lysate for both atopic and healthy PBMC.

Host defense peptides:
cBD3-like ELISA assay (Figure 4): Compared to healthy PBMC, a significantly higher amount of peptides were secreted by the atopic PBMC after exposure to PHA alone ($p=0.043$) or to 9µg/mL of resveratrol ($p=0.02$) alone or with 9µg/mL of resveratrol combined to PHA ($p=0.01$). However, there was no effect of resveratrol on cBD3-like secretion in either group (healthy or atopic) when compared to the baseline values (media or PHA only). A higher amount of cBD3-

like was seen at baseline (media only) in atopic PBMC when compared to healthy baseline PBMC, but this difference was not statistically significant (p=0.08).

cCath ELISA assay (Figure 5): The ELISA was not able to detect any significant difference between healthy and atopic PBMC or between resveratrol with or without PHA compared to the baselines (media or PHA alone).

Inflammatory markers:

The Milliplex MAP canine Multiplex® assay (Figure 6) was only able to detect seven of the thirteen cytokines tested (IL-6, IL-7, IL-8, IL-15, KC-like, MCP-1, and TNFα). In fact, the other cytokines were below detection limit. However, for most detectable cytokines the detectable values were above the limit of detection, thus the cytokines available for a full statistical analysis included only IL-8, KC-like and MCP-1. There was no statistical difference in IL-8 between healthy and atopic PBMC or between resveratrol with or without PHA compared to the baselines (media or PHA alone). There was no statistical difference in KC-like between healthy and atopic PBMC or between resveratrol with or without PHA compared to the baselines (media or PHA alone). There was a statistical reduction in MCP-1 between healthy and atopic PBMC after exposure to PHA and 1.5µg/mL of resveratrol. In addition, a significant reduction in MCP-1 was also seen in healthy PBMC after exposure to 9µg/mL of resveratrol when compared to baseline (media alone).

For another four cytokines (IL-6, IL-7, IL-15 and TNF-α) the only statistical comparison possible was in-between groups but not within-groups. For IL-6, when compared to healthy PBMC, atopic PBMC secreted significantly lower IL-6 at baselines (media and PHA alone; p=0.026 and p=0.044, respectively), as well when exposed to PHA with 1.5 and 6µg/mL of resveratrol (p=0.03 and p=0.02, respectively). For IL-7, when compared to healthy PBMC, atopic PBMC secreted significantly lower IL-7 when exposed to PHA with 1.5, 6 and 9µg/mL of resveratrol (p=0.049, p=0.048 and p=0.03, respectively). Although not statistically significant, a lower expression of IL-7 was also seen at baselines (media and PHA alone; p=0.055 and p=0.08, respectively) as well as when exposed to PHA with 3µg/mL of resveratrol (p=0.054). For IL-15, when compared to healthy PBMC, atopic PBMC secreted significantly lower IL-15 at baseline (media alone; p=0.004). For TNF-α, when compared to healthy PBMC, atopic PBMC secreted significantly lower TNF-α at baseline (media alone; p=0.04). Although not statistically significant, a lower expression of TNF-α was also seen after exposure to 1.5µg/mL of resveratrol (p=0.062).

DISCUSSION

This study confirmed the overall positive effects of resveratrol on circulating canine lymphocytes. One of the main findings of this study was the assessment of resveratrol on cell viability via LDH. Interestingly enough, resveratrol showed a significantly positive effect on PBMC viability with all the concentrations tested increasing the viability of healthy, but not atopic, PBMC. The effect was somehow dose-dependent up to 6µg/mL of resveratrol for then dropping the effect when PBMC were exposed to 9µg/mL of resveratrol. Such effect was also seen despite the exposure to PHA. Furthermore, these data showed how atopic PBMC are less viable than healthy PBMC. This is a new finding that was consistent throughout the entire experiment. In fact, a significantly lower cell viability (via LDH) was seen in atopic PBMC,

when compared to healthy PBMC, in all experimental conditions. Similar results were seen for the Cell Death assay, assessing the effect of resveratrol on apoptosis. In particular, the results of this study showed that healthy PBMC have a lower tendency to apoptosis when compared to atopic PBMC. However, when the atopic PBMC were exposed to PHA and 6µg/mL of resveratrol, the apoptotic tendency significantly decreased suggesting some protective effect of resveratrol in this setting. Altogether, these data confirm that resveratrol has beneficial effects on immune cells, in particular canine PBMC, increasing their viability. Such effect was mainly evident in healthy rather than atopic PBMC suggesting a potential for a dose-dependent effect with atopic cells needing more resveratrol to achieve the same behavior seen in healthy cells.

The second aim of this study was to assess the antioxidative effect of resveratrol on canine PBMC. We tested catalase and superoxide dismutase as two markers of oxidative stress. Unfortunately, using supernatant and cell lysate, we were not able to detect superoxide dismutase in any of our samples. As far as catalase, we did see a significant increase in catalase activity in healthy PBMC when exposed to 1.5 and 3µg/mL of resveratrol when compared to media alone. Such effect was not seen in atopic PBMC or in either group of cells when exposed to PHA and resveratrol simultaneously.

The third aim of the study was to assess the effect of resveratrol on canine HDPs secretion. Unfortunately, we were not able to show any direct effect of resveratrol on the secretion of either HDP tested. However, compared to healthy PBMC, we did see a significant increase in cBD3-like secretion in atopic PBMC when exposed to PHA, to 9µg/mL of resveratrol, and to 9µg/mL of resveratrol with PHA.

Finally, when we were looking at the amount of proinflammatory cytokines we were able to detect seven of the thirteen cytokines tested (IL-6, IL-7, IL-8, IL-15, KC-like, MCP-1, and TNFα); the other cytokines were below detection limits. Of those detectable, most cytokines were above the limit of detection leading to a potential alteration of the results. The cytokines available for a full statistical analysis (within and in-between groups analysis) were IL-8, KC-like and MCP-1. For the other four cytokines (IL-6, IL-7, IL-15 and TNF-α) the only statistical comparison possible was in-between groups, but not within-groups. The only cytokine on which resveratrol showed a significant anti-inflammatory effect was MCP-1. For this cytokine, it was evident a significant decrease in secretion by healthy PBMC, after exposure to 9µg/mL of resveratrol. Such effect was not evident in any other experimental condition. Of interest, atopic PBMC constantly secreted less cytokines before or after exposure to resveratrol (different concentrations for different cytokines) and/or PHA when compared to healthy PBMC.

In conclusion, these preliminary data confirm the overall beneficial effects of resveratrol on healthy PBMC as expression of higher cell viability and reduced oxidative stress. However, at the concentrations tested, there was not a significant effect of resveratrol on the secretion of HDPs nor on pro-inflammatory cytokines, except MCP-1. Looking at the data, a potential explanation for the lack of positive results could be the high variability in some of the outcomes measured (e.g., HDPs, catalase, and some cytokines). Another explanation is that atopic PBMC would need a higher concentration of resveratrol to be positively "activated". The concentrations tested in this study were based on pharmacokinetic studies in dogs showing that 6µg/mL of resveratrol would be the pick concertation present in the blood after oral administration of resveratrol given at a dose of 600mg/kg/day; such dose was elected as No Observed Adverse Effect Level (NOAEL) dose in healthy Beagle dogs. The concentration of 9µg/mL of resveratrol would equate to an oral dose of 1,200 mg/kg/day, which was above the NOAEL safety dosing. For these reasons, if the effect of resveratrol is dose-dependent and a higher blood concentration

of resveratrol is needed to see a positive effect of atopic PBMC, this may not be feasible in live animals due to potential renal side effects. An alternative to this would be testing resveratrol as topical compound in which case, we may not need to worry about systemic absorption. Finally, once administered orally, resveratrol is rapidly metabolized to its sulfate and glucuronide metabolites in dogs with a pick concentration up to 30 times higher than resveratrol. Unfortunately, in this study we did not test such metabolites, which may be the compounds that actively stimulate PBMC.

Appendix 1: Figures

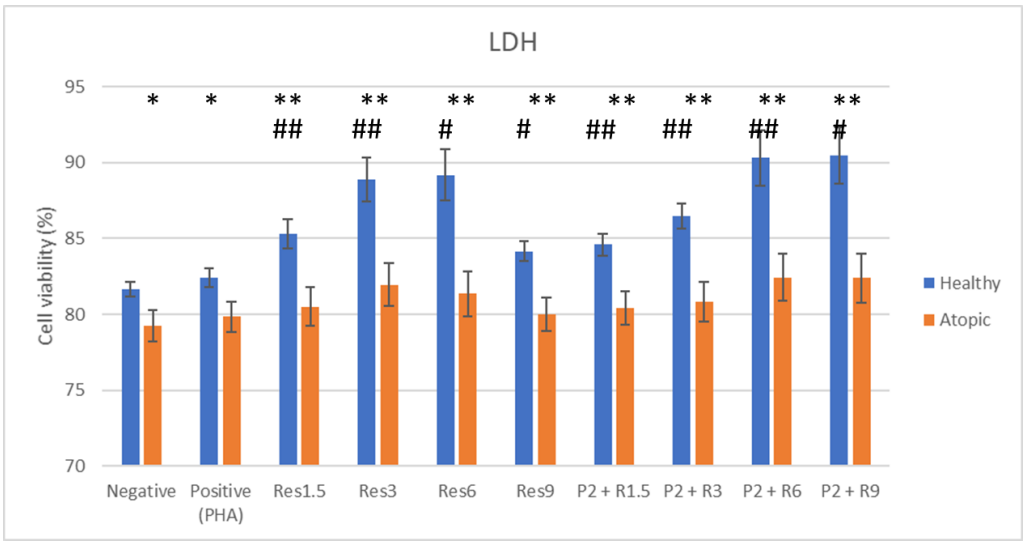

Figure 1: Average PBMC cell viability (LDH assay) before (negative control = media) and after exposure to resveratrol at different concentrations, phytohemagglutinin (PHA), or combination of both for 24 hours. Res: resveratrol; P2: PHA in combination with resveratrol at different concentrations; 1.5, 3, 6, and 9: 1.5, 3, 6, and 9 µg/mL of resveratrol.
#: $p<0.05$ and ##: $p<0.01$ = significantly different from baseline (media or PHA alone); *:$P<0.05$ and **: $p<0.01$ = significant difference between healthy and atopic. Bars: standard error of the mean.

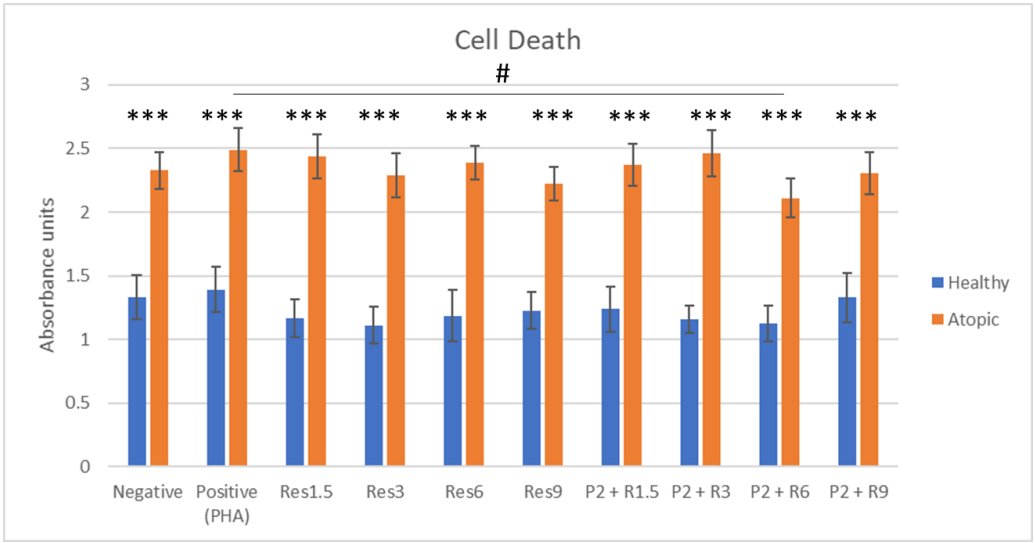

Figure 2: Average PBMC cell viability (Cell Death apoptosis assay) before (negative control = media) and after exposure to resveratrol at different concentrations, phytohemagglutinin (PHA), or combination of both for 24 hours. Res: resveratrol; P2: PHA in combination with resveratrol at different concentrations; 1.5, 3, 6, and 9: 1.5, 3, 6, and 9 µg/mL of resveratrol.
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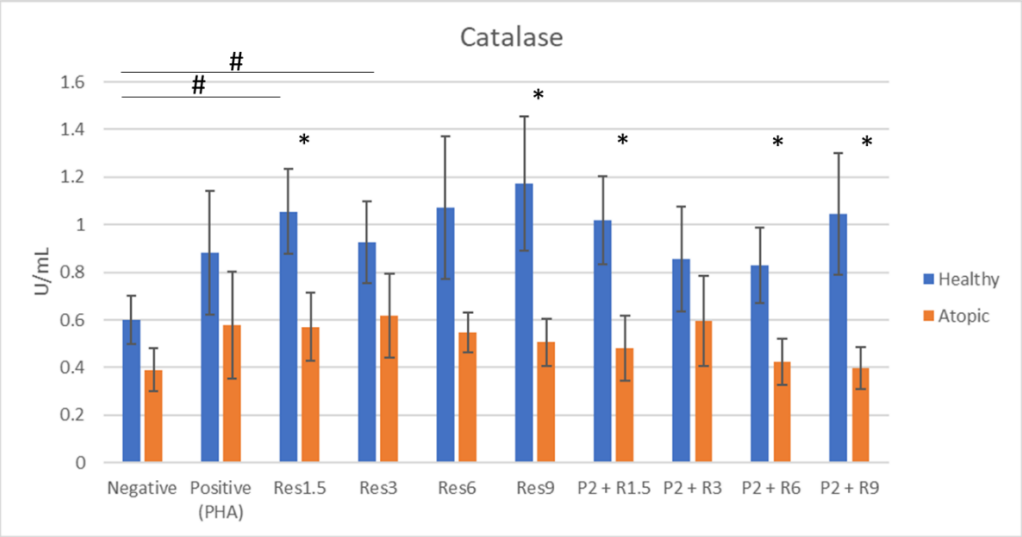

Figure 3: Average PBMC catalase activity before (negative control = media) and after exposure to resveratrol at different concentrations, phytohemagglutinin (PHA), or combination of both for 24 hours. Res: resveratrol; P2: PHA in combination with resveratrol at different concentrations; 1.5, 3, 6, and 9: 1.5, 3, 6, and 9 µg/mL of resveratrol.
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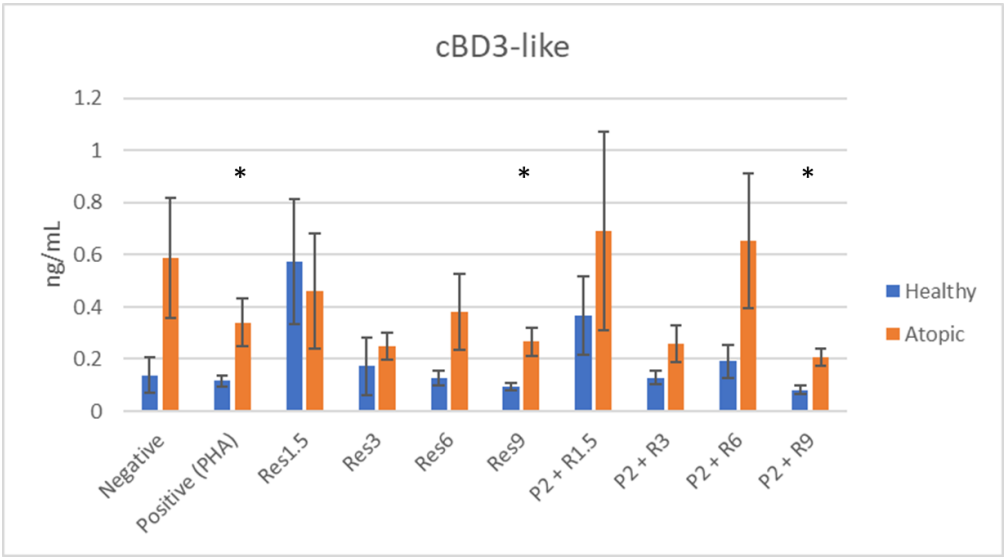

Figure 4: Average PBMC canine β-defenin (cBD)3-like secretion before (negative control = media) and after exposure to resveratrol at different concentrations, phytohemagglutinin (PHA), or combination of both for 24 hours. Res: resveratrol; P2: PHA in combination with resveratrol at different concentrations; 1.5, 3, 6, and 9: 1.5, 3, 6, and 9 µg/mL of resveratrol.
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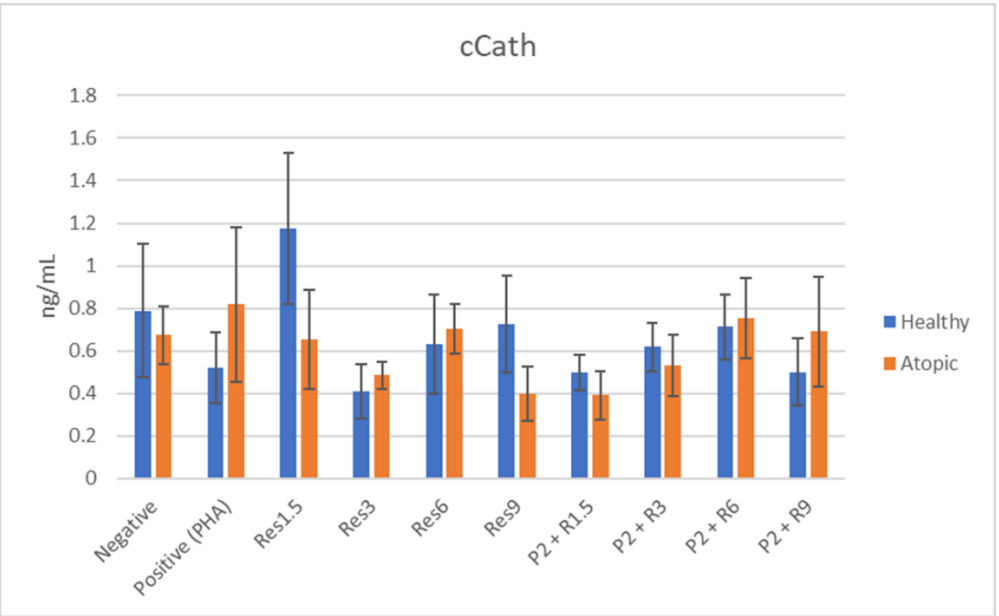

Figure 5: Average PBMC canine cathelicidin (cCath) secretion before (negative control = media) and after exposure to resveratrol at different concentrations, phytohemagglutinin (PHA), or combination of both for 24 hours. Res: resveratrol; P2: PHA in combination with resveratrol at different concentrations; 1.5, 3, 6, and 9: 1.5, 3, 6, and 9 µg/mL of resveratrol.
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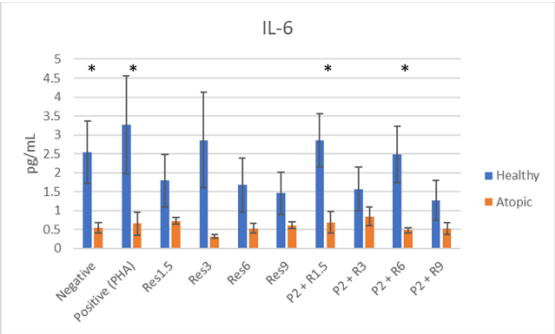

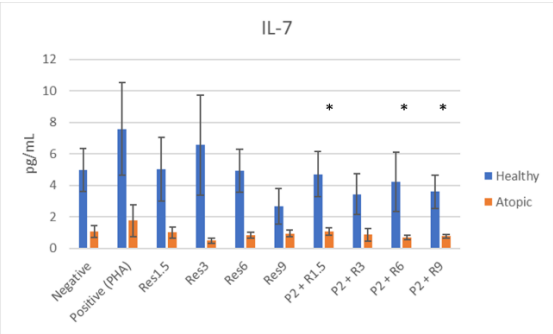

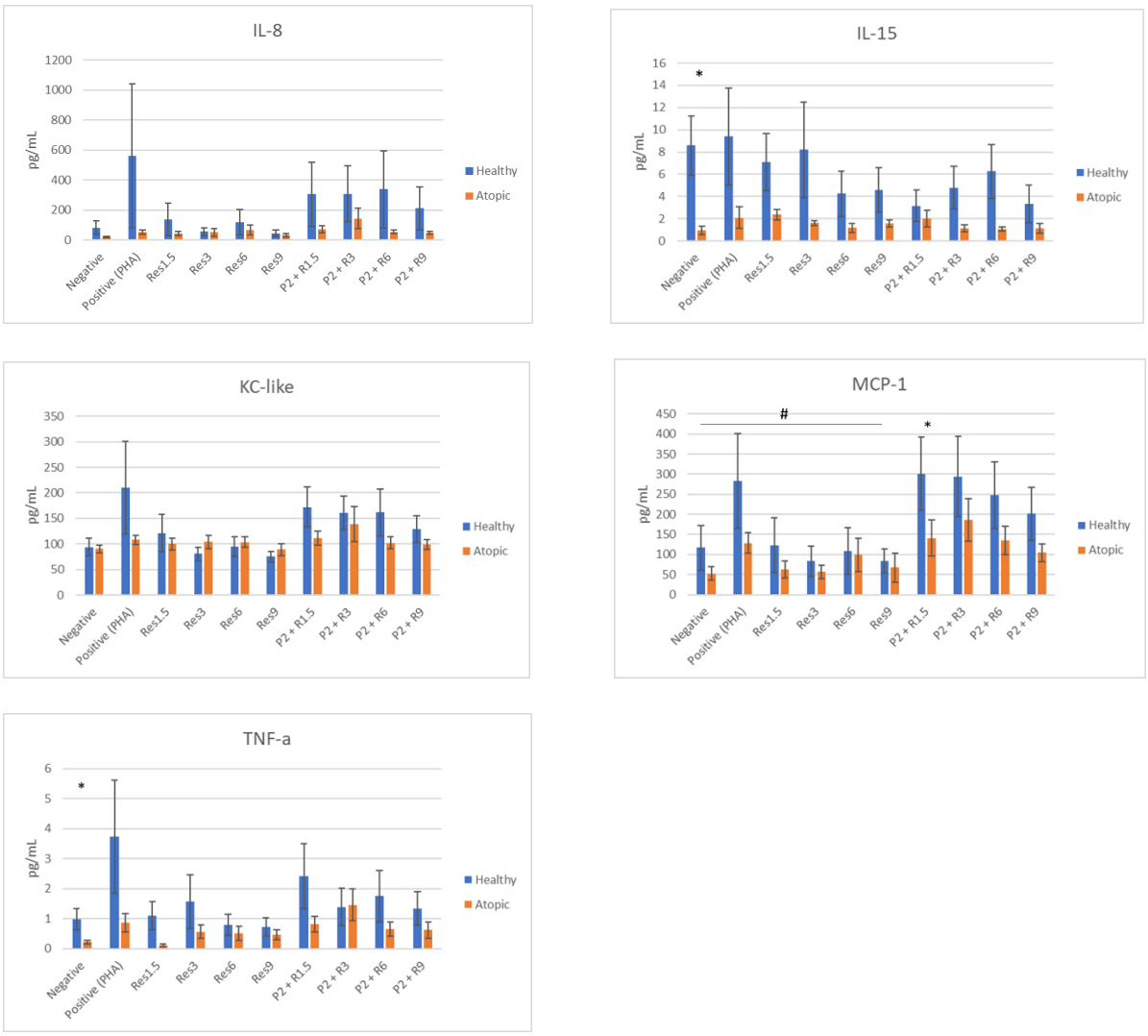

Figure 6: Average PBMC inflammatory cytokine secretion before (negative control = media) and after exposure to resveratrol at different concentrations, phytohemagglutinin (PHA), or combination of both for 24 hours. Res: resveratrol; P2: PHA in combination with resveratrol at different concentrations; 1.5, 3, 6, and 9: 1.5, 3, 6, and 9 µg/mL of resveratrol.
*: $p<0.05$ = significant difference between healthy and atopic. Bars: standard error of the mean.