The Benefit of Resveratrol and Quercetin Co-treatments in Canine Osteosarcoma

By
KayLynn Carver
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Allegheny College
Department of Biology

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KayLynn Carver
Biology Department

A Senior Comprehensive Project in Partial Fulfillment of the Requirements for a Bachelor of Science Degree from Allegheny College

I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor Code and to maintain the integrity of both myself and the College community as a whole.

KayLynn Carver
Pledge

Dr. Ann Kleinschmidt
Dr. Tricia Humphreys
April, 2017

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Abstract

Phytochemicals from grapes and berries, including resveratrol and quercetin, have been studied for their chemotherapeutic properties. Quercetin and resveratrol, used individually, have caused decreases in proliferation of various cancer cell lines ranging from breast cancer to human osteosarcoma. Co-treatments of resveratrol and quercetin have led to a synergistic, more than additive, decreases in cell proliferation through apoptotic pathways, synergism which lead to a more efficient cancer cell decrease than if the chemicals were merely additive. In this study it was of interest to determine whether resveratrol and quercetin, alone or in combination, would have anticancer effect on OSCA-40, an osteosarcoma cell line. Cells were treated with resveratrol and quercetin independently and in combination, a decrease in proliferation was observed. To determine if the proliferation decrease occurred due to apoptosis, the treated cell were stained using DRAQ 5, a DNA stain, and analyzed using an imaging flow cytometer. The analysis concluded that the co-treatment of resveratrol and quercetin lead to a significant increase in cells with a sub G_1 level of DNA, signifying apoptosis. The data supports that resveratrol and quercetin in combination are able to induce apoptosis of OSCA-40 cells.

Introduction

Phytochemicals in everyday foods can provide chemotherapeutic benefits (Singh et al., 2016). Many of the phytochemicals are beneficial because they cause modifications in cell signaling pathways that influence cell viability (Debatin, 2004). Since phytochemicals are able to decrease cell proliferation, researchers have investigated whether this decrease occurs through the induction of an apoptotic pathway (Davidson et al., 2016; Signorelli et al., 2015; Yang et al., 2009). An indicator of apoptosis induction is cell cycle arrest, which can be induced by chemicals such as resveratrol, quercetin, and ellagic acid (Benitez et al., 2007; Mertens-Talcott & Percival, 2005; Priyadarsini et al., 2010; Ranelletti et al., 2000; Suh et al., 2010).

The exact mechanism by which apoptosis is induced in cancer cells by phytochemicals has become a common focus in research. However, because many different pathways can be affected including NfκB, MAPK, and caspase cascades, it can be challenging to determine the specific change occurring when cells are treated (Debatin, 2004). Some combinations of chemicals can cause a synergistic effect by influencing different pathways that lead to apoptosis, ultimately causing a better than additive decrease in proliferation (Singh et al., 2016). By combating the cancer cells through multiple pathways the efficacy of the treatment increases while the chances of drug resistance decrease. For example, phytochemicals found in berries and grapes, known as flavonoids, have been found to re-sensitize drug resistant cells to chemotherapeutics, this would be an additional beneficial to combat cancer cells that may be chemo-resistant (Chen et al., 2010).

It has been found that grape extracts inhibit growth of cancer cells, and when extracts of 14 different grape strains were investigated, it was found that they were capable of decreasing proliferation of $HepG_2$, colon, Caco-2, and breast, MCF-7, cancer cells (Yang et al., 2009). In an

attempt to identify anti-cancer compounds, extracts from muscadine grapes were chemically analyzed and found to contain the highest combined levels of resveratrol and quercetin (Ector et al., 1996; Talcott & Lee, 2002) Resveratrol (3, 5, 4'-trihydroxystilbene, RES) and Quercetin (3, 3′, 4′, 5, 7-pentahydroxyflavone, QT) RES is produced mainly in the grape vines and skins as a response to environmental stress such as wind (Gambini et al., 2015). QT is a flavonoid in grapes, which is found in higher levels within grapes exposed to sun (Price et al., 1995).

Previous studies have found that RES decreases cell proliferation of cancer cells (Alkhalaf & Jaffal, 2006; Benitez et al., 2007; Chow et al., 2014; Gogada et al., 2011; Prabhu et al., 2013; Li et al., 2009). In many cases the decrease in proliferation was linked to the induction of programmed cell death as determined by analysis of a variety of known apoptotic markers (Alkhalaf & Jaffal, 2006; Benitez et al., 2007; Chow et al., 2014; Gogada et al., 2011; Prabhu et al., 2013). DNA analysis can be used to determine apoptosis since it can quantify the amount of DNA within the cell, so for eukaryotic cells a normal DNA level would be 2n or 4n, an apoptotic DNA levels would be less than 2n (Darzynkiewicz et al., 2010). Most cells normally arrest at the cell cycle check points to make sure the previous process was completed correctly before progressing further, however cancer cells do not always have working checkpoints leading to continuous progression even if there are flaws at each step (Johnson & Walker, 1999). The loss of functioning G_1 to S that commonly occurs in cancer cells leads to unregulated cell cycle progression, making chemicals that can inhibit this progression ideal for chemotherapeutics (Fig. 1). Cyclin D, which governs the checkpoint between G_1 and S, and its' pathway is a preferred target for chemotherapeutics, as it has been found chemicals that reestablish a working checkpoint can combat cancer (Johnson & Walker, 1999).

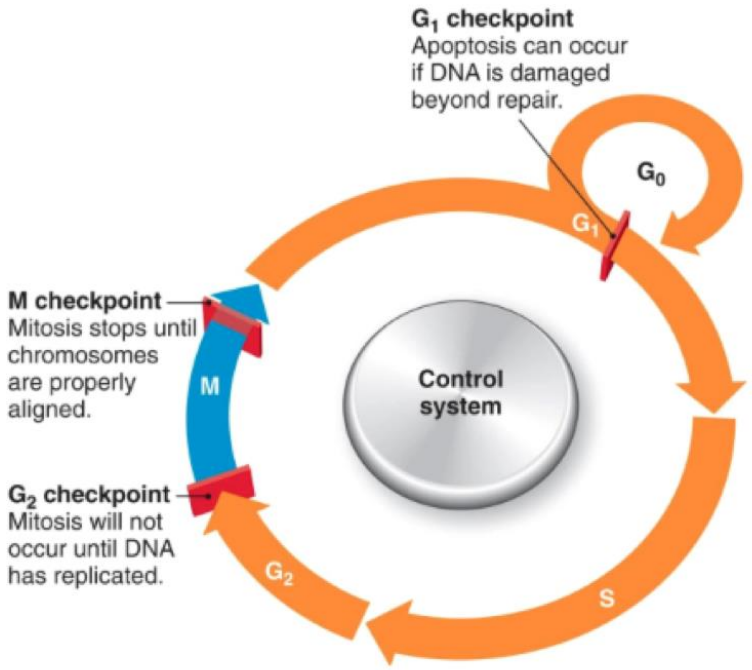


Figure 1. Cell cycle with check points marked (https://www.studyblue.com/notes/note/n/lecture-12-the-cell-cycle/deck/5949750)

When prostate, leukemia, or osteosarcoma cancer cells were treated with RES (Fig. 2A), proliferation decreased and levels of G_1 to S cell cycle regulators, p-21 and p-53 increased (Alkhalaf & Jaffal, 2006; Benitez et al., 2007; Mertens-Talcott & Percival, 2005). The alteration in these cell cycle modulators along with caspase activation supported that RES has the capability to induce apoptosis in various cancer cell lines. Although there has been evidence of apoptosis, there has also been evidence showing RES treatments inducing autophagy in human nasopharyngeal, breast, and prostate carcinoma cells (Chow et al., 2014; Prabhu et al., 2013).

Autophagy, which is often induced by cell starvation and can be detected through quantifying autophagosome and autolysosome levels (Chow et al., 2014), can act as an antagonist of apoptosis or to induce cell death. Autophagy can promote cell survival by limiting cell stress responses that would initiate apoptotic pathways (Eisenberg-Lerner et al., 2009). Autophagy can also promote cell death as it uses similar proteins as apoptosis such as BCL, and p-53 to induce cell death (Chaabane et al., 2013; Eisenberg-Lerner et al., 2009). Cancer cells,

however, may continue to proliferate when autophagy acts as an antagonizer of apoptosis that provides protection of cancer cells from cell death (Jin & White, 2006). Since RES can elicit both harmful and beneficial effects for cancer cells, cell lines in which autophagy promotes cancer cells to survive RES treatment can affect the efficacy of RES treatment may be less than optimal in some cancers.

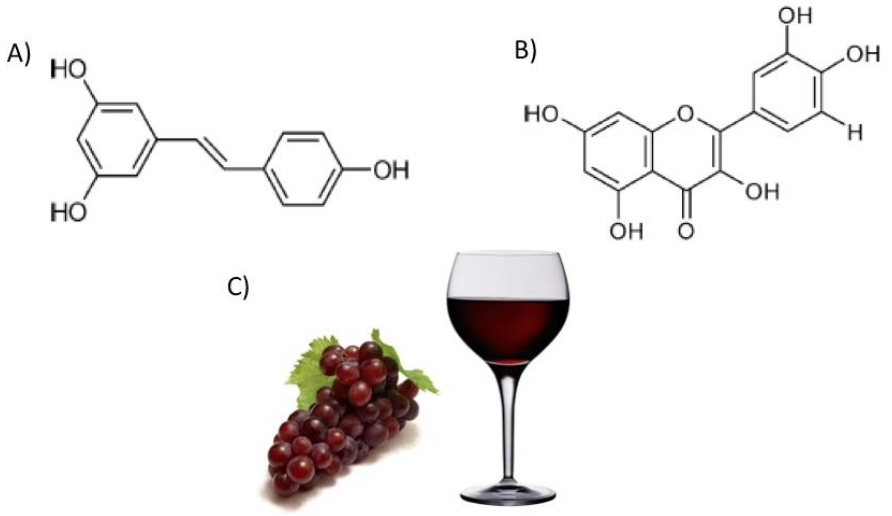


Figure 2. Chemical structures of A) Resveratrol and B) Quercetin (Taken from Mertens-Talcott and Percival, 2005) C) Red grapes and wine, where both Resveratrol and Quercetin are found (Singh et al., 2016; Yang et al., 2009)

Another dietary phytochemical providing a less toxic option for chemotherapy, also found in grapes, is quercetin (QT). QT (Fig. 2B), similarly to RES (Fig. 2A), has been found to induce cell death in human colon, hepatoma, lymphoma, and cervical cancer cells (Cincin et al., 2015; Granado-Serrano et al., 2006; Maurya & Vinayak, 2015; Priyadarsini et al., 2010). Like RES, QT has been shown to induce apoptosis, and, many studies suggest that cell cycle arrest occurs in these cells before apoptosis is induced (Mertens-Talcott & Percival, 2005; Priyadarsini et al., 2010; Ranelletti et al., 2000; Suh et al., 2010). The cell cycle arrest phase differed for cell types. In osteosarcoma, which is a cancer that occurs in bone generating cells, QT treatments lead to an accumulation of cells in the G_1/S phase that then progressed to apoptosis through

down regulation of cyclin D1 (Suh et al., 2010). In prostate cancer cells QT treatments inhibited colony formation of cells that had moderate and high metastasis potential. Since QT is able to inhibit colony formation, which often leads to increased cancer cell proliferation (Nair et al., 2004), it may be able to inhibit this formation in other cancer cells that metastasize like osteosarcoma (Fenger et al., 2014).

Treatment of leukemia cells with a combination of RES and QT causes a synergistic response, which is the observation of a greater effect than the sum of the individual chemical effects. Not only did the chemicals work at leading to cell death but they also caused temporary cell cycle arrest (Mertens-Talcott & Percival, 2005). In rat glioma cells, C-6, decreased cell proliferation was observed with co-treatments of RES and QT, although this decrease was not deemed synergistic (Zamin et al., 2009). Nude mice with xenografts of breast cancer cells were used to study the *in vivo* dietary levels of RES, QT, and catechin, a phytochemical in grapes, had on tumor growth. The result was that even at low levels these chemicals caused a decrease in tumor growth (Schlachterman et al., 2008).

The benefits of combining phytochemicals that act in synergism as expressed in the literature demonstrates that these combinations of chemicals can decrease proliferation more sufficiently then the two chemicals additive effect. The evidence of synergism between these two chemicals suggests that in leukemia cells the specific apoptotic pathways used by RES and QT are different (Mertens-Talcott & Percival, 2005). In experiments using rat glioma cells, caspase-3/7 induction was found to occur with treatments of RES and not QT, despite being enhanced when cells were co-treated with RES and QT (Zamin et al., 2009). Since caspase- 3/7 activation is involved in the initiation of some apoptotic pathways (Porter & Jänicke, 1999), the fact that

RES and QT could induce apoptosis without both affecting caspase-3/7 means that the pathways used by each drug differ.

Additional evidence supporting RES and QT influencing different pathways that induce apoptosis involved an experiment conducted on pancreatic cancer cells; where caspase- 3 activity inhibition was studied using cyclosporine A and aristolochic acid. These inhibitors interfere with different steps that affect membrane permeability and procaspase activity (Cai et al., 1998). The caspase-3 activity caused by QT was inhibited with cyclosporine A alone, whereas resveratrol required both inhibitors, this signified that the upstream targets that RES and QT affected were different but their pathways ultimately intersected and resulted in caspase activity (Mouria et al., 2002). Despite the evidence that RES and QT may be eliciting a synergistic effect in some of the research, the differences seen in cell types and pathways used to induce apoptosis supports the need for further studies on the combination of RES and QT.

Studying the effects RES and QT co-treatments have on osteosarcoma cells would expand the research to include an additional type of cancer cell. The variation seen in different cancer cell lines response to RES and QT, alone and combination demonstrates the importance of researching other cell lines. It is also of interest to see if co-treatments of RES and QT elicit a synergistic effect in osteosarcoma cells. Canine osteosarcoma is a great candidate as canines have a higher prevalence of osteosarcoma and common environmental factors make it an adequate animal model for the human disease (Fenger et al., 2014). Osteosarcoma (OSA) is a form of bone cancer, that occurs when there is an over proliferation of cells that synthesize bone, known as osteoblasts (American Cancer Society 3, 2016). This bone cancer is often seen in adolescent children in low rates; despite the low rates individuals diagnosed with osteosarcoma face a grim prognosis due to lack of research (American Cancer Society 1, 2016; American

Cancer Society 2, 2016). Using canines as a model for osteosarcoma is beneficial since dog owners are willing to have their animals test new therapy treatments (Fenger et al., 2014). Studying natural phytochemical combinations in canine osteosarcoma cells may provide a better understanding of how these chemicals alter cancer cell proliferation, which in turn can help the development of safe chemicals that would benefit children in which osteosarcoma is often diagnosed. The aim of this study it so determine whether co-treatments of resveratrol and quercetin decrease proliferation of canine osteosarcoma, OSCA-40, through synergism.

Methods

Cells and Cell Growth

Osteosarcoma 40 cells, OSCA-40, were obtained from Dr. Jaime Modiano (University of Minnesota, Minneapolis, MN). Cells were cultured in sterilized high glucose Dulbecco's Modified Eagle's Medium (DMEM: Gibco), 10% Fetal Bovine Serum (FBS; Fisher), 0.02 M HEPES, and 1 X Primocin, an antimicrobial reagent that prevents contamination, at 37°C in a humidified atmosphere with 5% CO_2.

Proliferation Assays

Cells were seeded, 5.0 x 10^4 cells/mL, (100 µL) per well, on ninety-six well plates in medium and treated with ethanol as control, resveratrol, 10µM, 25µM, 50 µM, 100 µM, quercetin, 10µM, 25µM, 50µM, 75µM, or combination of resveratrol, 100µM, and quercetin, 75µM for 70 hours. Stock solutions of 10 mM RES (Cayman Chemical) and 5mM QT (Cayman Chemical) were diluted into 100µL of medium at twice the final concentration and added to the wells already containing an equal volume of medium. 20µL CellTiter 96 AQ_{ueous} One Solution Reagent (Promega), which becomes a colorimetric indicator of cell metabolism due to cell byproducts of metabolism, is added to each well after treatment and incubated for 2 hours. To

evenly distribute the CellTiter solution the plate was put on a shaker for 5 minutes prior to incubation and plate reading. The plate was read using a microplate reader with Ascent software using a 492 nm filter. All concentrations were tested in triplicates and the experiment was replicated 3 times. These data sets were analyzed using a random block design ANOVA (JMP Pro 12) to determine significance and Tukey's HSD was run to determine where differences occurred.

Analysis of Apoptotic Cells

Cells were plated on 100mm plates for 30% plate coverage and incubated for 3 hours prior to treatment. Cells were treated with ethanol as control, resveratrol, 100 μM, quercetin, 75μM, and the combination of resveratrol, 100 μM, plus quercetin, 75μM, for 21 hours, in order to capture the DNA fragmentation before it progressed too far. Chemicals were diluted into medium at six-times the final concentration in 1 mL of medium, 1 mL was added to the plate with 5 mL of medium.

After treatment the medium of each plate was removed and placed in conical tubes, then put on ice to be used again later for collection after trypsinization. This resulted in neglecting to collect the floating cells, which were still of interest. To remove remaining cells from the plate surface, the medium was removed, 2 mL of 0.05% Trypsin, EDTA (Gibco) was added and distributed evenly on plate by rocking, removed, and 2 mL additional trypsin was added. The plates were incubated 25°C for 4-6 minutes and the trypsin was quenched with the medium, however, in the last trial trypsin was quenched using the DMEM and 5 % FBS rather than the medium of the respective plates, no observable difference was seen. The trypsin plus cells were added to the tube containing the medium from the plates. Cells were then centrifuged at 1,500 X G for 10 minutes at 4ºC. The supernatant was removed; cells were suspended at a concentration

of 10^7 cells/mL in phosphate-buffered saline (PBS) and 2 mM ethylenediaminetetracetic acid (EDTA). Cells were then stained with 2- 5 μM DRAQ5 (Biostatus), DNA stain, at room temperature in the dark for 20 minutes. Cells were centrifuged at 1,500 X G for 10 minutes and the supernatant was removed. Cells were suspended at 10^7 cells/mL in 2 % formaldehyde diluted in PBS from the 8% formaldehyde stock (Electron Microscopy Supplies, Hatfield, PA).

Flow Cytometer analysis of DRAQ5 stained cells

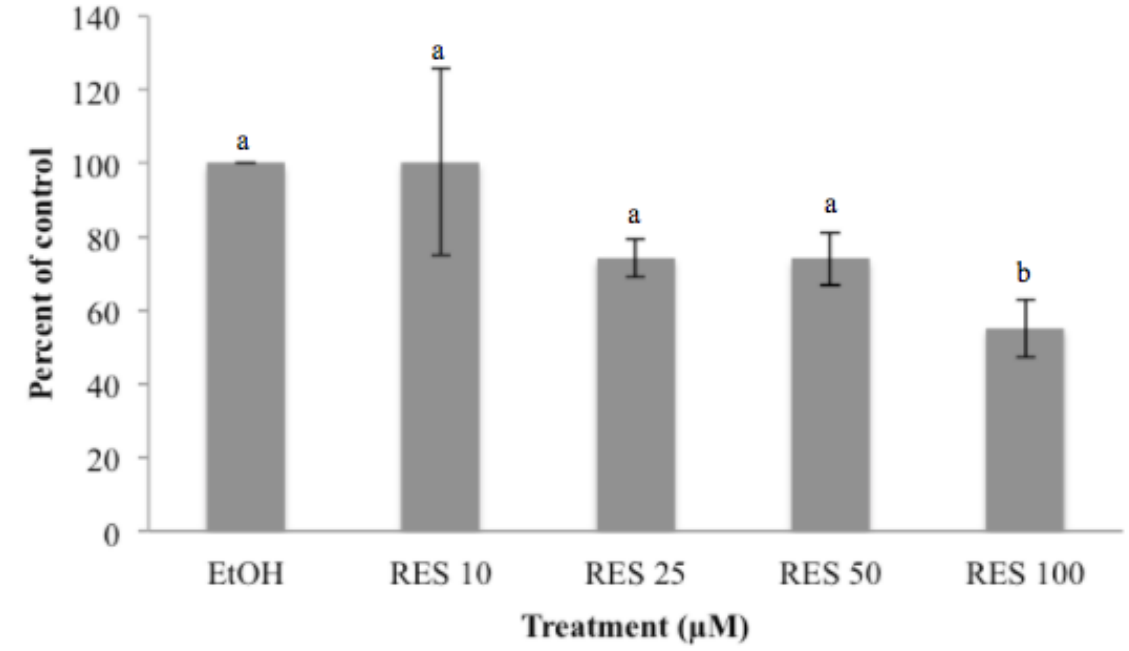
The DRAQ5 stained cells were analyzed using the ImageStream Mark II Imaging Flow Cytometer (Amnis) with INSPIRE™ software. DRAQ5 staining was analyzed using the 488 laser at various intensities ranging from 65-200 mW due to differing staining control intensities. Compensation for each control (1000 count) was completed by acquiring cells without the brightfield and side-scatter laser. The compensation determines the amount of fluorescence in all channels that is caused by a single fluorescence. Compensation matrices were made using Ideas 6.2 software (Amnis) after data acquisition. Cells (6000 count) of all treatments, control, RES 100 μM, QT 75μM, and RES 100 μM + QT 75μM, were collected for analysis after gating away from the beads. These data were analyzed using Ideas 6.2 (Amnis) software and were gated to isolate focused single cells for analysis using the begin analysis wizard. The remaining cells were graphed based on the intensity of the DRAQ5 staining; these cells were then gated based on being low in fluorescence, Sub G_1, or average and high, G_1-M phases. The quantities of cells in the gates were given using the statistical feature of the Ideas 6.2 (Amnis) software. A One-way ANOVA (JMP Pro 12) was used to analyze the significance, along with a Tukey's HSD to determine where the differences occurred.

Results

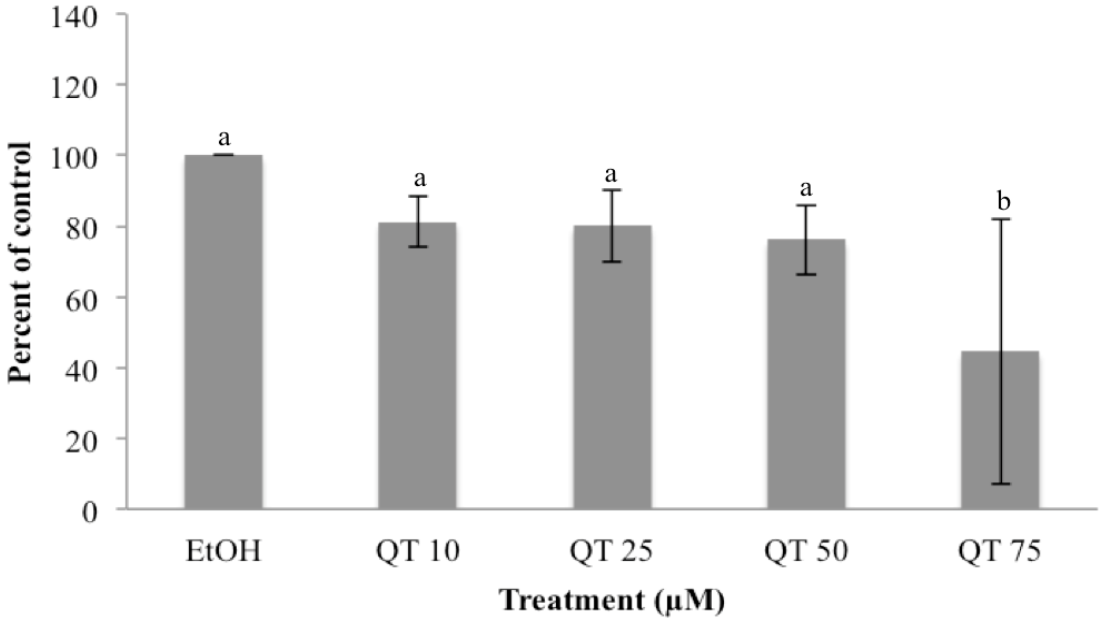
Treatments of Resveratrol and Quercetin Decrease Proliferation in OSCA-40 Cells

In order to study the chemotherapeutic effect of independent and co-treatments of resveratrol (RES) and quercetin (QT) proliferation of canine osteosarcoma 40 (OSCA-40) cells were analyzed. Sensitivity of OSCA-40 has not been studied, thus preliminary proliferation was examined to find concentrations of RES and QT that caused a 40 % decrease independently. The highest concentrations of RES and QT proved to be significantly different (Fig. 3 A&B), so they were used for the further analyses. Co-treated OSCA-40 cells resulted in a significant decrease in cell proliferation (Fig. 3 C, $P=<0.001$). To better understand if decrease in proliferation was caused by an additive or synergistic interaction a cQR value was determined using a formula cQR= Control-[(Control –R) + (Control –Q)] (Mertens-Talcott & Percival, 2005). In this study the value of decrease for the co-treatment was 60.4 whereas the cQR value was 65.1, the additive value, ultimately there was no significant difference (Fig. 3 C). This was then verified with a two-way ANOVA, which showed the interaction of the chemicals was not significant thus meaning synergism was not occurring. To determine if the decrease in proliferation was through apoptosis, the DNA of the treated cells was stained and analyzed.

A)


140
120
100
80
60
40
20
0
Percent of control
a
a
a
a
b
EtOH
RES 10
RES 25
RES 50
RES 100
Treatment (µM)

B)


140
120
100
80
60
40
20
0
Percent of control
a
a
a
a
b
EtOH
QT 10
QT 25
QT 50
QT 75
Treatment (µM)

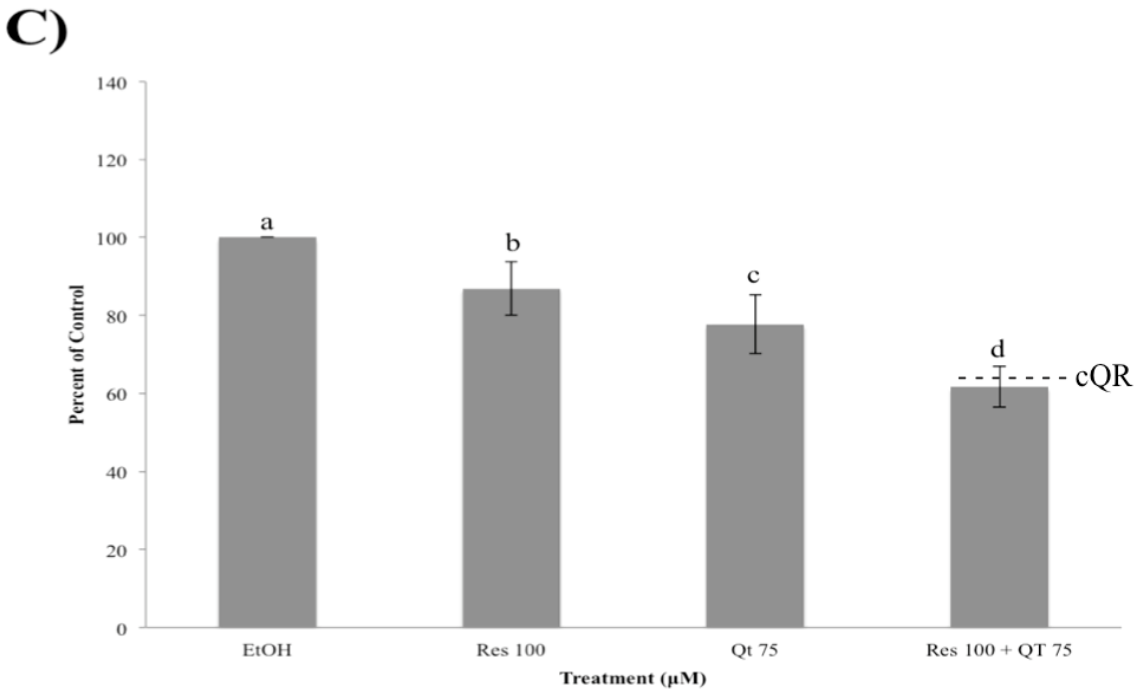


Figure 3. Independent and co-treatments of Resveratrol and Quercetin lead to decreased proliferation in OSCA-40 cells. **A)** OSCA-40 cells were treated with ethanol and various concentrations of resveratrol **B)** OSCA-40 cells were treated with ethanol and various concentrations of quercetin. **C)** Canine OSCA-40 cells were treated with ethanol, resveratrol, quercetin, and resveratrol plus quercetin for 69 hours. Proliferation was analyzed using CellTiter 96®AQueous One Solution Cell Proliferation Assay kit. Values are expressed as percent of control; this involved subtracting the control average of triplicates from the treatment average and dividing by control average for the same trial. Experiments were completed in triplicate and repeated 3 times for **A** & **B** and 4 times for **C**. A random block ANOVA analysis was performed on the absorbance values. P values of <0.05 were considered statistically significant. Different letters represent significant differences in level of proliferation. cQR represents the value for the additive decrease of cell proliferation of phytochemical treatments.

Accumulation of OSCA-40 cells in sub G0 with co-treatments of resveratrol and quercetin

Since cells in different stages of the cell cycles have varying amounts of DNA levels, 2n in G_1 to 4n in M and back to 2n in the S phase (Darzynkiewicz et al., 2010) cells that have fragmented DNA there will be less than 2n DNA staining. The level of apoptosis, as measured by the fraction of cells within the sub G_1 level of DNA, was analyzed in cells treated with independent and co-treatments of RES and QT as used in the proliferation assays (Fig 3 C). An interesting observation was that cells treated with phytochemicals, especially with the co-treatment of RES+ QT took longer to release themselves from the plates. The cell quantify for the sub G_1 levels when treated with co-treated cells (69%) was significantly different than the

control (21%)(Table 1). Subsequently the levels of cells in G_1-M for resveratrol (59%) and co-treated (32%) OSCA cells were significantly different than control (79%)(Table 1, P=0.0352).

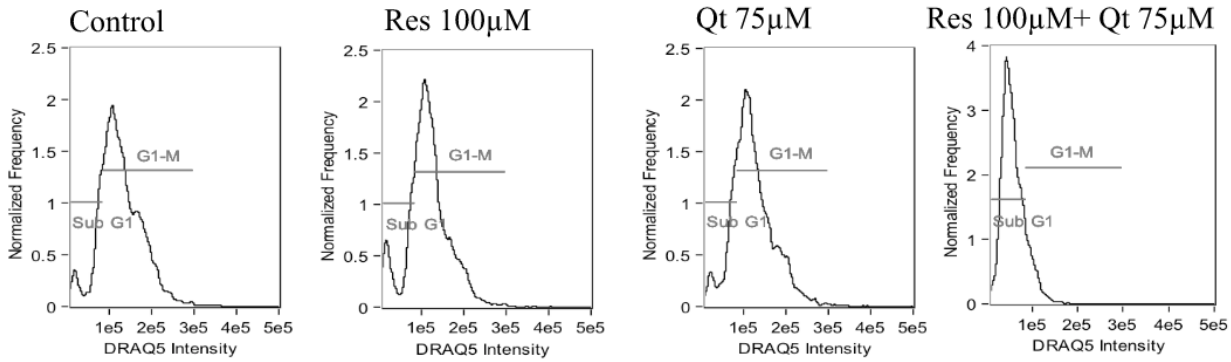


Figure 5. OSCA-40 cells in sub G1 increase with co-treatments of resveratrol and quercetin and independent treatment of resveratrol. Canine OSCA 40 cells were treated with ethanol, resveratrol, quercetin, and resveratrol plus quercetin for 21 hours. Relative DRAQ5 intensity was analyzed using an ImageStream Mark II Imagine Flow Cytometer as described in the methods. Experiments were completed independently and repeated 3 times. Relative DRAQ5 intensity for a trial using 5mM DRAQ5 is represented in order to show the gating of Sub G1 and G1-M.

Table 1. Quantity of OSCA-40 cells in Sub G1 and G1-M gates after phytochemical treatments•.

	% of cells in	
	Sub G1	G1-M
EtOH	20.7 ± 2.9	78.7 ±3.8
100µM RES	41.3 ±18.2	58.7 ±18.2
75µM QT	31.3 ±10.3	68.7 ±10.2
100µM RES+ 75µM QT	68.5 ±18.7 *	31.5 ±18.7*

•OSCA cells were treated with resveratrol, quercetin, and resveratrol plus quercetin for 21 hours. Percent of cells in the table was obtained by averaging the DRAQ5 intensity levels. These levels were determined using an imaging flow cytometer and by using the total the cells of sub G1 plus G1-M rather than total cells collected. * signifies P-value <0.05

Discussion

Phytochemicals have recently become a hot button topic in cancer research as their chemotherapeutic and preventative properties are frequently analyzed. Combinations of chemicals can be extremely beneficial in combating diseases such as cancer; especially if the chemicals are synergistic (Davidson et al., 2016). Phytochemicals within grapes contain the ability to decrease cell proliferation of cancer cells (Signorelli et al., 2015; Yang et al., 2009). Resveratrol (RES) and quercetin (QT) are two phytochemicals in grapes that have been found to independently decrease cell proliferation in human colon, leukemia, and osteosarcoma cells (Alkhalaf & Jaffal, 2006; Gogada et al., 2011; Mertens-Tallcott & Percival, 2005; Ranelletti et al., 2000; Suh et al., 2010). These two phytochemicals have been found to increase caspase-3 activity in a synergistic manner, leading to a more sufficient method of killing cancer cells (Mertens-Tallcott & Percival, 2005). This study found that RES and QT could independently cause a significant decrease in OSCA cell proliferation in a dose-dependent manner (Fig 3. A & B). Additionally, this study demonstrated that co-treatments of RES+ QT could significantly decrease cell proliferation of OSCA cells (Fig 3. C), however, this decrease did not show synergism. These decreases in proliferation could be a result of inducing apoptosis, which was investigated using cell cycle analysis.

The decrease in proliferation caused by treatments of RES and QT is often through an apoptotic mechanism. RES and QT have been shown to increase activity of caspase- 3, Bax, and p53, which are known proteins involved in apoptotic pathways that are not highly expressed during normal cell function (Gogada et al., 2011; Granado-Serrano et al., 2006; Maurya & Vinayak, 2015). Consistent with previous literature, resveratrol and quercetin were independently able to decrease proliferation of the OSCA-40 cells in a dose-dependent manner

(Fig. 3 A&B). This study found differences in the significant concentration needed to decrease OSCA proliferation than other studies of cell lines such as breast cancer cells, which only needed 5µM and 20µM of each chemical (Schlacterman et al., 2008). These differences may be due to the fact that each cell line is different from each other and not every method is universal.

Studies conducted on human osteosarcoma, which in theory should have more similarity to OSCA-40 than breast cancer cells, saw a decrease in proliferation with lower resveratrol and quercetin concentrations than used in this study (Alkhalaf & Jaffal, 2006; Liang et al., 2011; Li et al., 2009). In several cell lines of osteosarcoma, 10µM resveratrol was sufficient in decreasing cell proliferation in a time dependent manner (Alkhalaf & Jaffal, 2006; Li et al., 2009). In MG-63, a human osteosarcoma cell line, 20µM QT treatments decreased in cell proliferation within a shorter time frame then my study (Liang et al., 2011). A reason for this difference in significant concentration could be due to the fact that osteosarcoma in humans and canines has heterogeneity that has been shown to lead to having multiple subtypes of this cancer. The genetic characteristics of canine osteosarcoma are not as well-known as human osteosarcoma and although there are similarities there are no universal characteristic OSCA-40 has been (Scott et al., 2011;Varshney et al., 2016). An additional reason for the disparities between my study and past literature could be that my proliferation data varied quite a bit which may have lead my lower concentrations to be insignificant (Fig. 3 A &B).

It has been found that for some cancers the effects of co-treatment with RES and QT are additive (Del Follo-Martinez et al., 2015) and in others, synergistic (Mertens-Tallcott & Percival, 2005; Schlacterman et al., 2008; Zamin et al., 2009). A finding that was consistent with the literature was the ability of co-treatments of RES+ QT to decrease cell proliferation in an additive manner (Fig. 3C). Despite the lack of synergism found in decreased cell proliferation

and cell cycle analysis it is possible that the chemicals work in a synergistic manner to induce other biological factors, such as caspase activity like seen in leukemia cells (Mertens-Tallcott & Percival, 2005). The variation among each trial in both proliferation and cell DNA analysis could have led to the co-treatment not being synergistic in this study. Future experiments could investigate whether OSCA-40 with co-treatments of RES and QT would act in synergism to activate different biological factors that signify that apoptosis is occurring.

The pathways by which RES or QT induce apoptosis, thus decreasing cancer proliferation, has varied among cancer cell lines and this may be due to the genetic mutations in cell lines that cause cancer of that cell. Some examples are the activation of the MAPK pathway by RES in HOS, a human osteosarcoma cell line, and activation mitochondrial-dependent along with the caspase-3-dependent pathway by QT treatments (Alkhalaf & Jaffal, 2006; Li et al., 2009: Liang et al., 2011). With so many different pathways to study and difficulty of making comparisons across different cell lines there is a need of further studies with OSCA-40. These studies would provide a better understanding of which pathway co-treatments of RES and QT utilize to cause the decrease in cell proliferation.

In many studies a decrease in cancer cell proliferation because of treatments of RES, QT, and RES+QT was accompanied by G_0/G_1 cell cycle arrest in a dose and time-dependent manner (Benitez et al., 2007; Del Follo-Martinez et al., 2015; Mertens-Tallcott & Percival, 2005; Ranelletti et al., 2000). Sub G_1 DNA intensity, which arises from a reduction in the amount of DNA and thus staining, is an indication of apoptosis (Darzynkiewics et al., 2010; Robertson et al., 2000). Human osteosarcoma and pancreatic cancer cells have shown increases in sub G_1 DNA staining when treated with RES, QT, and RES+QT (Alkhalaf & Jaffal, 2006; Del Follo-Martinez et al., 2015; Mouria et al., 2002; Suh et al., 2010). In this study, OSCA-40 cells co-

treated with RES+ QT demonstrated a significant increase of cells in the sub G_1 gate, consistent with previous literature (Table 1). The differences seen in my study and the literature may be due to the fact that there was a large amount of variability within my trials (Table 1), so further studies would need to be conducted to finalize a more consistent method for the DRAQ5 staining that may produce different or similar results to what I observed.

Overall this study was able to determine that RES and QT treatments are able to decrease OSCA-40 cell proliferation. The decrease of cell proliferation with co-treatments of these chemicals was significantly more than the independent treatments. However there was no evidence of synergism between RES and QT, but rather these chemicals worked in an additive manner to decrease cell proliferation. Through DNA staining analysis, treatments of RES and RES+QT were found to cause a significant accumulation of cells in sub G_1, an indication of apoptosis. Further research is needed to verify that these chemicals induce apoptosis in OSCA-40.

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