The carotenoid astaxanthin has antioxidant properties and is considered the most potent antioxidant in vitro because it has >10 times the antioxidant effect of vitamin E.\textsuperscript{1,2} All carotenoids are derived from lycopene and consist of a structural polyene backbone that can have various terminal benzene rings at each end of the molecule.\textsuperscript{1,2} These ring structures can have oxygen atoms or hydroxyl groups attached, classifying them as xanthophylls, and the benzene ring structures of astaxanthin contain both in addition to the polyene structure, adding to the ability to scavenge free radicals. Astaxanthin can exist as a cis or trans isomer; the trans isomer is more stable and abundant in nature.\textsuperscript{2} The compound is naturally present in various marine fish, algae, and microorganisms and is most commonly extracted from \textit{Phaffia} yeast and \textit{Haematococcus pluvialis}.\textsuperscript{1,3} When extracted from biological sources, it is usually in trans iso-

### Objective
To determine the effects of the antioxidant astaxanthin on growth of canine osteosarcoma cells with and without concurrent chemotherapeutic or irradiation insult.

### Sample Population
Cells from 3 established canine osteosarcoma cell lines (D17, OS 2.4, and HMPOS).

### Procedures
Growth-curve kinetics and cell cytotoxic effects were assessed by means of various treatment combinations and a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Western blotting was performed to examine previously identified signaling pathways that astaxanthin reportedly affects. Additionally, cell-cycle kinetic evaluations, soft agar colony-forming assays, and antioxidant assays were performed to better understand the effect of astaxanthin on cell growth and function.

### Results
Exposure to astaxanthin alone resulted in a mild to pronounced attenuation of cell proliferation in vitro, depending on the cell line, and did not interfere with the cell-death response to doxorubicin, irradiation, or peroxide-mediated insult. In some instances, astaxanthin acted in an additive fashion to augment cell death. Astaxanthin exposure increased the antioxidant potential of cells, whereas peroxide-mediated cell stress increased the antioxidant potential to the same degree as astaxanthin exposure or greater. No dramatic changes in phosphorylation of protein kinase B or upregulation of connexin 43 were detected.

### Conclusions and Clinical Relevance
Findings suggested that astaxanthin administration may be beneficial in treatment of dogs for osteosarcoma. Its actions as an antioxidant did not improve osteosarcoma cell survival during chemotherapeutic or irradiation insults, warranting further research into this natural compound as an adjuvant, antiproliferative treatment for osteosarcoma in dogs. (\textit{Am J Vet Res} 2010;71:89–96)
upregulation of production of the gap junction protein connexin 43, which has been associated with a decrease in cell proliferation in vitro.\textsuperscript{10,11,17} Additionally, astaxanthin exposure can dampen the inflammatory cascade through inhibition of nitric oxide formation and nuclear signaling of nuclear factor-\text{kB} and can reduce the degree of AKT phosphorylation, leading to an increase in apoptosis and a decrease in cell proliferation in vitro.\textsuperscript{18,19} Not only does this dampening of signaling pathways possibly slow tumor growth, but it also seems to affect the ability of the immune system to regulate metastasis because astaxanthin administration can disrupt metastasis or tumor implantation in mice.\textsuperscript{6,12–14}

To our knowledge, the effects of astaxanthin administration on veterinary disease processes have not been scientifically evaluated. However, nutraceuticals are available and a product specifically designed for dogs was recently introduced to the veterinary market to treat inflammation associated with osteoarthritis.\textsuperscript{a} There has been only 1 study\textsuperscript{b} in which the antioxidant properties of astaxanthin were evaluated, but the clinical effectiveness of the drug with respect to inflammation or neoplasia (2 conditions in which astaxanthin treatment may be beneficial) was not examined. As many as 40\% of dogs will receive some form of nutraceutical treatment\textsuperscript{c}; however, oncologists discourage such supplementation during chemotherapy because of the possible protective advantages that the supplement may impart on the cancer itself.\textsuperscript{21,22} The purpose of the study reported here was to better understand how astaxanthin influences chemotherapeutic and irradiation-induced cell death by treating 3 osteosarcoma cell lines with doxorubicin, irradiation, or hydrogen peroxide and astaxanthin. Specifically, we sought to determine whether astaxanthin would augment or diminish cell death and to determine the direct effects of astaxanthin on osteosarcoma cell proliferation in general.

**Materials and Methods**

**Osteosarcoma cells**—Osteosarcoma cells from 3 cell lines were obtained: OS 2.4,\textsuperscript{4} HMOPOS,\textsuperscript{4} and D17.\textsuperscript{c} Cell lines were maintained in 10\% FBS in RPMI medium plus 1\% antimicrobial-antimycotic solution at 37°C and 5\% CO\textsubscript{2} for all experiments and for passage of cells.

**Cell culture media**—For cell proliferation assays, RPMI medium\textsuperscript{a} with 10\% FBS\textsuperscript{a} and 1\% antimicrobial-antimycotic solution\textsuperscript{a} was used. For growth-curve assays, 2\% FBS-supplemented RPMI medium was used. Final concentrations of tetrahydrofuran never exceeded 0.5\% vol/vol because higher concentrations adversely affect cell growth.\textsuperscript{23}

**Astaxanthin**—All-trans-astaxanthin\textsuperscript{1} was diluted in tetrahydrofuran\textsuperscript{1} (vehicle) and stored under nitrogen gas at \textdegree 80°C. The astaxanthin stock solution was prepared biweekly at a concentration of 5mM, which was completely soluble when added to aqueous medium.

**Determination of cell-growth curves**—Cells were treated with 10, 5, 2.5, and 1.25\,\mu M astaxanthin solubilized in culture medium for comparison with cells treated with vehicle or medium alone. Cells were plated at a density of 1,000 cells/well in 96-well tissue culture–treated plates in RPMI with 2\% FBS and 1\% antimicrobial-antimycotic solution. An MTT\textsuperscript{3} assay was performed every 2 days for a total of 8 days as described elsewhere.\textsuperscript{24} In brief, cell growth was measured by use of MTT reduction in live cells spectrophotometrically. Each treatment concentration was evaluated in triplicate during 3 experiments, and the results were averaged at each time point.

**Evaluation of cell cytotoxic effects**—Cells were treated with tetrahydrofuran alone, or 5\,\mu M astaxanthin in RPMI with 10\% FBS and 1\% antimicrobial-antimycotic solution. The cytotoxic agents used to induce cell death were doxorubicin\textsuperscript{1} (2 mg/mL), irradiation,\textsuperscript{25} or 30\% hydrogen peroxide.\textsuperscript{26} Doxorubicin treatment consisted of addition of 10, 1, or 0.1\,\mu M doxorubicin for 4 hours. The medium was then changed to the initial concentrations of astaxanthin for the remainder of the incubation period. Irradiation of the cells was accomplished with a 6-MV linear accelerator at a dose rate of 200 cGy/min. Plated cells were irradiated in conditions of full dose buildup with 1.5 cm of tissue-equivalent bolus material placed in the path of the radiation beam. Single radiation doses ranged from 2 to 10 Gy in 2-Gy increments, and cell proliferation was examined 48 hours after irradiation by means of MTT assays. Peroxide-mediated cell death was induced with 30\% hydrogen peroxide diluted in the aforementioned cell proliferation medium from 100 to 12.5\,\mu M. When percentages of surviving cells were between 40\% and 70\% of control values after cytotoxic exposures, the effect was defined as mild to modest cellular insult. All experiments were performed in triplicate 3 separate times, and results for each type of experiment were averaged.

**Cellular antioxidant activity**—Culture dishes (100 mm) were treated with vehicle, astaxanthin, 200\,\mu M 30\% hydrogen peroxide plus vehicle, or 200\,\mu M 30\% hydrogen peroxide plus astaxanthin at concentrations to induce cellular stress. After 24 hours of astaxanthin exposure and 12 hours of cellular stress, overall antioxidant effects were examined in all 3 cell lines by use of an antioxidant assay kit\textsuperscript{27} that measures total antioxidant ability. Preparation of cellular lysates was performed in accordance with the manufacturer's protocol. Protein concentrations of the cell lysates were measured in duplicate and averaged by use of the Bradford technique.\textsuperscript{25} Lysates were evaluated in quadruplicate, and results were plotted against the internal standard curve by the manufacturer to determine total antioxidant potential. This total antioxidant potential was averaged and then divided by the lysate total protein concentration to yield the total antioxidant potential per microgram of protein.

**Flow cytometric evaluation**—Cells from all 3 osteosarcoma cell lines were treated with vehicle, 10\,\mu M astaxanthin, or 5\,\mu M astaxanthin for 48 hours. Cells were then trypsinized, washed twice with PBS, and fixed with cold 95\% ethanol. Cells were stained with propidium iodide,\textsuperscript{26} treated with ribonuclease,\textsuperscript{26} and subjected to flow cytometry as described elsewhere.\textsuperscript{26} Each treatment was performed in duplicate for each cell line. Each cell line was gated to count 10,000 events by use of a flow cytometer.\textsuperscript{26} Cell populations were identified...
by their distinctive position on forward and side scatter plots. Data were analyzed for total counts within G1, S, or G2M phase and averaged for each treatment to yield the final percentage of cells within each stage of the cell cycle.

**Cell lysis and western blot analysis**—Cells were grown and treated for various amounts of time (0 and 30 minutes and 1, 4, 8, 24, 48, and 96 hours) with vehicle or 10μM astaxanthin. Cells were lysed at each point as described elsewhere.27 Cell lysates were collected, and protein concentration in each sample was measured by use of the Bradford technique. Lysates were equilibrated to a common volume (μg/μL) in lysis buffer and loading buffer. Western blot analysis was performed with 8% and 12% SDS-PAGE, 30 μg of protein/well was loaded, and products were transferred to a polyvinylidene difluoride membrane and immunoblotted.28 Immuno blotting was performed to detect expression of ser 473 phosphorylated AKT, p42-p44 ERK, and total connexin 43 because expression and phosphorylation of these proteins can be modified by astaxanthin exposure.16,17,29 After membrane blocking with 10% nonfat dried milk, the cells were incubated with an anti–phosphoserine 473 AKT primary antibody, anti–connexin 43 antibody16 or anti–p42-p44 ERK antibody28 overnight at 4°C. Membranes were washed twice with TBST and then incubated at room temperature (approx 27°C) for 1 hour with 1:5,000 dilutions of appropriate anti-rabbit or anti-mouse antibodies.9 Blots were again washed 3 times with TBST and made visible with an imaging station.9 All blots were then stripped with western-blot stripping reagent,9 and immunoblotting procedures were repeated with anti-AKT,7 actin,2 or anti–pan ERKα antibodies to ensure the total protein loaded was equivalent for all lanes for comparison.

**Soft agar**—Soft agar analysis was performed as described elsewhere.29 In brief, cells were plated in soft agar30 at a concentration of 5,000/well and treated with 5μM astaxanthin or vehicle alone in soft agar medium. Agar replenishment was performed every 3 days for 2 weeks. At day 16 of the experiment, the number of cells that formed colonies were counted in triplicate for each treatment and averaged.

**Statistical analysis**—All data were analyzed with parametric statistics. Multiple group comparisons were made via ANOVA, and a Tukey post hoc comparison was performed at each experimental end point. All growth curves were compared at day 8 of growth to evaluate reductions in growth kinetics, whereas cytotoxic effects and antioxidant final results were analyzed similarly at completion of experiments (24 to 48 hours after treatment). A value of P ≤ 0.05 was considered significant for all analyses.

**Results**

**Cell-growth curves**—Astaxanthin exposure inhibited growth of cells from all 3 cell lines (OS 2.4, HMPOS, and D17). The most pronounced effect was evident in the D17 cell line, in which treatment with 10μM astaxanthin nearly halted proliferation (Figure 1). However, after 8 days of incubation, proliferation significantly decreased in cells from all 3 lines after treatment with astaxanthin at concentrations of 5 or 10μM, compared with proliferation in cells treated with culture medium or tetrahydrofuran vehicle alone. In the HMPOS and OS 2.4 lines, cell growth was also significantly inhibited after treatment with 2.5μM astaxanthin, whereas the same was not true for the D17 cell line.

**Doxorubicin cytotoxic effects**—Doxorubicin exposure at concentrations of 1 and 10μM induced death in 30% to 60% of cells from all 3 lines within 48 hours. Optimal inhibitory concentrations were 1μM for HMPOS and D17 cells, whereas OS 2.4 cells were moderately doxorubicin resistant and required 10μM treatment for cell-death induction. No increase in the proportion of surviving cells was detected after treatment
with 10 and 5µM astaxanthin in all 3 cell lines. However, treatment with 10µM astaxanthin (but not 5µM) increased the amount of cell death in the D17 cell line, compared with that evident in the OS 2.4 and HMPOS cell lines (Figure 2).

Irradiation cytotoxic effects—Irradiation-induced cell death was examined 48 hours after irradiation. All 3 cell lines responded to doses of 2 or 4 Gy with 30% to 70% cell death. Interestingly, the D17 cells had no additional response at higher radiation doses, providing evidence of marked resistance to irradiation-mediated cell death, whereas HMPOS and OS 2.4 cells had nearly complete cell death at doses of 6 Gy and higher. There were no significant detrimental or beneficial effects of astaxanthin treatment in any of the cell lines examined (Figure 3).

Peroxide-induced cytotoxic effects—Cells were examined after 24 hours of exposure to hydrogen peroxide. Concentrations of hydrogen peroxide required to achieve 30% to 70% cell death varied among the 3 cell lines. To achieve measurable cell death, D17 cells required exposure to 75µM and HMPOS cells required exposure to 50µM, whereas OS 2.4 cells required exposure to 25µM. Overall, treatment with astaxanthin yielded no protective (decrease in amount of cell death) or detrimental (increase in amount of cell death) effects during hydrogen peroxide exposure (Figure 4).

Antioxidant potential of astaxanthin—Because astaxanthin appeared to provide little appreciable protection from peroxidative damage, we examined whether astaxanthin treatment yielded any increase in cell antioxidant potential. The antioxidant potential was significantly (P < 0.02) increased for cells from all 3 cell lines with 10µM astaxanthin treatment, compared with 10 and 5µM astaxanthin in all 3 cell lines. However, treatment with 10µM astaxanthin (but not 5µM) increased the amount of cell death in the D17 cell line, compared with that evident in the OS 2.4 and HMPOS cell lines (Figure 2).

Figure 2—Mean ± SD percentage of cells from 3 canine osteosarcoma cell lines (OS 2.4, D17, and HMPOS) that survived doxorubicin exposure after treatment with culture medium alone (control), tetrahydrofuran vehicle (THF), or astaxanthin (AST; 10 and 5µM). *Values differ significantly (P < 0.05) between astaxanthin treatment and control and vehicle treatment for the indicated concentration of astaxanthin.

Figure 3—Mean ± SD percentage of cells from 3 canine osteosarcoma cell lines that survived radiation exposure at 2 or 4 Gy after treatment with culture medium alone, THF vehicle, or AST (10 and 5µM). Values did not differ significantly (P > 0.05) between treatments. See Figure 2 for remainder of key.

Figure 4—Mean ± SD percentage of cells from 3 canine osteosarcoma cell lines that survived treatment with hydrogen peroxide (HP) after treatment with culture medium alone, THF vehicle, or AST (10 and 5µM). Values did not differ significantly (P > 0.05) between treatments. See Figure 2 for remainder of key.

Figure 5—Mean ± SD percentage change in antioxidant potential from control-cell values in AST-treated (10µM) and THF-treated canine osteosarcoma cells (lines D17, OS 2.4, and HMPOS) with or without exposure to HP (200µM for 12 hours). Astaxanthin-alone treatment resulted in a significant (P < 0.05) increase in antioxidant potential from THF-treated cell values in all cell lines. *Indicates a significant difference between the THF value and those for AST alone and AST+HP treatment. †Indicates a significant difference between the THF value and only that for AST+HP treatment. ‡Indicates a significant difference between the THF value and only that for AST alone.

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with vehicle control treatment. However, when these cells were treated with a submaximal toxic dose of hydrogen peroxide alone for 12 hours, the endogenous antioxidant potential increased to equal or beyond that detected with astaxanthin alone for all 3 cell lines ($P < 0.01$; Figure 5). When astaxanthin was added to cells during exposure to hydrogen peroxide, the overall antioxidant potential did not significantly change for the HMPOS cells but did significantly ($P < 0.03$) increase for OS 2.4 and D17 cells.

**Flow cytometry**—Analysis of cells from all 3 cell lines revealed no shifts in cell-cycle dynamics during G1, S, or G2M attributable to treatment with vehicle or astaxanthin (10 or 5μM). The mean proportion of OS 2.4 cells in various phases were as follows: G1, 68.5%; S, 14.9%; and G2M, 15.7%. The mean proportion of HMPOS cells in various phases were as follows: G1, 70.7%; S, 12.8%; and G2M, 16.5%. The mean proportion of D17 cells in various phases were as follows: G1, 62.6%; S, 17.6%; and G2M, 19.3%. No appreciable deviations from these percentages were evident.

**Western blot analysis**—Results of experiments within the first 24 hours indicated no differences in expression or phosphorylation status of AKT and ERK and in expression of connexin. Therefore, cells were examined after 48 hours of treatment with astaxanthin. Cells from all 3 lines expressed AKT (64-kDa) protein and had obvious differences in phosphorylation status of AKT, which did not change dramatically with astaxanthin or vehicle treatment. However, HMPOS cells had a modest decrease in phosphorylation of AKT after long-term (48-hour) treatment with astaxanthin, resulting in a 39% decrease in phosphorylation, compared with the degree of phosphorylation after vehicle treatment. In addition, overall phosphorylation of AKT was highest in HMPOS cells, compared with that in OS 2.4 and D17 cells (Figure 6). Degree of AKT phosphorylation did not correlate with ERK activity, given that the initial degree of ERK phosphorylation appeared highest in OS 2.4 cells, with modest phosphorylation in HMPOS cells and little phosphorylation in D17 cells. Degree of connexin-43 expression was assessed after 48 and 96 hours of treatment with 10μM astaxanthin, and there were no obvious changes, whereas overexpression at gap junctions at similar measurement points appeared associated with the antiproliferative effects of astaxanthin. Connexin expression was less in OS 2.4 and D17 cells than in HMPOS cells, whereas HMPOS cells expressed more protein at 48 and 96 hours.

**Soft agar**—Cells from all 3 cell lines grew successfully in soft agar. However, only D17 and HMPOS cells had a significant ($P < 0.05$) decrease in colony formation when treated with 10μM astaxanthin (Figure 7), compared with colony formation in the same cell lines treated with culture medium alone or with vehicle. On the other hand, the OS 2.4 cells had no significant decrease in colony formation with 10μM astaxanthin treatment.

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**Figure 6**—Photographs of results of western blot analysis indicating expression of various proteins (total AKT, phosphoserine 473 AKT [pAKT], total ERK, phosphoERK [pERK], connexin 43, and β-actin) in canine osteosarcoma cells (lines OS 2.4, HMPOS, and D17) after 3 weeks of treatment with culture medium alone (control), vehicle (0.05% THF), and 10μM AST. Cells were harvested for analysis after 48 (A) or 96 (B) hours of treatment. See Figure 2 for remainder of key.

**Figure 7**—Mean ± SD number of colonies of canine osteosarcoma cells (lines OS 2.4, HMPOS, and D17) after 3 weeks of growth on soft agar. *Values were significantly ($P < 0.05$) different between D17 and HMPOS cells treated with 10μM astaxanthin, and there were no obvious changes, whereas overexpression at gap junctions at similar measurement points appeared associated with the antiproliferative effects of astaxanthin. Connexin expression was less in OS 2.4 and D17 cells than in HMPOS cells, whereas HMPOS cells expressed more protein at 48 and 96 hours.
Discussion

In cell culture models of prostate and breast cancer, astaxanthin treatment reportedly has antiproliferative properties. Proliferation in prostate carcinoma cells was suppressed by 24% to 38%, whereas proliferation in breast cancer cells was nearly 100% inhibited.\textsuperscript{30,31} However, to date, astaxanthin has not been evaluated for antiproliferative properties in sarcoma cell lines. Our data suggested that treatment with 10μM astaxanthin can reduce proliferation of sarcoma cells, specifically osteosarcoma cells, by 30% to 97%, depending on the cell line examined. Therefore, regardless of the similar highly proliferative biological nature of osteosarcoma cells clinically, their reactivity to antineoplastic agents can be quite different, and additional investigation into differences in antiproliferative effects of astaxanthin in these cells is warranted.

One possible reason for the sensitivity of the D17 cells to astaxanthin may be the increased uptake of this carotenoid, compared with uptake in OS 2.4 and HMPOS cells. Although the amount of astaxanthin internalized by cells was not within the scope of our experiments, it was evident after lysis of cells from all 3 lines in the presence of 10μM astaxanthin that the D17 cell line may have accumulated more astaxanthin internally. Another study\textsuperscript{25} revealed that cells accumulate astaxanthin intracellularly in a variable pattern that appears to be cell-line specific.\textsuperscript{23} Additionally, other studies\textsuperscript{31,32} have revealed there is considerable cytoplasmic and intranuclear accumulation of astaxanthin. Consequently, the effects we detected in the D17 cells may have been related to intracellular concentration of astaxanthin, localization of astaxanthin, or expression of proteins that interact with carotenoids.\textsuperscript{31,32}

Our primary goal was to assess whether astaxanthin treatment could protect cells from doxorubicin- or irradiation-induced cell death. This protective effect would presumably be attributable to the antioxidant potential of astaxanthin in cells. Treatment with irradiation or doxorubicin generates free radicals within cells, and this is believed to be part of the mechanism for toxic effects.\textsuperscript{33} Surprisingly, astaxanthin treatment did not protect cells of any cell line from doxorubicin or irradiation-induced death. In fact, there appeared to be an additive effect of astaxanthin in the D17 cell line when cells were treated with doxorubicin, which is understandable given that astaxanthin had a pronounced antiproliferative effect in that cell line.

The aforementioned findings led us to investigate whether astaxanthin treatment yielded a protective effect in canine osteosarcoma cells during a potent free radical–mediated cell-death response to hydrogen peroxide exposure. Surprisingly, astaxanthin treatment resulted in no distinct protective effects after peroxide treatment for 24 hours. Although we expected astaxanthin to promote cell survival in the event of free radical damage, it may have been that the astaxanthin concentrations used did not provide any additional antioxidant effects for the cells in vitro. These findings are similar to those of a few other studies\textsuperscript{34,35} that revealed astaxanthin and other carotenoids do not potentiate cell survival through their antioxidant properties; however, the compounds did elicit an inhibitory effect on cell proliferation.

Additional examination of antioxidant potential was performed by use of 10μM astaxanthin in cell culture. In this set of experiments, astaxanthin treatment yielded a significant increase in antioxidant potential in cells from all cell lines, and this increase was most pronounced in the D17 cell line. However, during peroxide-mediated cell stress, the endogenous antioxidant potential in each cell line increased significantly. When cells were treated with astaxanthin and peroxide, astaxanthin treatment resulted in improved antioxidant potential, but this was primarily in D17 cells. This effect may have been attributable to increased cellular uptake in the D17 cell line, as previously mentioned. However, the lack of enhanced antioxidant effects in the other 2 cell lines suggested that astaxanthin yielded little protection from antioxidant damage, likely because of its pro-oxidative functions or because the upregulation of natural antioxidant potential masked any positive antioxidant effects astaxanthin might have had. This may explain the lack of overall protection against peroxide- and irradiation-induced cell damage.

More surprising was the strong antiproliferative effect of astaxanthin on D17 cells. Whereas OS 2.4 and HMPOS cells were far more resistant than D17 cells to the antiproliferative effects of astaxanthin, astaxanthin treatment reportedly influences mitogenic signaling from connexins and inhibits activation of AKT and nuclear factor-κB.\textsuperscript{36,37} In many instances, these regulators of cell proliferation and survival will be phosphorylated at specific residues such as ser 473 of AKT, which can promote cell proliferation. Our examination of phosphorylated AKT revealed that astaxanthin had little effect on AKT phosphorylation and that OS 2.4 and HMPOS cells had high endogenous phosphorylation of AKT in low serum concentrations, whereas D17 cells did not. Additionally, 48 hours of astaxanthin treatment mildly downregulated activity of phosphorylated AKT in HMPOS cells; however, HMPOS cells did not appear to have any less overall degree of AKT expression. The high degree of AKT activation may play a role in the resistance of HMPOS cells to astaxanthin inhibition. Activation of ERK also plays a role in osteosarcoma cell proliferation and appeared to have a certain degree of constitutive activation in all 3 cell lines in our study; however, there was no change in ERK phosphorylation status attributable to astaxanthin treatment. Interestingly, the D17 cell line, which was the most sensitive to high doses of astaxanthin of all 3 cell lines, did not have high constitutive activity of AKT or ERK, suggesting that the response to astaxanthin may have been inversely related to activation of these proliferative pathways.

Evaluation of the gap-junction protein connexin 43 was performed because of another report\textsuperscript{43} that suggested astaxanthin treatment can increase expression of connexin 43, which is important in gap-junction communication for preventing overproliferation through enhanced contact-contact inhibition.\textsuperscript{38} We found no appreciable changes in connexin-43 expression in the 3 cell lines examined, and there was variable expression of connexin 43 in each of the cell lines, suggesting that its role in cell proliferation may be limited.

Lastly, examination of the potential for soft agar growth of cells from all 3 cell lines when treated with
astaxanthin was remarkably different than findings of the cell proliferation experiments. The OS 2.4 cells that were modestly inhibited by 10µM astaxanthin during cell proliferation experiments seemed resistant to similar treatment in colony-formation soft agar experiments, therefore suggesting a variable response depending on the assay used to assess the antiproliferative effects of astaxanthin treatment. However, astaxanthin treatment significantly inhibited colony formation in HMPOS and D17 cells, suggesting that astaxanthin can significantly affect anchorage-independent growth, which is often a preliminary step toward providing evidence of the pro- or antiproliferative effects of a compound in vivo.

Overall, astaxanthin treatment at a concentration of approximately 10µM appeared to result in a modest to strong antiproliferative signal for canine osteosarcoma cells; however, the extent of that response was cell-line dependent. The antioxidant potential of astaxanthin seemed to provide no survival advantage for these cells when undergoing doxorubicin- or irradiation-mediated cell death. In fact, use of astaxanthin, depending on the cell line examined, has the potential to enhance the antiproliferative effects of current chemotherapeutic modalities. In canine osteosarcoma cells, the mechanism of astaxanthin inhibition appeared not to include differential regulation of AKT or ERK phosphorylation or expression of connexin 43. Although HMPOS cells treated with astaxanthin had mildly inhibited expression of phosphorylated AKT, it is likely that astaxanthin inhibits other cell proliferation pathways yet to be determined, warranting further examination of the antiproliferative effects of astaxanthin on osteosarcoma cells in vitro and in vivo.

References