Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org

Kafkas Univ Vet Fak Derg
27 (4): 465-473, 2021
DOI: 10.9775/kvfd.2021.25728

RESEARCH ARTICLE

Cytotoxic and Apoptotic Effects of Curcumin on D-17 Canine Osteosarcoma Cell Line [1]

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[1] *This work was supported by Scientific Research Project Coordination Unit of Aydın Adnan Menderes University (Project number: VTF-18006)*

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Article ID: KVFD-2021-25728 Received: 09.03.2021 Accepted: 24.07.2021 Published Online: 28.07.2021

Abstract

Cancer is a major health problem in dogs. Types of cancer seen in dogs include melanoma, Non-Hodgkin lymphoma, osteosarcoma, soft tissue sarcomas and prostate, breast, lung and colorectal carcinomas. Osteosarcoma (OSA) is the most common malignant primary bone tumor in domestic dogs. It constitutes 85% of skeletal tumors. It is derived from primitive bone cells that occur in both the appendicular (~75%) and axial (~25%) skeleton. The present study was intended to determine the cytotoxic and apoptotic effects of curcumin administration at certain doses and in certain periods on D-17 canine osteosarcoma cells. Canine osteosarcoma cells were treated with curcumin and the effects of it on proliferation were determined by WST-1, apoptosis by caspase 3/7 activity (MuseCaspase 3/7) and the ratio of proapoptotic Bax gene to antiapoptotic Bcl-2 gene expression level by qRT-PCR. Our data demonstrated that curcumin decreased cell proliferation and viability, ultimately inducing caspase 3/7 mediated apoptosis in treated D-17 canine osteosarcoma cells. Furthermore, the application of curcumin on canine osteosarcoma cells downregulated the expression of Bcl-2 and upregulated the expression of proapoptotic gene Bax. Thus, these results may provide a basis for further study of curcumin in the treatment of canine osteosarcoma.

Keywords: *Apoptozis, bax/bcl-2, Canine osteosarcoma, Caspase 3/7, Curcumin*

Kurkuminin D-17 Köpek Osteosarkom Hücre Hattı Üzerindeki Sitotoksik ve Apoptotik Etkileri

Öz

Kanser, köpeklerde büyük bir sağlık sorunudur. Köpeklerde görülen kanser türleri arasında melanom, Non-Hodgkin lenfoma, osteosarkom, yumuşak doku sarkomları ve prostat, meme, akciğer ve kolorektal karsinomlar bulunur. Osteosarkom (OSA) evcil köpeklerde en sık görülen kötü huylu birincil kemik tümörüdür. İskelet tümörlerinin %85'ini oluşturur. Hem apendiküler (~%75) hem de aksiyal (~%25) iskelette meydana gelen ilkel kemik hücrelerinden türemektedir. Bu çalışmanın amacı, belirli dozlarda ve belirli periyotlarda kurkumin uygulamasının ardından D-17 köpek osteosarkom hücreleri üzerindeki sitotoksik ve apoptotik etkilerini saptamaktır. Kurkumin uygulanan köpek osteosarkom hücrelerinde proliferasyon üzerindeki etkileri WST-1 analiziyle, apoptotik etkileri ise kaspaz 3/7 aktivitesi (MuseCaspase 3/7) ve proapoptotik Bax geninin antiapoptotik Bcl-2 gen ekspresyon düzeyine oranı ile belirlendi. Verilerimiz kurkuminin D-17 köpek osteosarkom hücrelerinde hücre proliferasyonunu ve canlılığını azalttığını, kazpaz 3/7 aracılığıyla apoptozu indüklediğini göstermektedir. Bunun yanında kurkuminin köpek osteosarkom hücrelerine uygulanması Bcl-2'nin ekspresyonunu azalttı ve proapoptotik gen Bax ekspresyonunu arttırdı. Bu nedenle, bu sonuçlar, köpek osteosarkoma tedavisinde kurkumin ile ilgili daha fazla çalışma için bir temel sağlayabilir.

Anahtar sözcükler: *Apoptoz, bax/bcl-2, Köpek osteosarkom, Kaspaz 3/7, Kurkumin*

INTRODUCTION

Osteosarcoma (OSA) is the most common type of malignant bone cancer found in dogs, and is about 10-50 times more common in dogs than in humans [1]. OSA, which constitutes approximately 85% of primary canine bone tumors, arises from mesenchymal cells and is mostly seen in long bones such as knee, hip and shoulder [2,3]. The tumor grows rapidly inside the bone and becomes more painful as it grows outward [4]. In humans, it is predominantly seen

How to cite this article?

Ekren Aşıcı GS,Kıral F, Bayar İ, Bildik A, Ulutaş PA: Cytotoxic and apoptotic effects of curcumin on D-17 canine osteosarcoma cell line. *Kafkas Univ Vet Fak Derg,* 27 (4): 465-473, 2021.
DOI: 10.9775/kvfd.2021.25728

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in adolescents and young adults and the most common incidence is between the ages of 10-15. Unlike human OSA, canine OSA is more common in older dogs [5] and it accounts for 80-85% of bone tumors in dogs 2 to 15 years old [6]. It is locally invasive and highly metastatic and this case makes it difficult to treat [4]. While it usually metastasizes to the lungs, it also spreads to other bones. In dogs, tumors spread hematogenously into the lungs earlier than in humans and they begin to develop micro-metastases [7]. While lower than 45% of the affected dogs can survive only in the first year despite appropriate surgical and chemotherapeutic protocols [8], less than 20% of them can survive more than 2 years after diagnosis [9].

Treatment of OSA is very difficult both in humans and in dogs. For this reason, despite the advanced cancer treatment methods, the annual survival rate in dogs is around 45%, while this rate in people with OSA has not changed lately [10]. The low survival rate in dogs with OSA highlights the need for new therapeutic approaches [11,12]. Today, osteosarcoma is usually treated with the preoperative chemotherapy, with the amputation or tumor resection for limb salvage and with the postoperative chemotherapy [3]. However, the use of chemotherapeutic agents is limited due to severe toxicity. Therefore, the ultimate goal of this study is to demonstrate the need for new studies to discover effective chemotherapeutics with appropriate and minimal toxicity.

Apoptosis is a physiological process responsible for the elimination of cells that have completed certain functions or that damage the growth and development of the organism. Cell apoptosis plays a central role in the control of cell proliferation and therefore has a significant role in preventing tumor growth [13]. Two characteristic apoptotic pathways, called as extrinsic and intrinsic pathways, have been identified [14]. The intrinsic pathway is activated as a result of death signals that may occur due to DNA damage, growth factor deficiency and oxidative stress, and these signals are carried to the mitochondria by two proapoptotic members of the Bcl-2 family (Bax, Bad), creating a large pore formation on the surface of the mitochondria [15,16]. Thus, there is a large increase in mitochondrial outer membrane permeability and cytochrome-c release is performed by breaking the mitochondrial outer membrane. The Bcl-2 family contains some pro-apoptotic (eg: BAK and BAX) and some anti-apoptotic proteins (eg: BCL-2, Bcl-xl) [17,18]. Among these, Bcl-2 and Bax are key proteins for apoptosis or survival [19].

Curcumin, [(1E, 6E) -1, 7-bis (4-hydroxy-3-methoxyphenyl) -1,6-heptadiene-3,5-dione or diferuloylmethane] is a yellow -orange phenolic compound obtained from the ribosomes of the turmeric plant *(Curcuma longa* L.) [20]. This compound has anti-inflammatory, antioxidant, and chemopreventive properties, and chemotherapeutic potential with no obvious side effects [21,22]. It exhibits beneficial effects on numerous diseases such as diabetes, allergies, asthma, hepatic diseases, arthritis, Alzheimer's disease, cardio-vascular diseases and cancer [23-25]. Curcumin has been shown to modulate multiple cell signaling pathways such as apoptosis, proliferation, angiogenesis, and inflammation [19].

Preclinical studies have proven that curcumin inhibits cell proliferation in various cancer cell lines, including breast, cervical and pancreatic cancers [19]. In addition, recent studies have shown that curcumin, alone or in combination with other anticancer agents, can effectively induce apoptosis [26]. Curcumin has been proven to display antitumoral effects on a wide variety of human cancer cell lines and to induce apoptotic cell death [27-29].

Chemotherapeutic agents used in veterinary oncology are generally applied by making use of the information obtained in human medicine. However, this practice is controversial due to the interspecies differences in pharmacokinetic parameters and the sensitivity of tumor cells to cancer therapeutic compounds [30]. Consequently, for the treatment of canine osteosarcoma, it is of great importance to develop and/or discover low cost, less toxic and highly efficient therapeutic agents that increase survival rates. Although the antiproliferative effect of curcumin has been shown in many cell lines, our study will contribute to the literature since it is a specific dose study that can be used in veterinary oncology, since it was performed with a dog cell line.

In our study, we were going to explore that curcumin exhibits antineoplastic potency in a metastatic canine osteosarcoma cell line (D-17) *in vitro*. To assess this hypothesis we exposed canine OSA cell lines to curcumin and determined *in vitro* measures of proliferation. Furthermore Bax/Bcl-2 expression ratio was determined by qRT-PCR as an indicator of apoptosis, and apoptosis was confirmed by determination of caspase 3/7 activity.

Material and Methods

Cell Culture and Treatment

The canine osteosarcoma cell line D-17 was purchased from the American Type Culture Collection (ATCC, Manassas, VA). Osteosarcoma cells were cultured in Minimum Essential Medium Eagle medium (MEM; Sigma, M4655) supplemented with 10% heat in-activated fetal bovine serum (FBS, Sigma, F0804), 0.1% 10.000 U/mL penicillin/ streptomycin (Gibco), 1% nonessential amino acids, 0.11 g/L pyruvic acid (sodium salt) and incubated at 37ºC in a humidified atmosphere of 5% CO_2 in air. The medium was refreshed every 2-3 days. After about 90% of confluence, the cultured cells were detached with 0.25% trypsin-EDTA (Sigma T4049) and sub-cultured.

Curcumin (Sigma, C1386) was dissolved in culture medium with 1% DMSO, the solution was added to the culture medium to reach final concentrations of 2.5, 5, 10, 25,

40, 50, 75, 100 µM. Control group (concentration 0) were cultured without curcumin and medium containing 1% DMSO.

Cell Proliferation Assay

The effect of curcumin on D-17 canine OSA cell proliferation was initially determined by cell proliferation analysis using commercial cell proliferation kit 2-(4-iodofenil)-3-(4-nitrofenil)-5-(2,4-disülfofenil)-2H-tetrazolyum or WST-1 which is one of the proliferation tests based on metabolic activity. Cells ($1x10^4$ cells per 200 µL medium per well) were seeded in 96-well plates and allowed to attach for 24 h. There after, medium was changed and cells of the experimental groups were treatment with 2.5-5-10-25-40-50-75 or 100 µM curcumin whereas the control group was treated with DMSO (1%). After 24, 48, and 72 h incubation, 100 µL WST-1 solution was added to each well and the cell was incubated for 3 h at 37ºC in a humidified atmosphere of 5% CO_2 in air. After incubation and mixing gently for one minute on an orbital shaker, the absorbance of each well was measured using a microplate-reader (Multiskan™ FC Microplate Photometer, Thermo Fisher, Finland) in absorbance mode at a wavelength of 450 nm. The mean absorbance of the four-time repeated measurements per curcumin concentration was related to the mean absorbance of the control and expressed as the percentage of control. Dose–response curves and IC_{50} (The half maximal inhibitory concentration) were established with Graphpad Prism.

RNA Isolation and cDNA Synthesis

Total RNA isolation was performed according to the manufacturer's instructions with a Hybrid-RTM RNA Isolation kit (GeneAll R Biotechnology, South Korea) after 24 and 48 h incubation times from canine OSA D-17 cells treated with curcumin at specified concentrations (0, 10, 25, 40 and 50 µM). cDNA synthesis was achieved using 2 µg total RNA using the HyperScript First Strand Syntesis Kit (GeneAll Biotechnology, South Korea). Then, 1/20 of the resultant cDNA was used for each PCR reaction in a total volume of 20 µL.

Real-Time Quantitative Polymerase Chain Reaction (RT-PCR)

Gene expression was evaluated by Quantitative real-time PCR (qPCR). qRT-PCR was carried out using SYBR® Green PCR Master Mix (Applied Biosystems kit) and LigthCycler®480 (Roche Diagnostics GmbH, Mannheim, Germany). Specific primer sets designed and utilized for canine bax, bcl-2 and house keeping gene gapdh were listed in *Table 1*. The annealing temperature for this primer was 60°C. Fluorescence was determined by Step One Plus (Applied Biosystems) at each amplification cycle and analyzed by Step One Software 2.1 (AppliedBiosystems). C_t values representing the number of amplification cycles were obtained for all samples. The expression levels for all the genes analyzed were normalized to GAPDH. The protocol was performed in triplicate.

Table 1. *Primers for RT-PCR*

Gene	Primer Base Sequences (5´—>3´)	Amplicon Length (bp)
Bax	F: TTCCGAGTGGCAGCTGAGATGTTT R: GCTGGCAAAGTAGAAGAGGGCAA	79
Bcl-2	F:CATGCCAAGAGGGAAACACCAGAA R: GTGCTTTGCATTCTTGGATGAGGG	76
Gapdh	F: AGTCAAGGCTGAGAACGGGAAA R: TCCACAACATACTCAGCACCAGC	114

Caspase 3/7 Activity Assay

Canine OSA D-17 cells were seeded in 12-well plates at a density of $2.5x10^5$ cells/well and incubated overnight. Cells were treated with various curcumin concentrations (5, 10, 25, 40, 50 µM) for 24 and 48 h. Wells with media only were included as controls. All experiment were done in triplicates for each dose treatment. Apoptotic and necrotic cell ratios in the cell suspension obtained after incubation were determined by Muse Cell Analyzer (Merck Millipore) using the MuseCaspase 3/7 (MCH100108) kit according to the manufacturer's protocol.

Statistical Analysis

All experiments were done in triplicates for each dose treatment. All data were presented as mean values ± standard error (SE). A P value of <0.05 was considered as significant. A Shapiro-Wilk test was used for evaluation of the normality of distributions. The one-way analysis of variance (ANOVA) test (for Windows Release 22.0 Standart Version Copyright© SPSS Inc. Chicago, IL, USA) was used for evaluating the differences in the data. Statistical significance ($P<0.05$) was established by the post hoc Tukey's pairwise comparison.

The IC_{50} values were calculated by four-parameter non-linear regression using GraphPad Prism v.5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). The data were performed replication in four-time for each dose in cell proliferation assay, and data are presented as mean ± standard error (SE).

Results

Curcumin Inhibits Population Growth in Canine OSA Cells

Effect of curcumin on canine OSA cell viability was evaluated by WST-1 method. Canine OSA D-17 cells exposed to curcumin resulted in a significant decrease in viable cells in a time and dose-dependent manner *(Fig. 1-A,B,C)*. A dose-effect curve was created using the GraphPad Prism 5 program with the dose-dependent viability results obtained by WST-1 viability analysis *(Fig. 2-A,B,C)*. IC_{50} values of canine OSA D-17 cells were 61.51, 47.80 and 39.41 µM

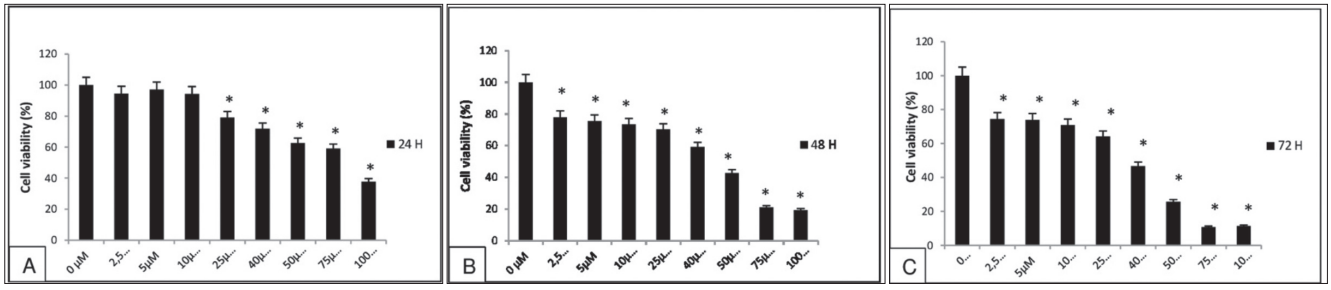


Fig 1. Effect of curcumin on cell viability of canine OSA D-17. Cells were treated with different concentrations of curcumin (0, 2.5, 5, 10, 25, 40, 50, 75, 100 µM) A- 24 h, B- 48 h, C- 72 h. The results represent the mean±standard deviation, * P<0.001 relative to control

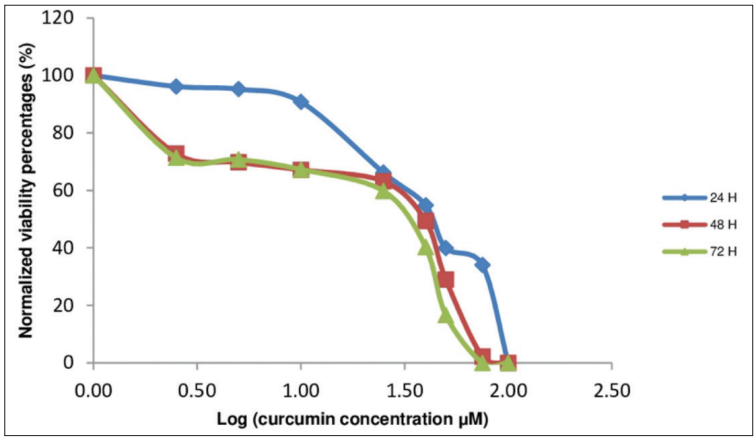
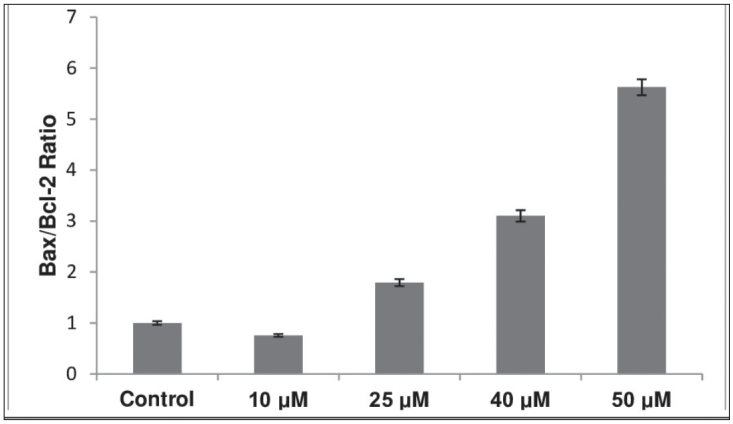


Fig 2. A dose-effect curve was created using the GraphPad Prism 5 program with the dose-dependent viability results obtained by WST-1 viability analysis of curcumin-administered D-17 canine osteosarcoma cell

Fig 3. Effect of curcumin on the Bax/Bcl 2 ratio in canine OSA D-17 cells. Cells were treated with different concentrations of curcumin (10, 25, 40 and 50 µM) for 48 h



at 24, 48 and 72 h, respectively. There was observed no significant effect on cell proliferation at 0-10 µM curcumin concentration during the 24 h incubation period (P>0.05). After 48 and 72 h incubation times, there was a decrease in cell viability in low doses (2.5, 5 and 10 µM) compared to the control group, but no statistically significant difference was found between the doses. Curcumin caused significant decreases in cell viability due to dose and time in increasing doses, and this decrease was found to be statistically significant (P<0.001).

Curcumin Increases the bax/Bcl2 Ratio in Canine OSA D-17 Cells

Expression of Bax (proapoptotic) and Bcl-2 (antiapoptotic) proteins were determined by RT-PCR method to investigate the apoptotic effect of curcumin on canine OSA D-17 cells and thus bax/bcl-2 ratios were evaluated. Canine OSA D-17 cells were treated with different concentrations of curcumin (0, 10, 25, 40, 50 µM) for 24 and 48 h. There was no significant change in Bax/Bcl-2 ratios compared to the control (0 µM) in 24-h incubation period (P>0.05), (Data not shown). End of 48 h incubation with curcumin, bax/bcl-2 ratio was found to be 1.79, 3.10 and 5.6 fold increase compared with the control cells for 25, 40 and 50 µM curcumin doses, respectively (P<0.05), *(Fig. 3)*.

Curcumin Toxicity is Associated with Caspase Activation in Canine OSA D-17 Cells

Cell viability in canine OSA D-17 cells treated with 5, 10 and 25 µM curcumin during 24 h was not significantly

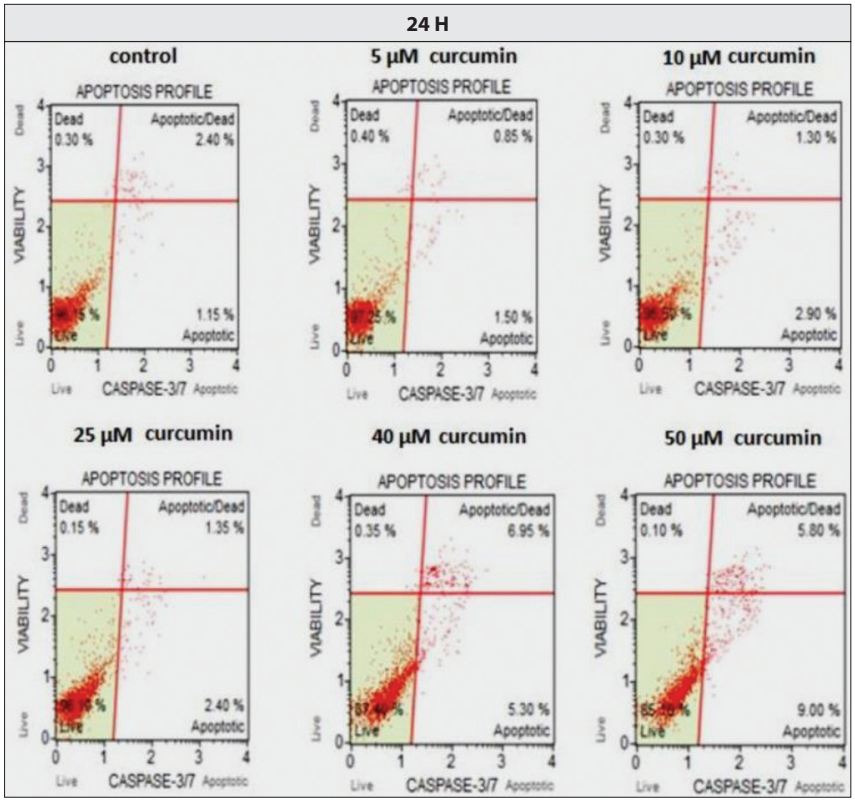


Fig 4. Caspase 3/7 activity after treatment with different concentrations of curcumin (0, 5, 10, 25, 40 and 50 µM) for 24 h in canine OSA D-17 cells-

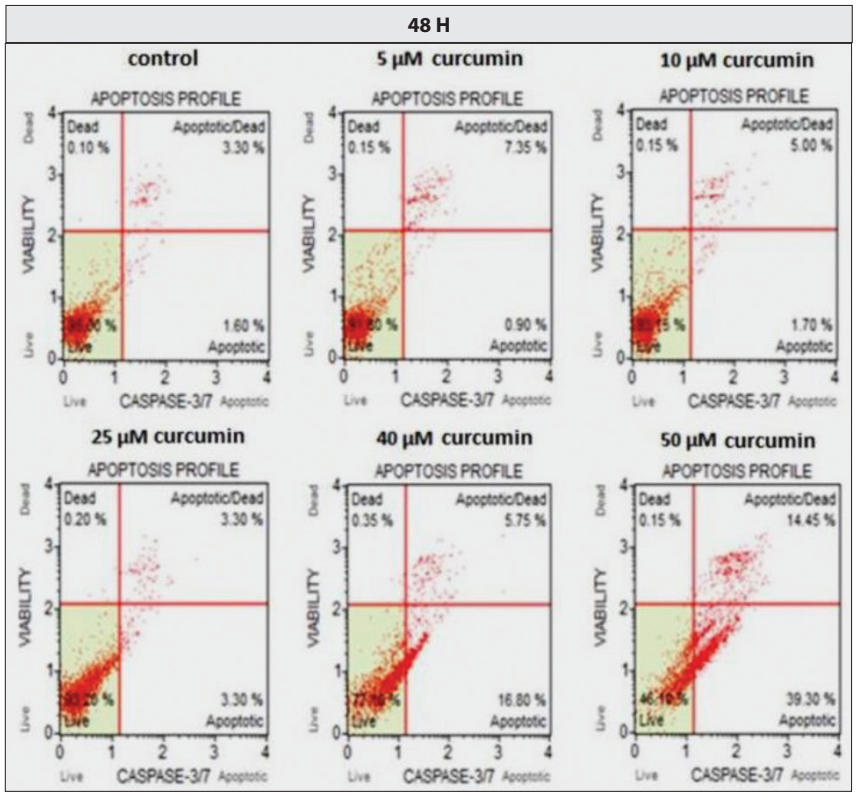


Fig 5. Caspase 3/7 activity after treatment with different concentrations of curcumin (0, 5, 10, 25, 40 and 50 µM) for 48 h in canine OSA D-17 cells

changed that due to caspase 3/7 activity compared to the control group (P>0.05). The percentage of early apoptotic cells induced by 40 µM and 50 µM curcumin increased to 5.73% and 9.51%, and the percentage of late apoptotic cells increased to 6.75% and 6.25%, respectively (P<0.05). The application of 5, 10 and 25 µM curcumin for 48 h did not affect viability significantly (P>0.05) however the percentage of early apoptotic cells 15.27% and 39.85% and late apoptotic cells 5.25% and 13.95% at 40 µM and 50 µM curcumin doses, respectively during 48 h (P<0.05). As a

Table 2. *Live/apoptotic/necrotic cell ratios in canine D-17 OSA cells treated for 24 h with 5, 10, 25, 40 and 50 µM of curcumin and in control group cells*

Group	% Live	% Apoptotic	% Dead
Control	95.45±1.26[a]	3.25±0.74[a]	0.68±0.19
5 µM	97.25±1.61[a]	2.58±0.87[a]	0.43±0.13
10 µM	96.27±1.43[a]	3.60±0.52[a]	0.25±0.048
25 µM	96.65±0.41[a]	2.77±0.81[a]	0.32±0.062
40 µM	87.25±0.89[b]	12.48±1.15[b]	0.36±0.14
50 µM	83.56±2.56[c]	15.76±2.03 [c]	0.10±0.011

[abc] *Statistical difference between groups with different letters in the same column is significant*

Table 3. *Live/apoptotic/necrotic cell ratios in canine D-17 OSA cells treated for 48 h with 5, 10, 25, 40 and 50 µM of curcumin and in control group cells*

Group	% Live	% Apoptotic	% Dead
Control	95.00±1.16[a]	4.95±1.24[a]	0.10±0.007
5 µM	94.65±1.23[a]	5.28±1.27[a]	0.13±0.013
10 µM	94.27±1.78[a]	5.60±1.50[a]	0.15±0.008
25 µM	93.65±0.91[a]	6.17±0.21[a]	0.12±0.043
40 µM	79.15±1.19[b]	20.62±1.15[b]	0.33±0.13
50 µM	47.16±1.56[c]	53.8±1.12 [c]	0.15±0.03

[abc] *Statistical difference between groups with different letters in the same column is significant*

result of the analysis based on the measurement principle of caspase 3/7 activity, it was observed that 40 and 50 µM curcumin application decreased viability and caused apoptosis in proportion according to total apoptotic ranges as 12.48% and 15.76% for 24 h and 20.62% and 53.8% for 48 h ($P<0.05$) *(Fig. 4, Fig. 5; Table 2, Table 3)*.

Discussion

Today, veterinarians' current options in the treatment of osteosarcoma are palliative therapy, limb-sparing anti-cancer therapy, preparation for surgery by reducing the size of the tumor to remove it, or prevent metastasis to other tissues. In addition, post-operative treatments prevent recurrence of the tumor [9,11,12,31]. The purpose of chemo-therapy and radiation treatments among these applied cancer therapies is to kill tumor cells because these cells are more sensitive to the effects of these drugs and methods, at least in adults, since they grow at a much faster rate than healthy cells [32]. However, losses occurring in healthy cells as well as tumor cells are a disadvantage of the treatments applied. For this reason, new treatment searches are required for healthy cells in the treatment of cancer that provide the induction of apoptosis by targeting molecules that have less toxicity but are located on apoptotic pathways in cancer cells [33]. It has been proven in many studies that phytochemicals affect gene expression of many different target molecules that modulate the signal transduction pathway, cell cycle, cell metabolism and apoptosis, and have antitumor effects. Therefore, more emphasis has been placed on phytochemicals and their antitumor effects in recent years [33,34].

Turmeric *(Curcuma longa)* is a plant that has medicinal value in both human and animal health [35]. Curcumin is a phytochemical compound in the class of flavonoids with various pharmacological properties and is obtained from the roots of the Curcuma longa plant that is used as a herbal diet and medicine [33]. Curcumin has a wide pharmacological action and low toxicity and is well tolerated by humans [3,36]. Curcumin is a highly pleiotropic molecule that modulates the activation of transcription factors, kinases, cytokines, various enzymes (eg, MMP, iNOS, GST, and ATPase), cell cycle (Cyclin D1 and cyclin E), growth factors (eg, EGF, NGF, HGF, and PDGF) and numerous targets involved in invasion, migration, angiogenesis, and apoptosis [37-39]. Curcumin can affect the skeletal system as many of the mentioned curcumin targets participate in the regulation of bone remodeling [20].

The effects of curcumin on osteoarcoma have been investigated in human osteosarcoma cell lines such as MG63, U2OS, MNNG/HOS, SAOS-2 and KHOS, and it has been determined that it has antiproliferative and apoptotic effects [3,20,40-43]. However, studies on canine osteosarcoma and specifically the D-17 cell line are limited. When our data is evaluated, it will be seen that curcumin inhibits the proliferation of canine OSA D-17 cells in a dose and time dependent manner.

It was found that D-17 canine OSA cells treated with curcumin had an IC_{50} value of 61.51 µM in the first 24 h and in short-term exposure to curcumin, cytotoxic activity increased at high doses. At 48 and 72 h, IC_{50} values were determined as 47.80 and 39.41 µM, respectively, and it was

concluded that the cells were more sensitive to long-term curcumin exposure. Our results are in line with the results of some studies based on the antiproliferative efficacy of curcumin with human and canine OSA cells. Lee et al.[41] reported that when they treated human osteosarcoma (HOS) cells with 0-20 µg/mL curcumin for 48 h, a dose-dependent reduction in cell proliferation occurred. Fossey et al.[44] investigated the anti-proliferative and apoptotic effects of FLLL32 compound obtained by modification of curcumin in order to improve the potential and biochemical properties of curcumin and after administration of FLLL32 particularly at concentrations above 0.75 µm (2.5 and 7.5 µm), there were significant reductions in the proliferation of both canine (OSA-8, 16 and D-17) and human (SJSA and U2OS) OSA cell lines. Canine cell lines have been found to be somewhat resistant, while human cell lines are more sensitive to curcumin treatment. Yu et al.[45] found that the proliferation of MG-63 cells, which were incubated with curcumin for 24 h, 36 h and 48 h in the range of 0-40 µM and at different concentrations, were inhibited significantly in a dose and time dependent manner. In another study comparing the effects of curcumin on the viability of MG-63 osteosarcoma cells and healthy human osteoblasts on different concentrations, when exposed to 5 and 10 µM curcumin, no statistical difference was observed in the density of healthy osteoblast cells, while the density of osteosarcoma cells decreased 0.6 and 5.3 times, respectively, compared to the control group [42]. The effect of Lipocurc™ formulation, designed to enhance the bioavailability of curcumin, on cell viability in canine OSA, melanoma and mammary carcinoma cell lines was investigated. A concentration-dependent decrease in cell viability was determined in all cell lines at concentrations above 2000 ng/mL, and the D-17 cell line was found to be significantly less sensitive to Lipocurc™ than both MG-63 and U2OS cell lines [46].

Induction of apoptosis, an actively regulated cellular process that causes cell death after specific stimuli are received in tumor cells, without cytotoxic effect in healthy cells in cancer therapy [47], is an approach on which many studies have been conducted in recent years [15,26]. Regulation of the level of some pro-apoptotic and anti-apoptotic proteins involved in the apoptotic process or activation of apoptotic enzymes causes apoptosis to occur in cells. This is achieved by the Bcl-2/Bax gene family and the activation of caspases [48,49]. While proapoptotic Bax protein interacts with membrane pore proteins to increase cytochrome c release, antiapoptotic Bcl-2 inhibits apoptotic signals by restricting proapoptotic Bax and thus the cytochrome c release [15]. Cells having a high Bax/Bcl-2 ratio are more sensitive to certain apoptotic stimuli [50]. Therefore, changes in Bax and Bcl-2 levels are important in determining whether cells will undergo apoptosis. The effects of apoptosis induction are more dependent on the ratio between these two proteins (Bax/Bcl-2) rather than the amount of Bcl-2 or Bax [51]. In our study, a dose-dependent increase in the Bax/Bcl-2 ratio was observed 24 h after treatment with curcumin in canine OSA D-17 cells. However, after 48 h of incubation, a significant increase in Bax/Bcl-2 ratio was detected, especially at 40 and 50 µM concentrations. Our results show that the strong anticancer activity of curcumin is associated with the concordant modulation of the expression rate (Bax/Bcl-2) of two major proteins that play a critical role for apoptosis induction by the intrinsic pathway. Curcumin has been reported to cause a decrease in cellular levels of Bcl-2 and an increase in cellular levels of Bax in various cancer cells [52,53]. Shankar et al.[52] and Anto et al.[54] determined that the regulation of Bcl-2 and Bax proteins by curcumin administration is a major factor for apoptosis caused by curcumin. Jin et al.[43] showed that curcumin can induce apoptosis in U2OS cells by increasing mitochondrial membrane permeability by downregulation of Bcl-2 and upregulation of Bax, Bak and p-Bad, and the treatment of U2OS cells with curcumin resulted in a significant inhibition of cell growth. Curcumin has been proven to induce apoptosis associated with the regulation of Bax/Bcl-2 protein expression in human hepatoma cells (SMMC7721) [33]. In addition, Jun et al.[3] found that there was a significant increase in Bax/Bcl-2 ratios depending on the dose after the human osteosarcoma cell line U2OS and MG-63 had been exposed to curcumin for 24 h.

Effector caspases (caspase 3, 7) also act on a range of different target proteins that play important roles in mediating the apoptotic response [55]. The results we obtained show that high doses of curcumin (40-50 µM) can induce apoptosis based on Caspase 3/7 activation. The highest apoptotic rates were determined for a dose of 50 µM curcumin in 48 h, indicating the long incubation time and high dose caspase-induced apoptotic pathway. Subramaniam et al.[56] found that the activation of effector caspase 3 and caspase 7 increased within 24 h in esophageal adenocarcinoma TE-7 cell line treated with curcumin. Another study showed that curcumin could activate caspase 3/7 in a dose-dependent manner (0, 10, 20, 30 µmol/L) within 24 h, thereby inducing apoptosis in human colon cancer HCT116 and SW480 cell lines [57]. Levine et al.[58] determined that caspase 3/7 activation was also induced in 3 different canine cell lines (C2 mastocytoma, CMT-12 mammary carcinoma and D-17 OSA) to which they applied turmeric root, which is the source of curcuminoids. They determined that there was a significant increase in 2 and 2.5 times in apoptotic cells, especially in C2 and CMT-12 cell lines. McNally et al.[59] determined that when doses of curcumin above 25 µm were administered to human liver cancer HUH7 cells, it reduced vitality depending on the increased concentration within 24 h. Furthermore, it was detected that caspase 3/7 activity increased at doses of 50 µM and above, and the decrease in survival was associated with caspase activation. In a study, it was observed that caspase-3, JNK and AMPK molecules were activated as a result of the application of curcumin at various

concentrations (20, 50 and 100 µM) to D-17 OSA cells, and the extrinsic apoptosis pathway mediated by caspase, JNK and cAMP/AMPK was induced [60]. The conducted studies reveal that curcumin exhibits an effective antitumoral potential by inducing the apoptotic pathways.

As a result, we demonstrated that canine OSA D-17 cells treated with curcumin decreased cell proliferation in a dose and time dependent manner, inducing apoptosis by increasing Bax/Bcl-2 protein expression and caspase 3/7 activation. Although various treatments are available, curcumin is predicted to be a candidate with a promising chemorherapeutic effect by inducing apoptosis for canine osteosarcoma with poor clinical results.

Acknowledgements

The authors would like to thank the Scientific Research Projects Unit of Adnan Menderes University and the Department of Biochemistry, Faculty of Veterinary Medicine, Adnan Menderes University.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this article.

Author Contributions

FK funded acquisition and conceptualizationed the study. GSEA and İB performed laboratory analysis and drafted manuscript. GSEA also analyzed data with specific software, wrote the manuscript. AB and PAU investigationed that literature and methodology, revised the manuscript.

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