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Curcumin promotes extrinsic apoptosis in canine osteosarcoma cells

Curcumina promove apoptose extrínseca em células de osteossarcoma canino

Curcumina promueve la apoptosis extrínseca en células de osteosarcoma canino

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Abstract

Canine osteosarcoma is the most common bone tumor in dogs. It has an intense metastatic capacity, and the patient survival rate is low in this disease. Curcumin, the most important compound derived from the plant *Curcuma longa* L., has been widely studied and has shown considerable antineoplastic effects against many tumors. This study aims to identify the activation of specific proteins of apoptosis pathways, tumor survival, and poor prognosis of this disease in D-17 osteosarcoma cells. For this, the cells were cultured and treated with curcumin at concentrations of 20μM, 50 μM, and 100 μM in prepared and fixed slides. Subsequently, we performed the immunocytochemical analysis with anti-caspase3, anti-JNK, anti-AMPK, anti-p53, anti-AKT, and anti-mTOR antibodies. Curcumin activated the cell-death proteins caspase-3, JNK, and AMPK, reduced the expression of the mutated p53 protein, and did not alter the AKT and mTOR proteins in canine osteosarcoma cells in vitro. Thus, curcumin induces extrinsic apoptosis mediated by caspase, JNK, and cAMP/AMPK in canine osteosarcoma cells. Besides, it has the potential to improve the tumor prognosis of this disease by inactivating the mutated p53. However, it does not interfere with AKT/mTOR expression, related to tumor proliferation and survival. Such results will serve as a basis for future studies that analyze the effect of curcumin in vivo on this disease.

Keywords: Cytotoxicity; D-17; Immunocytochemistry; Cell death.

Resumo

O osteossarcoma canino é o tumor ósseo mais comum em cães. Ele apresenta intensa capacidade metastásica e a sobrevida do paciente é baixa nessa doença. A curcumina, o composto mais importante derivado da planta *Curcuma longa* L., tem sido amplamente estudada e mostrou efeitos antineoplásicos consideráveis contra vários tumores. Este estudo tem por objetivo identificar a ativação de proteínas específicas de vias de apoptose,

sobrevivência tumoral e mau prognóstico dessa doença em células OSC da linhagem D-17. Para isso, as células foram cultivadas e tratadas com a curcumina nas concentrações de 20μM, 50 μM e 100 μM em lâminas, que foram preparadas e fixadas. Posteriormente, foi realizada a técnica de imucitoquímica, com os anticorpos anti-caspase3, anti-JNK, anti-AMPK, anti-p53, anti-AKT e anti-mTOR. Foi observado que a curcumina ativou em células de osteossarcoma canino in vitro as proteínas de morte celular caspase-3, JNK e AMPK, reduziu a expressão da proteína p53 mutada e não alterou as proteínas AKT e mTOR. Assim, verificou-se que a curcumina promove apoptose extrínseca mediada por caspase, JNK e cAMP/AMPK em células de osteossarcoma canino. Além disso, tem o potencial de melhorar o prognóstico tumoral dessa doença por inativação da p53 mutada. No entanto, ela não interfere na expressão de AKT/mTOR, relacionado a proliferação e sobrevivência tumoral. Tais resultados servirão de base para estudos futuros que analisem o efeito da curcumina in vivo nessa doença.

Palavras-chave: Citotoxicidade; D-17; Imunocitoquímica; Morte celular.

Resumem

El osteosarcoma canino es el tumor óseo más común en perros. Tiene una intensa capacidad metastásica y la supervivencia del paciente es baja en esta enfermedad. La curcumina, el compuesto más importante derivado de la planta *Curcuma longa* L., ha sido ampliamente estudiado y ha mostrado efectos antineoplásicos considerables contra varios tumores. Este estudio tiene como objetivo identificar la activación de proteínas específicas de las vías de apoptosis, supervivencia tumoral y mal pronóstico de esta enfermedad en células OSC del linaje D-17. Para ello, las células se cultivaron y trataron con curcumina a concentraciones de 20 μM, 50 μM y 100 μM en portaobjetos, que se prepararon y fijaron. Posteriormente, se realizó la técnica de inmunocitoquímica con anticuerpos anti-caspasa3, anti-JNK, anti-AMPK, anti-p53, anti-AKT y anti-mTOR. Se observó que la curcumina activó en células de osteosarcoma canino in vitro las proteínas de muerte celular caspasa-3, JNK y AMPK, redujo la expresión de la proteína p53 mutada y no alteró las proteínas AKT y mTOR. Así, se encontró que la curcumina promueve la apoptosis extrínseca mediada por caspasa, JNK y cAMP / AMPK en células de osteosarcoma canino. Además, tiene el potencial de mejorar el pronóstico tumoral de esta enfermedad al inactivar el p53 mutado. Sin embargo, no interfiere con la expresión de AKT / mTOR, relacionada con la proliferación y supervivencia tumoral. Tales resultados servirán de base para futuros estudios que analicen el efecto de la curcumina in vivo sobre esta enfermedad.

Palabra clave: Citotoxicidad; D-17; Inmunocitoquímica; Muerte celular.

1. Introduction

Canine osteosarcoma is the most common bone tumor in dogs and has a high capacity to invade normal tissues, in addition to spreading in the body through metastasis (Szewczyk et al. 2015). Mutation in tumor suppressor genes may be one of the causes of this disease, as well as in other tumors (Greenblatt et al. 1994). However, TP53 gene mutation, which encodes the genome guardian p53 protein, is reported as a factor in poor prognoses of osteosarcoma and relates to decreased survival time, increased likelihood of metastasis (Tsuchiya et al. 2000; Kirpensteijn et al. 2008; Mirabello et al. 2015), and resistance to chemotherapy (Cavalcanti et al. 2004).

The current treatment of this disease combines surgery (primary tumor removal), radiotherapy, and chemotherapy (induces the death of remaining and metastatic cells) (Szewczyk et al. 2015). Cell death, caused by chemotherapy, can occur in several ways and is classified morphologically as apoptosis, necrosis, or autophagy. However, apoptosis is reported in different mechanisms of action of antitumor substances (Tait & Green, 2010; Galluzzi et al. 2016).

Apoptosis occurs via two main pathways: intrinsic or extrinsic. The intrinsic pathway works by losing the integrity of the outer mitochondrial membrane. This process is caused by the inhibition of gene expression of anti-apoptotic proteins Bcl-2 and Bcl-Xl and activation of pro-apoptotic proteins Bax and Bak, which trigger caspase proteins that cause cell death. The extrinsic pathway occurs through the binding of cell death receptors to the plasma membrane, which activates caspases (Galluzzi et al. 2016; Amarante & Green, 1999) or the JNK protein pathway (Ray et al. 2011).

In the constant search for new agents, curcumin, one of the main active compounds of *Curcuma longa* Linn, has been widely studied in recent decades for its antineoplastic effects against multiple types of cancer, such as prostate, breast, colorectal, pancreas, head, and neck cancers, both in vitro and in vivo (Anand et al. 2008; Vallianou et al. 2015; Tomeh et al. 2019). Likewise, its efficacy and safety in cancer patients, alone or together with other chemotherapeutic agents, have been proven in several clinical studies (Tomeh et al. 2019).

Another relevant aspect of this molecule is that it can also sensitize tumor cells to targeted therapeutic agents and reverse resistance to chemotherapeutic drugs (Hu et al. 2018). In vitro studies show curcumin's remarkable ability to induce apoptosis in different cell lines.

It promotes cell death through both the intrinsic pathway, by disturbing the potential balance of the mitochondrial membrane (Balasubramanian & Eckert, 2007), and the extrinsic pathway, by binding to the TNF receptor (tumor necrosis factor) (Moragoda et al. 2001; Ashour et al. 2014).

This study aims to identify the effect of curcumin on specific proteins of apoptosis pathways, on mutated p53 protein, and tumor survival protein, in addition to testing the hypothesis that curcumin promotes extrinsic apoptosis in canine osteosarcoma cells in vitro.

2. Material and Methods

The experiment was developed at the Multi-User Laboratory for the Evaluation of Molecules, Cells, and Tissues, of the Veterinary and Animal School of the Federal University of Goiás, through quantitative analysis and methodological studies to test the hypothesis that curcumin promotes extrinsic apoptosis in canine osteosarcoma cells, on the labeling of antibodies that may be involved in this pathway. The methodology used follows a universal standard, which allows the reproducibility of the experiments Pereira et al. (2018).

Plant drug and cell culture

The osteosarcoma cells (D-17, BCRJ 0276, Lot 000573, Passage 239, Species Canis familiaris), originating from the ATCC (American Type Culture Collection - Manassas, VA, USA), were purchased from the Rio de Janeiro Cell Bank (BCRJ - Rio de Janeiro, Brazil). During cultivation, they were kept in a humidified incubator at 37 ºC with an atmosphere of 5% CO2, and cultured in Dulbecco's Modified Eagle Medium (DMEM) plus 10% fetal bovine serum, 1% L-glutamine, amphotericin B, penicillin G, and 0.1% streptomycin.

Curcumin (C21H20O6) (NCBI, 2020), from C. longa, has a molecular weight of 368.4 g/mol, and was obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany), diluted in 1 mM dimethyl sulfoxide (DMSO) and stored at -20 °C.

Immunocytochemistry

The cultured cells were seeded on cell culture slides with a Falcon™ chamber (BD Falcon CultureSlides) at 1x104 density, then incubated for substrate adhesion for 24 hours.

The cells were treated at concentrations of 20 µM, 50 µM, and 100 µM, for 24 hours. For each antibody tested in the experiment, we performed a positive control, free of curcumin, and negative control, free from the primary antibody.

After the end of the treatment, the cells were fixed on the slide with 4% paraformaldehyde for 30 minutes, and later, the slides were frozen at -80 ºC.

In the next phase, we performed an immunocytochemistry reaction on the slides at room temperature, using anti-Caspase-3 antibodies (SC 7148 rabbit polyclonal), anti-JNK (SC 6254 mouse monoclonal), anti-Phospho-AMPKα (monoclonal/Rabbit, Thr172 - 40H9), anti-p53 (SC71785 mouse monoclonal), anti-phospho-Akt (polyclonal/Rabbit, pSer473), anti-mTOR (Ser 2481 rabbit monoclonal), and secondary antibodies (anti-mouse and anti-rabbit) acquired from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc, Dallas, TX, USA), Cell Signal (Cell Signaling Technologies, Danvers, MA, USA), and Jackson (Jackson Immuno Research Laboratories, West Grove, PA, USA).

All steps were performed by the automated immunohistochemistry processor Bond-Max Fully Automated IHC-ISH (Leica Biosystems), using the following reagents: Bond Polymer Refine Detection, BondTM Wash Solution, BondTM Epitope Retrieval 1, Bond Dewax Solution, and BondTM Epitope Retrieval 2, Bond DAB (chromogen 3,3'-diaminobenzidine), and Bond hematoxylin.

Evaluation of protein expression

We used the scoring system proposed by Fedchenko & Reifenrath, 2014 to evaluate the protein expression. Initially, we performed the total cell count in each selected area and the percentage of positive cells marked by the antibody. Brown-labeled cells, partially or completely, were considered positive; unlabeled cells in shades of blue, due to counterstaining with hematoxylin, were considered negative. The ratio of positively stained cells (RPSC) was calculated by the ratio between the number of labeled cells and the total number of cells multiplied by 100.

With this value, categories 0 were assigned to 0%, 1 to <1%, 2 for 1-10%, 3 for 11-33%, 4 for 34-66%, and 5 for $\geq$67%. Semi-quantitative analysis was performed by three researchers to describe the expression intensity, using the scores from 0 to 3, according to the scale shown in Figure 1.

Figure 1. Reference tones for establishing the scores attributed to the different expressions of proteins by the immunocytochemistry analysis.

Labeling				
Scores	0	1	2	3
	None	Weak	Intermediate	Strong

Source: Own authorship.

Then, we adopted a combination of the semiquantitative scoring system (CSSS) and the Allred score, which combined the percentage of cells with the staining intensity (Fedchenko & Reifenrath, 2014). The two scores were added to a final value.

Statistical analysis was performed through a non-parametric Kruskal-Wallis test, with $p < 0.05$ to indicate a statistically significant difference, using the R software package easyanov (Team, 2013; Arnhold, 2013).

3. Results and Discussion

In this research, osteosarcoma cells in vitro were treated with curcumin to test the hypothesis that it promotes extrinsic apoptosis in these cells. The results on the labeling of antibodies that may be involved in this pathway, in the different concentrations of the tested substance, and their numerical representation are shown in Table 1.

Table 1. Mean results of the combined semiquantitative scoring system (CSSS) for the different antibodies tested in osteosarcoma cells in vitro, in response to treatment with curcumin. Kruskal-Wallis test, p <0.05.

ANTIBODY	CONCENTRATION	CATEGORY	SCORE	CSSS
Caspase-3	Control	5	3	8 A
	20 µM	5	2	7 B
	50 µM	5	1	6 C
	100 µM	5	2	7 B
JNK	Control	5	1	6 A
	20 µM	5	2	7 B
	50 µM	5	2	7 B
	100 µM	5	3	8 C
AMPK	Control	5	1	6 A
	20 µM	5	1	6 A
	50 µM	5	2	7 B
	100 µM	5	1	6 A
p53	Control	5	3	8 A
	20 µM	5	2	7 B
	50 µM	5	2	7 B
	100 µM	5	2	7 B
AKT	Control	5	3	8 A
	20 µM	5	3	8 A
	50 µM	5	3	8 A
	100 µM	5	3	8 A
Mtor	Control	5	1	6 A
	20 µM	4	1	5 A
	50 µM	5	1	6 A
	100 µM	5	1	6 A

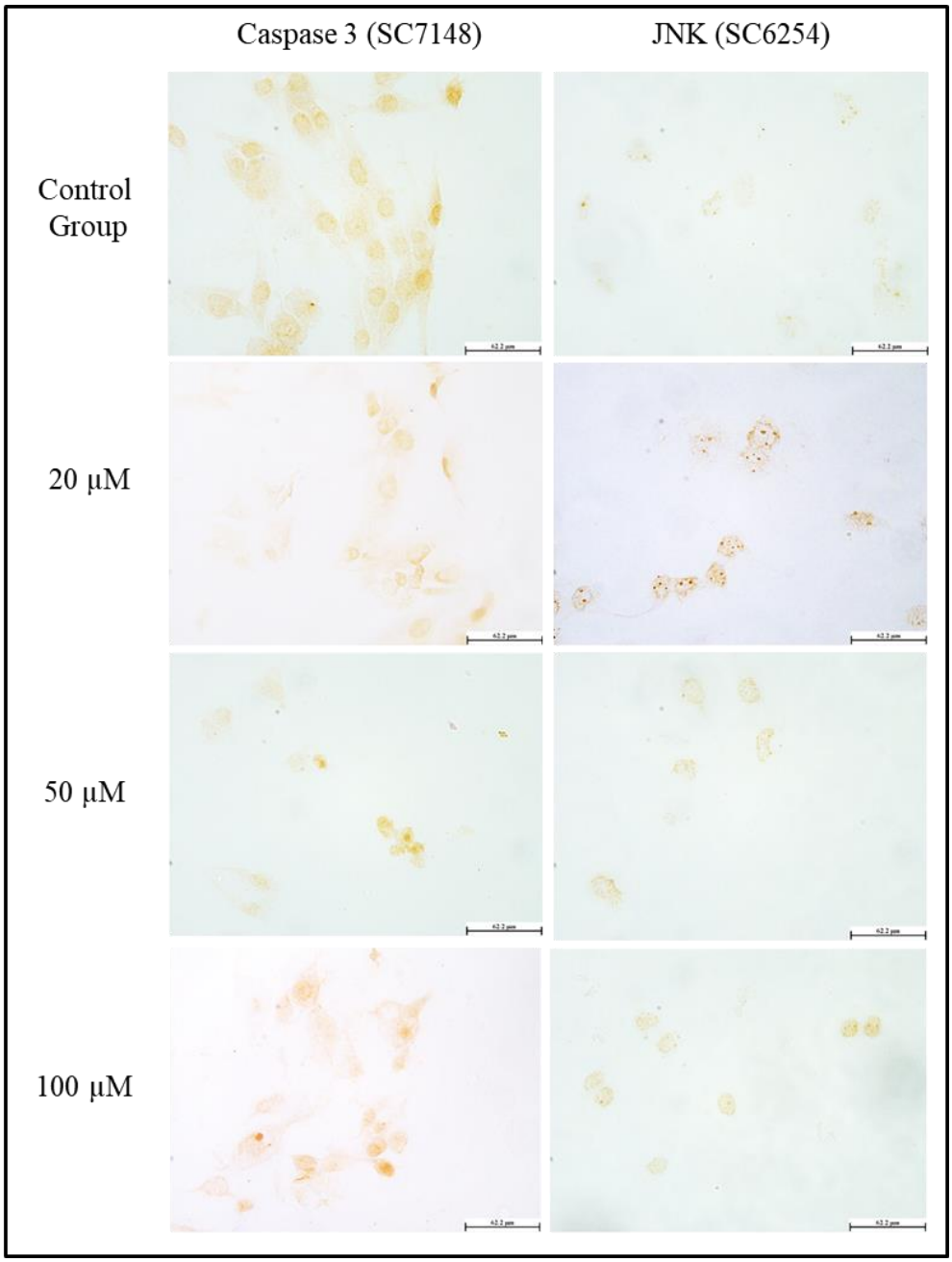
Category: the number that represents the percentage of labeled cells; Score: gradation of staining that reflects the intensity of the protein staining by the antibody; CSSS: the sum of the two results. Mean values followed by the same letter in the column are statistically equal by the Kruskal-Wallis test.
Source: Own authorship.

The antibody labeling the effector protein of apoptosis, caspase-3, showed a significant difference between control and treatments. Among the treatments, the dose of 50 µM was different from those of 20 µM and 100 µM, which were the same. In the labeling of the anti-JNK antibody, treatments were different from the control, where doses of 20 µM and 50 µM were the same and different from the treatment of 100 µM (Figure 2).

The treatment group of 50 µM curcumin showed a difference between the control and the other treatments in the anti-AMPK antibody. The p53 protein had its control labeling different from the doses, but there was no significant difference in concentrations between

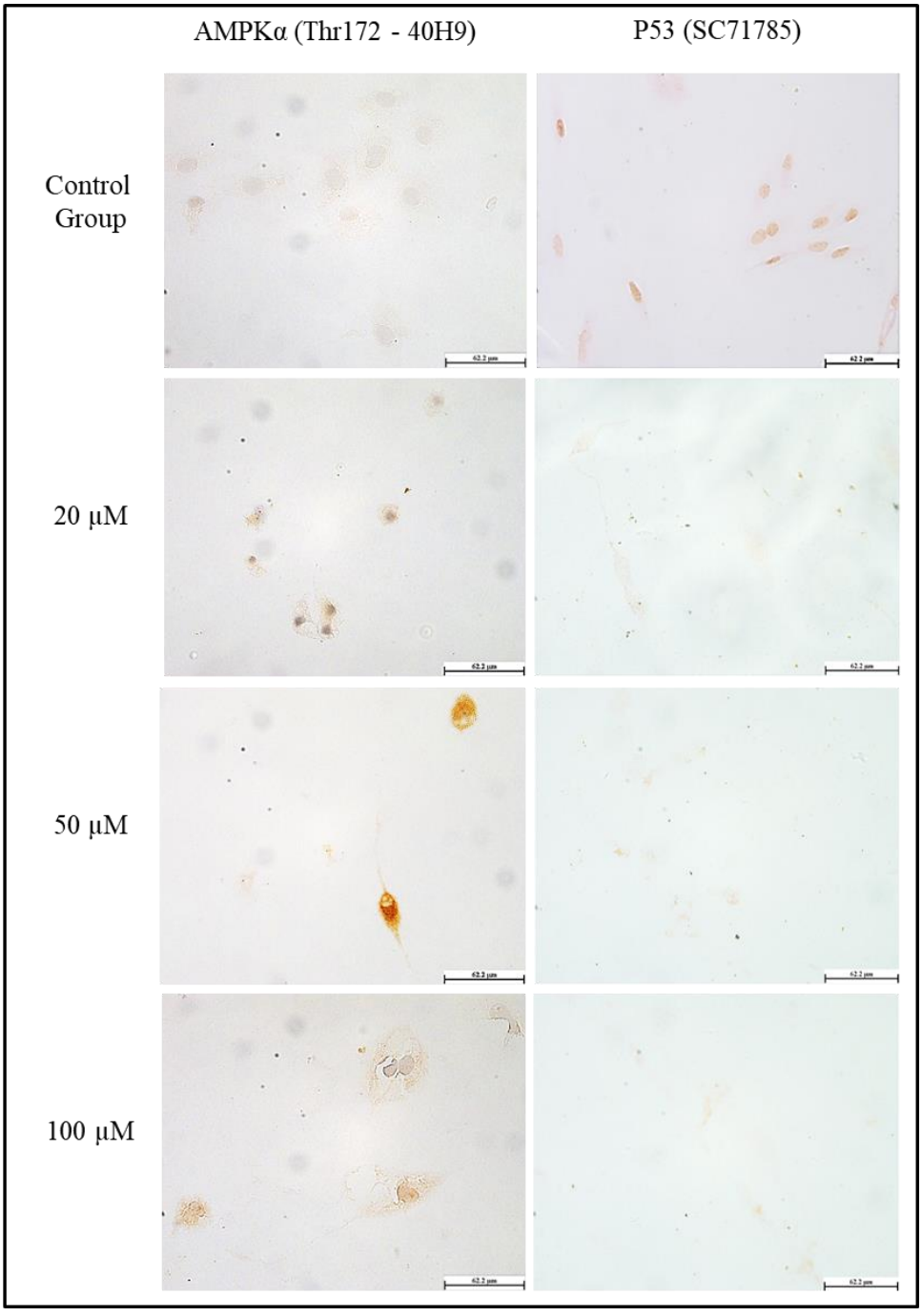
them (Figure 3). The labeling of anti-AKT e anti-mTOR antibodies showed no difference between the score assigned to control and treatments (Figure 4).

Figure 1. Photomicrographs of slides containing canine osteosarcoma cells, line D-17, treated with curcumin, in different concentrations, after the immunocytochemistry assay by the automatic Bond-Max processor (Leica), with anti-caspase-3 and anti-JNK antibodies. DAB, hematoxylin counterstaining. Scale = 62 µm.



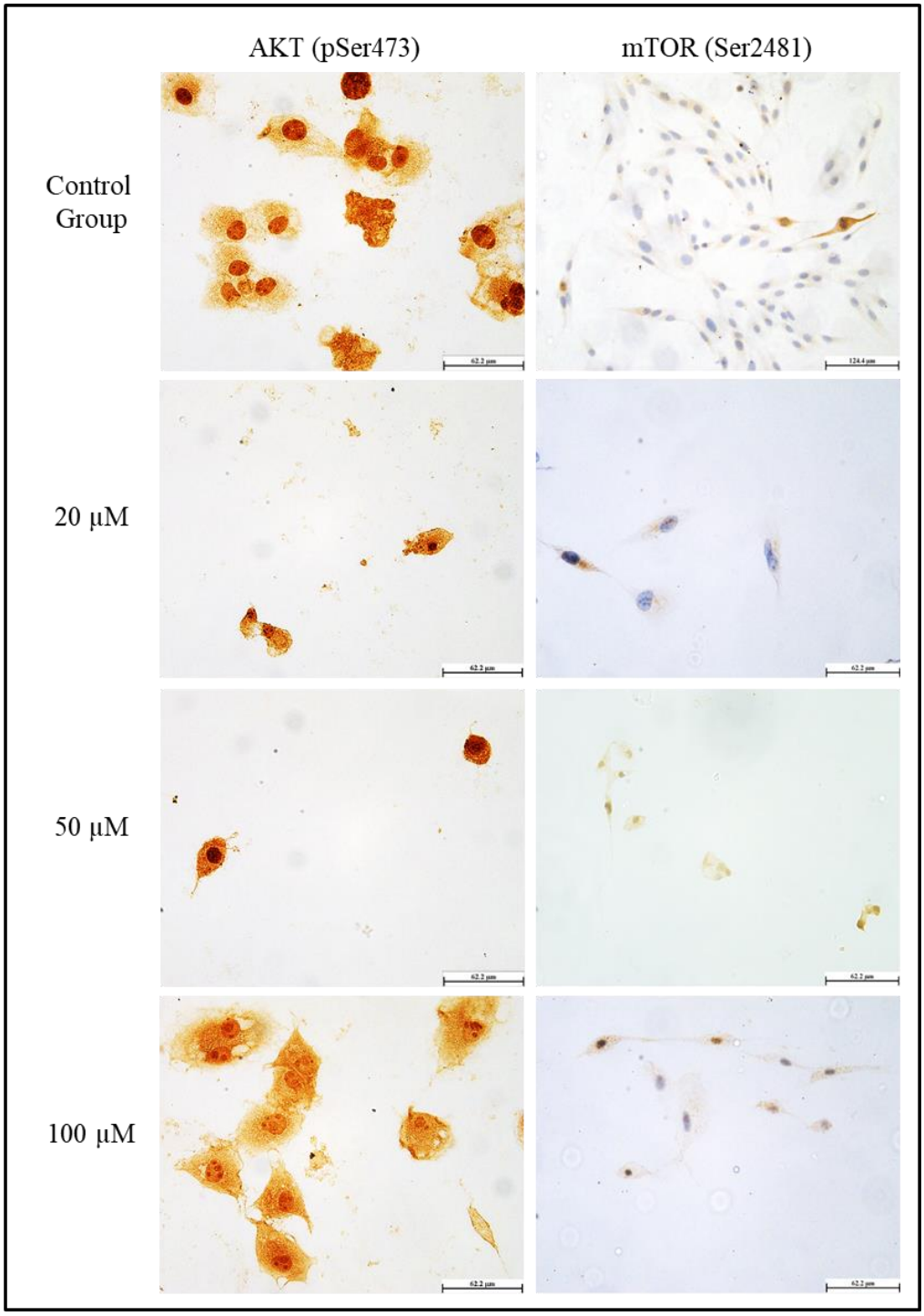
The labeling of anti-caspase 3 and anti-JNK antibodies is stronger and more evident in treatments than in control, which corroborates the data analyzed statistically. Also, notice the reduction in the number of cells in the treatments compared to the control.
Source: Own authorship.

Figure 3. Photomicrographs of slides containing canine osteosarcoma cells, line D-17, treated with curcumin, in different concentrations, after the immunocytochemistry assay by the automatic Bond-Max processor (Leica), with anti-AMPK and anti-p53 antibodies. DAB, hematoxylin counterstaining. Scale = 62 µm.



The labeling of anti-AMPK*α* antibodies is stronger and more evident in the 50 µM treatment than in the control and other treatments. For anti-p53 antibodies, it is possible to verify the highest marking tone, a score close to 3 in the control in relation to treatments, a score close to 1, based on the colorimetric table proposed by the respective study.
Source: Own authorship.

Figure 4. Photomicrographs of slides containing canine osteosarcoma cells, line D-17, treated with curcumin, in different concentrations, after the immunocytochemistry assay by the automatic Bond-Max processor (Leica), with anti-AKT and anti-mTOR antibodies. DAB, hematoxylin counterstaining. Scale = 62 μm.



It is possible to observe similar staining in the anti-AKT and anti-mTOR markers in the treatments and control groups, which corroborates the quantitative data. However, there is a reduction in the number of cells in the treatments.
Source: Own authorship.

There are several different approaches to establish the criteria for evaluation of staining by the antigen-antibody reaction in immunocytochemistry, and they are related to the experiment objective and the properties of the markers used. The method used in this study, for analyzing the labeling intensity, as a result of protein expression in cells, was based on the Allred score. This system is considered a "gold standard" in the evaluation and presentation of immunohistochemistry and immunocytochemistry data. It defines clear scores that improve the understanding of the data presented and reduces subjectivity, in addition to being widely accepted and recommended by the main associations and organizations of pathologists (Jin et al. 2001; Sappayatosok et al. 2009; Torlakovic et al. 2010; Fedchenko & Reifenrath, 2014; Fitzgibbons et al. 2014).

The results of the labeling of these proteins by their antibodies were based on the number of cells labeled by the total number of cells on the slide and the staining intensity presented. Thus, we did not consider the number of cells themselves present in each blade. However, we noticed a reduction in the number of cells between the control and treatments, which shows a decrease in cell viability caused by the treatment with curcumin (Figures 2, 3, and 4).

The extrinsic apoptosis pathway initiates by the binding of cell death receptors, which trigger caspase-8 that activates caspase-3. Caspase-3 acts as an endonuclease that promotes DNA strand breakage and causes disintegration of cell structures in the process of apoptosis (Fan et al. 2005).

Caspase-3 expression in canine osteosarcoma cells indicates this pathway activation in extrinsic apoptosis after curcumin treatment at different doses. Curcumin induced this apoptosis pathway in many cancer cells lines, such as acute monocytic leukemia cells (Zhu et al. 2016) and other leukemias (Gopal et al. 2014), prostate cancer (Jordan et al. 2016; Teiten et al. 2011), positive and negative squamous carcinoma of human papillomavirus (Khan et al. 2018), and malignant pleural mesothelioma in vivo (Zhang et al. 2018).

JNK is a protein of the MAPKs family (serine/threonine kinases), along with the P38 MAPK and ERK proteins. These molecules play an important role in extrinsic and intrinsic signal transduction pathways and induce the apoptosis of several cancer cells (Kim & Choi, 2010). The activation of P38 MAPK and JNK pathways is related to cell death, while the activation of ERK is often associated with survival (Zhu et al. 2013).

The sustained activation of JNK (c-Jun N-terminal kinase) can be a significant mediator of apoptosis in many types of cells, dependent on the activation of TNF (necrosis factor) receptors, a cell death receptor (Tang et al. 2001; De Smaele et al. 2001). Besides, it

inhibits crucial downstream nuclear proteins, such as c-Jun, which promotes tumor growth when it is most expressed in cancer cells (Behrens et al. 2000).

Our results indicate that the activation of JNK may play a role in this protein-dependent apoptosis of canine osteosarcoma cells induced by curcumin. Curcumin showed activation in the JNK-dependent apoptosis pathway in human colon cancer cells (Collett & Campbell, 2004), melanone (Yu et al. 2013), human monocytic leukemia (Yang et al. 2012), choriocarcinoma (Lim et al. 2016), and prostate cancer (Li et al. 2015; Guo et al. 2013).

Curcumin induced an increase in AMPK expression in canine osteosarcoma cells, with a concentration of 50 μM. AMPK (5'-AMP-activated protein kinase), activated by cAMP, can regulate a variety of cellular functions, from metabolism to activation of the ion channel, cell growth and differentiation, gene expression, and apoptosis (Chin et al. 2002).

This protein is recruited during metabolic stress, and recent findings suggest that AMPK activation strongly suppresses cell proliferation and induces cell apoptosis in many cancer cells (Hasima et al. 2012). The cAMP/AMPK pathway involves the activation of the protein substrates p38 and p53, and thus, leading to apoptosis. Curcumin activated this pathway in CaOV3 (Pan et al. 2008) ovarian cancer cells.

Therefore, the results showed here suggest that curcumin can increase the production of cAMP/AMPK, at a dose of 50 μM, and this molecule may be associated with apoptosis mediated by p38 and p53, promoted by curcumin in osteosarcoma cells.

Curcumin reduced the expression of mutated p53 in canine osteosarcoma cells. The p53 protein is a tumor suppressor, which assesses DNA damage and acts as a regulatory transcription factor for genes that control cell growth, DNA repair, apoptosis (Fridman & Lowe, 2003; George et al. 2011), and senescence (Itahana et al. 200; Qian & Chen, 2013). TP53 gene mutations cause the inactivation of the p53 protein. Thus, genetically modified cells cannot be stopped at the cell cycle (phase G1) checkpoints or sent to apoptosis, which leads to the formation of tumors, resulting in cancer cells (Prokocimer & Rotter, 1994; Shackelford et al. 1999).

Such mutations are common and can play a role in canine osteosarcoma tumorigenesis (Kirpensteijn et al. 2008). The incidence of mutated p53 in this disease ranges from 24% to 47%. Most of them are located in highly conserved domains and are almost identical to those reported in human osteosarcoma (Van Leeuwen et al. 1996; Johnson et al. 1998). Besides, it is related to the worsening prognosis of canine and human osteosarcomas, decreased survival, and increased metastasis (Mirabello et al. 2015).

This question is significant, since D17 is an established cell culture lineage, originating from the primary extraction of mutated P53 lung metastasis of canine osteosarcoma. Thus, our results demonstrate that curcumin can help the treatment of osteosarcoma associated with p53 mutation, improving its prognosis, decreasing the chance of metastasis, and increasing the patient's survival. Furthermore, it is a hope for the treatment of that tumor metastasis.

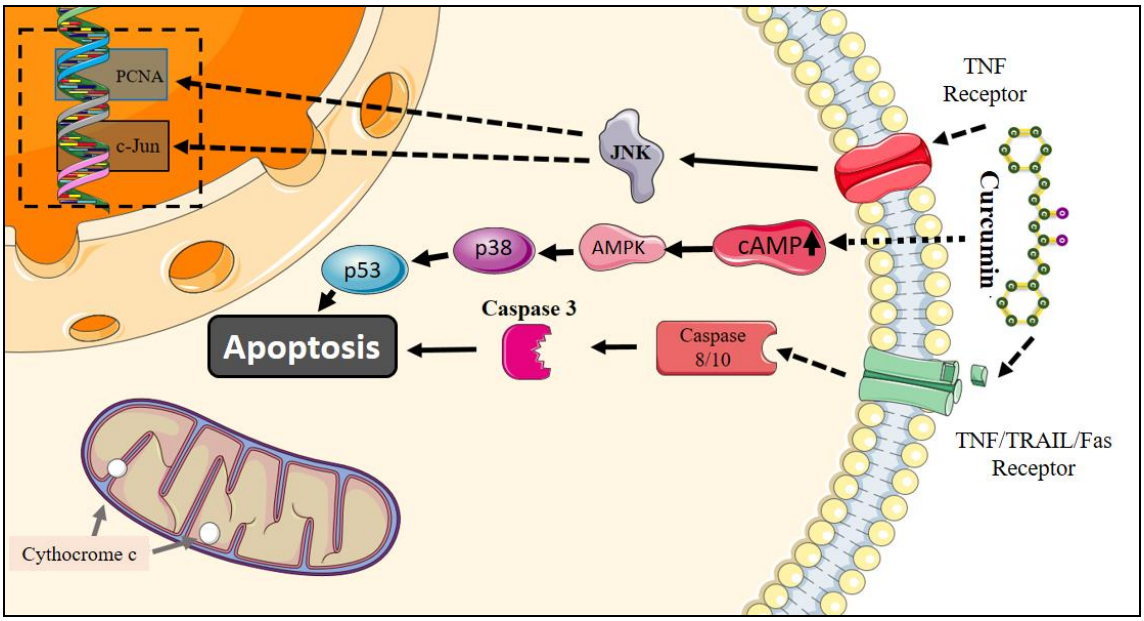
However, curcumin did not affect the expression of AKT proteins (also called PKB, protein kinase B) and mTOR (mechanistic target of rapamycin). This result was different from treatments with curcumin in other tumor cells (Yu et al. 2008; Zhang et al. 2018). The PI3K/Akt/mTOR signaling pathway is activated in cancer and is known to play a significant role in several cellular processes, including inhibition of apoptosis, cell proliferation, and angiogenesis (Zhang et al. 2018). Thus, in this research, curcumin did not alter the proliferation and survival of osteosarcoma cells via this pathway.

Therefore, in our hypothesis of the cell death pathway, curcumin activated the cell death receptors, which activated the protein JNK in the MAPK pathways, possibly inhibiting the PCNA protein linked to cell survival and activating the c-Jun protein linked to apoptosis. Besides activating the caspases 8 or 10, which activated caspase-3, promoting apoptosis.

AMPK increased at the 50 µM curcumin concentration, which may have led to another pathway of apoptosis, which activated p38 and p53. Moreover, curcumin probably reduced the mutated p53 in these cells and did not interfere with the AKT/mTOR pathways.

By analyzing the expression of the tested proteins, we can infer that cell death caused by curcumin treatment in canine osteosarcoma cells in vitro is mediated by caspase-3, JNK, and AMPK. Figure 5 shows a likely hypothesis of the main pathways triggered by curcumin in these cells.

Figure 5. Hypothesis illustration of the cell death mechanism induced by curcumin in D17 canine osteosarcoma cells in vitro, with multiple signaling pathways. The dotted table indicates the possible activation of gene expression, which is not elucidated in this research.



Model diagram proposed for the associations of curcumin and their respective involvement in the metabolic pathways of canine osteosarcoma cells, line D-17; after expression of key antibodies, evaluated by immunocytochemistry.
Source: Own authorship.

Curcumin changed the expression of the caspase-3, JNK, AMPK, and p53 proteins. However, it did not affect the proteins that act in the cell survival and tumor proliferation pathway, AKT/mTOR, in canine osteosarcoma cells in vitro. Thus, we infer that this substance promoted apoptosis in these cells through the activation of caspase-3 and JNK, which are proteins crucial in extrinsic apoptosis and reduction of cell survival. It also led to apoptosis cAMP/AMPK at a concentration of 50 µM.

Furthermore, curcumin may play an important role in improving the prognosis of canine osteosarcoma tumors because it has reduced the expression of mutated p53. However, clinical studies are needed to prove this function in vivo.

4. Conclusion

Curcumin promoted extrinsic apoptosis in canine osteosarcoma cells by activating the JNK and caspase-3 proteins. Besides, apoptosis was mediated by cAMP/AMPK, at a concentration of 50 µM. Finally, there was a reduction in mutant p53 and no interference in the AKT/mTOR pathways.

This study demonstrates at the molecular level the effects of curcumin on proteins involved in the apoptosis of canine ostessarcoma cells, however, it is necessary that future studies test the clinical effects of this substance so that its action is valid at an organic level.

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