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Original Article

The anti-proliferative and apoptotic effects of curcumin on feline mammary gland tumor cells *in vitro*

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Abstract

Background: Feline mammary gland tumors (FMGTs) are the third most diagnosed tumors in cats. Feline mammary gland tumors have aggressive biological behavior and poor response to both surgical and medical treatments, thus, new therapeutic approaches are essential to improve. Curcumin (CUR) is a polyphenol component exhibiting anti-cancer effects and induces apoptosis through different mechanisms especially in human breast cancer. However, there is no study investigating the effects of CUR on FMGTs. **Aims:** The aim of this study was to determine the anti-proliferative and apoptotic effects of CUR on primary cell lines from FMGT tissue samples of two cases classified as carcinoma-simple, tubular type (grade III). **Methods:** The cytotoxic effect of CUR was determined by water-soluble tetrazolium salt-1 (WST-1) assay. Annexin V, cell cycle, and acridine orange (AO) analyses were performed to determine the apoptotic effect of CUR. **Results:** Our results showed that CUR had an anti-proliferative and apoptotic effect through induction of apoptosis and cell cycle arrest (G0/G1) on FMGT cells. **Conclusion:** Therefore, this is the first study that shows the effects of CUR on FMGTs. However, further molecular studies are required to compare the effects of CUR on different histopathological phenotypes and to determine the further molecular mechanisms including the potential apoptotic and cellular pathways affected by CUR.

Key words: Apoptosis, Cell cycle, Curcumin, Feline mammary gland tumor

Introduction

Feline mammary gland tumors (FMGTs) are the third most diagnosed tumors in cats, following haemopoietic and cutaneous neoplasms. The malignancy rate of FMGTs ranges from 80-96% (Moulton, 1990). The most important prognostic factors in FMGT cases comprise Tumor size, lymph node involvement, histologic tumor subtype and grade, clinical stage, the existence of metastases, the extent of surgical intervention, estrogen receptor status, expression of human epithelial receptor-2, proliferating cell nuclear antigen (PCNA), argyrophilic nucleolar organizer region and Ki-67 (Preziosi *et al*., 1995; Giménez *et al*., 2010; Zappulli *et al*., 2015). Surgical approach is the main recommended treatment for FMGTs. However, cats with large tumors (>3 cm in diameter) are reported to have <1-year survival time after surgery (Ito *et al*., 1996; Matos *et al*., 2012). Adjuvant chemotherapy is also recommended, especially in cats with large tumors, which is applicable in presence of distant metastases, high histological grade tumors with an aggressive histological type of tumors such as solid carcinoma, cribriform carcinoma, and carcinosarcoma (Matos *et al*., 2012; Cassali *et al*., 2018). However, most of the chemotherapeutics are toxic to rapidly dividing tumor cells and normal cells. Therefore, treatments with single or combined chemotherapy have a major impact on the morbidity and mortality in cats with FMGTs (Hahni *et al*., 1994). Due to the highly aggressive biological behavior of FMGTs and poor response to both surgical and medical treatments, new therapeutic approaches are needed to improve.

Curcumin (CUR) is a polyphenol component that is extracted from the rhizomes of *Curcuma longa*. Curcumin exhibits the anti-cancer, anti-viral, antioxidant, and anti-inflammatory effects on different diseases (Chattopadhyay *et al*., 2004; Maheshwari *et al*., 2006; Rahmani *et al*., 2014; Deguchi, 2015; Tomeh *et al*., 2019). Among them, CUR modulates growth factors, enzymes, transcription factors, kinase, inflammatory cytokines, and apoptotic proteins through multiple signaling pathways in a variety of human breast cancer

cell lines, *in vitro* and *in vivo* (Wang *et al*., 2016; Calaf *et al*., 2018; Guney Eskiler *et al*., 2019). However, there is no study to investigate the effects of CUR on FMGTs. Therefore, the objective of this *in vitro* study was to determine the anti-proliferative and apoptotic effects of CUR on primary cell lines obtained from two cases of FMGT.

Materials and Methods

Ethical approval

The cats were operated on for cancer treatment, not for the experimental procedure. Ethics committee approval was received from the Faculty of Veterinary Medicine, Istanbul University-Cerrahpasa, with the number 2020/25, 24/07/2020.

Case description

Two cats were admitted to the Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Istanbul University-Cerrahpasa with palpable masses in mammary glands.

Case 1

An 11-year-old neutered (at age of 5) mix breed cat was presented to the clinic with a palpable 3 cm mass in the mammary gland. On clinical examination, no abnormalities were detected in regional lymph node palpation, heart rate, respiratory rate, and rectal temperature of the patient. Abdominal ultrasonography, thoracic radiography, serum biochemistry, and complete blood count did not reveal any significant abnormalities.

Case 2

A 9-year-old, intact mix breed cat was presented to the clinic with a palpable 3 cm mass in the mammary gland. Clinical examination revealed a visible and palpable enlargement of the axillary lymph node. Abdominal ultrasonography, serum biochemistry, and complete blood count did not reveal any abnormalities. However pulmonary metastases were determined via thoracic radiography.

A tentative diagnosis of mammary gland tumor was made, and a mastectomy procedure was performed on the cats.

Tissue sampling

Tissue samples were taken from surgically removed masses for histopathological and immunohistochemical (IHC) examinations and primary cell isolation. The tissues submitted to histopathological examination were fixed in 10% buffered formalin. Other parts of the tissue were kept Roswell Park Memorial Institute 1640 (RPMI-1640) medium in aseptic conditions.

Histopathological and IHC examination

For histopathology, tissue samples were fixed in 10% neutral buffered formalin solution, processed routinely, embedded in paraffin, cut in 4-5 µm by rotary microtome, and stained with haematoxylin and eosin (H&E). All sections were examined using a light microscope and a histologic grading system for the feline mammary tumors was applied according to the classification of Goldschmidt *et al*. (2011).

For histologic grading status of lymphovascular invasion inside the tumoral tissue, a nuclear form of atypical epithelial cells, and mitotic count in 10 × 40 high-power field (HPF) was taken into consideration (Mills *et al*., 2015).

To perform IHC, tissue sections from paraffin blocks were collected into positively charged slides. They were put through deparaffinization, dehydration, and antigen retrieval (citrate buffer pH = 6), then incubated with antibodies to PCNA (Santa Cruz Biotechnology, sc-56, diluted 1:200, 1 h, room temperature). Afterward, they were treated with a commercial secondary antibody kit (Abcam, Mouse and Rabbit Specific HRP/DAB IHC detection Kit, micro-polymer, ab236466) and marked with 3,3'-Diaminobenzidine (DAB) chromogen (included in the kit). Finally, the sections were counterstained with Mayer's haematoxylin. Negative control sections were incubated only with antibody diluent (ab64211) instead of the primary antibody. PCNA-positive cells were used to determine the proliferative index. Sections were examined using a light microscope (Olympus BX50F4) to quantify the proliferative index (percentage of PCNA-positive cells in 800-1000 cells).

Primary cell culture

In this study, two primary cell lines from FMGT tissue samples of two cases were used. For this purpose, tissue pieces obtained from tissue samples of two cases (C1: Case 1, and C2: Case 2) were treated with trypsin (Multicell, USA) and then incubated for 30 min. After the treatment, tissue pieces were centrifuged at 1500 × g for 5 min and the cells were cultivated in T_{25} flasks. Two primary cell lines obtained from FMGT of two cases were cultured in Dulbecco's Modified Eagle Media (DMEM, Capricorn Scientific, USA). After the cell cultivation, 10% fetal bovine serum and 1% penicillin-streptomycin were added to the medium and cultured at 37°C in a 95% humidity and 5% CO_2. To obtain the maximum number of cells, the confluent cells in a T_{25} flask were transferred to a T_{75}. This process was carried out in approximately 2-3 passages.

The water-soluble tetrazolium salt-1 (WST-1) assay

To investigate the anti-proliferative effect of CUR on C1 and C2 cells, WST-1 analysis was performed. Additionally, 1 mM stock solution of CUR (Sigma Chemical, St. Louis, USA) was prepared and kept as an aliquot at -20°C until the experiments were carried out. C1 and C2 cells were seeded into 96-well plates at 2 × 10^5 cells/well and then incubated for 24 h to reach 80% confluence. Then, the cells were treated with CUR (0.1, 0.5, and 1 mM) in DMEM for 24 and 48 h. Both cells were incubated with WST-1 reagent (BioVision, USA) at 37°C for 30 min in the dark condition. The cell viability

of the treatment groups was determined with an ELISA reader (Allsheng, China) at 450 nm absorbance according to the control group (100%) viability.

Apoptotic analysis

Annexin V assay

We performed Annexin V assay using the Muse Annexin V and Dead Cell Assay kit (Millipore, Germany) to observe the apoptotic effect of CUR in C1 and C2 cells. Both cells were seeded into 6 well plates at 2×10^4 cells/well and then treated with CUR (0.1, 0.5, and 1 mM). After 24 h incubation, cells were centrifuged at 1500 × g for 5 min and washed twice with a cold phosphate buffer saline (PBS, Multicell, USA). Afterward, cells were stained with Annexin V kit and stained cells were analyzed using the Muse® Cell Analyzer after 30-min incubation (Millipore, Germany).

Cell cycle assay

To evaluate the apoptotic effect of CUR, we performed cell cycle assay using the Cell Cycle kit (Millipore, Germany) in C1 and C2 cells. Cells were seeded into 6 well plates at 2×10^4 cells/well and then treated with different concentrations of CUR (0.1, 0.5, and 1 mM) for 24 h. Then, cells were fixed with 70% cold ethyl alcohol and incubated at -20°C. The fixed cells were centrifuged at 1500 × g for 5 min. Both cells were subsequently stained with a Cell Cycle kit (Millipore, Germany) and stained cells were analyzed using the Muse Cell Analyzer (Millipore, Germany).

Acridine orange (AO) staining

To determine the effects of CUR on cell morphology, we performed AO staining in C1 and C2 cells. Cells were seeded into 6 well plates at 2×10^4 cells/well and then treated with CUR (0.1, 0.5, and 1 mM) for 24 h. After incubation, cells were fixed with 4% paraformaldehyde (PFA) and stained with AO (100 mg/ml) for 30 min. Finally, stained cells were imaged and photographed with the EVOS Floid Cell Imaging System (Thermo Scientific, Waltham).

Statistical analysis

To evaluate the data for multiple comparisons, one-way analysis of variance (ANOVA) with Tukey's post hoc test was analyzed using the software GraphPad Prism. $P<0.05$ was considered significant. All experiments were conducted in triplicate.

Results

Histopathological and IHC findings

In the histopathologic examination (Figs. 1A and B) both cases were classified as carcinoma-simple, tubular type (grade III) and they presented atypical tubule formations with epithelial cells showing structures ranging from cubic to prisms. Those epithelial cells had

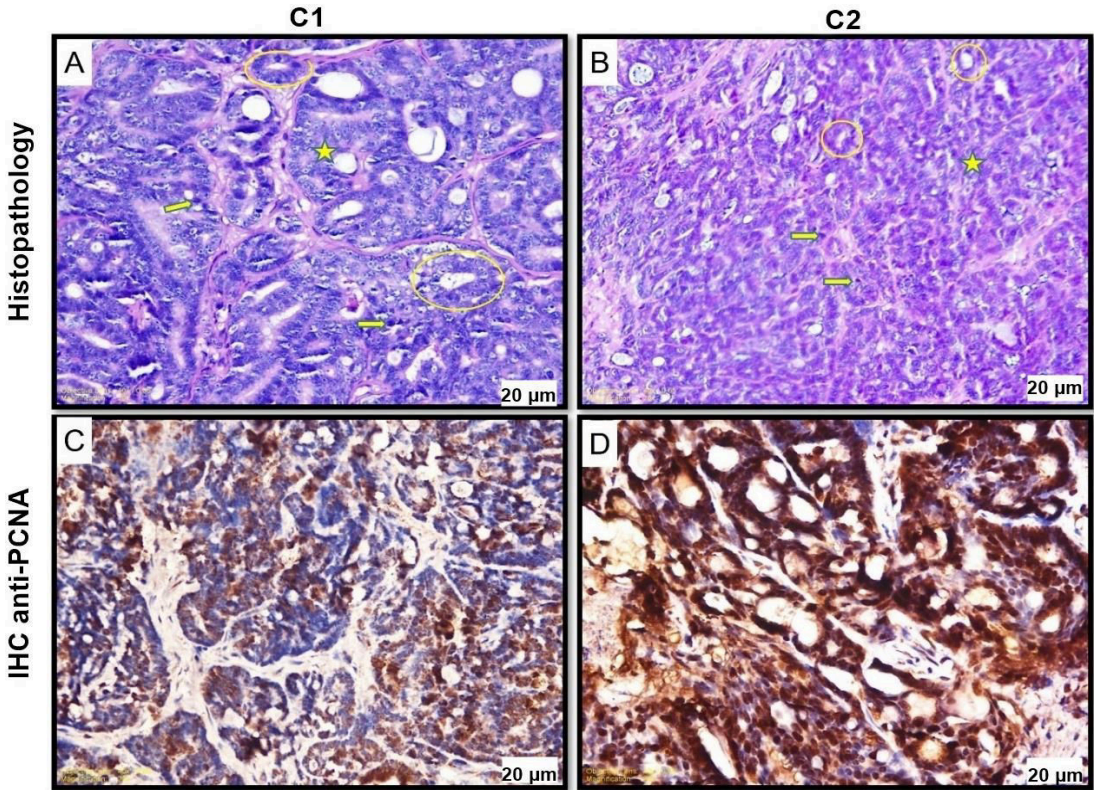

Fig. 1: Histopathology of the cases (H&E); the tubules (yellow circle) are lined by cuboidal epithelium with large, oval, vesicular nuclei, and a prominent nucleolus. (**A**) Has little nuclear and cellular pleomorphism (yellow star) and few mitotic figures, while (**B**) Demonstrates little tubular differentiation, considerable pleomorphism, with prominent nucleoli, and numerous mitoses (yellow arrows: mitosis). (**C**) and (**D**) Immunohistochemistry, dense and nuclear staining in the epithelial tumor cells. IHC: Immunohistochemistry, PCNA: Proliferating cell nuclear antigen, C1: Case 1, and C2: Case 2

large, oval, vesicular nuclei with prominent nucleolus and they showed numerous mitosis. Although tubular differentiation of C2 cells was less than C1 cells, both cases scored numerically between 2-3 and they were classified in grade III, high grade. In this classification, the numeric grading system was used as defined by Mills *et al*. (2015). There were tumor cell emboli inside 1 or 2 small lymphatics surrounding the main tumor tissue. Besides, wide tumor necrosis was observed in both cases. The intensity of PCNA expression was dense and nuclear (Figs. 1C and D). In C1 and C2 the percentage of PCNA-positive cells was found 73 (728/1000 cells) and 91 (912/1000 cells), respectively.

The effect of CUR on cell viability of FMGT cells

The water-soluble tetrazolium salt-1 assay was performed to determine the effect of CUR on the viability of primary cell lines from FMGT tissue samples of two cases after 24 h. The obtained results demonstrated a significant cytotoxic effect of CUR on C1 and C2 cells in a dose dependent manner (P<0.001, Fig. 2). However, the cytotoxic effect of CUR was more effective in C2 cells than C1 cells. According to the results, the viability of C1 cells significantly reduced to 84%, 64%, and 56% at 0.1, 0.5, and 1 mM CUR after 24 h, respectively (P<0.001). While the C2 cell growth was significantly reduced to 58%, 38%, and 35% after treating 0.1, 0.5, and 1 mM CUR for 24 h, respectively (P<0.001). Therefore, our results showed that CUR mediated apoptosis of FMGT cells.

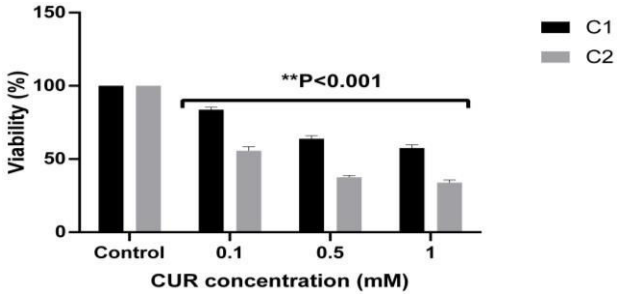

Fig. 2: Dose-dependent effect of CUR on cell growth in two primary cell lines from feline mammary gland tumors (FMGTs) tissue samples of two cases after 24 h. Relative cell viability was measured after treatment with different CUR concentrations after 24 h. C1: Case 1, C2: Case 2, CUR: Curcumin, and mM: Millimolar. ** P<0.001

CUR effect on apoptotic cell death of FMGT cells

Phosphatidylserine (PS) is located on the cytoplasmic surface of the plasma membrane in healthy cells, but PS is translocated from the inner to the outer leaflet of the plasma membrane in apoptotic cells. Therefore, fluorescent conjugates of Annexin V are commonly used to identify apoptotic cells. Curcumin-induced apoptotic effect was analyzed by Annexin V and cell cycle analysis in primary cell lines of FMGT of two cases after 24 h. The results demonstrated that CUR significantly induced apoptotic effect in C2 cells compared to C1 cells in a dose dependent manner (Fig. 3A). According to the

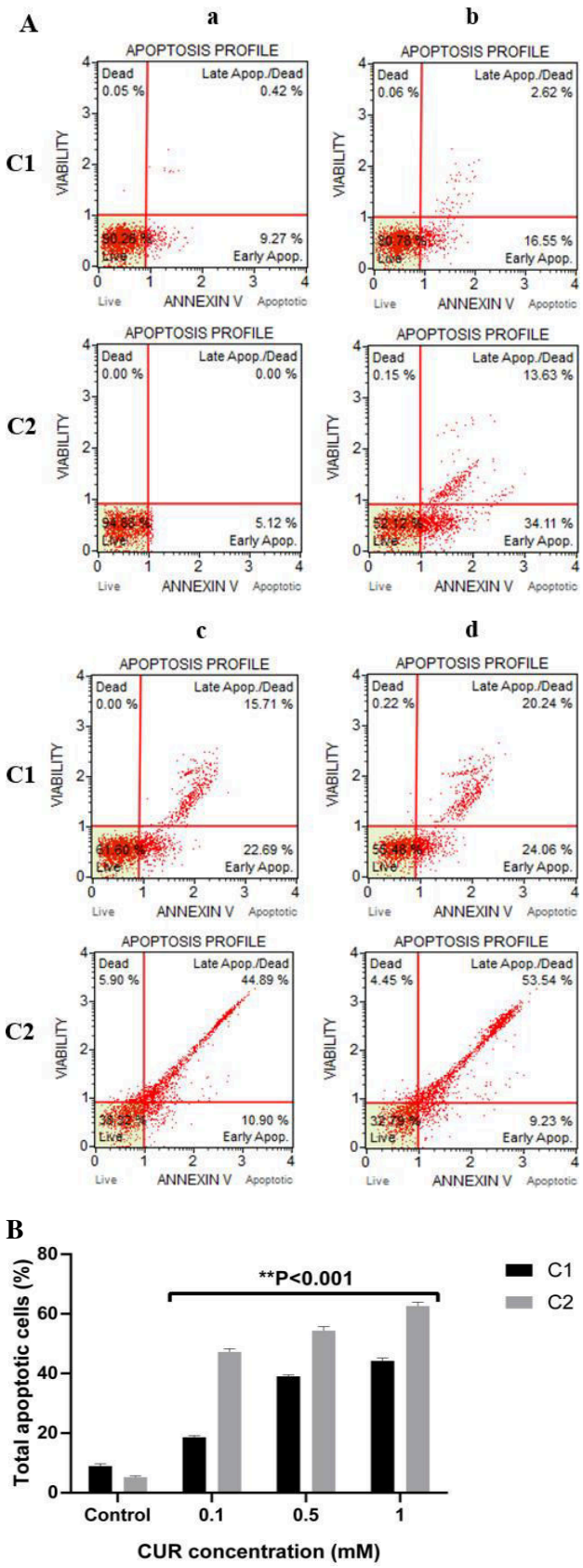

Fig. 3: The apoptotic effects of CUR on feline mammary gland tumors (FMGTs) cells from two cases after 24 h. (**A**) Histograms of Annexin V analysis in FMGT cells (a) Control, (b) 0.1, (c) 0.5, and (d) 1 mM CUR, respectively. (**B**) Statistical comparison of total apoptotic cells in FMGT cells. C1: Case 1, C2: Case 2, CUR: Curcumin, and mM: Millimolar. ** P<0.001

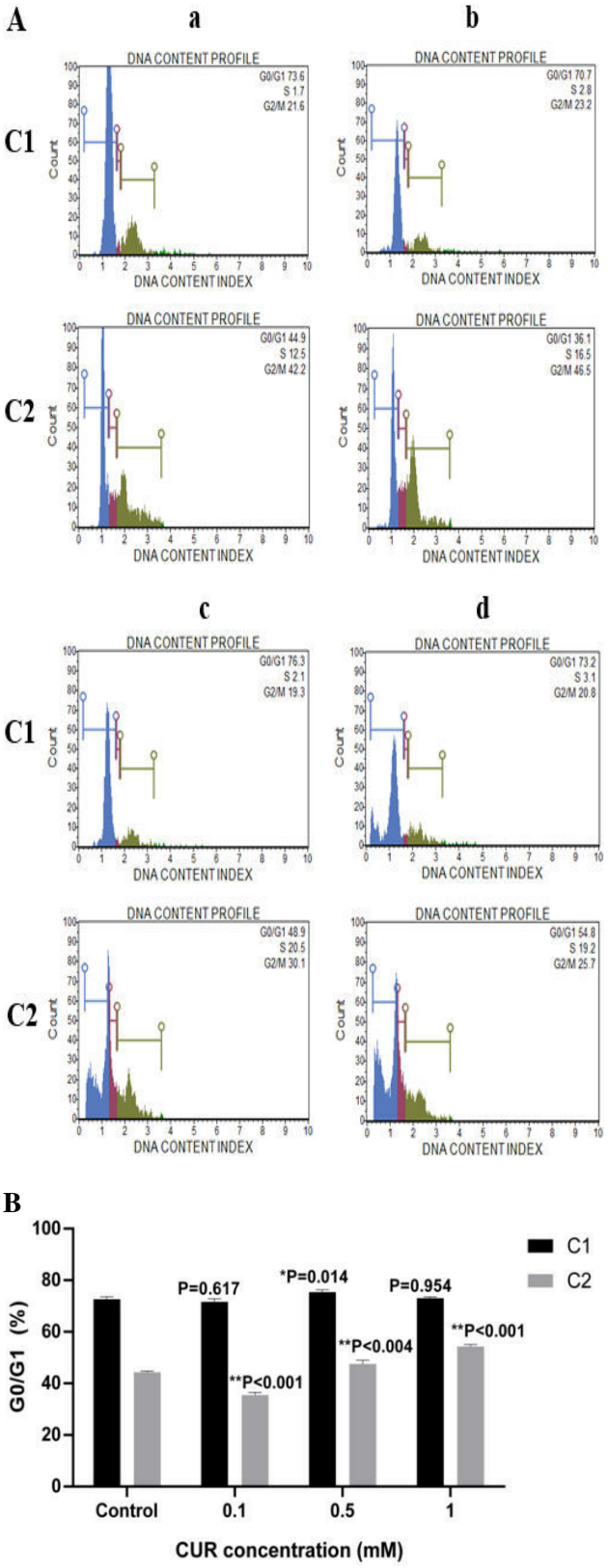

Fig. 4: The effects of CUR on cell cycle distribution in two primary cell lines from feline mammary gland tumors (FMGTs) of two cases after 24 h. (**A**) Histograms of cell cycle analysis in C1 and C2 cells (a) Control, (b) 0.1, (c) 0.5, and (d) 1 mM CUR, respectively. (**B**) Statistical comparison of accumulation in G0/G1 phase cells in FMGTs. G0/G1: Quiescence/First growth phase of cell cycle, C1: Case 1, C2: Case 2, CUR: Curcumin, and mM: Millimolar. ** P<0.001

Annexin V results, after 24 h treatment of 0.1, 0.5, and 1 mM of CUR, the total apoptotic C1 cells significantly increased from 9% to 18%, 39%, and 43% compared to the control, respectively (P<0.001, Fig. 3B). Additionally, 0.1, 0.5, and 1 mM of CUR treatment exhibited a significant increase (from 5% to 47%, 54%, and 62%, respectively) in the proportion of total apoptotic cells in C2 cells compared to the control (P<0.001, Fig. 3B).

CUR effect on cell cycle of FMGT cells

According to the cell cycle analysis results (Fig. 4), CUR treatment resulted in cell cycle arrest after 24 h in all primary FMGT cells (Fig. 4A). The accumulation of C1 cells in the G0/G1 phase resulted in a low increase level from 72% to 75%, and 73% at 0.5 and 1 mM CUR after 24 h, respectively (P<0.001, Fig. 4B). Additionally, CUR treatment significantly increased the percentage of C2 cells at G0/G1 phase (from 44% to 47%, and 54% for 0.5 and 1 mM, respectively) after 24 h (P<0.001, Fig. 4B). However, 0.1 mM CUR treatment did not affect the accumulation of C1 and C2 cells in the G0/G1 phase arrest after 24 h.

CUR effect on the morphology of FMGT cells

To evaluate the apoptotic effects of CUR on morphological changes in two primary cell lines of FMGT tissue samples, AO staining was performed (Fig. 5). After 24 h treatment with CUR, cytoplasmic vacuolization, round shape, and apoptotic bodies were observed in both C1 and C2 cells. Additionally, some of the cells exhibited necrotic morphology at higher concentrations of CUR. However, the greater changes in cell morphology were observed at 1 mM CUR in both C1 and C2 cells. Therefore, CUR-induced apoptotic cell death in FMGT cells was supported by AO staining.

Discussion

CUR, a plant derived substance, suppresses the onset, progression, and metastasis of many human cancers (such as breast, colorectal, and lung) by modulating many biological molecules (such as multiple receptor growth factors, inflammatory cytokine kinase enzymes, and transcription factors) (Troselj and Kujundzic, 2014; Banik *et al*., 2017; Gupta *et al*., 2019; Komal *et al*., 2019). However, no study has been examined the anti-cancer effects of CUR on FMGTs in the literature, yet. Therefore, in the present study, we aimed to evaluate the anti-proliferative and apoptotic effect of CUR at different concentrations in FMGT primary cell lines from two cases, for the first time.

Feline mammary gland tumors are the third most common tumors in cats with high malignancy rates (80-96%) and are primarily observed in middle-aged to old female cats (MacEwen, 1990; Moulton, 1990). Endogeneous and exogeneous progestins, breed and familial predispositions, reproductive status (spayed/intact), and age at ovariohysterectomy are reported as major factors in the pathophysiology of

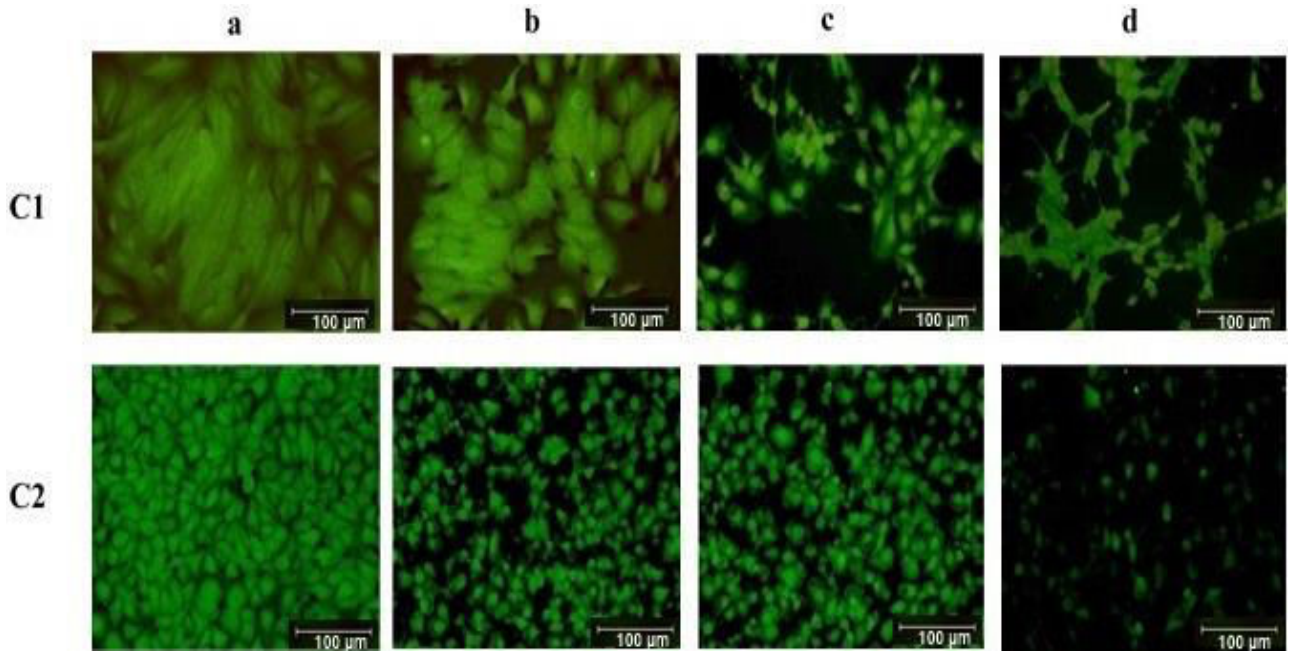

Fig. 5: Dose-dependent effect of CUR on cell morphology of two primary cell lines (C1, C2) from feline mammary gland tumors (FMGTs) after 24 h determined by acridine orange (AO) staining. Images were taken following the cells treatment with (a) Control, (b) 0.1, (c) 0.5, and (d) 1 mM (CUR) after 24 h at 510 ± 42 nm green filter. C1: Case 1, and C2: Case 2

FMGTs (Overley *et al*., 2005). It is hypothesized that under the influence of prolonged sex-steroid hormones, hyperproliferation of mammary epithelial cells starts and progresses to a preneoplastic state. Then, it grows and transforms into an invasive carcinoma, which breaches the basement membrane (Russo and Russo, 1998). Case 1 was a spayed cat, however, the age of the cat at spaying time was 5. Prolonged sex-steroid exposure might be the reason for the mammary gland tumor of the cats in this study. Tumor size, lymph node involvement, histologic tumor subtype and grade, clinical stage, the existence of metastases, the extent of surgical intervention, estrogen receptor status, expression of human epithelial receptor-2, PCNA, argyrophilic nucleolar organizer region, and Ki-67 are the most important prognostic factors in FMGT cases (Giménez *et al*., 2010; Zappulli *et al*., 2015). Other relevant histologic parameters including nuclear and cellular atypia, necrosis, lymphovascular emboli, lymphoplasmacytic nfiltration, and adhesion to the underlying tissue also have prognostic values (Zapulli *et al*., 2015). In the presented study both mammary neoplasms were malign, and they were both classified as carcinoma-simple, tubular type (grade III). The nuclear pleomorphism, wide tumor necrosis, and lymphovascular emboli were obtained in both C1 and C2. Furthermore, we analyzed PCNA expression in these tumors. Proliferating cell nuclear antigen is an important factor for the replication of DNA, and therefore PCNA inhibition is accepted as a new anticancer strategy (Wang, 2014). For this reason, the anti-PCNA antibody was used for immuno-histochemistry to determine the replication rate. Interestingly, our findings showed different PCNA expressions in these tumors, although these neoplasms were classified and graded similarly. According to Prezios *et al*. (1995), PCNA expressions are not correlated with nuclear grade in dogs or cats, and the mitotic index of the cat needs further investigation because they could not determine a significant difference between benign and malignant growths in the number of mitoses in the cat, which can present the diversity in FMGT. In this context, further investigations are required to identify new biomarkers in biological behaviors (cell cycle, apoptosis ratio, etc.) of FMGT and a better understanding of different response rates to treatment.

Mammary gland tumors are commonly revealed in dogs and cats and have many similarities to human breast cancers. The current treatment options (drug combinations, radiotherapy, or mixed therapy) of FMGTs (especially in carcinomas) are insufficient due to the recurrence in approximately 80% of the patients resulting in death (Sorenmo *et al*., 2003; Song *et al*., 2019). For this reason, there is an urgent need to discover new effective alternative treatments for FMGTs. Some studies have shown that CUR is a potential alternative therapeutic candidate as an effective anticancer drug that regulates different signaling pathways and molecular targets involved in cancer development (Giordano and Tommonaro, 2019). Curcumin exhibits an immuno-modulatory effect through suppressing the AP-1 and NF-κB activities as proinflammatory transcription factors in the proliferation of breast cancer cells (Sethi and Tergaonkar, 2009; Shanmugam *et al*., 2015). Furthermore, CUR suppressed the expression of human epidermal growth factor receptor 2 (HER2) in breast cancer (Yim-im *et al*., 2014). Likewise, CUR can activate autophagy and the ubiquitin-proteasome pathway via suppressing the PI3K/Akt signaling pathway and regulate cancer cell growth and proliferation (Tan *et*

al., 2014; Guan *et al*., 2016). In addition to apoptotic effects, CUR exhibited anti-invasive activities through the downregulation of matrix metalloproteinase-2 (MMP-2) and the upregulation of TIMP metallopeptidase inhibitor 1 (TIMP-1) in MDA-MB-231 breast cancer cells (Shao *et al*., 2002). As an alternative potential treatment option, CUR has antitumor activity in different subtypes of human breast cancer cells (Giordano and Tommonaro, 2019; Guney Eskiler *et al*., 2019; Guney Eskiler *et al*., 2020). There is a study investigating the CUR efficacy in canine mammary tumors (CMT) in terms of veterinary medicine. In a study of CMT, CUR treatment in combination with cyclophosphamide exerted a strong synergistic growth inhibitory effect on CMT primary cells (Alkan *et al*., 2014). Furthermore, the cytotoxic effects of CUR on canine breast carcinoma cell lines through Caspase activation and Annexin-V staining have been determined (Levine *et al*., 2016). However, there is no evidence of the CUR impact on FMGT. According to our findings, CUR had an anti-proliferative and apoptotic effect on FMGT cells through induction of apoptosis and G0/G1 cell cycle arrest. The survival rate of FMGT primary cells significantly decreased dose-dependently and CUR caused an increase in total apoptotic cells and G0/G1 phase arrest in FMGT primary cells compared to our previous findings (Guney Eskiler *et al*., 2019; Guney Eskiler *et al*., 2020). Feline mammary gland tumor cells (F1 and F2 cells) were more sensitive to CUR (IC_{50} was 1.60 ± 1.34 µM, and 0.23 ± 0.75 µM, respectively) than human breast cancer cell lines MDA-MB-231 and HCC1937 (IC50 was 5.35 ± 0.83 µM, and 7.41 ± 1.23 µM, respectively) because a lower concentration of CUR treatment could result in significant cytotoxicity.

In conclusion, this is the first study that shows the effects of CUR on FMGTs in terms of the established similarities between human breast tumors and feline breast tumors in terms of behavior, and histological origin. However, further molecular studies are required to compare the effects of CUR on different histopathological phenotypes. Besides, further molecular mechanisms including the potential apoptotic and cellular pathways affected by CUR could be elucidated. CUR-induced apoptosis in FMGT cells rather than other types of death could be elucidated by further molecular analysis.

Conflict of interest

The authors declare that there is no conflict of interest.

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