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Citrate and celecoxib induce apoptosis and decrease necrosis in synergistic manner in canine mammary tumor cells

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Abstract

Celecoxib and citrate have been shown to possess antitumor activity in a variety of cancer cells. However, the antitumor activities of these agents in canine mammary tumors have not been well demonstrated. The aim of our study was to investigate the apoptotic and antiproliferative effects of citrate and celecoxib, individually and in combination, on canine mammary tumor cell line CF41-Mg. MTT assay was performed to determine cell viability, and Annexin-PI test was performed to evaluate apoptosis induction. MTT assay results revealed that compared with the control groups, treatment groups, as both single and combined treatments, showed significant inhibition of tumor growth in a dose-dependent manner. IC_{50} concentrations of citrate and celecoxib were defined 26mM and 22µM, respectively. In another set of experiment, significant increase in cell apoptosis was observed at IC_{50} concentrations of citrate and celecoxib after 48h incubation. In spite of that, simultaneous treatment of cells with citrate and celecoxib eventuated with meaningful toxicity augmentation and induction of apoptosis at lower concentrations. Also necrotic cells were decreased by coadministration of the two agents. In conclusion, the present study indicates significant cytotoxic and apoptotic effects of citrate and celecoxib coadministration on CF41-Mg cells, and proposes new strategies for counteracting cancer cells proliferation and overcoming chemo resistance.

Key words: Canine mammary tumors, Citrate, Celecoxib, Apoptosis.

Introduction

Mammary tumors are the most frequent malignant neoplasms in the female dogs and they make up 52% of all the tumors in this gender (1). Common treatments for these tumors include surgery, radiotherapy, chemotherapy or a combination of these methods. Resistance to current anti-tumor therapy and side effects of the common treatments warrant new remedial approaches to be defined.

Besides the constant production of adenosine triphosphate (ATP), cancer cells use a lot of co-factors such as H^+, NADPH and NAD^+, various macromolecules, lipids, glucose and amino acids (2). Therefore, it seems that interference in the metabolism of cancer cells can be used as one of the therapeutic strategies (3-5). Considering the wide range of effects of citrate on the metabolism of cancer cells such as glycolysis inhibition, enhancement of lipid and glucose production, inhibition of Krebs cycle, depleting the cell's energy and inhibition of NAD^+, H^+ and NADPH, it seems that citrate can be used to slow down or stop the proliferation of cancer cells and to increase the cancer cells death rate by apoptosis and/or necrosis. Among other compounds used in the treatment of cancer, NSAIDs can be referred (6). NSAIDs include a wide range of compounds with different pharmacological properties (7). Celecoxib is one of the specific inhibitors of the cyclooxygenase-2 enzyme which is used to treat pain and inflammation due to osteoarthritis and orthopedic surgeries (8, 9). Unlike the Cyclooxygenase-1 enzyme (Cox-1) which is constantly expressed in most body tissues and controls many physiologic processes, cyclooxygenase-2 (Cox-2) is induced by pre-inflammatory or mitogenic factors and is overexpressed in various types of cancers such as breast and bladder tumors (9-13). The overexpression of the enzyme causes an uncontrolled increase in proliferation, inhibition of apoptosis, increase in angiogenesis and metastasis (14-16).The molecular mechanisms involved in the anti-tumor effect of Cox-2 inhibitor drugs are not completely known. If these substances only act by modulating the expression of Cox_2, their use will be limited to tumors which express a considerable amount of Cox_2. Although some researchers have mentioned the anti-neoplastic effects of these drugs which are independent of the cyclooxygenase enzyme (11, 16-18). Therefore, studying the effects of celecoxib is of great importance and can be beneficial in the treatment of tumors lacking Cox_2 expression. CF41-Mg is a cancerous cell lines which is derived from *Canis familiaris* and currently is in used for investigations of cancer mammary gland (1). Canine mammary tumors have been proposed as a model to study human breast cancer owing to the great number of resemblances between them, from epidemiological data to the histological patterns of the neoplastic lesions. In addition, the same molecular properties such as overexpression of steroid receptors, proliferation markers, epidermal growth factor, p53 suppressor gene mutations, similar metalloproteinases and cyclooxygenases show similarities between the two (19, 20).

Due to the limitations of chemotherapy drugs in veterinary medicine, lack of information about the impact of celecoxib and citrate on canine mammary tumors as well as no Cox_2 expression in CF41-Mg

cancerous cells, it was decided to test new treatment strategies and to study the anti-tumor effects of these two compounds on CF41-Mg cell line.

Materials and methods

Cell culture and treatment

Canine mammary tumor cells CF41.Mg (CRL-6232™) were obtained from National Cell Bank in Pasteur Institute of Iran and were cultured as a single layer in Dulbecco's modified Eagle's medium (Gibco Company, USA) with 10% fetal bovine serum (Gibco Company, USA), 60 $IUml^{-1}$ penicillin G, 100 μgml^{-1} streptomycin and 1.5 μgml^{-1} amphotericin B (Sigma Aldrich Company, USA) and were kept in an incubator at a temperature 37 °C and 5% carbon dioxide concentration. In order to maintain the exponential cell growth the culture medium was replaced every three days and the cells were given passage, after they had reached 80-90% confluency. The TrypLE enzyme (Gibco Company, USA) was used for the passage.

Celecoxib were supplied by Abidi Pharmaceutical Company (Tehran, Iran) and was dissolved in sterile Di-methyl sulphoxide (Sigma Aldrich Company, USA) to make the stoke solution and was filtered using syringe tip filters with 0.2 µm pores (Orange Scientific Company, Belgium). This stoke solution was kept at -20°C. In order to make the desired concentrations (5, 10, 15, 20, 22, 40, 80µM), the stoke solution was diluted using the culture medium. The sodium citrate tribasic solution was obtained from the Sigma Aldrich Company (USA) (pH = 7.5) and different concentrations (5, 10, 15, 20, 26, 40mM) were made using the culture medium. Finally the cells were put in contact with the different concentrations of celecoxib (5, 10, 15, 20, 22, 40, 80µM), citrate (5, 10, 15, 20, 26, 40mM) and a combination of these two drugs (10 and 22µM of celecoxib with 10, 15, 20 and 26mM of citrate) for 48 hours. The control groups for celecoxib, citrate and their mixture received di-methyl sulphoxide (<0.1%), distilled water and a combination of di-methyl sulphoxide and distilled water, respectively.

Immunocytochemistry

Immunocytochemistry was used to further characterize CF41.Mg cells as the previous investigations with minor modification (21). Briefly, the cells were dissociated using trypsin and then were placed on salinized sterile slides. A sufficient amount of culture medium containing fetal bovine serum (FBS) was added to the cells. After 24 hours the culture medium was emptied and the cells were rinsed using PBS. Then cells were fixed using methanol-acetone and then antigen retrieval was performed with Tris-EDTA buffer (pH 9.0). Heating for two 5 min periods in a microwave oven at 750 W, was done followed by cooling at room temperature for 20 min. After rinsing with Tris-NaCl buffer, endogenous peroxidase was blocked by immersion in 3% hydrogen peroxide for 10 minutes. At this stage, the cells were rinsed using Tris-NaCl buffer and then the primary and secondary antibodies (Dako REAL™ EnVision™ Detection System, Rabbit/Mouse, K5007) and finally the Liquid DAB+ Substrate (Dako Company, Denmark) were added. Mouse anti-human -P63 (NCL-p63, Novocastra Company, United Kingdom), -Ki-67 (N1633, Dako Company, Denmark) and -Bcl_2 (610538, BD Biosciences Company, USA) antibodies were used as primary antibodies. Ki-67 and the secondary antibody were ready to use but other antibodies were used at 1/100 concentration. Incubation times for primary and secondary antibodies were 1.5h and 45min, respectively. Slides were counterstained using Hematoxylin Harris stain. As the negative control, the primary antibodies were replaced with irrelevant, isotype-matched antibody to control for nonspecific binding of the secondary antibody. Immunoreactions were analysed using Leica microscope DM500 (Leica Microsystems Company, Germany) and images were taken using ICC50 HD camera (Leica Microsystems Company, Germany). The intensity of immunostaining was graded on a scale of 0-3 where 0 = no staining, 1 = equivocal staining, 2 = moderate to intense staining and 3 = the highest intensity staining.

Analyzing cell morphology

Subsequent to sufficient cell growth in flasks (Orange Scientific Company, Belgium), the cells were disassociated using trypsin and were evenly added to 96 well plates (Orange Scientific Company, Belgium). After an overnight incubation period IC_{50} concentrations of the compounds being studied were added to the wells. The morphological changes of cells were analyzed using the Motic AE31 Elite inverted phase contrast microscope (Hong kong, China) after 48 hours.

MTT assay

When the effective concentration ranges of citrate and celecoxib were determined using Trypan blue dye exclusion assay, the cell viability was determined using MTT assay. The basis of the test is the enzymatic reduction of Tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide) to formazan (1-[4,5-dimethylthiazol-2-yl]-3,5-diphenylformazan) using the mitochondria of living cells (22). Cells at a density of 7 x 10^3 cells/200 µl were cultured in a 96 well plate (Orange Scientific Company, Belgium). After overnight incubation the culture medium was removed and was replaced by 200 µl of the new culture medium containing the different concentrations of celecoxib (5, 10, 15, 20, 40, 80µM), citrate (5, 10, 15, 20, 40mM) and a combination of these two drugs (10, 22µM of celecoxib and 10, 15, 20, 26mM of citrate) (5 wells for each concentration). The medium containing different concentrations of celecoxib and citrate was removed after 48 hours and the survival rate of the cells was analyzed using the MTT assay kit (Sigma Aldrich Company, USA). To summaries, 100 µl of the MTT solution (5mg/ml) was added to each of the wells and after 4 hours of plate incubation, the overlying fluid was disposed and 100 µl of DMSO was added to the wells (DMSO increases the permeability of the cellular membrane and solves formazan which causes the spread of the purple color in the well). The plates were shaked a few times and then the optical density of the wells were read at 490nm using the ELISA reader ELX808 (BioTek Company, USA). Finally in order to analyze the effect of different celecoxib and citrate concentrations on the growth of cancerous cells, the dose-response curve was

plotted and the concentration for a 50% inhibition of survival rate (IC_{50}) was calculated.

Annexin-PI test for analysis of apoptosis

The quantitative analysis of the transferred phosphatidylserine to the surface of cells undergoing apoptosis was carried out by the ApoFlowEx® FITC kit (ExBio Company, Czech Republic). The kit was designed to attach Annexin V to the phosphatidylserine transferred from the inner layer of the cell membrane to the outer layer during apoptosis (23, 24). The cells were cultured in 25cm^2 (Orange Scientific Company, Belgium) flasks. After reaching a confluency of 80-90%, the culture medium was removed and replaced by a new medium containing IC_{50} concentrations of celecoxib and citrate (22µM and 26mM, respectively). Another flask was assigned to analyze the amount of induced apoptosis by the celecoxib citrate combination (20mM of citrate and 10µM of celecoxib). After 48 hours of exposing the cells to the compounds, the cells were treated by trypsin and were rinsed at ice cold PBS. At the next step, the cells were suspended in binding buffer and then propidium iodide (PI) and annexin v were added. The suspension then shaked slowly and was incubated for 15 minutes at room temperature in dark. Then annexin v, binding buffer and PI were removed and binding buffer was added again to each tube and the cells were examined using the BD FACSCalibur flow cytometry device (BD Biosciences Company, USA).

Statistical analysis

The differences between groups were evaluated using Students t-test for analysis of MTT data and one-way analysis of variance (ANOVA) for analysis of apoptosis SPSS 16.0 software package. The level of significance was considered as $P<0.05$.

Results

CF41-Mg cells morphology and Immunocytochemistry results

CF41-Mg cells adhered to culture flasks presented mesenchymal-like morphology and characteristics (Fig1. A, B). Morphologic evaluation of these cells on H&E stained smear using invert microscope revealed large spindle cells with some malignant criteria such as nuclear molding, numerous and abnormally prominent nucleoli and increased nuclear/cytoplasmic ratio. Immunocytochemical results using anti-p63, Bcl-2 and ki-67 antibodies have been presented in figure1. The intensity of immunostaining was graded on a scale of 0-3 where 0 = no staining, 1 = equivocal staining, 2 = moderate to intense staining, and 3 = highest intensity staining. As shown in figure 1, CF41-Mg cells were positive to myoepithelial (P63) and proliferation (Ki-67)cell markers, with the highest intensity staining. However, Bcl-2 immunolabelling was equivocal.

Anti-proliferation effects of Celecoxib and Citrate

Celecoxib and citrate were tested to assess their growth-inhibiting action on CF41-Mg cells. After exposure of CF41-Mg cells to different doses of celecoxib (5, 10, 15, 20, 40, 80µM) and citrate (5, 10, 15, 20, 40 mM) for 48 hr, the cell viability was

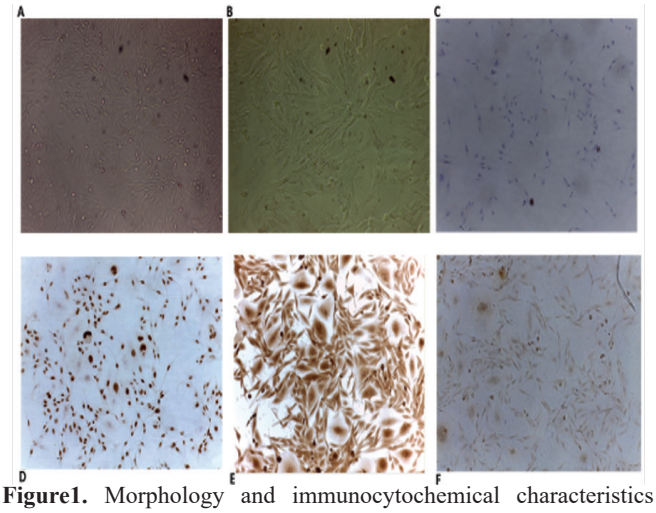

Figure1. Morphology and immunocytochemical characteristics of CF41-Mg cells. A. Low – magnification view, 100×. B. Higher magnification view, 400×. C, D, E and F are presented CF41-Mg cells immunolabelling using isotype-matched, anti-Ki-67 (highest intensity staining=3), -P63 (highest intensity staining=3) and -Bcl_2 (equivocal staining=1) antibodies, respectively.

measured using trypan blue dye exclusion and MTT assays (22). The mean percentages of cell survival in different doses of citrate were 96.97±3.26, 90.02±2.66, 86.04±5.58, 65.75±2.19, 39.99±1.42 and for celecoxib as 95.02±1.34, 90.48±1.86, 74.69±2.34, 55.02±1.09, 37.75±1.13 and 23.08±1.24, respectively. These results suggested that celecoxib and citrate treatment induced a dose-dependent inhibition of cell growth in CF41-Mg cells (Fig2). The IC_{50} values for celecoxib and citrate were considered 22µM and 26mM, respectively.

Celecoxib and Citrate - Induced apoptosis of CF41-Mg cells

Consistent with their anti-proliferative effects, the effects on apoptosis induction were observed after a 48hr exposure to IC_{50} doses of celecoxib and citrate. The apoptotic and necrotic cell number of CF41-Mg cells were presented in table1. As shown in figure3, IC_{50} concentrations of citrate and celecoxib decreased

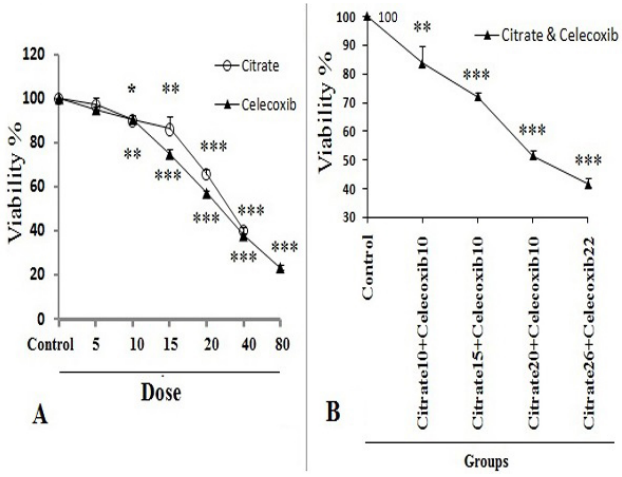

Figure 2. (A) The effects of citrate and celecoxib on the proliferation of the canine mammary tumor cell line (CF41-Mg) via the MTT method. Data are expressed as the percentage of inhibition compared with controls. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (n=5). (B) Antiproliferative effects of coadministration of citrate and celecoxib on canine mammary tumor cell line after 48h incubation. Data are expressed as the percentage of inhibition compared to control. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001 (n = 5)$. Concentration units for celecoxib and citrate are µM and mM, respectively.

Table 1. Effects of citrate, celecoxib and coadministration of these on necrosis, early and late apoptosis of CF41-Mg cells. Data are expressed as mean values ± standard error of means. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Groups	Early apoptotic cells	Late apoptotic cells	Necrotic cells
Control of Citrate	0.70 ± 0.21	5.09 ± 0.53	3.44 ± 0.65
Control of Celecoxib	0.73 ± 0.19	1.67 ± 0.44	6.64 ± 0.68
Control of Citrate & Celecoxib	1.13 ± 0.29	2.63 ± 0.58	6.47 ± 0.52
Citrate	15.97 ± 1.12***	23.50 ± 1.53***	12.15 ± 1.05***
Celecoxib	10.39 ± 2.00**	7.69 ± 0.76*	17.9 ± 0.81***
Citrate & Celecoxib	29.96 ± 3.55***	22.96 ± 2.11***	8.74 ± 0.73

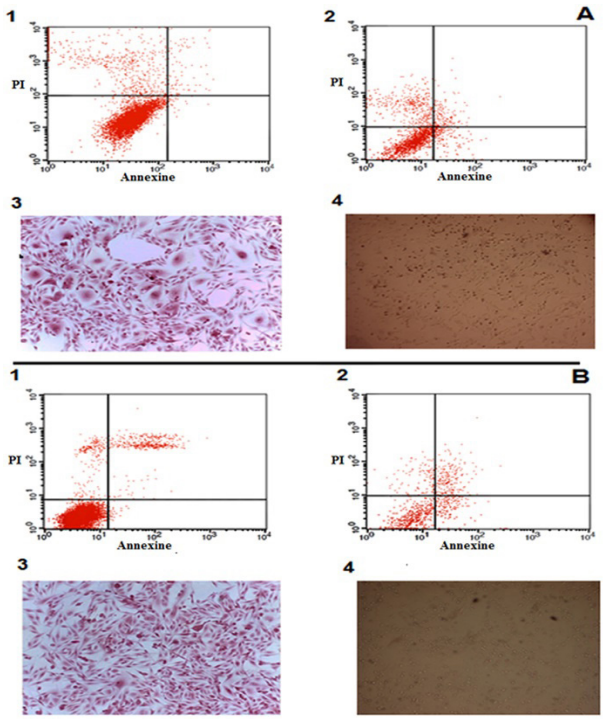

Figure 3. Effects of IC_{50} concentrations of citrate and celecoxib on CF41-Mg cells apoptosis. A_1&B_1: Flow cytometric histogram after CF41-Mg cells-treatment with celecoxib and citrate vehicles (DMSO for celecoxib [A1] and distilled water for citrate [B1]). A_2&B_2: Flow cytometric histogram of apoptotic-induction after 48h incubation by IC_{50} concentrations of celecoxib and citrate, respectively. A_3&B_3: Morphology of CF41-Mg cells after treatment with celecoxib and citrate vehicles, respectively (H&E staining). A_4&B_4: Morphological changes of CF41-Mg cells after treatment using IC_{50} concentrations of celecoxib and citrate, respectively.

the number of viable cells and increased the number of apoptotic cells, as a sum of early and late apoptotic cells, significantly ($p<0.001$).

Morphology of cancerous cells in the treatment and control groups are shown in Figure3. Cellular morphology did not exhibit any significant changes after treatment with distilled water and DMSO. But under the influence of IC_{50} concentrations of citrate and celecoxib, for 48h, apoptotic cells were more frequently seen. The most common apoptotic morphological changes observed in CF41-Mg cells included loss of normal shape and cellular disintegration along with the appearance of spherical cells with cytoplasmic shrinkage.

Citrate enhances the antitumor efficacy of Celecoxib

In another set of experiment, the effect of combined celecoxib with citrate on CF41-Mg cells was evaluated. CF41-Mg cells were exposed to different doses of celecoxib and citrate for 48h, then cell viability and flow cytometric analysis were performed. The findings displayed combined treatment had a synergistic growth inhibitory (figure2) and apoptotic (figures 4,5) effects on CF41-Mg cells ($p<0.001$). In addition, 50% inhibition of cell growth (IC_{50}) is achieved at fewer concentrations of citrate and celecoxib. Reduction in cell necrosis ($p<0.001$) after combined use of citrate and celecoxib was another important result of this study (figures 4, 5).

Discussion

Canine mammary tumors have been proposed as a model to study human breast cancer owing to the great number of resemblances between them, from epidemiological data to the histological patterns of

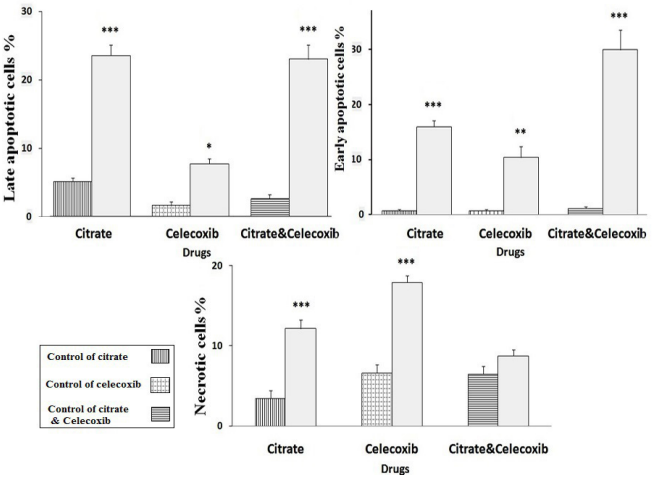

Figure 4. Flow cytometric analysis of vehicle (distilled water for citrate, DMSO for celecoxib and distilled water+ DMSO for citrate + celecoxib group), citrate/celecoxib and citrate + celecoxib treated cells stained with annexin V and propidium iodide (PI). Mean values of three experiments ± standard error of means are shown. *P* values represent significant differences between controls and test groups. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ ($n = 3$).

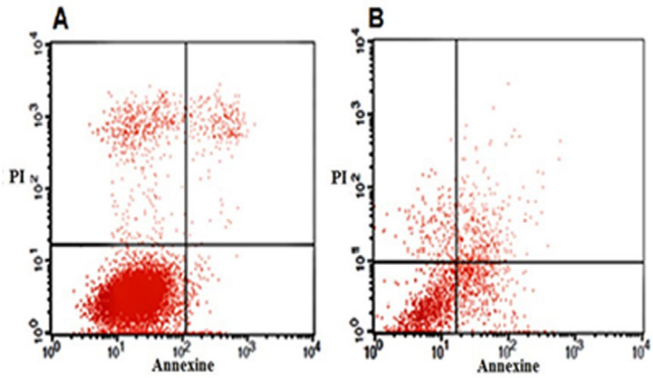

Figure 5. Flow cytometric histogram representative of apoptotic induction on canine mammary tumor cell line by simultaneous using of citrate and celecoxib (B) in comparison with control group (A) after 48h incubation.

the neoplastic lesions. In addition, the same molecular properties such as overexpression of steroid receptors, proliferation markers, epidermal growth factor, p53 suppressor gene mutations, similar metalloproteinases and cyclooxygenases show similarities between the two (19). The present study was performed to find more about the possible underlying mechanisms by which the studied drugs act.

Immunolabelling of CF41.Mg cells using anti-P63, Bcl_2 and Ki-67 antibodies showed that the studied cells, except for Bcl_2, expressed P63 and Ki-67 (Fig1). P63 has proven to be a potential myoepithelial cell marker, useful in the diagnosis to distinguish basal or myoepithelial cells from stromal myofibroblasts in the normal and neoplastic canine mammary glands (25, 26) . Considering the postulated functional role of p63 in normal breast development and maintenance of epithelial stem cell population, the presence of p63 protein which is exclusive to canine myoepithelium, might support a close link between myoepithelial and stem cells (26, 27). The Ki-67 nuclear antigen is one of the most commonly used immunohistochemical markers to evaluate tumor proliferative activity (28). It is expressed in all active phases of the cell cycle (G_1, S, G_2, M) but not in quiescent cells (G_0). In canine mammary tumors, high index values of Ki-67 were positively correlated with metastasis, death from neoplasia, low disease-free survival rates, and low overall survival rate (28). Bcl-2 is considered as an important anti-apoptotic protein and its expression is inversely related to the biologic aggressiveness, distant metastases, proliferation rates (Ki-67 expression) and tumor grade (29, 30). Knowlton et al., (1998) hypothesized that Bcl-2 might slow cell proliferation independently from its antiapoptotic effect, and showed this to be indeed the case in an experimental tumor model (31). This is confirmed by our present finding of an inverse correlation between Bcl-2 and Ki-67 expression. Previous studies have shown the anti-tumor effects of celecoxib and citrate on various cancers such as urinary bladder, gastric and colon cancers (11, 17, 32). Based on these studies, it seems that the cytotoxic effect of these compounds is cell type specific. Despite studies about anti-tumor effects of celecoxib and citrate in a variety of tumors, no report has was found about their effects, either alone or in combination, on the canine mammary tumor cells.

Cancer cells produce large amounts of lipids and macromolecules for cell reproduction and synthesis; these cells have to produce ATP and various co-factors constantly in order to keep the synthesis paths going. These cells also use a lot of glucose, amino acids and lipids (2-4). Citrate is a crucial sensor for energy level and is an important compound in metabolic pathways. It is assumed that citrate can contribute to cell growth inhibition as well as cell death. Of course the type of cell death (necrosis and/or apoptosis) depending on the cell being studied and the intensity of ATP depletion (2, 33). Celecoxib is a specific inhibitor of cyclooxygenase-2 and its anti-tumor activity has been demonstrated against some cancers (9, 11, 34, 35).

According to MTT assay results (figure2), both compounds had a dose dependent inhibitory effect on CF41-Mg cells viability. Citrate and celecoxib at concentrations greater than 10mM and 10μM respectively, could inhibit cell survival significantly ($P < 0.01$ and $P < 0.001$). Meanwhile, 50% inhibition in cell viability was observed at concentrations of 26mM and 22μM for citrate and celecoxib, respectively. 48 hours after cell treatment with IC_{50} concentrations of citrate and celecoxib, cancer cells demonstrated morphologic changes such as cellular disintegration along with the appearance of spherical cells with cytoplasmic shrinkage. To further investigate the inhibitory mechanisms of cell growth of the studied compounds and to determine the amount of apoptosis contribution in reducing the growth of cells, the apoptosis process in control and test groups were examined. Flow Cytometry graphs of cell apoptosis (early and late) following the use of celecoxib and citrate, alone or in combination, have been displayed in figures 3 and 5. Based on the obtained results, both compounds caused significant increase in apoptosis of CF41-Mg cells compared to the control group ($p<0.001$). Apoptosis is the pharmacodynamic endpoint of anticancer drug therapy as this phenomenon ensures that no chemotherapy resistance will occur. Moreover, apoptosis is an autonomous dismantled process to eliminate cellular components and avoids inflammatory effect normally associated with necrosis; thus no toxicity to the normal surrounding cells will occur when cells are subjected to apoptosis (36). The increase of early apoptotic activity conveys existence of static and non-proliferative cells and the increase of late apoptotic activity indicates that cells in the final stages of apoptosis and facing imminent death are present (37).

Different studies have shown that celecoxib has two types of effects. The first one depends on Cox which is achieved at low concentrations (cell growth inhibition and apoptosis induction by increasing IGFBP-3, PGE_2 inhibition, inhibiting MAP-kinase activation, reducing Bcl_2 level and inhibition of Cytochrome C activation) (11, 38) and the other effect is independent of Cox and is achieved at high concentrations (inhibition of NF-κB signaling through inhibition of IKB kinase B and attachment to PPAR nuclear receptors, accumulation of ceramide in cells and apoptosis induction, increase in Bax protein activation, and caspases) (11, 15-17, 35, 39). Therefore, it seems that Cox_2 activity is not always necessary for cell survival, and its inhibition does not lead to growth inhibition of all cancers expressing Cox_2. For example, Dhawan et al. found that different bladder cancer cells responded differently to celecoxib such that the proliferation of TCCSUP cells, which have a moderate rate of Cox_2 expression, is not inhibited. Celecoxib inhibits the proliferation of UMUC3 cells which do not express Cox_2 (11). Lack of relationship between anti-tumor effects associated with Cox_2 inhibitors and the expression level of the enzyme have also been proven in Cox_2 negative hematopoietic cell lines and canine naturally occurring invasive TCC (34, 40). Lack of Cox_2 expression in CF41-Mg cells was found in a survey conducted by Brunelle et al., (2006) (12). Based on the findings of the present study, the mechanism of inhibition of cell proliferation and apoptosis induction in CF41-Mg cells appears to be independent of Cox_2 expression. Further studies are required to discover the details of celecoxib's anti-tumor effects.

Citrate carries out its anti-neoplastic effects in two

ways: energetic and non-energetic. In the energetic path, citrate causes the inhibition of β-oxidation, inhibition of hexoKinase, pyruvate kinase, phosphofructokinase 1 and 2, pyruvate dehydrogenase (PDH) enzymes and succinate dehydrogenase (SDH) blocking which leads to the inhibition of glycolysis and Krebs cycle and eventually the cell energy production (ATP) is severely reduced (2, 32, 41). Inhibiting the hexokinase II enzyme in cancer cells may also stimulate apoptosis (2). Citrate also stimulates fatty acid synthesis and neoglucogenesis process (by increasing fructose 1,6-bisphophatase activity) which leads to the increased use of ATP and NADPH. This in turn leads to the depletion of intra cellular energy, cell growth inhibition and cell death. NADPH and H^+ are also necessary to keep the cytochrome C in reduced form. The lack of reduced cytochrome C causes apoptosis induction inhibition (2, 32). The possible mechanisms for the non-energetic method include the reduced expression of Mcl-1 (anti-apoptotic protein), stimulation of reactive oxygen species formation (ROS) and reducing the redox system activity (2, 32, 42). Icard et al., in a study, exposed two lines of human gastric cancer cells to citrate at 5-20mM concentrations. They concluded that citrate causes apoptotic cell death through the activation of Caspase 9 and the mitochondrial apoptotic pathway, in a time and dose dependent manner. Their proposed mechanism for apoptosis induction by citrate was Bcl-xl inhibition and reduction of Mcl-1 expression (2).

Several studies have shown that citrate and specific inhibitors of Cox_2 can cause cancerous cells to be sensitive to chemotherapy (41, 43-45). Among the proposed mechanisms for this ability of citrate, N-alpha-acetylation of proteins such as caspases, compromising DNA repair after damage caused through chemotherapy, compromising the detoxification of reactive oxygen species (ROS) and nullifying the discharge of chemotherapy drugs from inside the cell, can be noted (2, 41, 42). Cox_2 inhibitors also suppress transmembrane protein ATP dependent efflux pump and therefore cause glutathione transferase inhibition, chemotherapeutic efflux inhibition and decrease tumor cells resistance against chemotherapy drugs (45-49). Our data shows that the use of citrate and celecoxib combined has synergistic antitumor effect against CF41-Mg cells, so that the simultaneous use of two compounds, in addition to the increase in cellular apoptosis and a reduction in necrotic cells ($p<0.001$), can provoke more inhibition of cell proliferation process (figures 2,4,5). In addition, an increase in early and late apoptotic activity in combination therapy shows that apoptosis can be responsible for some of the effects of these compounds.

Given that using these two compounds either alone or together can cause inhibition of proliferation and apoptosis induction, it can be concluded that they can be used for treatment and reducing the tumor load in mammary tumors. However, before the therapeutic use of the compounds, clinical trials are needed to be performed.

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