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Research Article

Evaluation of the Apoptotic Effect of Sinapic Acid in D17 Canine Osteosarcoma Cell Line

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Abstract: Sinapic acid (SA), one of the hydroxycinnamic acid derivatives, has powerful antioxidant and anti-inflammatory characteristics. Moreover, SA has also been shown to have an apoptotic effect against various cancer cells. Here, we investigated the cytotoxic and apoptotic effects of sinapic acid on D17 canine osteosarcoma cell line. We measured the properties of SA on cell viability with the XTT test and found its IC_{50} dose at 750 µmol at 72 h. We analyzed the effects on gene expression of apoptosis pathways by qRT-PCR. qRT-PCR results revealed significant increases in the mRNA level expressions of BAX, CASP3, CASP7, CASP8, CASP9, FAS and P53; whereas, there was a statistically insignificant downregulation in CYCS level and increase in BCL2 level. Our findings show that SA can induce apoptosis in the D17 cell line.

Keywords: *Apoptosis, Canine osteosarcoma, Sinapic acid*

Sinapik Asidin D17 Köpek Osteosarkom Hücre Hattında Apoptotik Etkisinin Değerlendirilmesi

Öz: Hidroksisinnamik asit türevlerinden biri olan sinapik asit (SA), güçlü antioksidan ve antienflamatuar özelliklere sahiptir. Ayrıca sinapik asidin çeşitli kanser hücrelerine karşı apoptotik bir etkiye sahip olduğu da bilinmektedir. Bu çalışmada, sinapik asidin D17 köpek osteosarkom hücre hattı üzerindeki sitotoksik ve apoptotik etkileri araştırılmıştır. SA'nın hücre canlılığı üzerindeki özellikleri XTT testi ile belirlenmiş ve IC_{50} dozu 72 saatte 750 µmol olarak bulunmuştur. qRT-PZR ile apoptoz yolağında bulunan genlerin ekspresyonu üzerindeki etkileri analiz edilmiştir. qRT-PZR sonuçları; BAX, CASP3, CASP7, CASP8, CASP9, FAS ve P53'ün mRNA düzeyinde ifadelerinde anlamlı artış gözlenmiştir. CYCS düzeyinde istatistiksel olarak anlamsız bir baskılanma ve BCL2 düzeyinde ise istatistiksel olarak anlamlı olmayan bir artış tespit edilmiştir. Bulgular, SA'nın D17 hücre hattında apoptozu indükleyebileceğini göstermektedir.

Anahtar sözcükler: *Apoptozis, Köpek osteosarkomu, Sinapik asit*

Introduction

Osteosarcoma (OS) is one of the most common bone malignancy in both dogs and children having tendency for local invasion and pulmonary metastasis and cause death. Dogs oftentimes develop indigenous OS with higher prevalence comparing human. Both dog and human OS have similar aspects including clinical, pathophysiological and therapeutic management. Despite the treatments applied, the survival times have not been improved enough and dogs diagnosed with OS often die due to metastasis with in the first year [1-3].

Phenolic compounds have many positive effects on health because of their antioxidative and antibacterial properties [4]. Previous literature showed that these compounds exhibit anti-allergic, anti-inflammatory, antidiabetic, anti-microbial, antipathogenic and antiviral effects [5,6]. They are often defined as inhibitors and potential antioxidants of harmful oxidative processes associated with diseases

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including inflammation and cancer [7,8]. Sinapic acid (SA; 3,5-dimethoxy-4-hydroxycinnamic acid) exists in free or esters form. Hydroxynamic esters present as esters of sugar (glycosides) or other various organic compounds [9]. SA, as a phytochemical substance, originates from various plants including spices, citrus fruits, vegetables, cereals, oilseed crops and strawberry [5,10].

Oxidative stress is crucial for aging and physiopathology of several diseases such as cancer, atherosclerosis, diabetes and neurodegenerative disorders. It was stated that dietary antioxidants can protect against these oxidative stress related diseases [11,12]. Previous *in vitro* studies indicated that SA plays a role in cancer therapy. These anti-carcinogenic effects of SA are mainly attributed to its ability to remove free radicals and stimulation of cytoprotective enzymes [6,13-15]. The objective of this study was to evaluate anti-cancerogenic properties of SA in D17 canine osteosarcoma cell. For this purpose, the cytotoxic effect of SA in OS cells was determined and the expression levels of apoptosis related genes were investigated.

Material and Methods

Ethical Statement

Since this is an *in vitro* study and only cell line was used, there is no need for ethical approval.

Cell Line

D17 (ATCC® CRL-8468) osteosarcoma cell line was purchased from ATCC and were cultured in EMEM media containing 10% FBS, 1% L-glutamine, 100 IU/mL penicillin and 10 mg/mL streptomycin in a cell culture. The proliferation, passages and follow-up of the cells were monitored with an inverted microscope and the cells were maintained at 37°C in humidified atmosphere containing 5% CO_2.

Cytotoxicity Assay

In order to evaluate IC_{50} dose and cytotoxic effect of SA, XTT assay was used as previously described [8]. Briefly, approximately 1000 D17 cell/well were seeded in 96-well plate and treated with different doses of SA (250-4000 µmol) for 24, 48 and 72 h. After addition of XTT [2,3-bis (2-Methoxy-4-nitro-5-sulfophenyl) 2H tetrazolium-5-carboxanilide] solution, cell viability was determined with at 450 nm using a microplate reader.

RNA Isolation, cDNA Synthesis and qRT-PCR Analysis

Total RNA was isolated using TRIzol reagent (Invitrogen, USA), and Transcriptor First Strand cDNA Synthesis Kit (BIO-RAD) was used for cDNA synthesis. The primer sequences of apoptosis pathway and housekeeping genes were presented in *Table 1*. A 20 µL qRT-PCR reaction mix was set up including 10 µL 2X SyberGreen Supermix, 5

Table 1. *Primers sequences used for qRT-PCR analysis*

Gene	Primer Sequence	PCR Product Size (bp)
BAX	F:5-AGCAAACTGGTGCTCAAGG-3 R:5-GTGTCCCAAAGTAGGAGAGGA-3	151
*BCL2**	F:5-GTGGATGACTGAGTACCTGAAC-3 R:5-GAGACAGCCAGGAGAAATCAA-3	125
CASP3	F:5-CTCGGTCTGGTACAGATGTAGA-3 R:5-GCTTAGAAGCACGCAAACAAA-3	173
CASP7	F:5-TTTGTGCAGGCCCTGTG-3 R:5-CACATGGGATCTGCTTCTTCTC-3	150
CASP8	F:5-CTGACCTCTTACTTCACTGGTTC-3 R:5-GGACATCTTTCTCTTAGGCTCTG-3	296
CASP9	F:5-GGAAGCCCAAGCTCTTCTTTA-3 R:5-GGAGTGGGCAAACTAGACAC-3	184
CYCS	F:5-AAAGGGAGGCAAGCACAA-3 R:5-GGGATTCTCCAAATACTCCATCA-3	150
FAS	F:5-GCATGGCTTAGAAGTGGAAAG-3 R:5-CTCAAGGATTCCATGTTCACAC-3	136
P53	F:5-TGAGGAGGAGAATTTCCACAAG-3 R:5-TCAGCTCCAAGGCTTCATTC-3	140
*RPL32**	F:5-GGCACCAGTCAGACCGATATG-3 R:5-TGCGCACCCTATTGTCAATG-3	75
*YWHAZ**	F:5-TGTAGGAGCCCGTAGGTCATCT-3 R:5-TTCTCTCTGTATTCTCGAGCCATCT-3	102

**BCL2* [16], *RPL32* [17] *and YWHAZ* [17] *primers were obtained from previous literature. Other primers were designed in this study*

pmol of each primer and 2 µL cDNA. PCR amplifications were carried out with an initial denaturation at 95°C for 10 min, then 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. All resulting PCR products were evaluated using melting curve analysis and agarose gel electrophoresis.

Statistical Analysis

qRT-PCR data were normalized using Ct values of housekeeping genes (RPL32 and YWHAZ) and $2^{-\Delta\Delta Ct}$ values were calculated to compare fold change expression levels of control and treatment groups. Student *t*-test was used to assess statistically significant differences.

Results

Cytotoxic Effect of SA in D17 Cells

XTT assay was used to evaluate the ability of SA to inhibit the proliferation of D17 cells. *Fig. 1* illustrates that SA exhibited time- and concentration-dependent manner cytotoxic effect in osteosarcoma cells. The IC_{50} of SA in D17 cells was found to be 750 µmol for 72 h. Thus, dose of 750 µmol was used for in D17 cell in the following experiments.

Semiquantitative Real-Time PCR Analysis

Steady-state level expressions of apoptosis genes were evaluated using semiquantitative real time PCR in 750 µmol SA treated D17 cells and compared to the control. SA treatment in D17 cells resulted in the upregulated expressions of CASP3, CAS7, CASP8, CASP9, BAX, FAS and P53 ($P<0.05$). Increased BCL2 and downregulated CYCS expressions were observed but these differences were not statistically significant *(Fig. 2*, $P>0.05$).

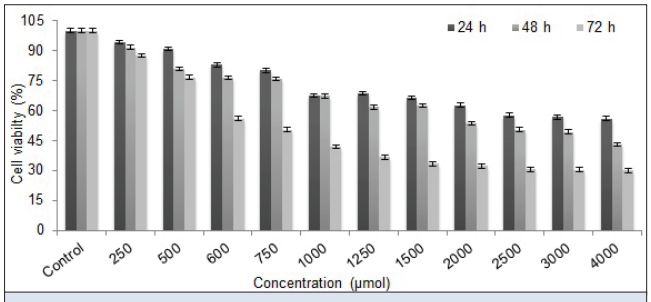

Fig 1. Effect of SA on the viability in the D17 cell. The cells were treated with SA and at different concentrations and time intervals and anti-proliferative effect was assessed by XTT assay. IC_{50} dose of SA in D17 cell line was found to be 750 µmol

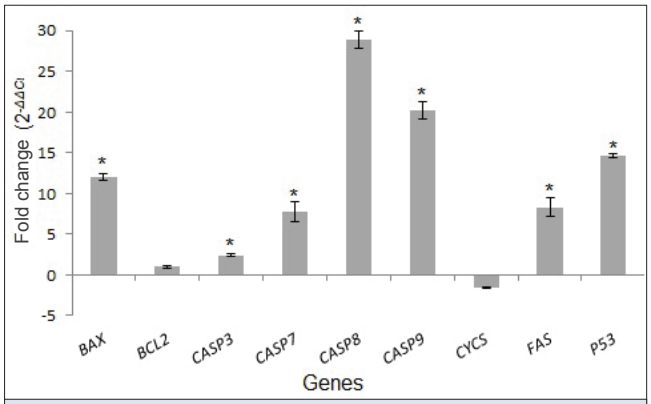

Fig 2. The changes in expression of genes that play an important role in apoptosis relative to the control group after SA treatment in D17 cell. * indicates statistically important ($P<0.05$) difference

Discussion

Using raw estimates of cancer incidence, 6 million new dog cancers cases are thought to be diagnosed each year [18] including lymphoma, adenocarcinoma, squamous cell carcinoma, osteosarcoma, mucosal melanoma, urothelial carcinoma and mast cell tumors [19,20]. These malignancies show strong similarities to human cancers, including histopathology, molecular mechanisms and therapy protocols [21]. According to the bone tumor database, OS is responsible for 98% of 1273 appendicular primary bone tumors in dogs [22]. Along with advancing science and technology, studies have focused on exploring complementary therapies as well as classical therapies in the fight against cancer. Regular consumption of whole grain products, which are natural antioxidant sources, reduces the risk of many chronic diseases such as diabetes, cardiovascular diseases and cancer [8].

In this study, the apoptotic effects of sinapic acid, which is known to have high antioxidative capacity and abundant in all cereal products, fruits and vegetables, in D17 canine osteosarcoma cell line were investigated. IC_{50} dose was determined by XTT test. Then qRT-PCR analysis of 9 different genes was performed to investigate the apoptotic effect of sinapic acid at the determined dose. Also, melting curve analysis was conducted and the resulting PCR products were electrophoresed using agarose gel.

In a previous study, the apoptotic effect of SA in PC-3 and LNCaP human prostate cancer cell lines was investigated. According to the XTT test results, the best half maximal inhibitory dose (IC_{50}) was 1000 µmol at 72 h [8]. In a previous literature, the effect of phenolic acids on LDL oxidation was investigated. It was reported that caffeic acid protects LDL against oxidation at 5 µmol (IC_{50} dose) and sinapic acid at 10 µmol (IC_{50} dose), preventing both hydroperoxide formation increasing apoprotein negative charge [23,24]. Liang et al.[25] examined the effect of gallic acid, which is defined as the main active fraction in herbal medicinal plants, on the growth inhibition of various cancer cells. According to the study, *in vitro* and *in vivo* anti-cancer properties of gallic acid on two human osteosarcoma cell lines were confirmed. It was stated that gallic acid induced apoptosis depending on the dose and time manner in human osteosarcoma cells. Walters et al.[26] investigated the anti-cancer effects of curcumin, another phenolic compound, against OS cells using vitality and apoptosis assays. It was reported that curcumin stimulates

apoptosis in OS cells [26,27]. It was showed that canola phenolic acid, orange peel extract (O-PMF) and O-PMF + Limonoid (1: 1) have the potential to inhibit OS growth in dogs [28]. In the present study, findings illustrated that SA can also inhibit D17 canine OS growth.

One of the most important tumor suppressor genes in the apoptosis pathway is P53. P53 plays a negative regulator role for cellular proliferation and suppresses cell growth and transformation [29]. Cell cycle and DNA replication were impaired in P53 loss of function. If this disruption cannot be repaired, it is resulted in increased BAX, FAS and APAF-1 expression and suppressed BCL2 and BCL-XL expression, thereby inducing apoptosis [30]. P53 mutations have involved pathogenesis and development of different cancers. Johnson et al.[31] reported that the P53 gene plays an important role in the generation of OS as a potential determinant of clinical prognosis, therapeutic response and therapy options. In the present study, it was observed that P53 level is elevated and promotes the cancerous cell to apoptosis.

Mainly, two molecular signal pathways lead to apoptotic cell death. The first, intrinsic pathway is associated with mitochondria. The second, extrinsic pathway involves binding of proapoptotic ligands to cell surface death receptors (DRs). Both initiates the enzymatic caspase cascade [32]. Wang and Youle, emphasized that levels of anti-apoptotic genes such as BCL2 should be decreased; however, proapoptotic genes for instance BAX should be increased in the mitochondrial membrane of cells undergoing apoptosis [33]. Eroğlu et al.[8] conducted qRT-PCR analysis to evaluate the effect of SA on the PC-3 and LNCaP human prostate cancer cell lines. According to the findings, it was stated that the upregulated CASP3, CASP7, BAX and CYCS expressions were statistically significant [8]. In the present study, the effects of sinapic acid on apoptotic and anti-apoptotic genes in D17 cells were evaluated at the mRNA level. Significant increases were observed in BAX (12-fold, P=0.000007), CASP3 (2.44-fold, P=0.0021), CASP7 (7.81-fold, P=0.0020), CASP8 (28.94-fold, P=0.0001), CASP9 (20.25-fold, P=0.00007), FAS (8.28-fold, P=0.0005) and P53 (14.62-fold, P=0.00001). Moreover, an increase in BCL2 (P=0.97) and a decrease in CYCS expression level (P=0.509) were found to be statistically insignificant.

In conclusion, it has been observed that SA, which is known to have strong antioxidative activity, shows apoptotic effect in D17 canine osteosarcoma cell line. Thus, SA have potential to act as an anti-cancer agent in canine osteosarcoma cells by showing anti-proliferative and apoptotic effects.

Availability of Data and Materials

The authors declare that the data that support the findings of this study are available from the corresponding author (Z. Bulut), upon reasonable request.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

ZB and HK designed the project. HK and CEG carried experiments. HK, CEG, MN, EK and ZB performed statistical analysis of data and wrote the article.

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