ORIGINAL ARTICLE

Eastern Journal of Medicine
East J Med 29(1): 18-21, 2024
DOI: 10.5505/ejm.2024.25564

Esculetin induce apoptosis in Human Cervical Cancer Cells

Tuğçe Duran[1*], Gözde Şahin[2], Ayşegul Kebapcilar[3], Çetin Çelik[4]

ABSTRACT

Despite the development of effective HPV screening and vaccination programs in women, cervical cancer ranks first in cancer-related deaths. In order to prevent the side effects of chemotherapeutic agents, studies on the use of natural compounds in the treatment of cervical cancer are becoming increasingly common. Esculetin, a coumarin derivative from natural compounds, is a pharmacological substance with anti-inflammatory, antioxidant, anti-tumor, neuroprotective effects. In various studies, esculetin has been found to induce apoptosis in colon cancer, breast cancer, and cervical cancer via JNK, ERK, ROS mediated mitochondrial pathway or ER stress activation pathways.

HeLa cells and HEK cells were cultured. MTT analysis was performed to determine the IC50 dose of the esculetin. Expression levels of apoptotic genes (BAX, BAK, BAD, APAF-1 and p53) and antiapoptotic genes (BCL-2, BCL-XL) were detected by qPCR.

The IC50 dose at which esculetin inhibited the viability of HeLa cells was determined as 800 µM. In addition, esculetin down-regulated antiapoptotic genes BCL-2 and BCL-XL, while up-regulated apoptotic genes such as BAK, APAF-1, p53, BAD, BAX.

Current findings showed that esculetin has apoptotic effects on HeLa cells.

Keywords: Apoptosis, cervical cancer, Esculetin, HeLa cell

Introduction

Cervical cancer is the fourth most common cancer among women. The HPV virus persistent infections, HPV 16 and HPV 18, are responsible for more than half of cervical cancer. Despite effective screening, vaccination and treatment strategies, cervical cancer is among the leading causes of death.

Natural compounds obtained from plants are being investigated in cancer treatment studies because of their low side-effect profile and toxicity. In recent years, apoptosis has come to the fore in cancer treatment strategies. Anticancer treatments and chemotherapeutics, activate different apoptotic mechanisms to eliminate tumor cells. Mitochondrial dysfunction is known to induce apoptosis in various cancer cells (1).

Esculetin is a herbal coumarin derivative. It is known that esculetin has anti-inflammatory, antioxidant, anticancer and neuroprotective effects, and its use in pharmacological treatment is advantageous due to its good tissue distribution and low side-effect profile. Esculetin has been found to induce apoptosis in various cancers (2).

In our study, we evaluated that esculetin may induce apoptosis in human cervical cells and can have anticancer activity.

Materials and Methods

In order to prove the apoptotic effect of esculetin in human cervical cancer cells (HeLa), the effective MTT dose (IC50) was determined. Expression levels of apoptotic and antiapoptotic genes were investigated by applying qPCR to intrinsic elements on the apoptosis pathway.

Cell culture: In our study, HeLa cell line and human embryonic kidney cell line (HEK293) were used as a control group. HEK 293 cell culture is used because it is easy to culture, is human original, is suitable for genetic treatments and its fast response time. The HeLa cell line was obtained from the American Type Culture Collection (ATCC), HeLa cell with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Germany) and 5% CO_2 in a humidified incubator at 37 °C with 1% penicillin/streptomycin solution. Cells were passaged and stored when they covered 80% to 90% of the flask surface.

MTT proliferation analysis: MTT proliferation analysis was performed with a standard method

*Corresponding Author: Gozde Sahin, Basaksehir Cam and Sakura City Hospital
E-mail address: sahin.gozde1983@gmail.com, Telephone: + 90 05337609568
ORCID ID: Tuğçe Duran: 0000-0002-7353-4527, Gözde Şahin: 0000-0003-3067-9125, Ayşegul Kebapcilar: 0000-0002-4188-2199, Çetin Çelik: 0000-0001-6165-5092
Received: , Accepted:

Table 1: Nucleotide Sequences of Primers Used For qPCR Analysis

Gene	Oligonucleotide sequence (5'-3')	Amplicon size
Bax	F-CCCGAGAGGTCTTTTTCCGAG R-CCAGCCCATGATGGTTCTGAT	155
Bad	F-CCCAGAGTTTGAGCCGAGTG R-CCCATCCCTTCGTCGTCCT	249
Bak	F-CATCAACCGACGCTATGACTC R-GTCAGGCCATGCTGGTAGAC	192
p53	F-CAGCACATGACGGAGGTTGT R-TCATCCAAATACTCCACACGC	125
Bcl-2	F-GGTGGGGTCATGTGTGTGG R-CGGTTCAGGTACTCAGTCATCC	89
Bcl-XL	F-GAGCTGGTGGTTGACTTTCTC R-TCCATCTCCGATTCAGTCCCT	119
APAF-1	F-AAGGTGGAGTACCACAGAGG R-TCCATGTATGGTGACCCAT	116
GAPDH	F-GGAGCGAGATCCCTCCAAAAT R-GGCTGTTGTCATACTTCTCAT	197

Table 2: Mtt Analysis. The effective IC50 Dose of the Esculetin

	ESCULETIN ADMINISTRATION DURATION			
CONCENTRATION	0 hour	24 hour	48 hour	72 hour
2,5 uM	2,73305	2,67665	2,848567	0,606817
5 uM	2,249817	2,37245	2,7159	0,6927
25 uM	2,864117	2,15585	2,1704	1,158117
50 uM	2,007067	2,0102	2,0490	1,14845
100 uM	2,9728	1,856967	2,0390	1,443817
200 uM	3,138033	1,865717	2,1909	1,084383
400 uM	2,48665	1,986083	1,8770	1,217217
800 uM	2,51545	2,080833	1,1180	1,060733
1000 uM	2,183633	1,557083	1,8361	0,80485
Control	3,053617	2,042667	2,2072	1,88935

for the viability of the cervical cancer cell and the effective IC50 dose of the esculetin. HeLa cells were seeded with 100 µl of fresh culture medium per well in 96-well plates (~4.5-5x10^3 cells). After culturing for 48 hours, cells were treated in esculetin wells at adjusted doses for 24, 48, and 72 hours at concentrations of 5 µM, 10 µM, 12.5 µM, 20 µM, 22.5 µM, 25 µM, 30 µM, and 50 µM. Esculetin-treated and untreated control cells were treated with 10 µl of 12 mM MTT solution (Sigma-Aldrich, Germany) and incubated at 37°C for 4 hours. The medium was then removed from the medium in a dark cell culture medium. DMSO (50 µl) was added to the wells and gently shaken for 20 minutes to dissolve the crystal blue solutions. The absorbance at 575 nm was recorded using the Multiskan Sky Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and the IC50 dose of the esculetin was determined to be 800 µM by MTT analyse .(**Table 2**).

Administration of IC50 active dose to cells, total RNA isolation and cDNA synthesis: HeLa and HEK293 cells were reprocessed 48 hours after treatment with an active IC50 dose of 800 µM esculetin in a cell culture medium. All cell groups, including the control group, were subjected to the classical RNA isolation procedure by applying the TRIzol (Sigma-Aldrich), chloroform and isoamyl alcohol method. The RNA pellet was precipitated with 75% ethanol (96%, v/v) and dissolved in nuclease-free water.

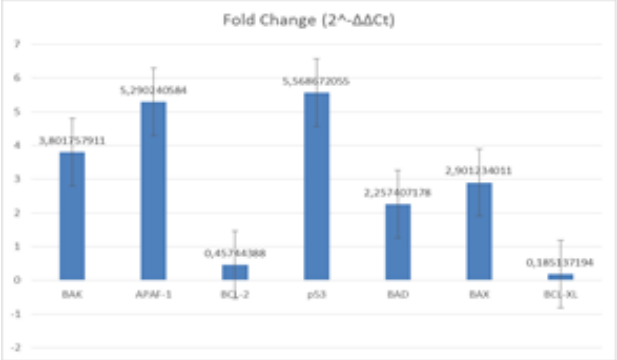

Fig. 1. Quantitative changes of genes showing increase and decrease

Fig. 2. Qualitative changes of genes showing increase and decrease

cDNAs were synthesized according to the manufacturer's instructions using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) from total RNAs equalized to 1 µg according to the manufacturer's instructions.

Quantitative Real Time PCR (qPCR): A qPCR analysis for all genes was performed in triplicate with the QuantStudio™3 Real Time PCR system (Applied Biosystems). Expression of *Bcl-2*, *Bcl-XL*, *BAK*, *APAF-1*, *p53*, *BAD*, *BAX* genes was evaluated using primers (Table 1). A thermal profile followed by melting curve analysis steps were performed at 95 °C for 15 minutes, 40 cycles at 95 °C for 15 seconds, 56-60°C for 30 seconds, and 72 °C for 15 seconds. The experiments are repeated 2 times

Statistical Analysis: Comparative Livak's ΔΔCT method (7) was used to calculate relative gene expression and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as housekeeping gene were used to normalize gene expressions. Cervical cancer (esculetin-treated) and control (untreated) comparisons within groups (HeLa and HEK293) were analyzed with the Statistical Package for the Social Sciences software, version 21 (IBM SPSS Corp.; Armonk, NY, USA) using the student's t-test. After normalization, ΔCt values were used in the analysis. Tests were considered a baseline significance level of $p<0.05$.

Results

Esculetin was found to inhibit the viability of HeLa cell lines in vitro. To show the effects of esculetin on cell viability; HeLa cells were exposed to different concentrations of esculetin (5, 10, 12.5, 15, 20, 22.5, 25, 30 and 50 µM) for 24, 48 and 72 hours and MTT analysis was performed for cell viability. The results showed that esculetin significantly blocked the viability of HeLa cells in a dose-dependent manner. Using these analysis results, the IC50 dose of esculetin was determined as 800 µM for 48 hours and it was shown that esculetin had a strong effect on the viability of HeLa cells at this dose (Table 2).

The apoptotic effect of esculetin on HeLa cells was evaluated by qPCR analysis of apoptotic genes such as *BAK*, *APAF-1*, *p53*, *BAD*, *BAX* and antiapoptotic genes such as *Bcl-2*, *Bcl-XL*. As seen in Figure-1, after esculetin administration in HeLa cells, antiapoptotic genes (Bcl-2 and Bcl-XL) were down-regulated, while apoptotic genes (BAK, APAF-1, p53, BAD and BAX) were up-regulated. The highest gene expression changes after esculetin we applied were on BAK, APAF-1, p53 and BAX (Figure-2).

Discussion

Cervical cancer is the fourth most common cancer among women after breast, lung, and colorectal cancers. Despite the use of Pap smear and HPV tests in screening and effective HPV vaccination, cervical cancer is among the deadly cancers (3). The high side effects and toxicity profiles of chemotherapeutics used in advanced disease have recently increased the interest in treatment with substances obtained from natural compounds.

Esculetin is a coumarin-derived compound found in plants such as cichorium intybus (Asteracea), artemisia capillaries (Compositae), and citrus limonia (Rutaceae) (4). Esculetin has antiproliferative effects against a wide variety of cancer cells. Esculetin also induces apoptosis through various signaling pathways.

Apoptosis is programmed cell death, and induction of apoptosis is one of the cancer treatment mechanisms. The BCL-2 protein family plays an important role in the control and regulation of the mitochondrial apoptotic pathway (5). The main feature of apoptosis is the release of cytochromes from mitochondria, which is regulated by the balance between proapototic and

antiapoptotic proteins of the BCL-2 family, initiator caspases such as caspase 8,9,10 and effector caspases such as caspase 3,6,7. Apoptosis results in the breakdown of the nuclear membrane by caspase 6 and the cleavage of many intracellular proteins and genomic DNA into nucleosomal structures. BAX is a pro-apoptotic protein that activates caspase 3 by inducing the permeability of the mitochondrial outer membrane and releasing cytochrome c into the cytoplasm, while BCL-2 inhibits apoptosis by preventing mitochondrial membrane permeability (6).

In this study, we determined that proapoptotic gene expression (BAK, APAF-1, P53, BAD, BAX) increased and antiapoptotic gene expression (Bcl-2, Bcl-XL) decreased in HeLa cells treated with esculetin (Figure-1, Figure-2). The highest gene expression changes after esculetin we applied were on BAK, APAF-1, p53 and BAX (Figure-1).

In conclusion,our study showed that esculin may have anti-proliferative effects and down-regulate anti-apoptotic genes by inhibiting viability in cervical cancer cells and increasing the expression of apoptotic genes such as BAK, APAF1, P53 and BAX. These findings indicate that esculetin may be a compound with anti-proliferative and apoptotic activity against cervical cancer cells, but also prove that it may be a new pharmacological agent for the development of herbal-derived cervical cancer therapies with a low side-effect profile.

Ethical Consent: Ethics committee approval was obtained from Selcuk University Faculty of Medicine Clinical Research Ethics Committee with the decision number 25 dated 16/01/2019.

Conflict of Interest: The authors have no conflict of interest regarding this study.

Financial Disclosure: This study was funded under the University BAP project.

Author Contributions: Concept (TD), Design (GŞ, TD), Data Collection and/or Processing (GŞ, TD, NK), Analysis and/or interpretation (GŞ, TD, AK, ÇÇ).

References

1. Cao A, Li Q, Yin P, et al. Curcumin induces apoptosis in human gastric carcinoma AGS cells and colon carcinoma HT-29 cells through mitochondrial dysfunction and endoplasmic reticulum stress. Apoptosis 2013; 11: 1391–1402.
2. Linlin Zhang |,Qingxuan Xie , Xiaofang Li. Esculetin: A review of its pharmacology and pharmacokinetics. Phytotherapy Research. 2022;36:279–298.
3. 3-Buskwofie A, David-West G, Clare CA. A Review of Cervical Cancer: Incidence and Disparities. J Natl Med Assoc. 2020; 112(2): 229-232.
4. 4- Chang WS, Lin CC, Chuang SC, Chiang HC. Superoxide anion scavenging effect of coumarins. Am J Chin Med. 1996; 24: 11–17.
5. 5- Shan M, Fan TJ. Cytotoxicity of carteolol to human corneal epithelial cells by inducing apoptosis via triggering the Bcl-2 family protein-mediated mitochondrial pro-apoptotic pathway. Toxicol In Vitro. 2016; 35: 36-42.
6. 6- Childs AC, Phaneuf SL, Dirks AJ, Phillips T, Leeuwenburgh C. Doxorubicin treatment in vivo causes cytochrome C release and cardiomyocyte apoptosis, as well as increased mitochondrial efficiency, superoxide dismutase activity, and Bcl-2:Bax ratio. Cancer Res. 2002; 62(16): 4592-4598.
7. 7- Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. methods, 25(4), 402-408.