The effects of resveratrol and tannic acid on apoptosis in colon adenocarcinoma cell line

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ABSTRACT

الأهداف: لفحص آثار عقار ريسفيراترول وحمض تانيك على موت الخلايا المبرمج، وبروتينات (Bak) و (FADD) في خط خلايا الورم الغدي السرطاني بالقولون (CaCo–2) بواسطة مقارنة آثارهم.

الطريقة: في هذه الدراسة الحالية، تم إعطاء عقار ريسفيراترول وحمض تانيك على خط خلايا الورم الغدي السرطاني بالقولون (CaCo–2) بمقدار الجرعات التالية (25، 50، 100µM). كان نمو ومتابعة خط خلايا الورم الغدي السرطاني بالقولون (CaCo–2)، في قسم الأحياء – جامعة اسكيسيهر عثمان غازي – اسكيسيهر – تركيا، خلال عام 2007م . تم تحديد آثار هذه العناصر على مدخلات موت الخلايا المبرمج بواسطة استعمال طقم تاك فوق الأكسيد لقياس موت الخلايا المبرمج وأثارها على معدل بروتينات (Bak) و (FADD) وبواسطة طريقة التصبيغ الكيميائي النسيجي المناعي عند 72–48–24 ساعة. تم حساب الخلايا المصبوغة وغير المصبوغة في 30 منطقة منفصلة لثلاثة حجر منفصلة تم إعدادها لكل مجموعة. تم وضع النتائج في صيغة واوجدنا النسبة المئوية. تلاها حساب نسبة موت الخلايا المبرمج وبروتين (Bak) و (FADD) مع مجموعة التحكم. كما تم إظهار متوسط القيم للتجارب الثلاثة.

النتائج: ازدادت قيم مدخلات موت الخلايا المبرمج ونسبة بروتين (Bak) و (FADD) المئوية لدى جميع المجموعات التي تلقت حمض تانيك وعقار ريسفيراترول عند المقارنة ضمن المجموعات. وجدت هذه الزيادة الوقت والجرعة المستقلة في جميع القياسات.

خاتمة: في الخاتمة، تظهر هذه النتائج أن الخلايا تخضع لموت الخلايا المبرمج في طريقتين في عقار ريسفيراترول وحمض تانيك المحرض لخلايا الورم الغدي السرطاني بالقولون (CaCo–2).

Objectives: To investigate the effects of resveratrol and tannic acid on apoptosis, and Bcl-2 homologous antagonist/killer (Bak) and fas associated death domain (FADD) proteins in the CaCo-2 cell line.

Methods: In the present study, resveratrol and tannic acid were administrated in the CaCo-2 cell line at doses of 25, 50, and 100 µM. The CaCo-2 cells were grown and cultured in the Medical Biology Department, Eskisehir Osmangazi University, Eskisehir, Turkey in 2007. The effects of these agents on apoptotic index were determined by Apop Taq peroxidase kit and their effects on the ratios of Bak and FADD proteins by the immunohistochemical staining method at 24, 48, and 72 hours. Stained and non-stained cells in 30 separate areas of the 3 separate chamber slides, prepared for each group, were counted. The percentage of apoptosis, and Bak and FADD proteins was calculated with the control. Mean ± standard error values were calculated for the 3 experiments.

Results: Apoptotic index, Bak protein percentage ratio, and FADD protein percentage ratio values in all groups that received tannic acid and resveratrol increased when compared within the groups. This increase was found to be time and dose independent in all parameters.

Conclusion: Cells undergo apoptosis in 2 pathways (mitochondrial and death receptor) in resveratrol and tannic acid induced CaCo-2 cells.

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Resveratrol (3,5,4' trihydroxystilbene) is found in many plants, including the ones known as Kojokon, the roots of *Polygonum cuspidatum*, grapes, red wine, peanuts, berries, and traditional oriental medicine plants.[1-3] Resveratrol is an anti-oxidant, anti-carcinogenic, anti-inflammatory, anti-coagulant, anti-mutagenic, anti-proliferative, anti-microbial, estrogenic, and vasodilator agent.[2-4] In addition, resveratrol suppresses the growth of breast cancer cell lines and inhibits the activity and the expression of several enzymes that have a key function in the regulation of the cell growth and apoptosis. Resveratrol increases the expression of apoptotic Bax and Bcl-2 homologous antagonist/killer (Bak), and downregulates antiapoptotic Bcl-2 and Bcl-xL in MCF-7. This means that the ratio of Bcl-2 to Bax is important for apoptosis induced by chemoprevention agents. The increased ratio of Bax to Bcl-2 might contribute to apoptosis induction in resveratrol-treated MCF-7 cells.[2] It is proposed that resveratrol affects death receptors and decreases cell proliferation by apoptosis in the CaCo-2 colon adenocarcinoma cell line.[5,6] Tannins are plant-derived polyphenolic compounds with a molecular weight between 500-3000 Da, which can be classified into 2 groups, namely, hydrolyzable and condensed tannins. The hydrolyzable tannins, commonly called tannic acid, contain either gallotannins or ellagictannins.[7] Polyphenols that contain tannin can function as anti-tumor agents, apart from functioning as antiviral, anti-HIV, inhibiting agents on lipid peroxidation and plasmin activity.[8] Phenolic phytochemicals such as tannins are natural constituents of tea, green tea, coffee, red wine, grapes, nuts, and other plant products.[9] Tannic acid has also been recently recognized to possess anti-carcinogenic, anti-oxidants, anti-mutagenic, anti-microbial, anti-allergic, anti-inflammatory, and astringent properties.[10] Tannic acid is also capable of inducing apoptosis in animal cells.[11] Inhibition of the proteasome by tannic acid in Jurkat T-cells results in accumulation of 2 natural proteasome substrates, the cyclin-dependent kinase inhibitor, p27Kip1 and the proapoptotic protein Bax, followed by growth arrest in G1 and induction of apoptotic cell death.[7] In this study, we aimed to investigate the effects of resveratrol and tannic acid on apoptosis, and Bak, and fas associated death domain (FADD) proteins in the CaCo-2 cell line.

Methods. The CaCo-2 colon cancer cell line was obtained from the Foot-and-Mouth Disease Institute, Ministry of Agriculture & Rural Affairs, Ankara, Turkey. The CaCo-2 cells were grown and cultured in the Medical Biology Department, Eskisehir Osmangazi University, Eskisehir, Turkey in 2007. The CaCo-2 cells were grown in a cell culture medium use to maintain cells in tissue culture called Eagle's minimal essential medium (EMEM) (Biowest, Nuaillé, France) supplement 10% fetal calf serum and penicillin-streptomycin. Cells were maintained in a 5% CO_2 atmosphere at 37°C. Tested groups were identified as control, 25, 50, and 100 µM doses of resveratrol and tannic acid. Resveratrol and tannic acid were dissolved in dimethyl sulfoxide. Measurements were carried out at 24, 48, and 72 hours for apoptosis assay, Bak, and FADD proteins in the CaCo-2 cell line. Cells fixed on the chamber slides' base were used for apoptosis assay and immunohistochemical analysis. Stained (for apoptotic and immunohistological index, see below) and non-stained cells in 30 separate areas of the 3 separate chamber slide (n=3), prepared for each group, were counted (**Table 1**). One hundred cells in the 30 different areas were evaluated in each slide. Results were placed into the formula (indicate the formula used), and percentage ratios were found. The apoptotic index was determined by Apop Taq Plus Peroxidase in Situ Apoptosis Detection Kit (Chemicon International, Huissen, The Netherlands) using the TUNEL (Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling) method. Apoptotic index (APOi): Number of apoptotic nuclei/total cell number x 100.[12] The Bak and FADD were determined by immunohistochemical detection kit (Lab Vision Corporation, Fremont, CA, USA) (Bak and FADD antibodies (Neomarkers, Fremont, CA, USA) using the streptavidin-biotin-peroxidase staining method. Immunohistochemical index: staining cell/total cell x 100. Evaluations of the stained preparations were performed using light microscopy by 2 of the authors, and they were scored independently. The percentage of apoptosis, Bak and FADD protein were calculated with the control. The mean ± standard error values were calculated for the 3 experiments. The results were compared by student-t test for double comparing and by one-way analyses of variance for more comparing. Multiple comparing was evaluated by the Holm-Sidak method. The statistical analyses were performed using the statistical software SPSS version 15.0 for Windows. A $p<0.05$ were considered statistically significant.

Results. Apoptotic index, Bak protein percentage ratio, and FADD protein percentage ratio values in all groups that received tannic acid and resveratrol increased when compared within the groups. This increase was found to be independent of time and dose in all parameters. The apoptotic index increased at a concentration of 100 µM resveratrol in the 48 and 72 hour groups, and 100 µM tannic acid in the

48 hour group compared with the control (**Table 1**). In particular, Bak protein percentage ratio increased at a concentration of 25 and 50 μM resveratrol in the 48 hour group, 25, 50, and 100 μM resveratrol in the 24 and the 72 hour groups, 100 μM resveratrol in the 48 hour group, and at a concentration of 25 μM tannic acid in the 48 hour group, 25, 50m, and 100 μM tannic acid in the 24 and the 72 hour groups, and 50 and 100 μM tannic acid in the 48 hour group compared with the control (**Table 1**). The FADD protein percentage ratio increased at a concentration of 50 μM resveratrol in the 48 and the 72 hour groups, 100 μM resveratrol in the 72 hour group, 25, 50, and 100 μM resveratrol in the 24 hour group, 25, and 100 μM resveratrol in the 48 hour group, and 25 and 50 μM tannic acid in the 24 hour group, 25 μM tannic acid in the 48 hour group, 50 μM tannic acid in the 72 hour group, 100 μM tannic acid in the 24 hour group, 50 and 100 μM tannic acid in the 48 hour group, and 100 μM tannic acid in the 72 hour group compared with the control (**Table 1**). Our results show that, in the groups where resveratrol or tannic acid is applied to the CaCo-2 cell line, there is no significant difference in terms of Bak and FADD protein ratios.

Discussion. Although there are studies investigating the effects of resveratrol[13-17] in the CaCo-2 cell line, there are few studies on tannic acid.[18,19]In a study performed on the CaCo-2 cell line, the effects of 12.5, 25, 50, 100, and 200 μM resveratrol doses on cell proliferation was evaluated at 24, 48, and 72 hours. There was no 100 μM/L dose effect at 72 hours, but the dose of 200 μM decreased the cell count. So, resveratrol was reported to increase cell proliferation in a dose and time dependent manner.[5] Investigating the studies performed, resveratrol was observed to increase apoptosis in a dose and time dependent manner. But, there are some differences at different doses and hours in the cell line.[13-16] In the studies performed, resveratrol was reported to be effective on the digestive system. Resveratrol may have an important role in preventing colon cancer by blocking excess division of epithelial tissue and inducing apoptosis.[5,17] In the studies on tannic acid and apoptosis, 2, 6, 12, and 24 μM condensed tannin was administrated to normal fibroblast lung (HEL 299), colon (CaCo-2), breast (MCF-7, Hs578T), and prostate (DU 145). After 24 hours, normal cells were alive, but the cancer cell death increased.[18] In another study performed on the prostate cancer cell line (LNCaP), tannic acid 5 and 10μmol/L increased apoptotic index significantly at 72 hours as compared to control.[19] In another study performed on human Jurkat T cells, tannic acid 50 and 100 μg/ml increased apoptotic cell death in a dose dependent manner at 24 hours.[7] The effect of tannic acid on apoptosis is different at different doses and hours at cell line. We observed in our study that tannic acid increased the apoptotic index in a dose independent manner at all hours in the CaCo-2 cell line, with the highest increase of 100 μM at 48 hours. Research indicates that there are 2 main apoptotic pathways: death receptor pathway and mitochondrial pathway. We also investigated the Bak proteins that are effective on the mitochondrial pathway and the FADD proteins that are effective on the cytosolic death receptor pathway. In a study that investigated the effects of resveratrol, lower doses

Table 1 - Apoptotic index was analyzed with terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assay. Bcl-2 homologous antagonist/killer (Bak) and fas associated death domain (FADD) protein were analyzed with immunohistochemical staining assay in CaCo-2 cell line. The percentage of apoptosis, Bak and FADD protein was calculated from the control.

Cell line/ groups	Apoptotic index			Bak (%)			FADD (%)		
	24th hour	48th hour	72nd hour	24th hour	48th hour	72nd hour	24th hour	48th hour	72nd hour
CaCo-2									
Control									
0 μM	7.863 ± 0.344	8.788 ± 0.531	9.362 ± 0.284	45.586 ± 0.793	51.126 ± 1.071	45.906 ± 0.811	46.880 ± 1.211	49.640 ± 1.099	51.446 ± 0.639
Resveratrol									
25 μM	5.760 ± 0.369*	10.288 ± 0.149*	10.500 ± 0.690*	64.886 ± 0.992**	60.863 ± 1.345‡	61.570 ± 1.346**	62.086 ± 1.031**	63.256 ± 1.602**	54.773 ± 1.244*
50 μM	6.076 ± 0.686*	11.448 ± 0.714*	9.677 ± 0.481*	64.723 ± 0.569**	59.923 ± 0.783‡	59.970 ± 0.199**	59.456 ± 0.584**	55.496 ± 1.214†	57.280 ± 0.373†
100 μM	6.102 ± 0.239*	12.190 ± 0.824†	12.389 ± 0.741†	63.060 ± 0.470**	64.073 ± 1.180**	61.746 ± 2.178**	56.993 ± 0.692**	65.310 ± 0.605**	61.136 ± 1.303‡
Tannic acid									
25 μM	7.351 ± 0.044	8.135 ± 0.741*	10.912 ± 0.456*	60.236 ± 0.391**	57.773 ± 1.247‡	60.610 ± 0.805**	59.480 ± 1.067‡	56.250 ± 0.425†	53.483 ± 0.575*
50 μM	8.903 ± 0.244*	7.507 ± 0.670*	10.658 ± 0.670*	60.420 ± 2.263**	70.163 ± 1.271**	67.573 ± 0.558**	59.226 ± 1.283‡	63.130 ± 0.815**	58.630 ± 1.101‡
100 μM	8.922 ± 0.189*	14.352 ± 1.575†	9.887 ± 0.544*	69.946 ± 1.161**	73.223 ± 0.085**	76.746 ± 1.024**	61.106 ± 1.437**	67.543 ± 0.693**	64.653 ± 1.452**

Mean ± S.E. values are shown for 3 experiments (n=3) (*not significant, †p<0.05, ‡p<0.01, **p<0.001)
CaCo-2 - human colon adenocarcinoma cell line

of resveratrol (≤4μM) increased cell proliferation at estrogen-receptor (ER)-positive human breast cancer cell lines for MCF-7 and higher doses of resveratrol (≥44 μM) inhibited cell proliferation. The inhibition of cell proliferation was thought to be caused by sub G1 phase fraction, up-regulation of Bak and Bax proteins, down regulation of Bcl-xL protein and activation of caspase 3 and the induction of apoptosis.[20] Resveratrol 10-100 μM was reported to activate caspase at SW480 colon cancer cells. This activation was associated with the accumulation of Bak and proapoptotic proteins like Bax.[6] In the studies performed, resveratrol was proposed to increase the expression of Bak and Bax proteins responsible in the mitochondrial pathway.[2,21-23] In study performed on colorectal cancer cell, Bax and Bak proteins disappeared by clonal selection, but the cells were reported to undergo apoptosis. Consequently, it is reported that, resveratrol may sensitize the cells to death receptor pathway mediated apoptosis induced by CD95 (Fas), tumor necrosis factor alpha (TNFα), and tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), the ligands of death receptors.[23,24] However, resveratrol can function as a sensitizer for death receptor pathway-mediated apoptosis triggered by the death receptor ligands Fas, TNFα, and TRAIL.[23,24] In another study, it is reported that resveratrol induced the recruitment and the redistribution of FADD, procaspase-8, and Fas in SW480 cells. The redistribution of Fas receptors on membranes by resveratrol contributes to the induction of apoptosis on colon cancer cells.[6] According to the results of our study, resveratrol guides the cells to apoptosis in 2 pathways. In a study on HepG2 liver cell line, epigallocatechin-3-gallate (EGCG), a tea polyphenol that is a similar to tannic acid, increased Bak and Bax proteins at different doses and different hours in a dose dependent manner.[25] Our results showed no significant difference between the percent ratios of Bak proteins and FADD proteins.

In conclusion, these results show that the cells undergo apoptosis in 2 pathways in resveratrol and tannic acid induced CaCo-2 cells. Additionally, we observed in our study that resveratrol and tannic acid increased the apoptotic index in a dose independent manner at all hours in the CaCo-2 cell line. Further studies may be needed to support our results.

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