Effects of Hydroxytyrosol on Expression of Apoptotic Genes and Activity of Antioxidant Enzymes in LS180 Cells

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Purpose: Colorectal cancer is the third–most commonly occurring cancer in developed countries. Hydroxytyrosol is a potent antioxidant that has several activities, such as oxidative-stress control, inhibition of cell proliferation, and induction of apoptosis. In this study, the effect of hydroxytyrosol on the expression of genes effective in apoptosis — BAX, BCL2, CASP3, P53, PPARG, and NFE2L2 — and antioxidant-enzyme activity in LS180 cells of human colorectal cancer was investigated.

Methods: The human colorectal cancer cell line LS180 was treated with different concentrations of hydroxytyrosol for 24 hours. Expression of BAX, BCL2, CASP3, NFE2L2, PPARG, and P53 was investigated using real-time PCR. The activity of antioxidant and malondialdehyde enzymes was measured by calorimetric methods.

Results: Analysis of gene expression showed that hydroxytyrosol significantly increased the expression of CASP3 and the BAX:BCL2 ratio in treatment groups compared to the control (P<0.05). Also, hydroxytyrosol significantly reduced the expression of the NFE2L2 gene (P<0.05). Calorimetric analysis showed that hydroxytyrosol increased activity of the antioxidant enzymes catalase, superoxide dismutase, and glutathione peroxidase in treatment groups significantly more than the control group and reduced thiobarbituric acid–reactive substances on an oxidative stress index (P<0.05).

Conclusion: Hydroxytyrosol may induce apoptosis in colorectal cancer cells by increasing the expression of CASP3 gene and increasing the BAX:BCL2 ratio. Also, hydroxytyrosol may increase the activity of antioxidant enzymes and reduce the proliferation of LS180 cells by changing the antioxidant-defense system in cancer cells.

Keywords: colorectal cancer, hydroxytyrosol, apoptosis, antioxidant enzymes, calorimetric analysis, malondialdehyde enzymes

Introduction
Colorectal cancer is the third–most common cancer in men (after lung and prostate cancer) and the second–most common in women (after breast cancer).1 The most important risk factors for colorectal cancer, which can be controlled and prevented by the individual, include environmental factors, lifestyle, and nutritional factors, such as low-fiber and high-fat diets, smoking, alcohol intake, inadequate activity, and lack of exercise. Inevitable risk factors include age, genetics, and inheritance.2

The most important risk factor for colorectal cancer is nutritional. It is known that the type of diet can affect the distribution of gastrointestinal cancers, and in the case of lifestyle changes and nutritional habits, up to 30% of cases of colorectal cancer can be
Methods
Cell Culture and Treatment with Hydroxytyrosol
In this study, the human colorectal cancer cell line LS180 was obtained from the cell bank of the Pasteur Institute of Tehran and grown in DMEM containing 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37°C with 5% CO₂. After treatment of cells with 50, 100, and 150 μM hydroxytyrosol (Sigma) for 24 hours, cells were collected by trypsinization.

RNA Extraction and cDNA Synthesis
RNA extraction was performed using an RNA-extraction kit (Gena Bioscience, Germany) using the protocol provided by the manufacturer. Quantitative RNA extracted from electrophoresis was viewed on 1.5% agarose gel and measured with a NanoDrop device. cDNA was extracted using a cDNA-synthesis kits (Gena Bioscience and Corbett) with the relevant primers (Table 1).

Evaluation of Gene Expression Using Real-Time PCR
Expression levels of P53, NFE2L2, PPARG, BAX, BCL2, and CASP3 were assessed using real-time PCR (Gena Bioscience and Corbett) with the relevant primers (Table 1).

Activity of Antioxidant and Malondialdehyde Enzymes
The calorimetric method was used to evaluate the activity of catalase in the enzyme reaction with methanol in the presence

Table 1 Forward- and Reverse-Primer Sequences

| Gene   | Sequences             | Product size |
|--------|-----------------------|--------------|
| BCL2-F | TCGCCCTGTTGATGACTGA   | 134 bp       |
| BCL2-R | CAGAGACAGCCAGGAAATCA  | 195 bp       |
| BAX-F  | TGGCAGCTGACATTTTCTGAC | 134 bp       |
| BAX-R  | TCACCCCAACCCCTGTCTTT  | 147 bp       |
| CASP3-F| TACCTGCGCTGTCTACCG    | 118 bp       |
| CASP3-R| TCAGTGTCTCCATGGATACCT | 132 bp       |
| NFE2L2-F| TCCGTGCGTCAGTAACT     | 167 bp       |
| NFE2L2-R| GGCACATTTCGCTGCGG     | 134 bp       |
| P53-F  | CTAGGATCAGTCGGCCGTC   | 132 bp       |
| P53-R  | TAAAGTCCCTCCGGCTGACC  | 134 bp       |
| PPARG  | GGGGTATGTTTTGAACTGA   | 147 bp       |
| PPARG-R| ACTGAATTTCCACCTCACTGA | 118 bp       |
| B2M-F  | AAGCAAGCAGCAAGTGGGTA  | 134 bp       |
| B2M-R  | AAGCAAGCAAGCAGAATTGGA | 134 bp       |
of oxygenated water, and the specific activity of the enzyme was calculated in terms of unit/mg protein. The activity of superoxide dismutase was analyzed by a Pyrogallol radical superoxide reaction using a spectrophotometer. The specific activity of the enzyme was calculated in terms of unit/mg protein. Measurement of glutathione peroxidase activity was performed based on calorimetry and glutathione oxide formation. Specific activity was also calculated in terms of unit/mg protein. Malondialdehyde assays were performed based on reactions with a thiobarbituric acid–reactive substrate.

Data Analysis

REST software was used to analyze the data obtained on gene expression. SPSS software was used to analyze the oxidative-stress index and antioxidant-enzyme activity. To describe the data, means, SD, frequency tables, one-way ANOVA, and Tukey’s post hoc test were used.

Results

Evaluation of Gene Expression

The results of this study showed that hydroxytyrosol at all concentrations significantly increased BAX expression (P<0.05, Figure 1A). Expression of BCL2 increased in the treatment group compared to the control, but this increase was not statistically significant (P>0.05, Figure 1B). Hydroxytyrosol increased the BAX:BCL2 ratio at all concentrations compared to the control group (P<0.05, Figure 1C). Hydroxytyrosol increased CASP3 expression at all concentrations compared to the control group (P<0.05, Figure 1D). Hydroxytyrosol did not significantly affect expression of P53 compared to the control group (P>0.05, Figure 2A). Furthermore, hydroxytyrosol reduced the expression of NFE2L2 in the treated group compared to the control group, which was statistically significant at a concentration of 50 μM (P<0.05, Figure 2B). The treatment group also had a significant reduction in the

![Figure 1](image_url)

**Figure 1** Expression of BAX, BCL2, and CASP3 and BAX:BCL2 ratio in groups treated with 50, 100, and 150 μM hydroxytyrosol and controls in the LS180 cell line. **Notes:** *P*<0.05, **P**<0.01. BAX:BCL2 ratio was greatest at 100 μM concentration whereas, CASP3 gene activity increased in a dose-dependent manner. (A) BAX expression was significant at all concentrations. (B) BCL2 expression increase was not significant. (C) The BAX:BCL2 ratio was increased at all concentrations. (D) Hydroxytyrosol increased CASP3 expression at all concentrations compared to control group, which was statistically significant.
expression of PPARγ at all concentrations by 1.7 (P<0.05, Figure 2C).

Effect of Hydroxytyrosol on Activity of Antioxidant Enzymes and Thiobarbituric Acid–Reactive Substances

Catalase-enzyme activity at 50, 100, and 150 μM hydroxytyrosol increased in comparison with the control group (P<0.05, Figure 3A). Similarly, all treatment groups had significantly increased activity of glutathione peroxidase and superoxide dismutase compared with the control group (P<0.05. Figure 3B and C). At all concentrations, hydroxytyrosol decreased levels of thiobarbituric acid–reactive substances compared to the control group (P<0.05, Figure 3D).

Discussion

Colorectal cancer is the most common cancer of the digestive tract. In recent years, efforts have been made to find natural products that can fight colorectal cancer. Among these compounds, polyphenols, secondary metabolites of plants that have beneficial effects on human health and reduce the risk of most cancers, have been widely recognized. Hydroxytyrosol is a natural polyphenol compound and a strong antioxidant. Recent studies have shown that this potent antioxidant has several biological activities, such as oxidative-stress control, cellular efficacy, and induction of apoptosis in several tumor-cell lines. Our study evaluated the effects of three doses of hydroxytyrosol — 50, 100, and 150 μM — on colorectal cancer cells. Various concentrations of hydroxytyrosol have been reported to have antioxidant, antiangiogenic, and apoptotic effects in different cancer-cell lines.

Several studies have confirmed the antitumor effects of hydroxytyrosol on several cancers, such as breast cancer, leukemia, and melanoma. Studies have shown that hydroxytyrosol induces G2/M cell-cycle end points and inhibits cell proliferation. It also induces apoptosis in cancer cells and inhibits Akt activity and the NFKB pathway. In a study on HL60 (promyelocytic leukemia) and HT29 (colon adenocarcinoma), it was shown that hydroxytyrosol slowed cell growth and proliferation and stopped the cell cycle and apoptosis in these cells. Various proteins are involved in the regulation of apoptosis. The family of BCL2 proteins,

Figure 2 Expression of P53, NFE2L2, and PPARγ genes in groups treated with 50, 100, and 150 μM hydroxytyrosol and controls in the LS180 cell line.

Notes: **P<0.05; ***P<0.01. NFE2L2 expression was significant only at 50 μM. (A) The expression of P53 gene compared to control group did not increase significantly. (B) The expression of Nrf2 gene was significant at a concentration of 50 μM. (C) Significant reductions in the of PPARγ expression at all concentrations were found.
which include proapoptotic and antiapoptotic proteins, is one of the major regulators of this process.\textsuperscript{31} Our study showed that hydroxytyrosol at all concentrations significantly increased expression of \textit{BAX} compared to the control group. Also, hydroxytyrosol increased \textit{BCL2} expression at all concentrations compared to the control. Similarly, the \textit{BAX}:\textit{BCL2} ratio seems to be a more appropriate indicator for inducing apoptosis and determining the cell-death rate toward apoptosis after exposure to cytotoxic agents. Our study showed that hydroxytyrosol at all concentrations significantly increased expression of \textit{CASP3} in the LS180 cell line compared to the control group. In a study conducted on uterine (FB2 and TPC1) and follicular thyroid cancer (WRO) cancer-cell lines in 2017, hydroxytyrosol reduced the expression of mRNA and cyclin D1 protein and increased the expression of p21 in uterine cancer cells. Hydroxytyrosol also increased the expression of caspase 3 and PARP1 in these cell lines.\textsuperscript{27}

\textit{P53} is the most widely known tumor-suppressor gene, and plays a crucial role in genomic stability and tumor suppression by inducing apoptosis, halting the cell cycle, aging, and angiogenesis.\textsuperscript{32} Studies have shown that hydroxytyrosol induces apoptosis by effecting the expression of \textit{P53}. The results of this study showed increased expression of \textit{P53} at different concentrations of hydroxytyrosol in the LS180 cell line. In a study on the breast cancer–cell line MCF7, it has been shown that oleuropein (from hydrolysis of oleruopin and hydroxytyrosol) increases the expression of \textit{P53} in treated groups compared to controls and resulted in apoptosis. It has also been shown in another study on uterine cancer cells (FB2 and TPC1) and thyroid cancer cells (WRO) that hydroxytyrosol increased the expression of p53 and BAD and induced apoptosis.\textsuperscript{27} Nrf2 is a key factor in mechanisms of cell defense against oxidative stress.\textsuperscript{33} The results of our study showed that hydroxytyrosol at all concentrations reduced the expression of Nrf2 compared to the control group. A study in 2011 showed that hydroxytyrosol significantly increased the expression of Nrf2 in the normal epithelial cell line MCF10A, but did not increase in the breast cancer–cell lines MDA-MB231 and MCF7 or was not effective.\textsuperscript{33} Our study results were consistent with this study.
During normal cell activity, reactive oxygen species (ROS) are produced, which also play an important role in apoptosis. Active oxygen species act as a redox messenger in low physiological levels in signaling and intracellular regulation, while at high levels they produce oxidative stress and induce oxidative changes in macromolecules. Each cell is equipped with an antioxidant defense system to prevent the excessive production of active radicals.\textsuperscript{34} In vitro studies have shown that the beneficial effects of phenolic compounds, such as hydroxytyrosol, on human health are due to their antioxidant properties. In a study on the CACO2 cell line, it was shown that hydroxytyrosol inhibited ROS-induced cytotoxicity in these cells and reduced malondialdehyde levels in dose-dependent groups.\textsuperscript{35}

The results of this study showed that hydroxytyrosol significantly increased the activity of cellular antioxidant enzymes, including catalase, superoxide dismutase, and glutathione peroxidase, in all treatment groups. Also, malondialdehyde concentrations in the treated groups were significantly lower than controls. Studies have shown that anticancer compounds that have antioxidant activity may have beneficial effects by balancing ROS levels, which not only prevent the proliferation of cancer cells but also lead to apoptosis. In a similar study, the semisynthetic derivative of an anticarcinogenic alkaloid, through the production of ROS and increased oxidative stress, led to toxicity to the MCF7 breast cancer–cell line, and upregulated the activity of antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase. The study concluded that this increase in activity of antioxidant enzymes could be a response to increased oxidative stress in treated cells.\textsuperscript{36} On the other hand, studies have shown that phenolic compounds are not only antioxidants but can also be prooxidants and result in the production of ROS.\textsuperscript{37} Based on the results of this study, hydroxytyrosol seems to act as a prooxidant in the LS180 cell line of human colorectal cancer and mediates the production of ROS, and thereby the increased activity of the antioxidant enzymes observed in this study could be a response to increased oxidative stress in treated cells.

**Conclusion**

Hydroxytyrosol can induce apoptosis in the LS180 colorectal cancer–cell line by increasing the expression of proapoptotic genes, such as BAX, CASP3, and P53, increasing the BAX: BCL2 ratio, and downregulating NFE2L2 expression. Also, the treatment of cells with hydroxytyrosol upregulates antioxidative activity in the colorectal cancer–cell line, marked by increased antioxidant enzymes. In vivo studies on the therapeutic effects of hydroxytyrosol on colorectal cancer in animal models can give better conclusions.

**Author Contributions**

All authors contributed to data analysis, drafting or revising the article, gave final approval to the version to be published, and agree to be accountable for all aspects of the work.

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**Disclosure**

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