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Effect of Sodium Butyrate and Epigallocatechin-3-Gallate on the Genes Expression of Intrinsic Apoptotic Pathway on PA-TU-8902, CFPAC-1, and CAPAN-1 Human Pancreatic Cancer Cell Lines

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Abstract

Background: Histone deacetylase inhibitors (HDACIs) are novel anticancer agents that induce cell death and cycle arrest. Several studies reported that HDACIs induce apoptosis via two well-defined intrinsic/mitochondrial and death receptor pathways. In addition to HDACIs, DNA methyltransferase inhibitors effectively revert the promoter hypermethylation of tumor suppressor genes and apoptosis induction. The current study aimed to investigate the effect of sodium butyrate and epigallocatechin-3-gallate (EGCG) on the genes expression of the intrinsic pathway (*BAX*, *BAK*, *APAF1*, *Bcl-2*, and *Bcl-xL*), *p21*, and *p53* on PA-TU-8902, CFPAC-1, and CAPAN-1 human pancreatic cancer cell lines. **Materials and Methods:** The PA-TU-8902, CFPAC-1, and CAPAN-1 cells were treated with sodium butyrate and EGCG. To determine cell viability, cell apoptosis, and the relative gene expression level, the 3-(4,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, flow cytometry, and real-time quantitative reverse transcription polymerase chain reaction were done, respectively. **Results:** Both compounds changed the expression levels of the mentioned genes in a p53-dependent and -independent manner, which induced cell apoptosis and inhibited cell growth in all three cell lines. **Conclusion:** We indicated that sodium butyrate and EGCG could induce apoptosis in human pancreatic cancer cell lines.**[GMJ.2022;11:e2248]** **DOI:10.31661/gmj.v11i.2248**

Keywords: Sodium Butyrate; Epigallocatechin-3-Gallate; Gene Expression Regulation; Pancreatic Cancer

Introduction

Histone deacetylases (HDACs) and histone acetyltransferases (HATs) are the enzymes that modify histone acetylation and regulate chromatin structure through removing and adding the acetyl group from the lysine residues, respectively. These chromatin modifications regulate gene transcriptions and are necessary for gene expression [1]. HDACs inhibitors (HDACIs) are novel anticancer agents that induce cell death, differentiation, and cell cycle arrest. These compounds represent a broad family that includes four main structural classes compressing short-chain fatty acids

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(valproic acid [VPA] and butyrate), hydroxamates (e.g., suberoylanilide hydroxamic acid [SAHA] or vorinostat, trichostatin A [TSA], LBH589 [panobinostat], oxamflatin, PXD101 [belinostat], and tubacin), benzamides (MGCD0103 and SNDX275), and cyclic tetrapeptides (e.g., trapoxin A, FK228 [romidepsin], and apicidin) [2]. It has been reported that HDACIs can induce apoptosis in various cancers such as breast, colon, prostate, bladder, lung, ovary, pancreas, and stomach cancer cell lines [3]. HDACIs can induce apoptosis through various mechanisms. Previously, we indicated that TSA could induce apoptosis by up-regulation of p21Cip1/Waf1/Sdi1, p27Kip1, and p57Kip2; and down-regulation of Class I HDACs (HDACs 1, 2, and 3) and Class II HDACs (HDACs 4, 5, and 6) in LS 174T colon cancer [4]. Further, we reported that this compound could play its apoptotic effect by estrogen receptor alpha up-regulation in hepatocellular carcinoma cell lines [5].

Several studies reported that HDACIs can induce apoptosis via two well-defined intrinsic/mitochondrial (via up-regulation of several numbers of pro-apoptotic BH3-only Bcl-2 family genes) and death receptor (DR)/extrinsic (through up-regulation of DR expression) pathways [6]. In many cases, the activation of the intrinsic apoptotic pathway is the predominant apoptotic mechanism of HDACIs [6].

In addition to HDACIs, DNA methyltransferase inhibitors (DNMTIs) have been shown to be effective in reverting the promoter hypermethylation of tumor suppressor genes (TSGs) and apoptosis induction. The human genome contains four DNA methyltransferase (DNMT) genes compressing DNMT1, DNMT2, DNMT3A, and DNMT3B [7]. Broadly, DNMTIs are divided into three classes: (1) nucleoside inhibitors (e.g. 5-azacytidine [5AC], and 5-aza-20-deoxycytidine [DAC], and pyrimidin-2-one b-ribofuranoside [zebularine]); (2) nonnucleoside inhibitors (such as epigallocatechin-3-gallate [EGCG]; and (3) rationally designed inhibitors [8-10]. Recently, we reported that the effect of DNMTI 5-Aza-2'-deoxycytidine (5-Aza-CdR) on DNMT1, and CIP/KIP family (*p21*, *p27*, and *p57*) genes expression, and apoptosis induction in SW480 colon cancer cell line [11]. Further, we indicated that 5-Aza-CdR decreases DNMT1, DNMT3a, and DNMT3b resulting in apoptotic induction in LCL-PI 11 hepatocellular carcinoma cell line [12] and also reactivates *p15INK4*, *p16INK4*, *p18INK4*, and *p19INK4* genes expression in HCC PLC/PRF/5 cell line [13]. Several researchers have shown that DNMTIs induce mitochondrial-mediated apoptosis through the up-regulation of pro-apoptotic genes (such as *BAX*) and down-regulation of anti-apoptotic genes (e.g., *Bcl-2*) [14]. Finally, it has been reported that DNMTIs induce cell apoptosis and cell cycle arrest through p53-dependent and -independent pathways [15, 16]. The current study aimed to investigate the effect of sodium butyrate and EGCG on the genes of intrinsic apoptotic pathway (*BAX*, *BAK*, *APAF1*, *Bcl-2*, and *Bcl-xL*), *p21*, and *p53* gene expression, cell viability, and apoptosis on PA-TU-8902, CFPAC-1, and CAPAN-1 human pancreatic cancer cell lines.

Materials and Methods

Materials

Human pancreatic cancer PA-TU-8902, CFPAC-1, and CAPAN-1 cell lines were purchased from the National Cell Bank of Pasteur Institute (Iran, Tehran). The sodium butyrate, EGCG, and Dulbecco's modified Eagle's medium (DMEM), total RNA extraction Kit (TRIZOL reagent), real-time polymerase chain reaction kits (qPCRMasterMix Plus for SYBR Green I dNTP), and 3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyl-2-tetrazolium bromide (MTT) assay Kit were purchased from Sigma (St. Louis, MO, USA).

The trypsin-EDTA (Invitrogen, Cergy-Pontoise, France) and other necessary materials and kits were purchased as previously indicated [17, 18].

Ethical Considerations

This study was approved by the Ethics

Committee of Jahrom University of Medical science with a code number of IR.JUMS.REC.1399.124.

Cell Culture and Cell Viability

The PA-TU-8902, CFPAC-1, and CAPAN-1 cells were cultured in DMEM supplemented with fetal bovine serum 10% and antibiotics at 37 °C in 5% CO_2 overnight, and then the cells were seeded into 96-well plates (3×10^5 cells per well). After 24 hours, the culture medium was replaced with a medium containing various concentrations of sodium butyrate (0, 1, 5, 10, 25, and 50 µM) and EGCG (0, 5, 10, 25, 50, and 100 µM). The control groups were exposed to an equivalent volume of solvent. After 24 hours of treatment, the treated and untreated cells were investigated by MTT assay according to standard protocols to determine cell viability as we described previously [19, 20].

Cell Apoptosis Assay

To determine cell apoptosis, the cell lines were cultured at a density of 3×10^5 cells/well and treated with sodium butyrate and EGCG, based on the half maximal inhibitory concentration (IC50) values and the control groups were exposed to an equivalent volume of solvents. Then, the cells were harvested by trypsin-EDTA washed with cold phosphate-buffered saline and re-suspended in a binding buffer (1x). Finally, 5 µL of Annexin V-FITC solution and 10 µL of PI solution were used according to the kit's protocol. Then, cells were incubated for 15 minutes at room temperature in the dark and measured with a Becton Dickinson FACScan flow cytometry (Becton Dickinson, Heidelberg, Germany). Each experiment was performed in triplicate.

qRT-PCR

To determine the relative expression level of the *BAX*, *BAK*, *APAF1*, *Bcl-2*, *Bcl-xL*, *p21*, and *p53* genes qRT-PCR was done. The PA-TU-8902, CFPAC-1, and CAPAN-1 cells (at a density of 3×10^5 cells/well) were treated with sodium butyrate and EGCG, based on IC50 value for 24 hours, and the control groups were exposed to an equivalent volume of solvents. Then qRT-PCR was performed based on the previous studies [21, 22]. The primer sequences are shown in Table-1 [23-30].

Statistical Analysis

Data were analyzed with a one-way analysis of variance (ANOVA) followed by Turkey test

Table 1. The Primer Sequences of Studied Genes.

Primer	Primer sequences (5' to 3')	Length (bp)	Reference
BAX	F: AGTAACATGGAGCTGCAGAGGAT R: GCTGCCACTCGGAAAAAGAC	77	23
BAK	F: CCTGCCCTCTGCTTCTGA R: CTGCTGATGGCGGTAAAAA	82	24
APAF1	F: TGCGCTGCTCTGCCTTCT R: CCATGGGTAGCAGCTCCTTCT	142	25
Bcl-2	F: TGGCCAGGGTCAGAGTTAAA R: TGGCCTCTCTTGCGGAGTA	147	26
Bcl-xL	F: TCCTTGTCTACGCTTTCCACG R: GGTCGCATTGTGGCCTTT	62	27
p21	F: CTGGAGACTCTCAGGGTCGAA R: GGATTAGGGCTTCCTCTTGGA	197	28
p53	F: ATGTTTTGCCAACTGGCCAAG R: TGAGCAGCGCTCATGGTG	153	29
GAPDH	F: TGTTGCCATCAATGACCCCTT R: CTCCACGACGTACTCAGCG	148	30

and t-test by using Graph Pad Prism Software (version 8.0, GraphPad Software Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a significant difference.

Results

Cell Viability

As shown in Figure-1, EGCG (A-C) and sodium butyrate (D-F) induced significant cell growth inhibition (P<0.001). Among treated cell lines with sodium butyrate, the CAPAN-1 has the lowest IC50 (10.56 µM, Table-2). Also, the lowest IC50 for the EGCG-treated cells was related to PA-TU-8902 (9.979 µM). Other details of IC50 are presented in Table-2.

Cell Apoptosis

As indicated in Figure-2, sodium butyrate induced cell apoptosis in the all cell lines. The maximal apoptosis (94.87%) was seen in the PA-TU-8902 cell line (Figure-2D, P<0.001). Also, EGCG leads to apoptosis in the all the cell lines (Figure-3). In comparison to sodium butyrate, EGCG induced lower apoptosis in the PA-TU-8902 (Figure-4).

Genes Expression Levels in Sodium Butyrate-Treated Cell Lines

Sodium butyrate up-regulated the *BAX*, *BAK*, *APAF1*, *p21*, and *p53* and down-regulated *Bcl-2* and *Bcl-xL* after 24 hours of treatment in all three cell lines (Figure-5A-C).

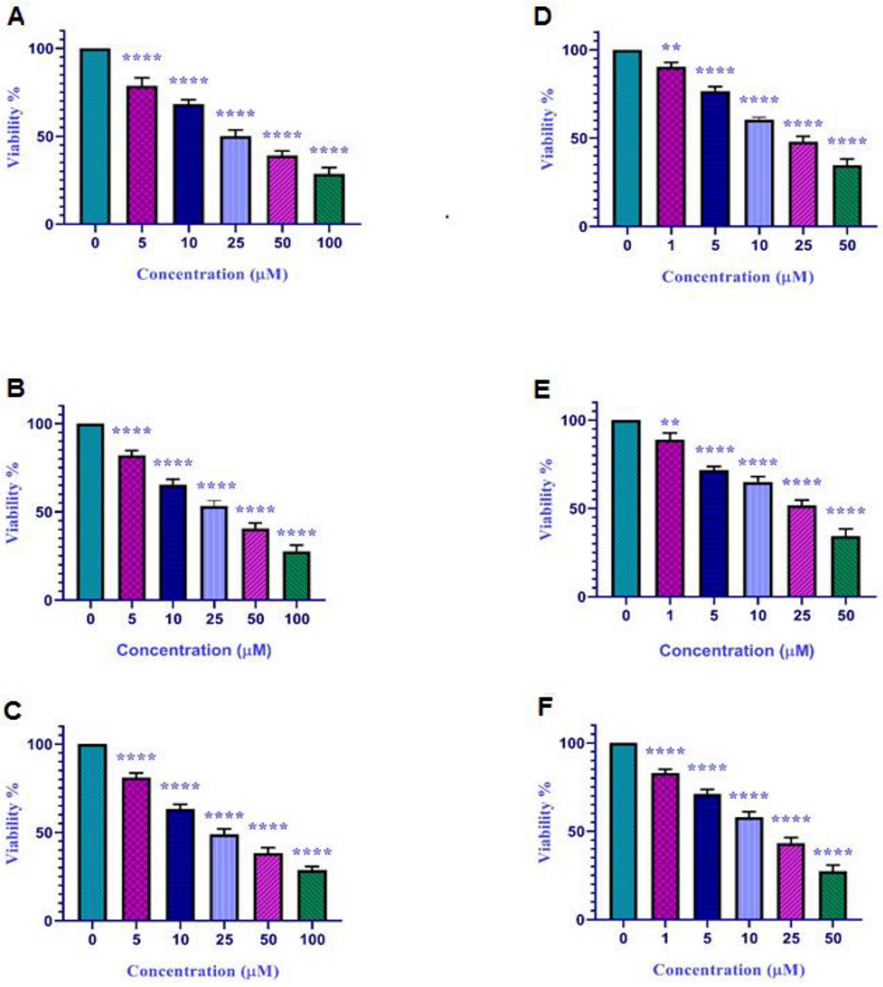

Figure 1. Effects of various concentration of EGCG (0, 5, 10, 25, 50, and 100 µM) on cell viability of PA-TU-8902 (**A**), CFPAC-1 (**B**), and CAPAN-1 (**C**) cell lines. Also, cell viability of sodium butyrate (0, 1, 5, 10, 25, and 50 µM) was determined by MTT assay at 24 hours on PA-TU-8902 (**D**), CFPAC-1 (**E**), and CAPAN-1 (**F**) cell lines. Both compounds significantly inhibited the growth of all three cell lines in a dose-dependent manner. **P<0.005 and ****P<0.0001 vs. control

Table 2. Half Maximal Inhibitory Concentration Values of Sodium Butyrate and Epigallocatechin-3-Gallate.

Cell lines	Treatment	IC50 (µM)	R squared
PA-TU-8902	EGCG	12.57	0.9855
	Sodium butyrate	9.979	0.9735
CFPAC-1	EGCG	13.49	0.9807
	Sodium butyrate	11.09	0.9486
CAPAN-1	EGCG	10.56	0.9895
	Sodium butyrate	11.61	0.9466

IC50: Half maximal inhibitory concentration; **EGCG:** Epigallocatechin-3-gallate; **µM:** Micromole; **R-Squared:** R^2 or the coefficient of determination

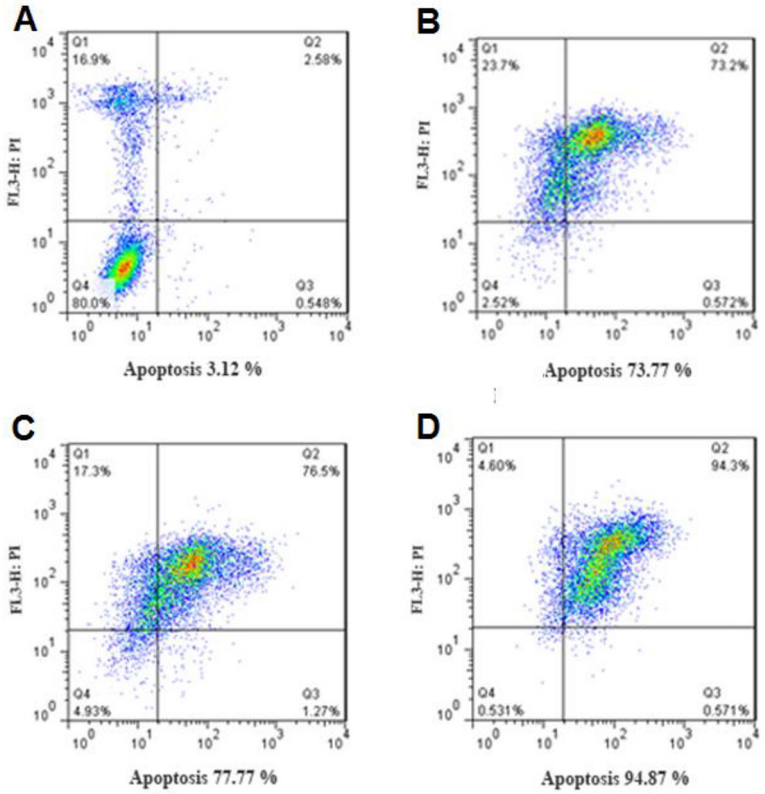

Figure 2. The apoptotic effect of sodium butyrate on control group (**A**) versus CFPAC-1 (**B**), CAPAN-1 (**C**), and PA-TU-8902 (**D**) cells at 24 hours. Maximal apoptosis was seen in the PA-TU-8902 cell line.

Genes Expression Levels in EGCG-Treated Cell Lines

The result showed that EGCG significantly up-regulated the *BAX*, *BAK*, *APAF1*, *p21*, and *p53* and down-regulated *Bcl-2* and *Bcl-xL* after treatment in the PA-TU-8902 cell line (Figure-5D). Also, up-regulation of the *BAX*, *BAK*, and *APAF1* as well as down-regulation of *Bcl-2* and *Bcl-xL* were observed in the CFPAC-1 cell line (Figure-5E). Regarding Figure-5, treatment with EGCG leads to significant up-regulation of *BAX*, *BAK*, *APAF1*, *p21*, and *p53* in the CAPAN-1 cell line (F); however, there were no significant changes in *Bcl-2* and *Bcl-xL* expression level.

Discussion

Cancer, a complex epigenetic disease resulting from mutation of TSGs, can be developed due to alteration of signaling pathways; it has been well known to have numerous links to apoptosis. Many of the

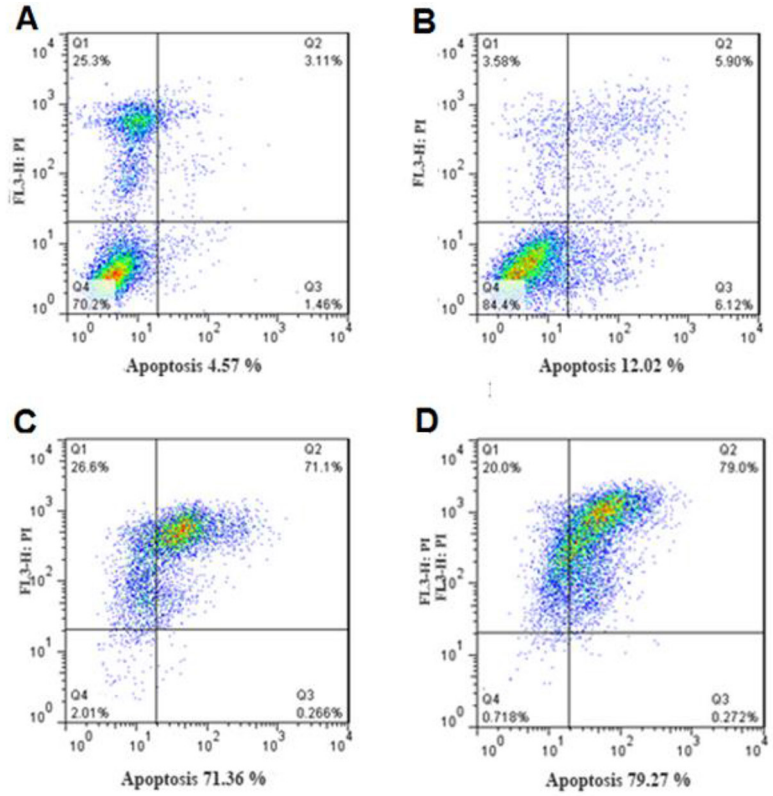

Figure 3. The apoptotic effect of EGCG on control group (**A**) versus CFPAC-1 (**B**), CAPAN-1 (**C**), and PA-TU-8902 (**D**) cells at 24 hours. Maximal apoptosis was observed in the PA-TU-8902 cell line.

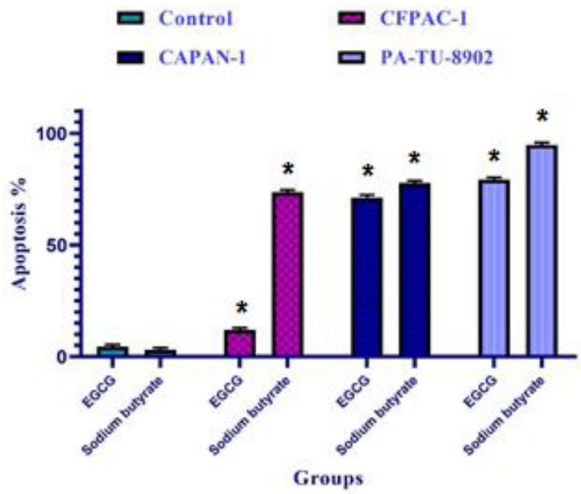

Figure 4. The apoptotic effects of EGCG compared with sodium butyrate on PA-TU-8902, CFPAC-1, and CAPAN-1cells. The sodium butyrate had a more significant apoptotic effect than EGCG. *P<0.0001 vs. control

genes that control the processes of apoptosis have been identified [31]. Two molecular pathways exist to induce apoptosis, the intrinsic (mitochondrial) and the extrinsic (DR) pathways. The intrinsic pathway leads to apoptosis under the control of mitochondrial pro-enzymes [31]. The extrinsic pathway is triggered by the Fas plasma membrane DR binding to its extracellular ligand. In both cases, outer mitochondrial membranes become permeable to internal cytochrome c, which is then released into the cytosol [32]. Cytochrome c recruits pro-caspase-9 and APAF1 to compose the apoptosome,

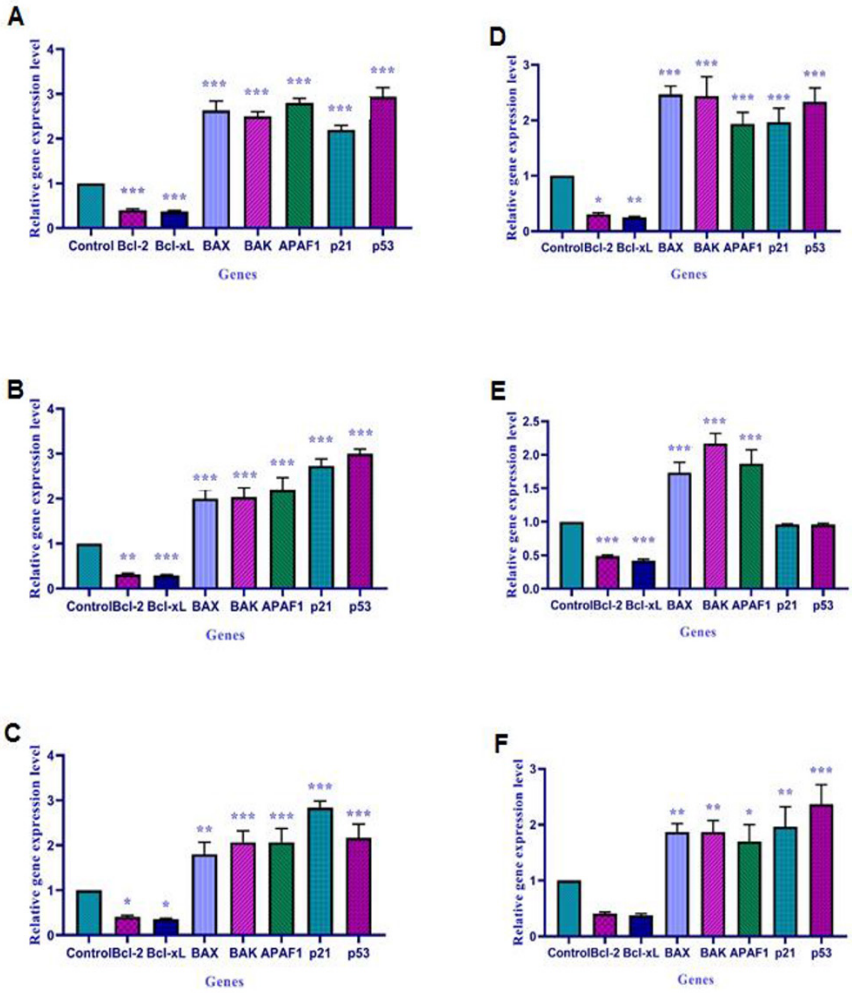

Figure 5. The relative expression level of BAX, BAK, APAF1, Bcl-2, Bcl-xL, p21, and p53 gene in the PA-TU-8902 (**A**), CFPAC-1 (**B**), and CAPAN-1(**C**) cell lines treated with sodium butyrate versus untreated control groups at 24 hours. Also, the effects of EGCG on the mentioned genes expression levels for PA-TU-8902 (**D**), CFPAC-1 (**E**), and CAPAN-1 (**F**) cell lines are presented. *P<0.05, **P<0.005, and ***P<0.0001 vs. control

culminating in apoptosis [31]. HDACIs and DNMTIs can induce apoptosis via the DR pathway [32, 33]. In the present study, we indicated that sodium butyrate and EGCG could induce cell apoptosis through the extrinsic pathway in human pancreatic cancer cell lines. These compounds up-regulated *BAX*, *BAK*, *APAF1*, *p21*, and p53 expression and down-regulated *Bcl-2* and *Bcl-xL* expression, resulting in apoptosis induction. Similarly, it has been reported that sodium butyrate can induce apoptosis in pancreatic cancer ASPC-1 and PANC-1 cell lines via the mitochondrial apoptotic pathway, *Bcl-2* and *Bcl-xL* down-regulation, depolarization of the mitochondrial membrane, cytochrome c release from mitochondria, activation of caspases (caspase-9 and -3) and apoptosis induction [34]. A similar effect has been shown in the HT-29 human colon cancer cell line [35]. In human COLO 205 colon adenocarcinoma cells, sodium butyrate can induce apoptosis by up-regulation of the *BAK* and reduction of anti-apoptotic BCL-xL, XIAP, and survivin proteins [36]. It has been demonstrated that sodium butyrate increases the expression levels of *p21* and *p53*, the release of cytochrome c, and alters the balance of anti- and pro-apoptotic Bcl-2 family proteins in HCT 116 human colorectal cancer cell line [37]. Additionally, in vitro studies have demonstrated that EGCG, as

DNMTIs, induces cell growth inhibition and apoptosis induction by the up-regulation of *Fas* and *Bax*, and down-regulation of *Bcl-2* leads to the activation of caspase-3, -7, -8, and -9 in B lymphoma [38]. In human laryngeal epidermoid carcinoma of the larynx (Hep2 cell line), it has been indicated that EGCG treatment increases the levels of *BAX* and *p53* with a corresponding decrease in *Bcl-2* level [39]. Besides, significant up-regulation of *BAX*, *p21*, *p27*, and *p53*, as well as down-regulation of *c-myc* and *Bcl-2* due to EGCG treatment has been shown in Sarcoma180 cells [40]. Recent in vitro studies have indicated that HDACIs and DNMTIs can induce apoptosis in a p53-dependent and -independent manner [41-43]. Inconsistent with the mentioned reports, we indicated that sodium butyrate and EGCG can induce apoptosis in human pancreatic cancer cell lines via a p53-dependent and -independent manner. EGCG treatment could not induce significant p53 up-regulation in the CFPAC-1 cell line. Further, minimal cell apoptosis was observed in this group. It could be concluded that a p53-dependent manner can induce more strong apoptosis. However, we do not evaluate protein levels; hence, it is recommended that future studies consider this issue.

Conclusion

We indicated that sodium butyrate and EGCG could induce apoptosis in human pancreatic cancer cell lines (PA-TU-8902, CFPAC-1, and CAPAN-1) through the intrinsic apoptotic pathway in a p53-dependent and -independent manner.

Acknowledgments

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Conflict of Interest

The authors report no conflict of interest.

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