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Chemerin-induced oxidative stress triggers apoptosis in HT-29 colon adenocarcinoma cells

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Colorectal cancer (CRC) remains one of the most common cancers and is the third leading cause of cancer-related deaths despite conventional treatments. Chemerin is an endogenous adipocytokine that plays a role in cancer, apoptosis, oxidative stress, and inflammation processes. The aim of this study is to investigate intracellular chemerin signalling in apoptosis, oxidative stress, and inflammation in HT-29 cells treated with exogenous chemerin. The study aims to reveal how chemerin affects these processes and potentially triggers apoptosis in colon cancer cells. Cell viability was determined using the XTT cell proliferation assay after HT-29 cells were treated with 5, 20, and 40 nM chemerin for 48 hours. Oxidative stress (TOS, TAS) and inflammation markers (TNFα and IL1β) were measured using ELISA, while NF-kB mRNA expression was assessed with qRT-PCR. Additionally, CMKLR1, cytochrome c, and caspase-3 gene expressions were quantified using qRT-PCR. PTEN and Bax/Bcl-2 protein expressions were evaluated through Western blotting. Chemerin dose-dependently reduced CMKLR1 levels. Notably, low-dose chemerin increased TOS, OSI, TNFα, IL1β, and NF-kB levels, and low doses were more effective in reducing TAS compared to other doses. Administration of chemerin in human HT-29 cells also resulted in the up-regulation of PTEN, Bax, cytochrome c, and caspase-3, and the down-regulation of Bcl-2. Overall, the study data demonstrated that chemerin induces apoptosis in colon cancer cells by increasing oxidative stress and inflammation through intracellular PTEN, Bax/Bcl-2, cytochrome c, and caspase-3 signalling pathways.

Keywords: Colorectal cancer, PTEN, Bax/Bcl-2, Cytochrome c, Caspase-3

Colorectal cancer (CRC) is the fourth most prevalent cancer type and ranks third in cancer-related mortality despite conventional treatment methods. Thus, molecular therapies that target intracellular signalling pathways have been promising in CRC in recent years[1]. Chemerin, a versatile adipocytokine, was associated with apoptosis[2-6], oxidative stress, inflammation[7,4], and various types of cancer[8,6,5]. Although plasma chemerin expression is upregulated in colorectal adenocarcinoma[9,10], it is downregulated in tumour tissues when compared to normal tissues[11]. Chemerin chemokine-like receptor 1 (CMKLR1/ ChemR23)[12], which is the primary functional chemerin receptor, is also expressed in cancerous colon cells in addition to macrophages, dendritic cells, adipocytes, and vascular cells[13,14]. It was reported that chemerin could modulate the tumour cell phenotype by interacting with CMKLR1 and inhibit the growth of cancerous cells in melanoma[11]. Furthermore, a study demonstrated that force-expressed chemerin activates CMKLR1 and releases phosphatase and tensin homologue (PTEN), a tumour suppressor protein, thus, inhibiting migration and metastasis in hepatocellular carcinoma[6]. Another study reported that exogenous chemerin activated PTEN in human prostate and sarcoma tumors[5]. These studies[9-11], show that there is a relationship between chemerin and CRC and that its receptor is involved in different cancers[11,6,5]. However, no study demonstrated the possible pro-anti-apoptotic effect of chemerin in colon cancer via an intracellular signalling pathway. It is known that cancer develops due to the increase in cell proliferation and the decrease in apoptosis[15]. The extrinsic pathway (death receptor) and the intrinsic pathway (mitochondrial pathway) are involved in apoptosis activation. Major intrinsic pathway factors include proapoptotic Bax and anti-apoptotic Bcl-2. Bcl-2 protects cells against apoptosis, while high Bax levels promote necrosis. Thus, upregulation of Bax and downregulation of Bcl-2 are critical indicators of cell fate[15]. As the anti-apoptotic regulator Bcl-2 prevents the passage of cytochrome c into the cytosol, the proapoptotic regulator Bax contributes to the passage of cytochrome c into the cytosol[16]. Both extrinsic and intrinsic pathways terminate in the final

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caspase-3 stage of apoptosis. Caspase-3 is one of the most important enforcers of apoptosis[15]. Apoptosis could occur after cells are exposed to reactive oxygen product (ROS) mediated oxidative stress or following inflammation[17]. In contrast to the detrimental effects of oxidative stress on various pathologies other than cancer, ROS accelerates the anti-tumorigenic signal and promotes apoptosis in tumour cells[18]. The extrinsic apoptosis pathway is activated by the binding of certain cytokines such as tumour necrosis factor alpha (TNFα) and their receptors[17]. The TNFα initiated death signal[17], mitochondrial outer membrane permeability changes, and TNFα triggers ROS more in this process. On the other hand, ROS-activated proapoptotic protein expression could be neutralized by nuclear factor kappa B (NF-kB), generally known as the survival signal[16] and vice versa[19]. When the signal flow shifts to the proapoptotic direction, caspase-3 is activated due to cytosolic cytochrome c release, leading to classical apoptosis. Thus, the last stage in the intrinsic and extrinsic pathways is caspase-3[16]. More importantly, there is evidence that chemerin triggers ROS and induces apoptosis by upregulating Bax/Bcl-2 and caspase-3[20]. The present study hypothesized that chemerin could exhibit an apoptotic effect by inducing oxidative stress in the HT-29 colon cancer cell line. Therefore, PTEN (a tumour suppressor protein), Bax/Bcl-2 proteins (important apoptosis markers), and proapoptotic cytochrome c and caspase-3 gene expression levels were examined in the study.

Materials and Methods

Cell culture and reagents

The human colon adenocarcinoma cell lines (HT-29, ATCC, HBT-38) were procured from Atlas Biotechnology (Ankara, Türkiye). After the cells frozen in the cryovial were thawed in a 37°C water bath, Modified Michel's fixative-Zeus medium (McCoy's 5A, Capricorn) that included 10% Fetal Bovine Serum (FBS, Capricorn), 1% Penicillin-Streptomycin (Capricorn), and 1% L-Glutamine were transferred into 25 cm^2 cell culture dishes. And these were placed in an incubator at 37^oC with 5% CO_2 and 100% humidity. The flasks in the incubator were checked with an inverted microscope during incubation to determine whether they were disturbed for the first two days, and cellular proliferation was observed. Cells were used in the experiment after they covered the flask base by 85-90%. Chemerin (149-157, Anaspec, cat no:2255045) peptide was dissolved with phosphate buffer serum (PBS) to obtain master stock (940 nM). Intermediate stocks in various concentrations were prepared by diluting the main stock and stored at -20°C.

XTT cell viability

HT-29 cell lines were inoculated into 96-well culture plates with 10,000 cells per well in a complete culture medium that included various chemerin concentrations (0, 5, 10, 20, 40, 80, and 160 nM). The cytotoxicity and apoptotic effects of chemerin were determined with a colourimetric assay sodium 30-[1-phenyl-aminocarbonyl)-3,4-tetrazolium] bis (4-methoxy-6-nitro) benzenesulfonic acid (XTT) cell viability kit (Biotium, Inc., cat no: 30007). The experimental protocol was determined based on manufacturer instructions. The viable cell count was measured with Heales MB-530 External Computer Elisa Microplate Reader Machine (Shenzhen, China) based on formazan. Cell viability was calculated with the following formula: Cell survival rate = [(experimental well-blank well)/(control well-blank well)] × 100. Where experimental well (medium containing cells, XTT study solution, and chemerin), control well (culture containing cells, XTT study solution, but no chemerin), blank hole (medium without cells, XTT study solution or chemerin)[21]. The IC50 curve was plotted to determine the IC50 dose that gives the chemerin concentration that inhibited half-maximum proliferation of HT-29 cells.

Experimental design and treatment

The selection of chemerin concentration and the exposure time was based on a previous where 6 nM chemerin activated PTEN after 48 h[5]. For further analysis, cells were randomly divided into four groups: Control group (cells were cultured without chemerin), Chemerin group (cells were cultured with chemerin (5 nM-low doses) for 48 h), Chemerin group (cells were cultured with chemerin (20 nM-moderate dose) for 48 h), Chemerin group (cells were cultured with chemerin (40 nM-high doses) for 48 h). Cells were harvested for molecular analysis after the time mentioned above.

Determination of cell lysate total oxidant status (TOS), total antioxidant status (TAS)

Total oxidant status (TOS) and total antioxidant status (TAS) were measured with Erel's method[22,23] in cell lysate. Rel Assay Diagnostics kit (cat no: RL0024, RL0017, Mega Medicine Ltd., Sahinbey/Gaziantep/ Turkey) was used in TOS and TAS measurements. The absorbance of the samples was determined with a spectrophotometer (ChemWell 2910, Awareness Technology, Palm City, USA). The study was repeated three times for all samples. TOS and TAS findings were presented in μmol H_2O_2 Equiv./L and Trolox Equiv./L, respectively.

Determination of HT-29 cell lysate oxidative stress index (OSI)

OSI was calculated to determine the oxidative stress level in HT-29 cell lysate. OSI is a unitless (AU: arbitrary unit) parameter calculated by the ratio of TOS to TAS. Before the calculation, the TAS (mmol TroloxEquiv./L) was converted to μmol TroloxEquiv./L. The findings are presented as a percentage based on the following formula: OSI = [(TOS, μmol H_2O_2 Equiv./L) / (TAS, μmol TroloxEquiv./L) × 100][24,25]. High OSI was accepted as the indicator of oxidation as opposed to antioxidant capacity.

Measurement of TNFα and IL1β levels in cell lysate with the ELISA method

Human TNFα and human IL1β ELISA kits (Sun Red, Shanghai Sunred Biological Technology Co., Ltd) were employed to determine TNFα and IL1β protein concentrations in cell lysate. The concentrations were calculated on the basis of sample readings on a spectrophotometer. (Chromate 4300, Awareness Technology, Inc. Martin Hwy. Palm City, USA) at 450 nm. The measurements were conducted with the Protein Quantification Kit (Sigma-Aldrich, cat no: 51254-1KT, USA). The TNFα and IL1β protein concentrations are presented in ng/mg protein and pg/mg protein, respectively.

Quantitative Real-Time PCR analysis of CMKLR1, NF-κB, Cytochrome c, Caspase-3 gene expression

After HT-29 cells were incubated with chemerin for 48 h, cells were harvested from the wells and pelleted with centrifugation. Immediately, total RNA isolation (A.B.T.™ Blood/Tissue RNA Purification Kit for Leukemia, cat no: I04-01-10) was conducted. ABT High-Capacity cDNA Reverse Transcription kit (cat no: C03-01-05) was employed to synthesize the cDNA. The targeted genes were amplificated with Real-Time PCR (Applied Biosystems™ 7500 Real-Time PCR System) with specific oligonucleotide primers. The primer sequences used in PCR are presented in Table 1. Real-Time PCR cycles were as follows: 95 °C for 5 min, 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s, and 40 cycles. The relative expressions of targeted genes were determined with the $2^{-\Delta\Delta Ct}$ method[26], and normalized with the *β-actin* gene.

Apoptosis assay: Cleaved PARP (asp 214) sandwich ELISA

PARP (Cleaved) [214/215] Human ELISA Kit (Invitrogen, cat no: KHO0741) was employed to determine the apoptotic effect of chemerin on the HT-29 cell line. Care was taken to ensure that the concentration of each sample was 0.156-10 ng/mL in the study. Poly ADP-Ribose Polymerase (PARP) activity increases with DNA damage. During apoptosis, the cleavage between Adp216 and Gly217, a conserved region in PARP, is conducted by the caspase-3 protein. Proteolysis of PARP to the stable 85 kDa fragment is an early marker of apoptosis. Thus, the 89 kDa fragment is a reliable biomarker of apoptosis. Based on the manufacturer′s instructions, all samples were read in triplicate on a microplate reader (Chromate 4300, Awareness Technology, Inc. Martin Hwy. Palm City, USA) at 450 nm[27,28].

Western blotting

HT-29 cells were harvested and extracted with the RIPA buffer (Thermo Scientific, Pierce RIPA Buffer, cat no: 89901). Protein content was determined with the Qubit® Protein Assay Kits (Thermo Fisher Scientific, cat no: Q33211) in a Qubit® 3.0 Fluorometer (Thermo Fisher Scientific, cat no: Q33216). An equal amount of protein (100 μg) was separated from each sample on a 4-12% Bis-Tris gradient gel (Invitrogen, NuPAGE 4-12% Bis-Tris Gel, cat no: NP0321PK2). The proteins were transferred to nitrocellulose membranes and blocked with membrane blocking solution (PBS containing 0.1% Tween-20 with 5% BSA). They were incubated with primary antibodies (Anti-PTEN: cat no: A11528, 1:500; Anti-Bax: cat no: sc-493, 1:500; Anti-Bcl-2: cat no: MA5-11757, 1:1000; Anti- β-Actin: cat no: sc-130656, 1:1000) and horseradish peroxidase-

Table 1 — Primer sequences for qRT-PCR

Genes	Forward (5'-3')	Reverse (5'-3')
CMKLR1	CTGTCCACACCTGGGTCTTC	ACGATGGTGAGGTAGCAAGC
NF-κB	CCGCTTAGGAGGGAGAGCC	TCTGCCATTCTGAGCTGGT
Cytochrome c	CGTTGTGCCAGCGACTAAAA	GCTTGCCTCCCTTTTCAACG
Caspase-3	TGCTATTGTGAGCCGGTTGT	TCCAGAGTCCATTGATTCGCT
β-actin	AGCAAGAGAGGCATCCTCACC	ACAGGGATAGCACAGCCTGGA

conjugated secondary antibodies (Anti-rabbit: cat no: R05072-500, 1:10000 or Anti-mouse: sc-2005, 1:5000), and the membranes were treated with enhanced chemiluminescence (ECL) solution (NZY Supreme ECL HRP Substrate, Nzytech, cat no: Mb19301). Bands were observed with ChemiDoc-It2, UVP, and band density was determined using ImageJ software. Protein expressions were standardized based on β-actin.

Statistical analysis

The study data were analyzed with the SPSS 22 software. The group data are presented in "mean ± standard deviation." One-way ANOVA (*post-hoc* LSD) was conducted for inter-group comparisons. $P<0.05$ was considered statistically significant.

Results

The effect of chemerin on cell viability

Cell viability percentages were 103.5±5.82, 101.67±3.4%, 99.04±9.39%, 89.26±6.16%, 56.52±3.28%, and 41.41±2.97%, in 5, 10, 20, 40, 80, and 160 nM chemerin treatments, respectively. The IC50 dose for chemerin in HT-29 cells was calculated as 126.64 nM. Although there were no significant differences between cell viability with 5, 10, and 20 nM chemerin doses when compared to the control (P=0.426, P=0.701, P=0.825, respectively), cell viability significantly was lower with 40, 80, and 160 nM doses (Fig. 1).

Effect of chemerin on oxidative stress and inflammation markers

TOS was significantly higher (P=0.001, P=0.011, P=0.001, respectively), and TAS was lower ($P<0.001$, $P<0.001$, $P<0.001$, respectively) in 5, 20, and 40 nM chemerin administrations when compared to the control group. (Fig. 2A& 2B). TAS was also higher in the 40 nM chemerin group compared to the 5 and 20 nM groups (P=0.001, P=0.004, respectively). Although OSI was the highest at 5 nM chemerin dose, it was significantly higher in 5, 20, and 40 nM chemerin administrations compared to the control group ($P<0.001$, $P<0.001$, P=0.001, respectively) (Fig. 2C). The OSI was significantly higher in the 5

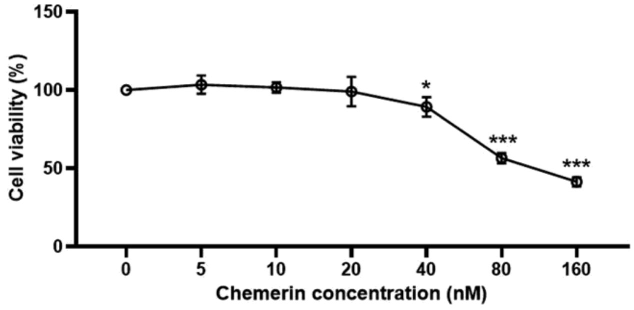

Fig. 1 — XTT measurements for chemerin administration for 48 h in HT-29 human colon adenocarcinoma cell line. [Data are presented as mean ± standard deviation of three independent experiments. *$P<0.05$ compared to the control; ***$P<0.001$ compared to the control]

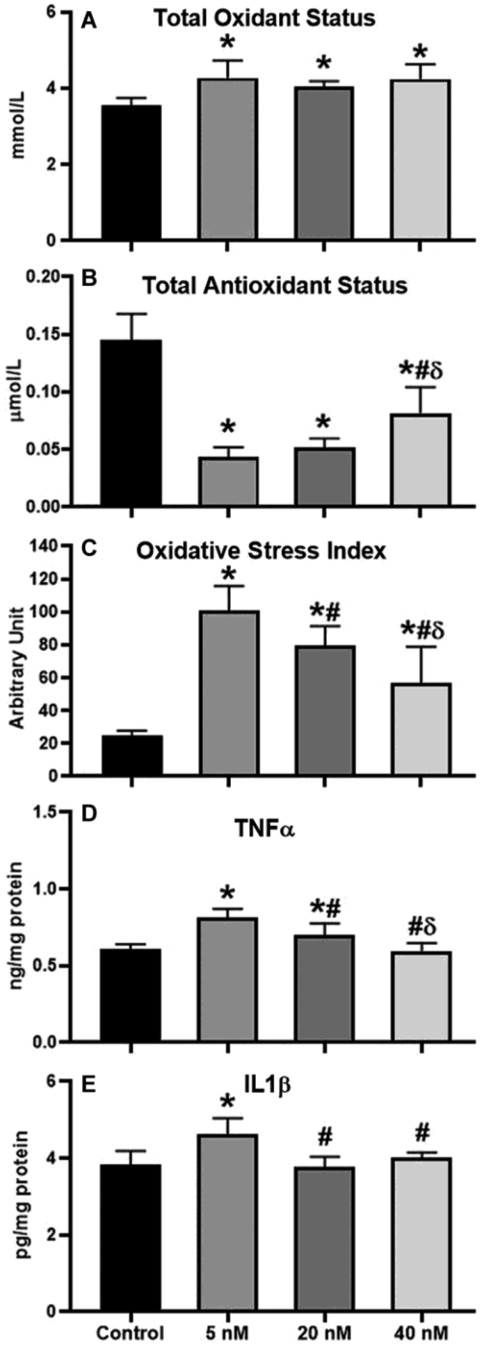

Fig. 2 — The effects of 5, 20, and 40 nM chemerin administrations on total oxidant level (A), total antioxidant level (B), oxidative stress index (C), TNFα (D), and IL1β (E) levels in HT-29 human colon adenocarcinoma cell line.[*$P<0.05$ compared to the control; #$P<0.05$ compared to the 5 nM chemerin group; δ$P<0.05$ compared to the 20 nM chemerin group]

nM chemerin group when compared to the 20 and 40 nM groups (*P*=0.018, *P*<0.001, respectively), and the OSI was also significantly higher in the 20 nM group when compared to the 40 nM group (*P*=0.01).

TNFα levels were higher in 5 and 20 nM chemerin treatments when compared to the control group (*P*<0.001, *P*=0.006, respectively) (Fig. 2D). TNFα level was higher in the 5 nM group when compared to the 20 and 40 nM groups (*P*=0.001, *P*<0.001, respectively), and TNFα was higher in the 20 nM administration when compared to the 40 nM group (*P*=0.003). IL1β level was significantly higher in the 5 nM chemerin group when compared to the control and 20 and 40 nM chemerin administration groups (*P*<0.001, *P*<0.001, *P*=0.003, respectively) (Fig. 2E).

Effect of chemerin on CMKLR1, NF-κB, Cytochrome c, and Caspase-3 mRNA expressions

CMKLR1 mRNA expression was higher in the 5 nM chemerin group (*P*<0.001) and lower in the 20 and 40 nM groups when compared to the control group. CMKLR1 expression was lower in 20 and 40 nM chemerin groups when compared to the control and 5 nM groups (*P*<0.001, *P*<0.001, respectively) (Fig. 3A). NF-kB mRNA expression was higher in the 20 nM chemerin group when compared to the control, 5 nM, and 40 nM chemerin groups (*P*<0.001, *P*<0.001, *P*<0.001, respectively) (Fig. 3B). Cytochrome c mRNA expression was higher in three chemerin administration doses (5, 20, and 40 nM) when compared to the control group (*P*=0.012, *P*<0.001, *P*=0.036, respectively). Cytochrome c expression was also higher in the 20 nM chemerin group when compared to the 5 nM and 40 nM treatments (*P*<0.001, *P*<0.001, respectively) (Fig. 3C). Caspase-3 mRNA expression was higher in 5, 20, and 40 nM chemerin groups compared to the control group (*P*<0.001, *P*<0.001, *P*=0.001, respectively). Caspase-3 expression was higher in the 20 nm group when compared to the 5 and 40 nM groups (*P*<0.001, *P*<0.001, respectively) (Fig. 3D). As a result, it can be interpreted that a 20 nM chemerin dose is more effective on cytochrome c and caspase-3, which are the markers that trigger apoptosis, compared to other chemerin doses.

Effect of chemerin on PTEN, Bax, and Bcl-2 protein expression

PTEN protein expression was higher in 5, 20, and 40 nM chemerin treatments when compared to the control group (*P*<0.001, *P*<0.001, *P*<0.001, respectively). PTEN expression was higher in the 5 nM group compared to the 20 and 40 nM groups (*P*=0.001, *P*=0.01, respectively) (Fig. 4A & 4B). Bax protein expression was significantly higher in 5, 20, and 40 nM chemerin doses when compared to the control (*P*<0.001, *P*<0.001, *P*<0.001, respectively). Bax expression was higher in the 20 nM chemerin group when compared to 5 and 40 nM groups (*P*<0.001, *P*=0.008, respectively) and higher in the 40 nM chemerin group when compared to 5 nM administration (*P*<0.001)(Fig. 4A& 4C). Bcl-2 was lower in all three chemerin administration doses (5, 20, 40 nM) when compared to the control (*P*=0.015, *P*<0.001, *P*=0.013, respectively). Bcl-2 was lower in

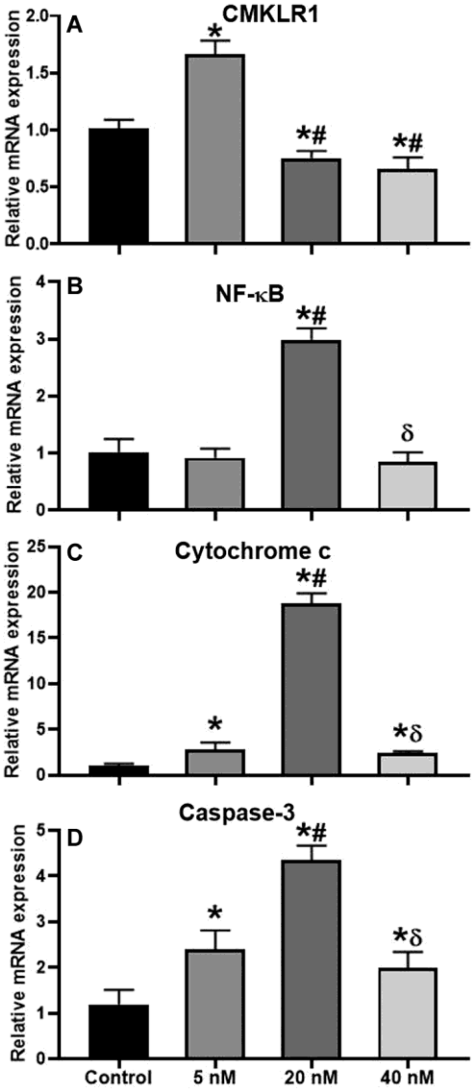

Fig. 3 — The effects of 5, 20, and 40 nM chemerin administrations on CMKLR1 (A), NF-kB (B), Cytochrome c (C), and Caspase-3 (D) mRNA expressions in HT-29 human colon adenocarcinoma cell line. .[**P*<0.05 compared to the control; #*P*<0.05 compared to the 5 nM chemerin group; δ*P*<0.05 compared to the 20 nM chemerin group]

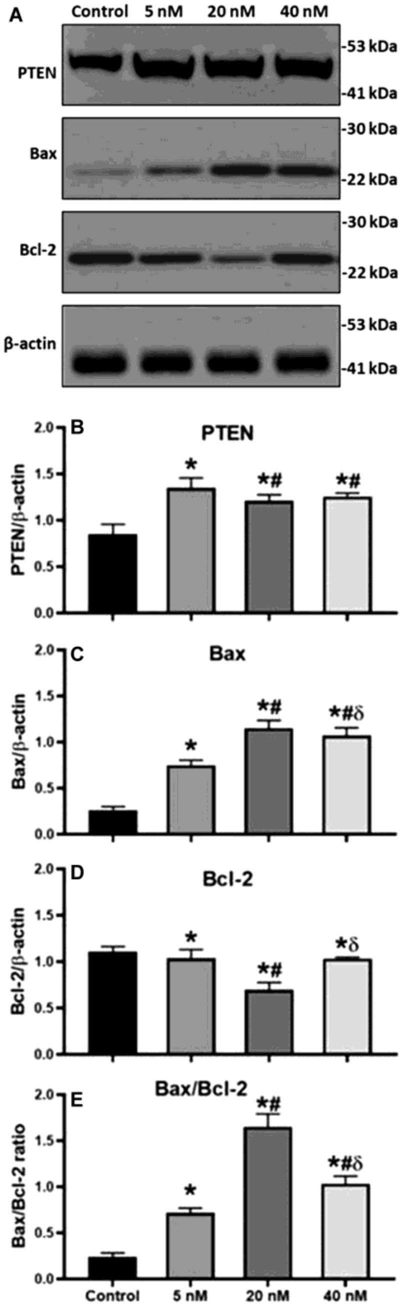

Fig. 4 — Representative Western blot findings for PTEN, Bax, Bcl-2, and β-actin in the HT29 cells treated with medium or 5, 20, 40 nM chemerin (A). PTEN (B), Bax (C), and Bcl-2 (D) protein expressions were normalized based on β-actin. Furthermore, Bax/Bcl-2 ratio was calculated with the ratio of Bax to Bcl-2 (E). .[*$P<0.05$ compared to the control; #$P<0.05$ compared to the 5 nM chemerin group; δ$P<0.05$ compared to the 20 nM chemerin group]

the 20 nM chemerin group when compared to the 5 and 40 nM groups ($P<0.001$, $P<0.001$, respectively) (Fig. 4A & 4D).The Bax/Bcl-2 ratio was significantly higher in 5, 20, and 40 nM chemerin doses when compared to the control ($P<0.001$, $P<0.001$, $P<0.001$, respectively). Bax/Bcl-2 was higher in the 20 nM chemerin group compared to the 5 and 40 nM groups ($P<0.001$, $P<0.001$, respectively). Also, the Bax/Bcl-2 ratio was higher in the 40 nM group when compared to the 5 nM chemerin dose ($P<0.001$) (Fig. 4A & 4E).

In summary, the increase in Bax protein expression and the decrease in Bcl-2 protein expression are more pronounced in the 20 nM chemerin group. Although the increase in PTEN protein was higher in the 5nM chemerin group than in other doses, it should not be ignored that the expression of PTEN was higher in the 20 nM chemerin group compared to the control group. Therefore, it is observed that the dose of 20 nM chemerin is more effective in the expression of these apoptosis-related proteins.

Effect of chemerin on apoptosis in HT-29 cells

Cleaved PARP was significantly higher in 5, 20, and 40 nM chemerin doses when compared to the control ($P<0.001$, $P<0.001$, $P<0.001$, respectively). The cleaved PARP was higher in the 20 nM chemerin group when compared to the 5 and 40 nM groups ($P<0.001$, $P<0.001$, respectively) and higher in the 40 nM group when compared to the 5 nM chemerin dose group ($P= 0.023$) (Fig. 5). Collectively, our data show that even the lowest chemerin administration (5nM) triggers apoptosis in colon cancer cells. However, it is noteworthy that the 20 nM chemerin application made the maximum changes in apoptosis

Discussion

One of the most important results of our study was that chemerin induced apoptosis in HT-29 cells by upregulating PTEN, Bax/Bcl-2, cytochrome c, and caspase-3. Also, low-dose chemerin administration was more effective in triggering markers of inflammation (TNFα, IL1β, NF-κB) and oxidative stress (TOS and OSI) than other doses. It was determined that apoptotic induction and markers increased significantly, especially in the moderate dose chemerin. As far as we know, our study findings are the first, as there has been no study to demonstrate the apoptotic cell signalling of chemerin in colon cancer.

In low-dose chemerin administration, the expression of the chemerin receptor CMKLR1 was increased, while its receptor expression was decreased at higher chemerin concentrations like in other studies[12,29]. Interestingly, the amount of PTEN protein, which is thought to be activated by interacting with CMKLR1, was also more expressed at the lowest dose of chemerin. As the chemerin dose increased, PTEN

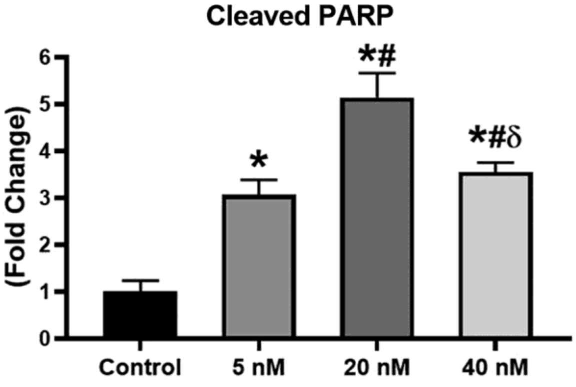

Fig. 5 — The effects of 5, 20, and 40 nM chemerin administrations on cleaved PARP (fold change) levels in HT-29 human colon adenocarcinoma cell line. .[$*P<0.05$ compared to the control; $\#P<0.05$ compared to the 5 nM chemerin group; $\delta P<0.05$ compared to the 20 nM chemerin group]

protein expression remained higher compared to the control group, although it was lower compared to the lower doses. A recent study reported that chemerin increased PTEN activity and exhibited tumour-suppressive properties in hepatocellular carcinoma[6]. In the study mentioned above, it was hypothesized that binding of exogenous chemerin to CMKLR1 weakens the PTEN-CMKLR1 interaction, thus releasing PTEN and increasing its tumour suppressor activity[6]. This hypothesis seems to explain the fact that while CMKLR1 expression decreased in our study, the amount of PTEN protein remained high compared to the control group. According to our estimations, the decreased expression of CMKLR1 may be associated with PTEN gaining tumour suppressor activity by dissociating from the receptor. However, further studies conducted on a higher number of effectors in downstream signalling are required to explore the details of CMKLR1-PTEN interaction and clarify their mutual regulation. In an *in vitro* study conducted on human umbilical vein endothelial cells, it was reported that chemerin increased PTEN activity[12]. Dhaou *et al*; showed that chemerin overexpression induces apoptosis in endothelial cells by negative regulation of the PI3-kinase/AKT pathway in a mouse model of proliferative retinopathy. Moreover, they pointed out that this situation returned to normal in mice phenotypically deficient in the CMKLR1 receptor[12]. In addition, Rodriguez-Penas *et al*. reported that chemerin induces apoptosis in mouse cardio-myocytes by reducing AKT phosphorylation and increasing caspase-9 cleavage[3].

In another *in vivo* study, force-expressed chemerin inhibited tumour growth in prostate and sarcoma tumour lines *via* transcriptional upregulation of PTEN[5]. Although these *in vivo* observations are not related to colon cancer cells, they complement the *in vitro* findings in our study and provide evidence that chemerin works for PTEN activation. PTEN is a versatile tumour suppressor. Its regulatory effects originate in its ability to restrain cancers by regulating PI3 Kinase signalling. Loss of the functionality of the PTEN is a critical event in the development of several human cancers. Although the catalytic activity of ROS-modulated PTEN led to the catalytic inhibition of oxidative stress[30], chemerin-induced oxidative stress did not cause PTEN inactivation in the present study, confirming that PTEN activation was induced by chemerin. Another significant result of this study was that chemerin increased oxidative stress in HT-29 cells. It is known that ROS production increases with conventional cancer treatments[18]. In contrast to its detrimental effects in healthy cells, oxidative stress accelerates anti-tumourigenic signalling and induces apoptosis in cancerous cells[31]. Therefore, targeting ROS in cancerous cells may be a different interface of cancer therapy. The primary cellular action of antioxidants is to balance redox[18]. Antioxidants scavenge excess ROS and develop resistance to ROS-induced apoptosis in tumour cells[32,33]. The clinical use of various chemotherapeutic drugs could induce resistance to these drugs. In short, medications with a single apoptosis target are ineffective in cancer treatment[18]. Thus, in the present study, high oxidative stress and low antioxidant capacity induced by chemerin contributed to chemerin-induced apoptosis in colon cancer cells.

High ROS initiates apoptotic signalling both intrinsically and extrinsically, stopping the cellular cycle and leading to the killing of cancerous cells[33]. Activation of apoptotic effectors, including the Bax/Bcl-2 protein family, cytochrome c, and caspase-3, is affected by excessive ROS production. DNA fragmentation occurs by the cleavage of poly ADP ribose polymerase (PARP)[34]. On the other hand, the increased level of ROS leads to cleavage of Bcl-2 protein downstream by binding cytokines such as TNFα to death receptors.

It also causes the penetration of Bax, a proapoptotic protein, into the inner layer of the mitochondria, causing the release of cytochrome c[17]. Cytochrome c creates a complex downstream that induces caspase-3 cleavage. In apoptotic signalling, anti-apoptotic Bcl-2 is inhibited, while proapoptotic Bax is activated (Graphical abstract). ROS-induced apoptosis could be attributed to the disruption of redox homeostasis by the increase in oxidants and loss of antioxidants[35]. In the present study, TOS and TAS parameters employed to demonstrate oxidative stress predicted the general state of the oxidant and antioxidant abilities of the body. OSI that increased with chemerin administration was used to analyze the redox balance[36]. The relationship between chemerin-induced ROS and apoptosis in human colon cancer cells has not been described. Thus, a possible molecular mechanism associated with chemerin-induced apoptosis in HT-29 cells could be ROS-triggered apoptosis (Graphical abstract) because our findings demonstrated that ROS-dependent Bax/Bcl-2 ratio, cytochrome c and caspase-3 gene expressions in the downstream apoptotic signalling increased with the chemerin administration. The study where it was reported that chemerin-induced ROS also led to apoptosis via Bax/Bcl-2 and caspase-3 upregulation in human granulosa-lutein cells[20] was consistent with the current study hypothesis.

Another significant finding was the induction of TNFα, IL1β, and NF-kB by chemerin in HT-29 cells. This effect of chemerin, known to have biphasic pro-inflammatory and anti-inflammatory properties[15], during apoptosis, was obviously not an expected finding. Systemic inflammation is strongly associated with the development of cancer. Inflammatory mediators such as cytokines and reactive oxygen species produced by tumour cells lead to a carcinogenic microenvironment that contributes to the initiation and progression of cancer. Elevated IL1β and TNFα levels in CRC were associated with tumour stages, survival rate, or distant metastasis in colorectal cancer patients. IL1β initiates pro-inflammatory cascades and facilitates the spread of tumours. TNFα signalling activates the transcription factor NFκB. Thus, it supports the expression of downstream inflammatory mediators involved in aberrant cell differentiation and cell proliferation. Clinical studies reported that the increase in TNFα levels increases the CRC risk, while the decrease in IL1β levels reduces it[37]. Recent studies reported a positive correlation between chemerin, IL1β, and TNFα[38,39,29]. There is an interaction between TNFα and chemerin. Chemerin is upregulated by TNFα[38,39,29] and increases IL1β levels[30]. Based on the current study cleaved poly ADP ribosome polymerase (c-PARP) findings, the strongest apoptosis was observed with the 20 nM chemerin dose. Bax/Bcl-2 ratio, cytochrome c, and caspase-3 activation were also indicated with the same dose. However, we observed that apoptosis continued at the lowest 5 nM chemerin dose. This finding could be explained with a hypothetical approach. Pyroptosis is a third type of programmed cell death besides apoptosis. Pyroptosis is a potent pro-inflammatory form of lytic cellular death inducted downstream of inflammatory complexes[40]. Pyroptotic cells release an inflammasome complex, which includes inflammatory IL1β and other cytokines, which is one of the driving forces of proptosis that leads to complex cell death[41]. TNFα is one of the main mediators of inflammation and is a double-edged sword for the tissues. There are two main pathways in TNFα signalling. The first pathway, along with the inflammatory factors, could lead to various pathological damages and promote the invasion and metastasis of tumour cells. The second pathway is proapoptotic, which entails TNFα-induced ROS downstream migration of Bcl-2 to mitochondria, cytochrome c release, and caspase-3 cleavage[42] (Graphical abstract). In the final stage of apoptosis, only c-PARP is activated when DNA is fragmented. PARP is a protein involved in DNA repair; however, its repair function could be inactivated by cleavage. Therefore, c-PARP is a clear marker of apoptosis in cells[43]. There is a study to explain the events that we have hypothesized so far. Although the study mentioned above is associated with apoptosis in hippocampal neurons, it supports TNFα-stimulated Bax/Bcl-2, cytochrome c, and caspase-3 apoptotic signalling in colon cancer[44]. However, the pathway hypothesized based on chemerin in colon cancers is a first in the literature.

NF-kB is a critical regulator of apoptosis and could serve as both a pro- and anti-apoptotic factor. Although NF-kB is generally anti-apoptotic, it was recently suggested that it could promote apoptosis in certain cases, particularly in response to cellular stress. Despite the evidence suggesting that NF-kB promotes apoptosis, the underlying mechanisms have not yet been determined[45]. A previous study indicated that TNFα and NF-kB induced by chemerin and its receptor CMKLR1 in response to cellular stress participated in apoptosis[46]. Activating inactive NF-kB in the cytoplasm could be initiated by a cytokine such as TNFα. By poorly understood processes, the NF-kB complex can also migrate to mitochondria. It is thought that NF-kB activation in mitochondria after this causes cytochrome c release and stimulates the intrinsic apoptotic pathway via caspase steps[19]. These data identified a distinct proapoptotic pathway and a novel mechanism by which chemerin could induce apoptosis due to its effects on NF-kB transcriptional activity, regulated by TNFα and NF-kB in colon cancer. Our current findings show that increases in oxidative stress and inflammation accompany increased apoptotic activity.

Conclusion

As a result, this study demonstrated that chemerin increases oxidative stress and inflammation in colon cancer cells and induces apoptosis by activating intracellular signalling pathways involving PTEN, Bax/Bcl-2, cytochrome c, and caspase-3.

Ethical approval

All procedures were approved by the Afyonkarahisar Health Sciences University non-interventional clinical studies by the ethics committee (Approval number: 2021/375).

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Conflict of interest

The authors have no conflicts of interest to declare.

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