Effects of colchicine on cell cycle arrest and MMP-2 mRNA expression in MCF-7 breast adenocarcinoma cells

Kolşisin'in MCF-7 insan meme adenokarsinoma hücrelerinde hücre döngüsü tutulumu ve MMP-2 mRNA ifade seviyesi üzerine etkisi

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ABSTRACT

Objective: Colchicine is a tricyclic alkaloid drug and it's been clinically used for a long time because of its anti-inflammatory effects. While colchicine has been safely used in oral applications, it's been demonstrated that colchicine is toxic at higher concentrations. So, the mechanism of effects of low dose colchicine has been studied in cancer related researches. Matrix metalloproteinase-2 (MMP-2) is a member of matrix metalloproteinase enzyme family and it's been demonstrated that MMP-2 expression is increased in cancer and it causes a metastasis of cancer cells through degradation of extracellular matrix. Because of this important effect of MMPs, the studies related to develop MMP inhibitor compounds have great importance in cancer investigations. At present study, it's aimed to investigate the effects of colchicine on cell cycle arrest and MMP-2 mRNA expression in MCF-7 human breast cancer cells.

Methods: In the study, MCF-7 human breast adenocarcinoma cells were purchased from ATCC. Cells were treated with 0.1, 10 ve 100 µg/ml colchicine and cell viability was determined via MTT assay. The effect of colchicine on cell cycle arrest was

ÖZET

Amaç: Kolşisin, trisiklik alkaloid bir ilaç olup klinikte anti-enflamatuvar etkisinden dolayı yaygın olarak kullanılmaktadır. Kolşisin uygun konsantrasyonlarda oral yolla kullanılmakta iken yüksek konsantrasyonlarda toksik etki gösterdiği tespit edilmiştir. Bu nedenle, kanser araştırmalarında düşük doz kolşisinin etki mekanizmaları üzerine çalışmalar yapılmaktadır. Matriks metalloproteinaz-2 (MMP-2), matriks metalloproteinaz enzim ailesinin bir üyesi olup kanserde ifade seviyesinin arttığı ve ekstrasellüler matriksi yıkıma uğratarak kanser hücrelerinin metastazına neden olduğu bildirilmiştir. MMP'lerin bu önemli etkisi nedeni ile kanser hastalığında metastazın önlenmesi amacıyla çeşitli bileşiklerin MMP üzerine inhibitör etkisinin değerlendirilmesi kanser araştırmalarında önemli yer tutmaktadır. Bu çalışmada, kolşisinin, MCF-7 insan meme adenokarsinoma hücresinde hücre döngüsü tutulumu ve metastazda önemli rol oynadığı bilinen MMP-2 protein ifadesi üzerine etkisinin araştırılması amaçlanmıştır.

Yöntem: Çalışmada, MCF-7 insan meme adenokarsinoma hücreleri kullanılmıştır (ATCC, HTB-22). Hücrelere 0.1, 10 ve 100 µg/ml konsantrasyonlarda kolşisin uygulanmış ve hücre canlılığı analizi MTT testi ile

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determined by Muse Cell Analyzer and the percent of cell populations at G0/G1, S and G2/M cycles were identified. The effect of colchicine on MMP-2 mRNA expression has been performed by real-time PCR (qRT-PCR) analysis.

Results: Colchicine has significantly inhibited cell viability at 10 and 100 µg/ml concentrations. It's been also demonstrated that colchicine has induced a cell cycle arrest at G2/M phase and downregulated MMP-2 mRNA expression of MCF-7 cells in all treated concentration ($p<0.0001$).

Conclusion: The results of this study illustrated that colchicine may be a candidate anticancer compound for breast cancer studies and further studies are required to identify the underlying mechanism of effects.

Key Words: colchicine, cell cycle, MMP-2 expression, anticancer, MCF-7

yapılmıştır. Kolşisin'in hücre döngüsü tutulumu üzerine etkisi analiz edilmiş ve G0/G1, S ve G2/M döngüsündeki hücre populasyonları yüzde (%) olarak ifade edilmiştir. Kolşisin'in MMP-2 mRNA ifade seviyesi üzerine etkisi ise gerçek zamanlı PCR (qRT-PCR) yöntemi ile tayin edilmiştir.

Bulgular: 10 ve 100 µg/ml konsantrasyonlarda Kolşisin MCF-7 meme kanser hücresinin canlılığını istatistiksel olarak anlamlı derecede inhibe etmiştir. Kolşisin'in tüm konsantrasyonlarda hücre döngüsünü G2/M fazında inhibe ettiği ve MMP-2 geninin ifade seviyesini anlamlı olarak azalttığı saptanmıştır ($p<0.0001$).

Sonuç: Çalışmadan elde edilen sonuçlar ile kolşisinin kanser çalışmaları için aday bir anti-kanser bileşik olabileceği gösterilmiş olup mekanizmanın aydınlatılabilmesi için ileri çalışmalara ihtiyaç bulunmaktadır.

Anahtar Kelimeler: kolşisin, hücre döngüsü, MMP-2 ekspresyonu, antikanser, MCF-7

INTRODUCTION

Colchicine is a lipophilic, plant derived alkaloid drug which is produced from Colchicum autumnale (1). Although the first approving by the US Food and Drug Administration was in 2009, colchicine is used more than 2000 years for Gout disease treatment (2,3). Colchicine has anti-inflammatory activity and therefore is used for diseases like Gout, FMF and other rheumatic diseases like Behcet's disease, idiopathic recurrent acute pericarditis etc. (4,5). Colchicine's most studied mechanism of action is microtubule disruption (5,6). Colchicine blockes the assembly and polymerization of microtubules and this ability of compound effects further lots of cellular processes such as maintenance of cellular shape, migration of the cell, vesicular actions inside the cell, secretion of cytokines, chemokines and ion channels (5). Recent studies indicated some other effects of colchicine, like inhibition of fibrosis and cardiovascular protective effects (5). Beside, recently published studies discuss that colchicine derivatives could be used as potent anti-tumoral molecules and there are many attempts to develop colchicine hybrids and bioactive drugs as a anti-cancer agents (3,7). A research group has investigated the anticancer effects of colchicine on hepatocellular carcinoma (HCC), cholangiocarcinoma and gastric cancer cells in different studies and has indicated that colchicine has achieved potential anticancer effects at acceptable concentrations and could be used as a palliative treatment in these cancer types (8-16).

MATERIAL and METHOD

Cell Culture and Treatment

Human breast cancer cell line MCF-7 (HTB22) was purchased from American Type Culture Collection. Cells were grown as monolayer cultured in DMEM (ATCC, 30-2002) media supplemented with 10% fetal bovine serum (FBS, Lonza), 1% penicillin/streptomycin (PAA, The Cell Culture Company) and 1% L-glutamine (PAA, Austria) and incubated in a 5% CO2 humidified atmosphere at 37°C. Colchicine was dissolved in % 0.1 DMSO and cells were treated with 0.1, 10 and 100 µg/ml colchicine for 24 hours as described by Sun et al. (17).

Cell viability

The effects of colchicine on cell growth of MCF-7 cells were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, a 180 µl of $5x10^4$ cells/ml were treated with 0.1, 10 and 100 µg/ml of colchicine for 24 h and the cells treated with DMSO were used as control. The cells were washed with PBS, 20 µl MTT solution was added to the wells and incubated at 37 °C for 4 h. Following incubation, the absorbance at 540 nm was recorded with microplate reader (Thermo, Germany). Data were obtained from four repeats per condition and represented mean±standard deviation (SD) of three independent experiments.

Cell Cycle Assay

Effects of colchicine on cell cycle analysis was performed using Muse Cell Cycle Assay Kit (Merck Millipore, Germany). The cells were treated with 0.1, 10 and 100 µg/ml of colchicine for 24 h and harvested by trypsinization. The cells were then fixed and prepared for analysis according to the manufacturer's instructions. The cell population at different stages of the cell cycle was measured by Muse Cell Analyzer (Millipore).

RT-PCR Analysis

MCF-7 cells were treated with 0.1, 10 and 100 µg/ml of colchicine and total cellular RNA was extracted using RNEasy plus mini kit (Qiagen, Germany) according to the manufacturer's instructions. The cDNA was synthesized from isolated total RNA using RT2 First Strand Kit (Qiagen, Germany), according to the manual's intructions. Briefly, 8 µl of RNA (adjusted to 100 ng) and 6 µl of genomic elimination buffer were mixed and incubated at 42 °C for 5 min and then place immediately on ice for at least 1 min. A 6 µl of reverse transcriptase was added to each tube and mixed gently by pipetting up and down. The tubes were incubated at 42°C for exactly 15 min. Then the reaction was immediately stopped by incubating at 95°C for 5 min. 90 µl RNase-free water was added to each reaction and mixed by pipetting up and down several times. The reactions were placed on ice and proceeded with the real-time PCR protocol. For real-time PCR analysis, reactions were performed in triplicate using Rotor Gene System (Qiagen, Germany). The primers used to amplify specific gene products were as follows: MMP-2 sense 5′-CATGTCGCCCCTAAAACAGA-3′; MMP-2-antisense,5′-CCATCAAACGGGTATCCATC-3′; GAPDH-sense, 5′-CCCATCACCATCTTCCAG-3′;GAPDH-antisense,5′ CAGTCTTCTGGGTGGCAG T-3′.

The PCR conditions were given below.

Cycles	Duration	Temperature
1	10 min	95°C
45	15 s	95°C
	1 min	60 °C

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 6.0 version (GraphPad Software Inc.). Data obtained from the cell culture experiments were expressed as mean ±SD and One-way ANOVA test was applied for multiple comparisons. The data from PCR studies were analyzed using the comparative ΔΔCt method calculating the difference between the threshold cycle (Ct) values of the target and reference

gene of each sample. The results of treated groups were compared to the results of non-treated control group.

RESULTS

The effect of colchicine on cell viability was determined by MTT test and the results were shown in Figure 1. Colchicine has significantly induced cell death at 10 and 100 µg/ml in MCF-7 cells when compared to control (p=0.0012 and p<0.0001, respectively). The viable cell amount was 77.48±1.91 at 10 µg/ml concentration, and 53.85±4.69 at 100 µg/ml.

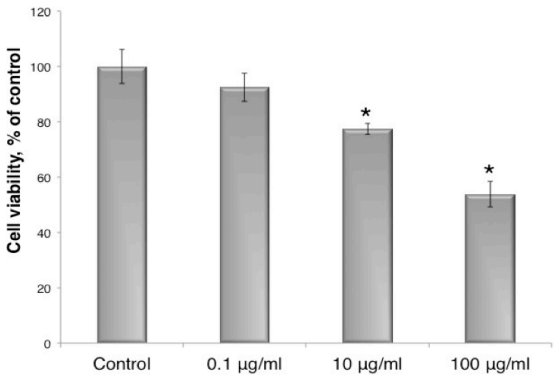

Figure 1. Effects of different concentrations of colchicine on MCF-7 human breast adenocarcinoma cell viability. The viable cell amount was determined by MTT assay. The cells were treated with 0.1, 10 and 100 µg/ml colchicine for 24 h. The results are expressed as percentage of live cells compared with untreated control. The data present the mean±SD of three independent experiments. The differences are * from control, (p<0.05).

In order to evaluate the possibility that colchicine may involve an arrest of cells at a specific cell cycle check point, we performed cell cycle analysis assay. The results indicated that colchicine induced a significant arrest of cell cycle at G2/M phase of the cell cycle at all treated concentrations (Figure 2). The percent amount of cell population at G2/M phase in control group was 62.98±0.98%, whereas the increased amounts were recorded as 63.70±2.50%, 73.20±2.10% and 80.00±2.20% for 0.1, 10 and 100 µg/ml colchicine-treated groups, respectively (p<0.0001). The increase of cell population amount at G2/M phase was accompanied with the decrease at G0/G1 and S population.

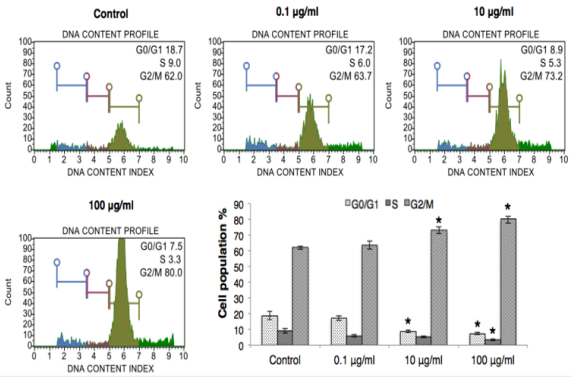

Figure 2. Colchicine induced a cell cycle arrest at G2/M phase in MCF-7 cells. Data belongs to the results of 0.1, 10 and 100 µg/ml colchicine treatments and non-treated group is accepted as control and data are representative of one experiment. The peak panels show cell population at check points of cell cycle. Bar graphs belong to quantified values of Muse Cell Analyser data. The graph shows the mean±SD of the three independent cell cycle experiments. The differences are given as compared to control, * p<0.0001.

The real-time PCR experiments have shown that MMP-2 mRNA expression has significantly decreased in colchicine-treated cells in a dose dependent manner (Figure 3) (p<0.0001).

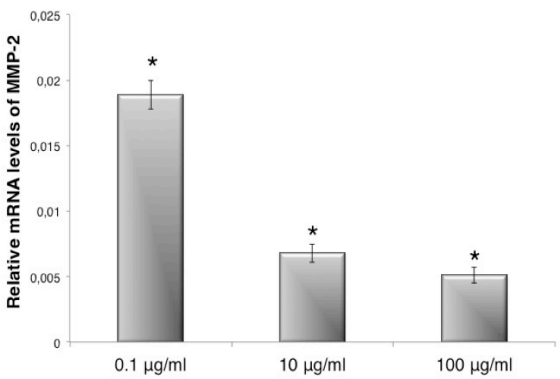

Figure 3. Colchicine inhibited MMP-2 mRNA expression in a dose dependent manner. Mean±s.d. are shown (n=3).
* p<0.0001.

DISCUSSION

Colchicine is an alkaloid drug compound and it's widely used in clinical applications. The studies have shown that colchicine prevents growth of cancer cells by antimitotic activity through interacting with microtubules, and contributes to the establishment of improved cancer therapies (11,12). Colchicine causes antiproliferative effects through the inhibition of microtubule formation, which leads to mitotic arrest and cell death by apoptosis (13). Previous studies also reported that colchicine and its potent derivatives showed a favorable therapeutic effect on hypopharyngeal, pancreatic, gastric, colon and hepatocellular carcinoma (10, 13-17).

At present study we evaluated antiproliferative and antimetastatic effects of colchicine in MCF-7 breast adenocarcinoma cells. We found that colchicine has significantly inhibited cell growth at 10 µg/ml and higher concentrations. The researchers have shown that colchicine induced a dose-dependent reduction in cell viability and early apoptosis in HT-29 colon cancer cells treated with 20 µg/ml colchicine (16). The anti-proliferative effect of colchicine at 2 and 6 ng/mL was observed on hepatocellular carcinoma and cancer-associated fibroblast cell lines (8). A study by Sun et al. showed that colchicine can inhibits proliferation of MCF-7 breast cancer cells and induce cell apoptosis, strongly at 40 µg/ml concentration for 72 h (17). In a similar manner, treatment with microtubule depolymerizing colchicine analogue arrested cell cycle at the G2/M transition in HCT-116 colon cells (16). Conversely, colchicine at 2.5 nM which is defined as clinically acceptable concentration caused no G2/M arrest in A549 lung cancer cells (18).

The susceptibility to colchicine can show differences among different cell lines (8,9,10). For instance, the antiproliferative effects of colchicine on gastric cancer cells were observed only at concentration of 6 ng/mL (10), whereas colchicine at 2 ng/mL showed significant antiproliferative effects on hepatocellular carcinoma and cholangiocarcinoma cells (8,11).

Moreover, colchicine-treated nude mice showed lower increased tumor volume ratios and tumor growth rates than the control mice (9,10). In clinical studies, colchicine administration prevented and delayed the development of hepatocellular carcinoma and decreased risk of incident all-cause cancers in male Taiwanese patients with gout (19,20).

During the carcinogenesis process, tumor cells interact with tumor microenvironment such as extracellular matrix (ECM), growth factors, ECM-related cytokins, endothelial cells, fibroblasts, macrophages, adipocytes, etc. (21,22). The studies have revelaed that the enzymes which degrade ECM are essential for tumor progression. Tumor cells are well organized to produce enzymes which have ability to degrade matrix barrier of tumor tissue, and so, they enable the metastasis of cancer to further tissues. For the last decades, MMPs have been accepted as potential enzymes for such tumoral activities since they can degrade all of the structural components of ECM. In addition, these enzymes have been up-regulated in nearly all human and animal tumors as well as tumor cell lines (23). Since there is limited data about the effects of colchicine on types of cancer and its mechanisms of effects, our study is the first to evaluate the effects of colchicine on MMP-2 mRNA expression in MCF-7 cells. According to our data, the downregulation of MMP-2 expression, has demonstrated that colchicine may be important features on preventing metastasis of cancer cells.

In conclusion, the results of our study indicate that colchicine can be identified as a potential anti-cancer compound for MCF-7 breast cancer cell line.

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