RESEARCH ARTICLE

Editorial Process: Submission:12/23/2023 Acceptance:04/11/2024

Evaluation of the Combined Effect of Caffeine and 5-Fluorouracil on Colorectal Cancer Cell Lines

Fatemeh Khavari[1], Mohammad Taheri[2,3], Hamed Manoochehri[4], Pejman Molaei[2], Fatemeh Nouri[2,5]*

Abstract

Background: Cancer is the second leading cause of death in the world. Colorectal cancer is the third leading cause of cancer death. Today, there are several options for treating colorectal cancer such as chemotherapy, surgery, radiotherapy, immunotherapy, and gene therapy. 5-Fluorouracil is known as a suitable candidate for the treatment of various cancers, especially colorectal cancer. However, the use of this drug is limited, so it is usually used in combination with other drugs and agents. Based on the evidence obtained, this study attempted to evaluate the combined effects of 5-fluorouracil and caffeine on colorectal cancer cells. **Methods:** In this study, initially HCT116 and HEK293 cell lines were cultured as cancer and normal cells, respectively. These cell lines were then evaluated for cytotoxicity, induction of apoptosis, and rate of cell migration. All data were analyzed by statistical methods. **Results:** The results indicated that a combination of caffeine and 5-FU augmented their cytotoxicity in HCT116 cells but reduced cytotoxicity in HEK293 cells. No reduction was observed in the migration of HCT116 cells that were treated with caffeine or a combination of caffeine and 5-FU. Also, it seems that caffeine reverses the apoptotic effect of 5-FU in HCT116 cells.

Keywords: Colorectal neoplasm- 5-Fluorouracil- Caffeine

Asian Pac J Cancer Prev, **25 (4)**, 1419-1424

Introduction

Colorectal cancer (CRC), which includes colon and/or rectum malignancies, is one of the most common cancers and is considered the third cause of cancer-related deaths [1]. Colorectal cancer, as a multifactorial disease, is associated with epithelial-mesenchymal transition (EMT) in more severe stages [2]. There are several options for treating colorectal cancer, the most important of which are chemotherapy, surgery, radiotherapy, and gene therapy. The choice of treatment depends on factors such as the location and size of the tumor and the status of the disease in the body. However, each treatment method has some side effects that limit its use [3].

Today, coffee is one of the most popular beverages used in all countries, which contains various chemical compounds. The most famous compound in coffee beans is caffeine or 1, 3, and 7 trimethyl xanthine [4-6]. The purine alkaloid caffeine is involved in numerous essential cellular processes, such as DNA repair, cell cycle regulation, and defense against oxidative stress. Caffeine is a neuroactive compound that is absorbed by the digestive system with high speed and percentage after consumption. Many studies have also shown that caffeine consumption plays an effective role in reducing the risk of breast, brain, liver, colorectal, and kidney cancers [7-9]. In colorectal cancer, caffeine is believed to exert a significant influence on both the prevention and pathogenesis of this malignancy. It achieves this by impeding the progression and metastasis of cancerous cells through the regulation of chronic inflammation, augmentation of free radical receptor activity, and modulation of key pathways, including PI3K/AKT/mTOR, MAPK, and TGF-β [10-13].

Chemotherapeutic agents such as oxaliplatin, 5-fluorouracil, capecitabine, and irinotecan are employed in the treatment of colorectal cancer (CRC). 5-fluorouracil (5-FU) stands as the primary chemotherapy option for patients in the early stages of the disease. This antimetabolite drug can be administered independently or in conjunction with other anti-cancer compounds. By inhibiting thymidylate synthase, an enzyme crucial for DNA replication, 5-FU induces DNA damage and

*[1]Student Research Committee, Hamadan University of Medical Sciences, Hamadan, Iran. [2]Research Center for Molecular Medicine, Hamadan University of Medical Sciences, Hamadan, Iran. [3]Department of Medical Microbiology, Faculty of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran. [4]The Persian Gulf Marine Biotechnology Research Center, The Persian Gulf Biomedical Sciences Research Institute, Bushehr University of Medical Sciences, Bushehr, Iran. [5]Department of Pharmaceutical Biotechnology, School of Pharmacy, Hamadan University of Medical Sciences, Hamadan, Iran. *For Correspondence: Fatemenouri1@gmail.com*

cell apoptosis. This disruption in the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) underlies its mechanism of action [14, 3]. Multidrug resistance (MDR) is an important challenge in the treatment of CRC patients so half of the patients are resistant to chemotherapy by 5-FU [15]. Scientists have concentrated on using this medication in combination with natural substances to lessen side effects and enhance chemotherapy as a solution to this issue. Based on a study by Wang et al. in 2019, it was found that in hepatocellular carcinoma cells, the combined effect of fluorouracil and caffeine compared to the single use of fluorouracil could significantly reduce intracellular reactive oxygen species (ROS), proliferation and induce apoptosis [16]. Based on the information obtained, in this study, the combined effect of the 5-FU as an anti-cancer drug and caffeine on colorectal cancer cells will be investigated.

Materials and Methods

Cell culture

HCT116 cells of colorectal cancer and HEK293 normal cells were purchased from Iran Pasteur Institute. These cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Farzan-Teb, Iran) with 10% (v/v) fetal bovine serum (FBS) (CinnaGen, Iran), 1% antibiotic (penicillin and streptomycin) and incubated at 37°C within a humidified incubator under 5% CO2. When cells reached 80% confluency harvested using trypsin–EDTA 0.25% to maintain the logarithmic growth phase.

Cytotoxic examination

HCT116 and HEK293 cells were seeded in 96-well plates with a density of 7000 and 9000 cells/well. After 24 hours of incubation at 37 ° C, cells were treated with different concentrations of 5-FU (1000, 500, 250, 125, 62.5, 31.25, or 15.625 µM), and with concentrations of caffeine (5000, 500, 250, 125, 62.5, 31.25, 15.625 or 7.8125 µM). Untreated cells are considered as a control group. All target groups were incubated for 24 hours and cell viability was evaluated by MTT assay.

MTT Assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) reagent was dissolved in phosphate buffer at a concentration of 5 mg/ml and 20 µl was added to each well, then incubated at 37 ° C for 4 h. After 4 hours, the supernatant culture medium of the cells was discarded and 100 µl of DMSO was added to each well to dissolve the formed formazide crystals. Finally, with the help of an ELISA reader, the absorption of solutions in each well at 570 and 630 nm was recorded and the amount of cell survival was obtained [16]. Throughout this study, cell culture at the growth and proliferation stage was used.

Cell Apoptosis Assay

Apoptosis was determined by flow cytometry using an Annexin V-FITC apoptosis diagnosis kit (MabTag GmbH, Germany) that uses FITC-Annexin V staining and propidium iodide. Cells were cultured in 6-well plates at the density of 5×10^4 per well and treated with IC50 concentration of caffeine and a combination of caffeine and 5-FU. A suspension of cells was prepared first, then 5 µl of FITC-Annexin V and 5 µl of propidium iodide solution were added to 100 µl. The solution was incubated on ice for 10 minutes in the dark. Then 400 µl of ice-cold binding buffer was added and gently stirred and flow cytometric analysis was performed [17].

Cell Migration Assay

At this stage of the work, the Scratch assay was used to examine cell migration. First, the cells were seeded into the bottom of the 24-well plate at the concentration of 1×10^5 cells/well. When HCT116 and HEK293 cells reached 100% confluency, a scratch occurred in the middle of the well with a sterile 10 µl pipette. The cells isolated from the plate surface were then removed by washing and the culture medium was added with 5000 µM caffeine, 62.5 µM 5-FU, or a combination of them. Next, the wells were observed under a microscope and images were obtained at 0 and 24 hours from each scratch. The images were further analyzed quantitatively using Image J software [18].

Statistical Analysis

Data was obtained from the study to compare each of the variables, one-way analysis of variance and one-way ANOVA were used. In this study, a value of P less than 0.05 will be considered statistically significant. If the analysis of variance is significant, appropriate follow-up tests will be used.

Results

A combination of caffeine and 5-FU augmented their cytotoxicity in HCT116 cells but reduced cytotoxicity in HEK293 cells

To examine the anti-proliferative effect of 5-FU and caffeine on HCT116 and HEK293 cell lines, the viability of cells was measured by MTT assay, after treatment with different concentrations of 5-FU and caffeine. The IC50 value of 5-FU was 39.03 µM and 65.37 µM for HCT116 and HEK293 cells, respectively (Figure 1a). Furthermore, the IC50 value of caffeine was 4720 µM and 20593 µM for HCT116 and HEK293 cells (Figure 1b). Next, cells were treated with the combination of different concentration of 5-FU and constant 5000 µM caffeine. In HCT116 cells the IC50 value of 5-FU was reduced to 6.712 µM but in HEK293 cells evaluated to 13439 µM when cells were treated with the combination of 5-FU and caffeine (Figure 1c).

Caffeine did not trigger apoptosis in HCT116 cells

To study whether caffeine can promote HCT116 cell apoptosis, flow cytometry analysis was conducted. The percentage of cells in the late apoptotic (UR) or early apoptotic (LR) phase, did not significantly change after treatment with IC50 concentration of caffeine and a combination of caffeine and 5-FU (Figure 2). It seems that caffeine reverses the apoptotic effect of 5-FU in HCT116 cells.

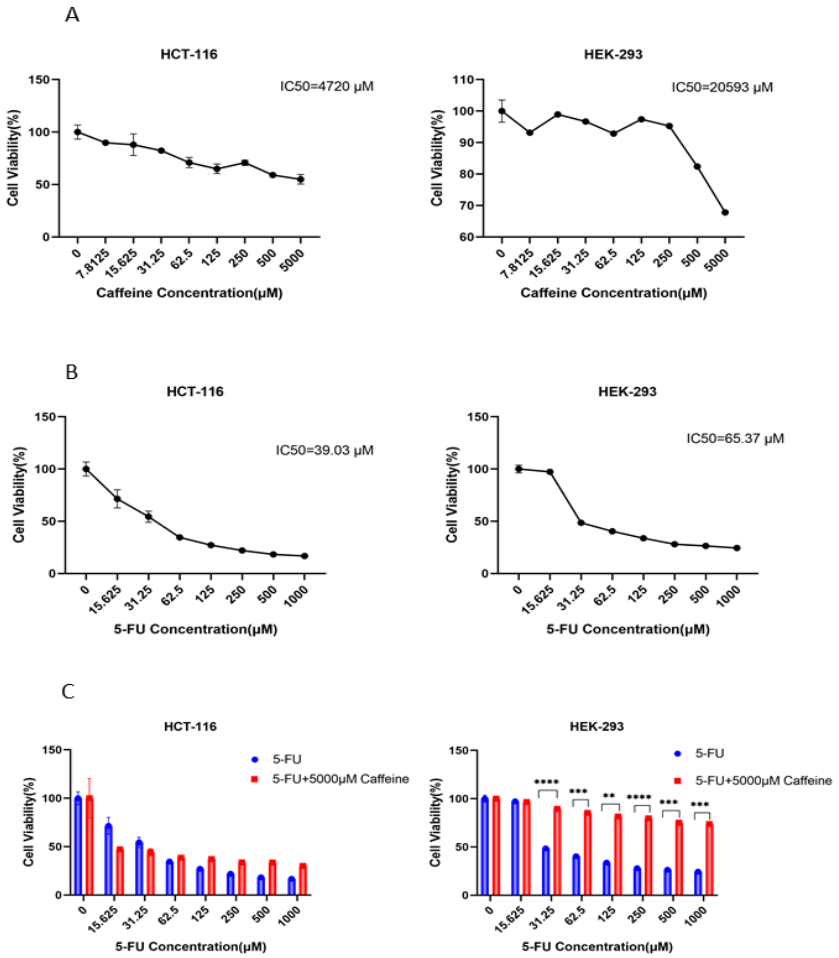

Figure 1. Cytotoxic Effects of (a) Caffeine, (b) 5-FU, and (c) a combination of 5000µM caffeine and 5-FU in various concentrations after 24 h incubation on HCT-116 colorectal cancer cell line and HEK-293 normal cell line. **P< 0.01, ***P< 0.001, ****P< 0.0001.

Cell migration assay

Wound healing assay results showed the migration of HCT116 and HEK293 cells was significantly (P value=0.0002 and <0.0001) reduced after 24 h incubation with 5-FU. Also, upon comparison with the control group the migration of HEK293 cells that were treated with caffeine or a combination of caffeine and 5-FU, significantly (P value=0.0017 and 0.0015) reduced. However, no reduction was observed in the migration of HCT116 cells that were treated with caffeine or a

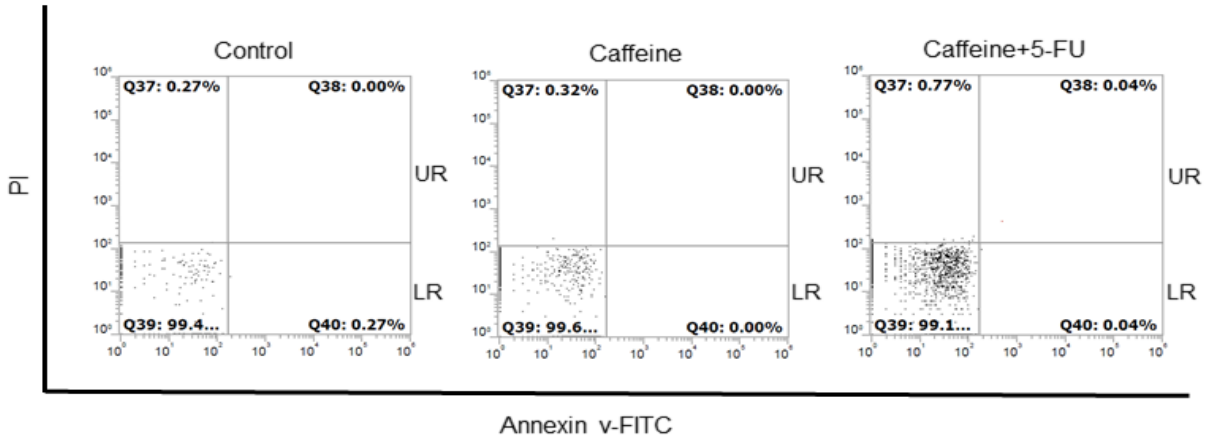

Figure 2. Effect of Caffeine on Apoptosis Cell Death. Representative flow cytometry plots of HCT116 cells stained with Propidium iodide/Annexin V-FITC at 48 h after treatment with IC_{50} concentration of caffeine and a combination of caffeine and 5-FU. UR shows the percentage of late apoptotic cells (PI-stained cells), and LR shows the percentage of early apoptotic cells (annexin V-stained positive cells).

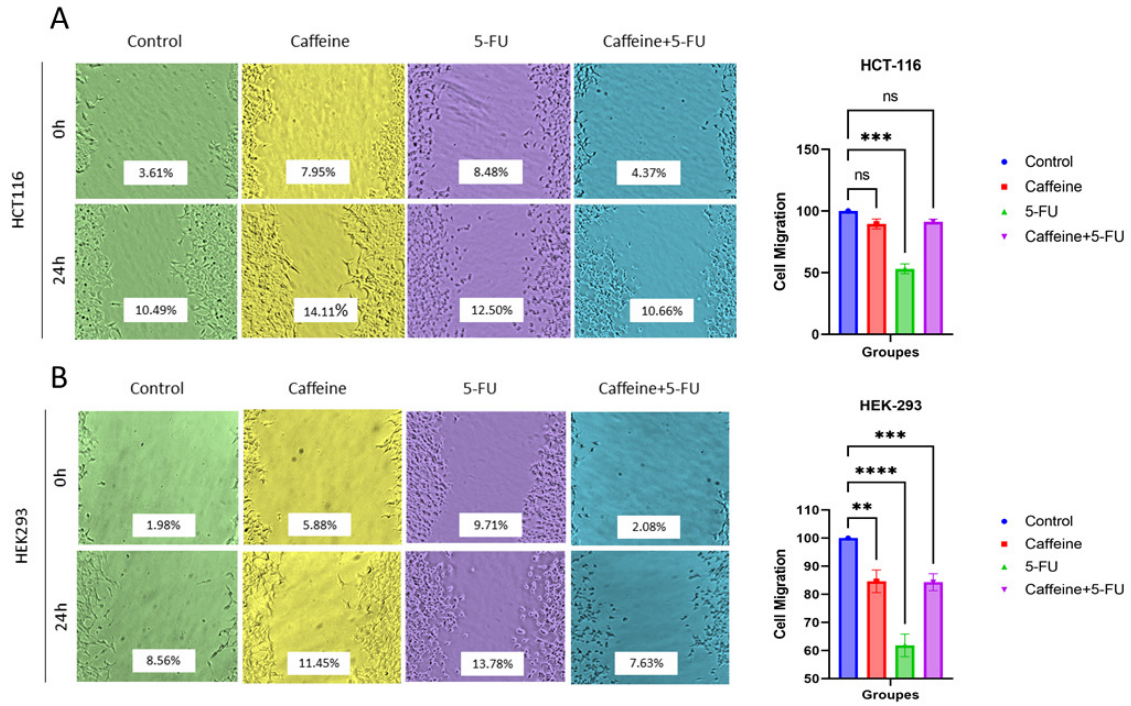

Figure 3. The effects of Caffeine, 5-FU, and a Combination of Caffeine and 5-FU on the migration of (a) HCT-116 and (b) HEK-293 cells were evaluated by scratch assay. **P< 0.01, ***P< 0.001, ****P< 0.0001.

combination of caffeine and 5-FU (Figure 3).

Discussion

Colorectal cancer has remained one of the most common lethal cancers all over the world. Chemotherapy with 5-fluorouracil (5-FU) is a suitable treatment for patients with advanced stages of CRC [19]. Due to adverse side effects and resistance to chemotherapy, cancer researchers are interested in the study of natural compounds' anticancer activity. Caffeine is a purine alkaloid that showed a broad range of effects on CRC pathogenesis [12]. In addition to coffee, caffeine is an important component in cocoa beans, tea leaves, and approximately 60 other plants [10].

In this study, the anti-tumor effect of 5-FU and caffeine combination therapy on HCT116 was evaluated. Compared with 5-FU or caffeine treatment, combination treatment significantly decreased the HCT116 proliferation. Unexpectedly, the co-treatment of HEK293 which was used as normal cell lines significantly increased cell proliferation compared with 5-FU treatment. Our recent data also showed that a combination treatment of HEK293 by caffeine and 5-FU significantly (P value=0.0015) reduces cell migration. However, no significant reduction was observed in HCT116 migration. Results of the cell apoptosis assay using flow cytometry showed that caffeine may lead to the reverse induction of apoptosis by 5-FU on colorectal cancer cell lines.

Several studies have confirmed the apoptotic effect of caffeine on various cancer cell lines [16, 20, 21]. For the first time, Wang et al. demonstrate that caffeine enhances the anti-cancer effect of 5 fluorouracil by regulating intracellular ROS production in hepatocellular carcinoma. Also, in vivo, results revealed that the tumor sizes in the combination group were significantly smaller than the caffeine or 5-FU groups [16]. Tonkaboni et al. caffeine can inhibit the proliferation of human head and neck carcinoma cell line HN5 and human esophagus carcinoma squamous cell line KYSE-30 [21]. Furthermore, caffeine induces apoptosis in JB6 Cl41 cells, mouse epidermal cell line, by activation of Bax, p53, and caspase 3 [20].

Mhaidat et al. found that pre-treatment of Colo25, a cell line of CRC, with caffeine increased the levels of Mcl-1, a member of the Bcl-2 family, and significantly inhibited paclitaxel-induced apoptosis [22]. Xu et al. found that caffeine inhibits the anticancer effect of paclitaxel in A549 and HeLa cells by down-regulation of α-tubulin acetylation. They suggested patients receiving paclitaxel should not consume coffee and other caffeinated beverages [17]. Choi et al. investigation showed that up to 200 μM caffeine has no significant cytotoxic effect in HT-29 human colon adenocarcinoma cells [23]. Shojaei-Zarghani et al. systematically reviewed the human and animal studies investigating the effect of caffeine on CRC. They found that based on previous studies there is no association between dietary caffeine intake and the risk of CRC [24]. It seems that the effect of caffeine combination therapy with 5-FU is ambiguous. According to our study in vitro results, it seems CRC combination therapy with 5-FU and caffeine has no significant effect on the induction of apoptosis, and prevention of CRC cell migration. Moreover, according to that caffeine reverses the apoptotic effect of 5-FU in HCT116 cells, it is suggested to investigate the role of caffeine in cancer chemoresistance studies.

In conclusion, the present study, showed that caffeine enhances the cytotoxic effect of 5-FU but reverses the apoptotic effect in HCT116 cells. These results suggest

that the consuming of caffeinated beverages should be avoided in CRC patients treated with 5-FU. However, the exact mechanism by which caffeine reverses the apoptotic effect of 5-FU may need more studies.

Author Contribution Statement

Idea: F.N, F.KH, and M. T; data gathering: F.N, M.T, F.KH; draft the manuscript: F.KH and H.M, revision and final approval: M.T, P. M, and F.N. All authors read and approved the final version of the manuscript..

Acknowledgements

We are thankful to Hamadan University of Medical Sciences, Hamadan, Iran.

Funding

This work was supported financially by Hamadan University of Medical Sciences, Hamadan, Iran (Grant No. 140011058999).

Availability of data and materials

The data that appeared in this study are already publicly available in the literature.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Hamadan University of Medical Sciences (IR.UMSHA.REC.1400.691).

Conflict of interest

The author(s) declared no potential conflicts of interest

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