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Hesperidin potentiates the chemosensitivity of HT-29 colon cancer cells to 5-fluorouracil

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Abstract

Aim: Hesperidin is an important flavonoid compound of citrus fruits. It has the potential to be evaluated as a cytotoxic agent against a variety of malignant human cancer cells, especially in colon, pancreatic, breast, and other cancer types. The practical application of combination therapies is commonly used to eliminate or reduce drug resistance.

Materials and Methods: In this study, hesperidin (0.001-300µM) and 5-fluorouracil (5-FU) (1-300 µM), a cytotoxic chemotherapy drug used for treating cancer, were applied both individually and in combination (100 µM) on the HT-29 cells (human colon cancer cell line). The effects of these compounds on HT-29 cell viability were revealed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method, and the genotoxic effects on HT-29 cells were revealed using single-cell gel electrophoresis analysis.

Results: The data obtained showed that the combination of 5-FU and hesperidin importantly decreased HT-29 cell viability compared to the group treated with 5-FU alone. These findings indicate that the combined application of these compounds is more effective than the individual administration of 5-FU or hesperidin alone.

Conclusion: The research suggests that hesperidin might possess the ability to counter drug resistance in cancer cells, offering promising prospects when used alongside current anti-cancer medications. Nevertheless, to validate these findings, more extensive research and clinical trials are imperative.

Introduction

Colorectal cancer (CRC) is one of the major causes of cancer-related mortality. CRC is the third most common cause of cancer mortality worldwide with more than 1.85 million cases and 850.000 deaths annually [1]. Although most CRCs are spontaneous, in addition to genetic factors, dietary habits and toxins are effective in the development of the disease.

Chemotherapy is the most important trump card in the treatment of cancer diseases. 5-fluorouracil (5-FU), an antimetabolite, is an important chemotherapeutic agent in the treatment of CRC [2]. In addition, other chemotherapeutic agents such as irinotecan, capecitabine, and oxaliplatin have been introduced into CRC treatment in recent years. The standard treatment approach in this disease group commonly includes the combination of 5-FU with irinotecan or oxaliplatin [3]. In addition to these, in recent years, there have been important developments in treatment as a result of the use of monoclonal antibodies. Despite all these advances, the five-year survival rate of CRC patients is just over 65% between 2012 and 2018 [4].

The emergence of drug resistance is the main cause of poor prognosis. Mutations in the p53 gene are common in many cancers, including CRC. Altered function of the p53 protein as a result of mutations mediates the development of drug resistance and increased survival of tumour cells. 5-FU-based chemotherapy is resistant in about half of metastatic CRC patients. Therefore, a main obstacle in the treatment of metastatic CRC is not overcoming drug resistance [5, 6]. Research efforts to understand and overcome resistance mechanisms are important to increase the chances of treatment success. At this point, the use of different agents in combination is a common choice. This approach can help overcome different resistance mechanisms in cancer cells, potentially leading to more effective treatment outcomes.

The use of herbal components to fight against cancer has been present in ancient traditional medicine of Asian,

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African, and European cultures [7]. Flavonoids are among the natural products that are considered to have potential effectiveness in fighting against cancer. These compounds have been shown to be effective, especially when used in combination with other drugs, against multidrug-resistant cells [8, 9]. Hesperidin is a flavonoid found in fruits and vegetables. This flavonoid is reported to have anti-inflammatory, antioxidant, and antimutagenic effects. Additionally, studies have shown that this compound can impact the division and death mechanisms of cancer cells. The pro-apoptotic effect of hesperidin is mediated by many different mechanisms. For example, hesperidin induces cell death through DNA fragmentation by causing an increase in the Bax/Bcl-2 ratio and caspase-7 activation in breast cancer cells [10]. It is also reported that this flavonoid exerts a cytotoxic effect by causing an increase in the intracellular reactive oxygen level in hepatocellular carcinoma cells [11]. Hesperidin may play a role in the process of cancer development by inhibiting angiogenesis and metastasis [12]. Studies show that hesperidin exerts direct or indirect effects on cancer cells.

Drug resistance is a significant obstacle in cancer treatment, and increasing the sensitivity of cancer cells to chemotherapeutics increases the chance of success in treatment. Researchers report that hesperidin has the potential to reduce or reverse drug resistance in cancer cells when used in conjunction with existing chemotherapeutics [13]. Considering what is known, we hypothesized that hesperidin may exert a synergistic effect with 5-FU. The aim of this study was to investigate the effect of the combination of hesperidin and 5-FU on HT-29 colon cancer cells with a mutated p53 gene. Study results showed that hesperidin and 5-FU combination had greater cytotoxic and genotoxic effects than 5-FU alone in HT-29 cells. We suggest that hesperidin, a natural compound for p53 mutant cells, may mediate the enhancement of the therapeutic efficacy of 5-FU.

Materials and Methods

Preparation of compounds

Doses of hesperidin (H5254, Sigma Aldrich, Germany) and 5-FU (A13456, Alfa Aesar, Germany) used in the study were prepared in dimethyl sulfoxide (DMSO). Samples were stored at +4°C for the duration of the analysis.

Cell culture

This research is an experimental study. HT-29 cells (ATCC) were used in the study. Cells were grown in RPMI-1640 medium (added with 10% fetal bovine serum and 1% solution of penicillin and streptomycin). 96-well plates were used for cytotoxicity assays. Approximately 15.000 cells were seeded in each well. The cell flasks were incubated every other day in a 5% CO_2 incubator (37°C) [14]. Then, the prepared doses of hesperidin and 5-FU were applied (final solvent volume 1µl). After 24 h, cell viability in the wells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [15]. The results were calculated as a percentage change in comparison with the control. Experiments were performed in 5 independent repetitions.

Genotoxicity analysis (Comet assay)

In the study used alkaline comet analysis [16]. For the analysis of genotoxicity, cells were grown in 6-well plates. The cells were treated with the hesperidin and 5-FU compounds, both individually and in combination. The highest doses of hesperidin and 5-FU that did not affect cell viability (100 µM for both compounds) were applied after the MTT analysis. The cells treated with the compound were then collected and approximately 10.000 cells were mixed with 1% low melting point agarose prepared in phosphate buffer. The suspension mixture was transferred to slides coated with 1% agarose and preparations were prepared by closing the coverslip. After the preparation dried, the coverslips were peeled off. Samples were left in the lysis solution for 1 h and then transferred to a horizontal electrophoresis tank.

The samples were left in the lysis solution for 1 h and then transferred to a horizontal electrophoresis tank. After electrophoresis (25 V, Max. 300 mA, 30 minutes), the neutralized slides were dried. Finally, the samples were stained with ethidium bromide for 15 minutes. Excess dye was removed with cold distilled water and the slides were viewed under a fluorescence microscope. At least 100 cells from each group were analyzed with TriTek Comet Software (Version 2.0) [17]. DNA damage levels were evaluated with tail DNA%, tail intensity (TI), tail length (TL) and tail moment (TM) parameters.

Statistical analysis

Data were analyzed using GraphPad Prism Software (Version 5). The Shapiro-Wilk test was used to understand whether the data were normal distribution. The Kruskal-Wallis H test was used to assess how the group means differed for the relevant variables, and Dunn's test was used for multiple comparisons. Pairwise comparisons were conducted using two-sample t-test. Results were summarized as mean ± standard error (SE), and $p<0.05$ was considered significant.

Results

Cytotoxicity analysis

The doses of 5-FU applied to HT-29 cells, with the exception of the two high doses, did not significantly alter cell viability in comparison to the control and solvent groups (Figure 1A). Following the application of 200 and 300 µM 5-FU, the viability level decreased by approximately 20% and 30% respectively, and this change was found to be significant ($p=0.0138$, $p=0.0018$). It was found that the applied doses of 200 and 300 µM hesperidin significantly reduced cell viability after 24 h of hesperidin application ($p=0.0038$, $p=0.0002$). The viability level of cells in the groups receiving other doses (0.01-100 µM) was similar to those of the control and solvent groups (Figure1B).

Comet assay

The genotoxic effects occurring in HT-29 cells after the application of 5-FU, hesperidin, and the combination of both compounds were determined using Comet analysis (Figure 2), and the results are presented in Figure 3. It was found that the application of 5-FU or hesperidin alone

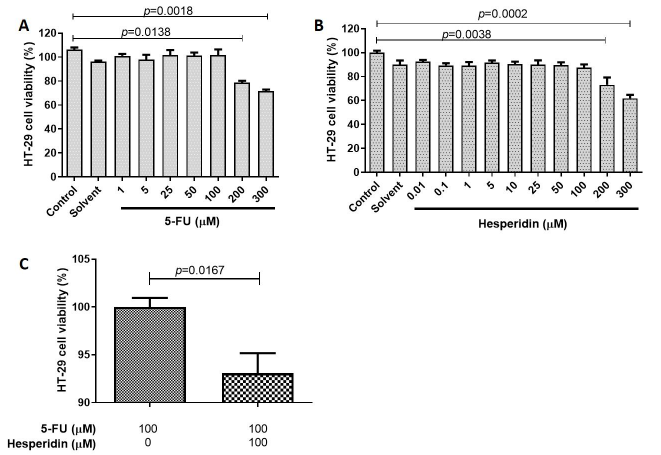

Figure 1. Cell viability level after hesperidin and 5-FU treatments. (A) 5-FU, (B) hesperidin, and (C) 5-FU and hesperidin combination shows the change in viability 24 h after applications.

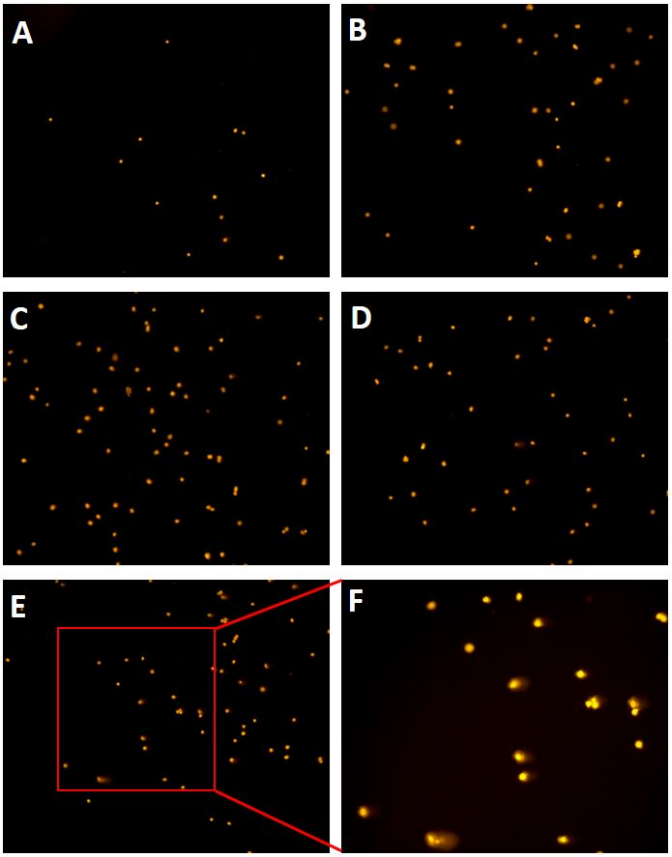

Figure 2. Images of DNA damage observed in cells after treatments. (A) Control, (B) Solvent, (C) 100 µM 5-FU, (D) 100 µM hesperidin, (E) 100 µM 5-FU+100 µM hesperidin groups microscopy images (100X). (F) 200X field view of the 100 µM 5-FU+100 µM hesperidin group.

did not create a significant difference in tail DNA%, TL, TM, and TI parameters compared to the control. However, in the groups treated with the combination of 5-FU and hesperidin, significant increases in these parameters were

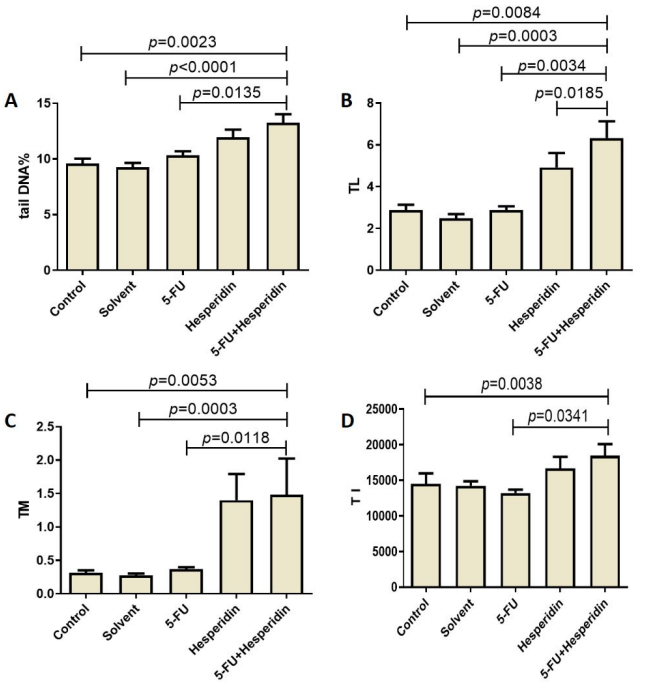

Figure 3. Comet analysis results of HT-29 cells after 24 h of 5-FU and hesperidin treatments.

observed ($p<0.05$).

Discussion

CRC is an important health problem and treatment strategies and treatment success rates are not yet at the desired level. The biggest obstacle to the treatment of this cancer group is drug resistance, which occurs as a result of gene mutations. Today, it is aimed to increase the success rate of treatment by finding new compounds with high efficacy and low toxicity. Hesperidin has been reported to have anti-tumor effects in many different types of cancer [18, 19]. In this study, we showed that hesperidin increased the cytotoxic activity of 5-FU in colon cancer cells with mutant-p53 gene.

Many of the studies on the anticancer effect of hesperidin report that the compound induces apoptosis in cancer cells. Palit et al. reported increased markers of apoptosis such as membrane phospholipid migration, DNA damage and caspase activation in MCF-7 cells treated with hesperidin [10]. In another study, Magura et al. demonstrated by flow cytometric analysis that 100 µg/mL hesperidin application increased the apoptotic cell population in MCF-7 cells [20]. Additionally, it has been reported that hesperidin enhances apoptosis in malignant cells through the NF-κB, mTOR, and PI3K/AKT pathways [21]. Cincin et al. showed that after hesperidin administration, proliferation decreased and caspase-3 expression increased in A549 and NCI-H358 cells in a dose and time dependent manner. The authors report that hesperidin exerts its effects by modulating FGF and NF-kB signaling pathways in cells [22].

The anticancer effects of hesperidin are not limited to inducing apoptosis in tumor cells. Studies have reported that hesperidin application prevents angiogenesis

and metastasis. Xia et al. showed that hesperidin administered to A549 cells suppressed the SDF-1/CXCR-4 pathway, reducing cell migration and invasion [23]. In another study, it was reported that hesperidin prevented vascular formation by blocking AKT/mTOR signaling pathways in HUVEC cell line [24]. Available information reflects the potential chemotherapeutic activity of hesperidin. However, studies on drug resistance mechanisms are limited.

Few studies reflect the interaction of hesperidin and other flavanoids with chemotherapeutics in drug-resistant cancer cells. Febriansah et al. showed that hesperidin+doxorubicin administration in doxorubicin-resistant MCF-7 cells caused a decrease in P-glycoprotein 1 expression, which is responsible for multi-drug resistance, without causing a change in the level of apoptosis [25].

Khamis et al. reports that tamoxifen and hesperidin exhibit synergistic effects in breast cancer cells. Researchers reported that the combination of hesperidin+tamoxifen caused stronger apoptosis induction and suppressed EGFR and Eα expressions [26]. We determined that hesperidin increased the cytotoxic and genotoxic activity of 5-FU in colon cancer cells containing mutant-p53 gene. We applied 5-FU and hesperidin to HT-29 cells and determined doses that did not alter viability. Cytotoxicity and DNA fragmentation were significantly higher in the combined 5-FU+hesperidin group.

Mutant-53 gene is frequently seen in CRC cases and this situation is directly associated with poor prognosis and drug resistance. Combined applications of chemotherapy are an alternative for the treatment of aggressive tumors. However, unwanted side effects are inevitable during the treatment process. Flavonoids are compounds with a strong potential to overcome some of the challenges encountered in cancer cells, such as drug resistance, uncontrolled proliferation, and evasion of apoptosis. Our results show that hesperidin not only exerts cytotoxic effects on cancer cells alone but also increases the effect of chemotherapeutics. Moreover, it demonstrated this synergistic effect in a specialized cancer cell containing a mutant-p53 gene, such as HT-29. Based on this evidence, we predict that hesperidin may contribute to the difficulties encountered in CRC treatment. Hesperidin supports the use of low doses of chemotherapeutics and may increase their effects. Thus, it can support the survival of healthy cells during the treatment process and increase the success rate of the treatment. We believe that the results of this study will be hope for new studies.

In vivo, and preclinical studies to be planned may elucidate the molecular mechanism of these beneficial effects of hesperidin and strengthen its potential in treatment processes.

Conclusion

Cytotoxicity and genotoxicity analyses showed that the combination of low doses of hesperidin and 5-FU increased cell death in colon cancer cells. Although these results show that hesperidin increases the effectiveness of chemotherapy in the treatment of CRC, new in vivo and clinical studies to be planned will help us understand the mechanism of the positive effect. Thus, the formulations obtained as a result of the studies can increase the chance of success in treatment and eliminate the negative effects of drug resistance.

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Ethical approval

It is a cell culture study. An ethics committee is not required.

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