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The Combination Effect of Ferulic Acid and Gemcitabine on Expression of Genes Related Apoptosis and Metastasis in PC-3 Prostate Cancer Cells

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ABSTRACT

Objective: Prostate cancer is the second most common cause of cancer-related deaths in men. Nowadays, new treatment approaches have been tested for cancer therapy including natural compounds with low toxicity. Ferulic acid (FA) is known as an abundant phenolic compound found in various fruits and vegetables. As a potent antioxidant, the anticarcinogenic effect of FA has been demonstrated in various cancer cell lines. The objective of this study was to investigate the combined effect of FA and gemcitabine on apoptosis and metastasis in PC-3 human prostate cancer cell lines.

Materials and Methods: Cell viability was determined using the XTT method after the cells were treated with gemcitabine or FA and gemcitabine. According to the results of cytotoxicity assays, PC-3 cells were treated with <IC50 doses of combination (200 µM FA and 35 µM gemcitabine) and IC50 dose of gemcitabine. Expressions of genes that are important in apoptosis and metastasis pathways were evaluated in dose and control groups by qPCR.

Results: According to the results, the combination of FA and gemcitabine affected the expression of more genes in apoptosis and metastasis with a higher fold change compared with the single treatment of gemcitabine in PC-3 human prostate cancer cell lines.

Conclusion: Our study indicates that FA can be an effective part of the combination treatments.

Keywords: Ferulic acid, gemcitabine, PC-3 cell line

INTRODUCTION

Prostate cancer is one of the most prevalent types of cancer in men with the highest mortality rate together with lung and colorectal cancer, especially in industrialized countries (1). When prostate cancer is diagnosed at an early stage, stage 1 and 2, the 5-year survival rate can reach 90%, while the likelihood of successful treatment at advanced stages such as stage 3 and 4 is low (2). Digital rectal examination (DRE) and measurements of prostate-specific antigen (PSA) level are often used for the diagnosis of early-stage prostate cancer, but these methods are inadequate because of low specificity (3). Androgen is one of the most important risk factors for the development of prostate cancer, so androgen deprivation therapy (ADT) is widely used as the main treatment method as well as surgery and radiation therapy (4,5). When patients don't respond to the ADT for a long time, chemotherapeutic approaches are applied in treatment (6). Unfortunately, resistance to treatments is eventually seen in most of the patients, and, for this reason, new therapeutic strategies are needed for the treatment of prostate cancer.

It is known that more than 60% of anticancer drugs currently used are derived from natural sources (7). Therefore, the development of more effective chemotherapeutic agents from natural products and the investigation of the abilities of inducing apoptosis or cancer prevention by different mechanisms are the most important focus points in cancer studies. The findings of research illustrated that various natural products had protective effects and could inhibit carcinogenesis by regulating the expressions of genes in apoptosis, invasion, angiogenesis and metastasis pathways (8).

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Ferulic acid (FA; 4-hydroxy-3-methoxycinnamic acid), is a caffeic acid derivative found in vegetables, fruit, some beverages (for example, coffee and beer) and is an effective component of some Chinese medicinal herbs, for instance, *Angelica sinensis*, *Cimicifuga racemosa* and *Ligusticum chuangxiong* (9). It has been reported that FA has various pharmacological effects including antioxidant, antimicrobial, antiinflammatory, antithrombotic and antihypercholesterolemic effects (10-14). Moreover, the anticancer effect of FA has been demonstrated through studies involving various cancer cell lines and its anticancer activity is attributed to an antioxidant property which is associated with its phenolic nucleus and unsaturated side chain (15). It has been shown that FA has cytotoxic effects on cancer cells and affects important processes such as apoptosis, cell cycle and metastasis in the studies conducted with human breast cancer (MCF-7), human pancreatic cancer, human prostate cancer (PC-3 and LNCAP), human lung cancer (H1299) and human osteosarcoma (143B and MG63) (16-20). However, little is known about its effectiveness as a part of the combination therapies in cancer treatment (21).

In studies involving the investigation of anticancer properties of natural products, demonstration of their apoptotic and anti-metastatic effects is a priority. The apoptosis process, which is an important part of the mechanism that regulates the death or survival of the cell, is controlled by various signaling pathways (22). The apoptosis pathway is divided into three categories (i) the pathway of death receptor or extrinsic induced by death receptors, (ii) the intrinsic or mitochondrial pathway, and (iii) the perforin/granzyme pathway which is induced by granzyme A and granzyme B from cytotoxic T cells (23). It is important that chemotherapeutic agents have inhibiting capabilities to prevent invasion and metastasis in prostate cancer cells in addition to inducing apoptosis because bone metastasis occurs in 80% of advanced prostate cancer patients and is one of the main causes of prostate cancer related deaths (24,25). Tumor metastasis is a multistep process that includes the development of new blood vessels, detachment of metastatic cells from the primary tumor, invasion to stroma, intravasation to the blood and lymphatic vessels and extravasation to the target organ and growth of secondary tumor (26). All these steps are mediated by different factors and, especially, molecules involved in cell-cell and cell-matrix interaction, and the proteases responsible for the degradation of extracellular matrix components which are the most important participants in this process (27).

Gemcitabine is a deoxycytidine analog that exhibits anticancer activity against various solid tumors such as pancreatic cancer, lung cancer and prostate cancer (28,29). It is also thought that gemcitabine can be part of combination therapies based on the phase studies involving combinations with various chemotherapeutic agents in prostate cancer (30,31). The aim of this study is to investigate the combined effect of FA and gemcitabine on apoptosis and metastasis by evaluating the expression levels of genes important in apoptosis and metastasis in PC-3 prostate cancer cell lines.

MATERIAL AND METHODS

Cell Culture

A PC-3 (ATCC® CRL-1435™) human prostate cancer cell line, obtained from the ATCC (Manassass,VA, USA), was cultured in RPMI-1640 medium containing 2 mM L-glutamine supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO_2-95% air. FA and gemcitabine were purchased from Sigma-Aldrich Chemical Company (USA).

Cytotoxicity Assay

The cytotoxic effects of gemcitabine and its combination with FA on PC-3 cells were determined by XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) assay. The cells ($1x10^3$ cells/well) were seeded into 96-well plates and incubated for 24 h. The cells were treated with 0-70 µM gemcitabine for 48 h after the incubation. And then XTT solution was added to each well and incubated at 37°C for 4 h. The absorbance was read at 450 nm (reference wavelength 630 nM) in a microplate reader. The concentration of gemcitabine which inhibited 50% of cell viability (IC_{50}) was determined. The combination doses were detected according to IC_{50} doses of gemcitabine and FA. IC_{50} dose of FA was previously indicated by Eroglu et al. (18). Then cells were treated with gemcitabine and FA at various concentration (0-150 µM doses and 0-900 µM doses, respectively). According to the combined effect of gemcitabine and FA on cell viability, two groups were formed for subsequent experiments. Therefore, PC-3 cells were treated with 200 µM FA and 35 µM gemcitabine (<IC_{50} doses), and 50 µM gemcitabine (IC_{50} dose) for 48 h.

RNA Isolation and qPCR Analysis

Total RNAs were extracted from PC-3 cells using TRIzol Reagent and reverse transcription was performed using iScript™ cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions.

The primer sequences of target and reference genes were designed using IDT PrimerQuest (https://eu.idtdna.com/site). Primers used in the qPCR reaction are presented in Table 1. The effects of FA and, gemcitabine on apoptosis and metastasis were evaluated using qPCR (Biorad CFX Connect). Each qPCR mix was set up in 20 µl final volume containing 10 µl 2X SYBR Green Master Mix, 5 pMol of each primer and 1 µl cDNA. The following PCR profile was used: denaturation at 95°C for 10 min, followed by 35 cycles consisting of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s.

Statistical Analysis

The gene expressions analysis of the groups was determined by using the $2^{-\Delta\Delta CT}$ method. The volcano plot analysis, from RT² Profiles™ PCR Array Data Analysis, which is assessed statistically using Student's t test, was used in the comparison of the groups.

RESULTS

Anti-proliferative Effects of Gemcitabine and Combination of Gemcitabine and FA in PC-3 Cells

The cytotoxic effects of gemcitabine and combination of gemcitabine and FA were determined using the XTT assay. Gemcitabine inhibited the cell viability of PC-3 cells in a dose depen-

Table 1. Primers sequences used qPCR analysis

Gene	Primer sequence
GAPDH	F:5-TGAACGGGAAGCTCACTGG-3
	R:5-TCCACCACCCTGTTGCTGTA-3
CASP3	F:5-GAGCCATGGTGAAGAAGGAATA-3
	R:5-TCAATGCCACAGTCCAGTTC-3
CASP7	F:5-CGAAACGGAACAGACAAAGATG-3
	R:5-TTAAGAGGATGCAGGCGAAG-3
CASP8	F:5-GCCCAAACTTCACAGCATTAG-3
	R:5-GTGGTCCATGAGTTGGTAGATT-3
CASP9	F:5-CGACCTGACTGCCAAGAAA-3
	R:5-CATCCATCTGTGCCGTAGAC-3
BCL2	F:5- GTGGATGACTGAGTACCTGAAC-3
	R:5- GAGACAGCCAGGAGAAATCAA-3
BAX	F:5- GGAGCTGCAGAGGATGATTG-3
	R:5- GGCCTTGAGCACCAGTTT-3
FAS	F:5- GTGATGAAGGACATGGCTTAGA-3
	R:5- GCCCAAACTTCACAGCATTAG-3
CYCS	F:5- GGAGAGGATACACTGATGGAGTA-3
	R:5- GTCTGCCCTTTCTTCCTTCTT-3
TNF	F:5- CCTCCTCTCTGCCATCAAG-3
	R:5- CCAGATAGATGGGCTCATACC-3
PPARG	F:5- TGGGTGAAACTCTGGGAGAT-3
	R:5- AAGTTGGTGGGCCAGAATG-3
MMP-2	F:5-TGGCAGTGCAATACCTGAA-3
	R:5-GCATGGTCTCGATGGTATTCT-3
MMP-9	F:5-GCAGACATCGTCATCCAGTT-3
	R:5-ACAACTCGTCATCGTCGAAAT-3
TIMP-1	F:5-GCGTTATGAGATCAAGATGACCA-3
	R:5-AACTCCTCGCTGCGGTT-3
TIMP-2	F:5-GCTGCGAGTGCAAGATCA-3
	R:5-CTCTTGATGCAGGCGAAGAA-3
CDH1	F:5-GAGAGCGGTGGTCAAAGAG-3
	R:5-AGCTGGCTCAAGTCAAAGT-3
CDH2	F:5-GCTGACCAGCCTCCAAC-3
	R:5-CATGTGCCCTCAAATGAAACC-3
COL4A2	F:5-AAGTACAGCTTCTGGCTGAC-3
	R:5-AGCGGCTGATGTGTGTG-3
VEGFA	F:5-CGCAGACAGTGCTCCAG-3
	R:5-CACCCAAGACAGCAGAAAGT-3
HIF1A	F:5-ACCCTCTGATTTAGCATGTAGAC-3
	R:5-TTCACCCTGCAGTAGGTTTC-3

dent manner as shown in Figure 1. The IC_{50} dose of gemcitabine was found to be 50 μM in the PC-3 cell line for 48 h and the IC_{50} dose of FA have been determined as 300 μM in the PC-3 cell line for 48 h in a previous study (18).

For determining the cytotoxic effect of combinations, cells were treated with gemcitabine and FA simultaneously for 48 h at various concentrations. The combination of gemcitabine and FA inhibited the cell viability of PC-3 cells in a dose dependent manner as shown in Figure 2. Considering these results, combination doses used for subsequent experiments were determined as 35 μM gemcitabine and 200 μM FA. The PC-3 cells were treated to IC_{50} doses of gemcitabine and combination $<IC_{50}$ doses of both for 48 h.

Effects of Gemcitabine and FA on Expressions of Genes Associated with Apoptosis

The effects of gemcitabine and combination of FA and gemcitabine on expressions of genes are important in apoptosis including *CASP3*, *CASP7*, *CASP8*, *CASP9*, *BCL2*, *BAX*, *FAS*, *CYCS*, *TNF* and *PPARG*, and were determined using qPCR analysis, after total RNA isolation and cDNA synthesis from control and dose group cells.

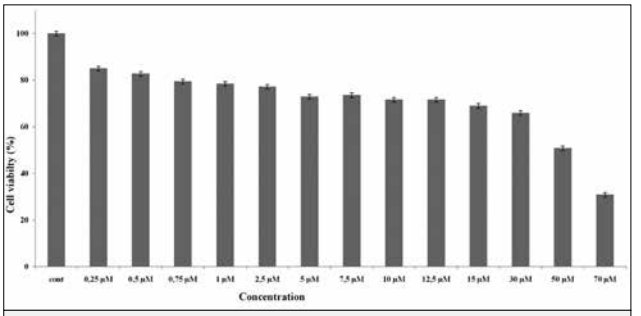

Figure 1. Effect of gemcitabine on the viability of PC-3 cells for 48 h. The cells were treated with gemcitabine with different concentrations for 48 h and cell proliferation was determined by XTT assay. The IC50 dose of gemcitabine was found to be 50 μM for 48 h in the PC-3 cell line. The data is the average results of three independent experiments.

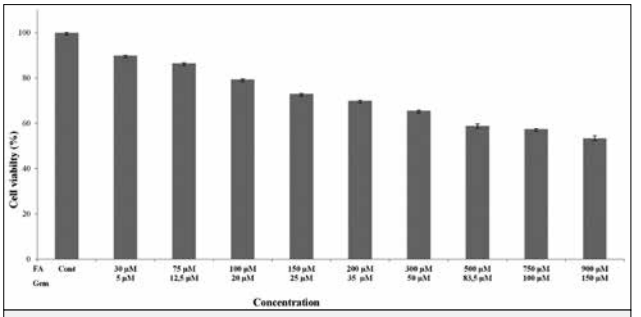

Figure 2. The combined effect of gemcitabine and FA on the viability in PC-3 cells for 48 h. The cells were treated with gemcitabine and FA with different concentrations for 48 h and the cytotoxic effect of combination doses was determined by XTT assay. The data is the average results of three independent experiments.

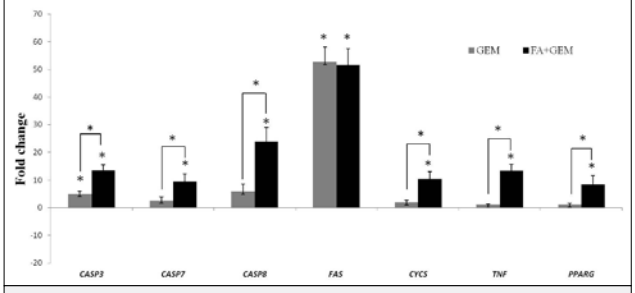

Figure 3. The effect of gemcitabine and FA on the expression of important genes in apoptosis for 48 h. Values represent the mean ± SD of three independent experiments. *p<0.05.

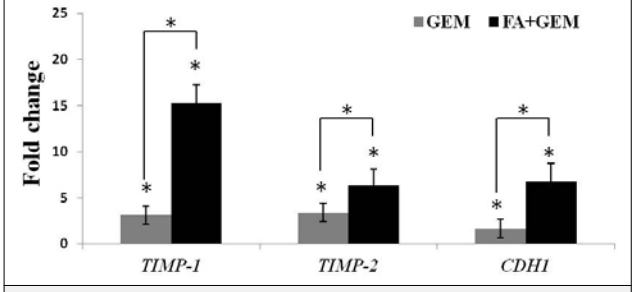

Figure 4. The effect of gemcitabine and FA on the expression of important genes in metastasis for 48 h. Values represent the mean ± SD of three independent experiments. *p<0.05.

According to the qPCR results, when the cells were treated with the IC_{50} dose of gemcitabine, the expression of *CASP3* (5.01 fold) and *FAS* (52.8 fold) genes were significantly upregulated. After the treatment with a combination of FA and gemcitabine, significant increases in the expression of *CASP3*, *CASP7*, *CASP8*, *FAS*, *CYCS*, *TNF* and *PPARG* were observed as 13.55, 9.48, 23.83, 51.63, 10.34, 17.86 and 14.63 folds, respectively compared with the control group (p<0.05). On the other hand, *CASP3*, *CASP7*, *CASP8*, *CYCS*, *TNF* and *PPARG* expressions were significantly elevated in combination treatment when compared with the gemcitabine treatment (p<0.05) (Figure 3).

Effects of Gemcitabine and FA on Expressions of Genes Associated with Metastasis

After the treatment with gemcitabine or combination of FA and gemcitabine, the expression levels of important genes associated with metastasis including *MMP-2*, *MMP-9*, *TIMP-1*, *TIMP-2*, *CDH1*, *CDH2*, *COL4A2*, *VEGF* and *HIF1A* were determined using qPCR analysis.

As shown in Figure 4, after the treatment with gemcitabine, the expressions of *TIMP-1* (3.13 fold), *TIMP-2* (3.41 fold) and *CDH1* (1.66 fold) genes were significantly elevated compared with the control group. On the other hand, a significant increase in the expression of *TIMP-1*, *TIMP-2* and *CDH1* genes was observed when a combination of gemcitabine and FA was used as 14.24, 6.30 and 6.73 folds, respectively (p<0.05). Furthermore, significant changes in expressions of *TIMP-1*, *TIMP-2* and *CDH1* genes were determined in combination treatment compared with the gemcitabine treatment (p<0.05). No significant change was observed in the other genes.

DISCUSSION

It is known that the consumption of vegetables, fruits, whole grains reduces the incidence of chronic diseases and various cancers especially stomach, esophagus, lung, oral cavity, pancreatic and colon (32,33). The anticancer activities of these foods are associated with the phenolic compounds which are secondary metabolites with a common aromatic ring possessing one or more hydroxyl group (34,35).

Various studies, including those on prostate cancer, have shown that phenolic compounds can induce apoptosis by affecting important signaling pathways or can inhibit invasion and metastasis. Furthermore, some of these compounds have been found to have a synergistic effect with chemotherapeutic agents used in standard therapy (36-38). According to these results, the investigation of the anticancer properties of natural compounds by cell culture and animal studies has become an important focus for the development of new therapeutic strategies against cancer.

FA, a natural phytochemical which is found in rice, wheat, barley, orange, coffee, apple and peanuts, was investigated for its possible synergistic effect with gemcitabine on prostate cancer cells in present study (39,40). Although there is only a limited number of studies investigating the synergistic effect of FA with various agents in cancer, previous literature illustrated that FA can be part of the combination therapies in various situations. For example, Pan et al. reported that FA and Z-ligustilide which is another major component of *Angelica sinensis,* have a synergistic effect on cold-induced vasospasm by regulating cold-sensing proteins TRPM8 and TPRA1 (41). Another study indicated that low doses of FA combined with a subthreshold dose of piperine, a bioavailability enhancer, have synergistic antidepressant-like effect on depression-like behaviors in mice (42). Canturk has shown that FA exhibits a synergistic anticandidal and apoptotic effects in combination with caspofungin against *C. albicans* (43). The potential protective effect of FA on splenic toxicity was investigated and it has been reported that its combination with ascorbic acid has a significant recuperative effect on aniline induced spleen toxicity in rats (44). The effectiveness of FA in combination treatments has also been demonstrated in studies with cancer cells. The combination of FA and δ-tocotrienol (δ-T3), another important component of rice bran, significantly reduces the proliferation of human prostate carcinoma, human breast adenocarcinoma and human pancreatic carcinoma cells as compared to single treatment (45). Likewise, in another study, the same researchers demonstrated that the combination of FA and δ-T3 significantly decreases cellular telomerase activity in colorectal adenocarcinoma cells. Moreover, it was thought that FA increases the bioavailability of δ-T3 (46). In a study conducted with breast cancer cells, it was shown that FA renders cancer cells more hypersensitive to ABT-888, poly

(ADP-ribose) polymerase (PARP) inhibitors, compared to stand alone treatment of ABT-888 (21).

In this study, to demonstrate the efficacy of FA in combination therapy, a combination dose was primarily determined considering the IC_{50} doses of FA and gemcitabine. The cytotoxic effect of gemcitabine in PC-3 cell lines was detected in a time- and dose-dependent manner using the XTT method. The IC_{50} dose of gemcitabine in PC-3 was found to be 50 µM for 48 h. IC_{50} doses of FA have been determined as 300 µM for the same period in a previous study. Then, combination doses were determined at lower concentrations to demonstrate the possible additive or synergistic effect of FA and gemcitabine in PC-3 cells. After the treatment of the prescribed doses to the cells, expressions of genes, which are important in apoptosis and metastasis, were analyzed in dose and control groups.

Apoptosis, described as a physiological process, plays an important role in the maintenance of hemostasis and the control of the cell proliferation in normal tissue. It is known that defects that occur in this mechanism cause cancer development (47). Molecules involved in this process are important for demonstrating the efficacy of newly developed agents. In the present study, the expressions of genes coding of molecules in both intrinsic and extrinsic pathways of apoptosis have been analyzed. According to the results, in the group treated with gemcitabine, the expressions of *CASP3* and *FAS* genes significantly increased. After the treatment with A combination of FA and gemcitabine, a significant increase was observed in the expressions of *CASP3*, *CASP7*, *CASP8*, *FAS*, *CYCS*, *TNF* and *PPARG* genes compared with the control group ($p<0.05$). In addition, the increases in the expressions of *CASP3*, *CASP7*, *CASP8*, *CYCS*, *TNF* and *PPARG* genes are significant compared with the gemcitabine treatment. According to the results, it can be concluded that the combination treatment affected expression of more genes in apoptosis compared with the single treatments in PC-3 human prostate cancer cell line.

Epithelial to mesenchymal transition (EMT), a biological process in which polarized epithelial cells undergo various biochemical changes resulting in increased cell migration, invasiveness and resistance to apoptosis, is the most important stage of metastasis (48). At this stage, there is a decrease in the expressions of various epithelial junction proteins such as E-cadherin, α-catenin, and γ-catenin; while, there is an increase in nonepithelial cadherins such as N-cadherin (49). In addition, various enzyme groups, especially matrix metalloproteases (MMP), contribute to this process by destroying the extracellular matrix components (50). In this study, the anti-metastatic effect of FA and gemcitabine was investigated by analyzing the expression levels of gene encoding molecules that are important in the EMT process. According to the results, the expression of *TIMP-1*, *TIMP-2* and *CDH1* genes significantly increased after the gemcitabine treatment compared with the control group. Likewise, after the treatment with a combination of FA and gemcitabine, a significant increase in the expression of *TIMP-1*, *TIMP-2* and *CDH1* genes was found compared with the control group ($p<0.05$). However, the increases in the expression of genes were higher than single treatments of gemcitabine. Moreover, the increases in the expressions of *TIMP-1*, *TIMP-2* and *CDH1* genes are significant compared with the gemcitabine treatment.

Chemotherapeutic agents are known to be toxic and have serious side effects. Natural products may exhibit a synergistic effect with the agents used in the treatment as well as reducing these side effects. In the present study, we demonstrated for the first time that a combination of FA with gemcitabine, an agent used in standard therapy, synergistically inhibited apoptosis and metastasis by regulating genes associated with these processes in prostate cancer cells. These results show that FA can promotes efficacy of gemcitabine in prostate cancer cells and FA can be an effective part of the combination treatments.

Conflict of Interest: The authors have no conflict of interest to declare.

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