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Combination of 5-fluorouracil and Lipopolysaccharide Synergistically Induces Cytotoxicity and Apoptosis in MCF-7 Human Breast Cancer Cells

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ABSTRACT

Several studies have been conducted to find suitable combinations of drugs to increase the efficacy of chemotherapy and reduce the resistance of tumor cells to treatment. Lipopolysaccharide (LPS), as a ligand for Toll-like receptor 4 (TLR-4), can modify immune responses in different cancers. Although multiple studies have been performed in this area, the effect of LPS on tumor cells remains controversial. In the present study, the cytotoxic effects of 5-fluorouracil (5-FU), with or without LPS, were evaluated in human breast cancer cell line (MCF-7) on apoptosis and gene expression in downstream signaling pathways.

MCF-7 was obtained from the Pasteur Institute of Iran. The effects of LPS and 5-FU on cytotoxicity, apoptosis, and gene expression in NF-κB, ERK, and AKT signaling pathways were evaluated by MTT assay, Annexin V/propidium iodide (PI) apoptosis assay, and qRT-PCR, respectively.

Our findings showed that LPS alone did not significantly affect cytotoxicity or apoptosis, compared to the control cells (untreated cells), while combined with 5-FU, it caused a significant increase in the apoptosis of cancer cells and decreased cell viability. It was also concluded that LPS in combination with 5-FU increased TLR-4 expression and down-regulated gene expression in NF-κB, ERK, and AKT pathways (p=0.001).

Although the role of LPS in tumor inhibition or progression remains controversial, our findings suggest that LPS can be considered a novel complementary approach intranslational oncology research of breast cancer therapy.

Keywords: Breast cancer; Cytotoxicity; Lipopolysaccharides; Toll-like receptor 4; 5-Fluorouracil

INTRODUCTION

Breast cancer is one of the most common causes of cancer-related death in women worldwide. According to GLOBOCAN estimates, nearly 2.1 million new cases of breast cancer were reported in 2018 worldwide (prevalence, 11.6%). In Iran, the prevalence of this cancer has been estimated at 12.5% in both

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sexes and all age groups.[1,2] Different strategies are used to prevent the spread of breast cancer, such as surgery, chemotherapy, and radiation therapy. However, conventional chemotherapy approaches may lead to chemoresistance and recurrence of tumors in many patients.

5-Fluorouracil (5-FU) is one of the chemotherapeutic agents, which is commonly administrated for the treatment of colorectal, head and neck, pancreatic, and breast cancer.[3-6] However, the main problem of long-term treatment with 5-FU is chemo-resistance.[7] Therefore, finding an appropriate therapeutic approach to increase 5-FU sensitivity and reverse resistance to this drug can be useful.[8] A large body of research indicates that Toll-like receptors (TLRs) potentially contribute to a variety of cancers. Previous studies revealed that among 13 discovered TLRs, TLR-4 is expressed in a variety of cancers, such as lung, neuroblastoma, prostate, and breast cancers.[9-12]

There are various ligands for TLR-4, includingHeat shock protein 60 (HSP60), HSP70, Hyaluronan, High mobility group box 1 (HMGB1) proteins, and Lipopolysaccharide (LPS).[13] LPS is a major component in the cell wall of Gram-negative bacteria,which is recognized by TLR-4 and canmodify immune responses in different cancers.[14-16] The activation of TLR-4 by its ligand, LPS, results in the recruitment of adaptor molecules, which modulate signaling pathways, includingnuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), mitogen-activated protein kinase (MAPK),c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and interferon regulatory factor (IRF) pathways.[17-19]

Previous studies revealed that LPS has a dichotomous mechanism in cancer therapy. On one hand, LPS exhibits anti-tumor activities in some cancers, such as colorectal and lung cancers,[20-22] but on the other hand, LPS can increase invasion and angiogenesis in hepatocellular cancers.[23-25] According to some investigations, LPS exerts its oncogenic or tumor-suppressive effects dose-dependently in a variety of cancers. Low concentrations of LPS, injectedintravascularor subcutaneously, can have therapeutic effects in cancer patients.[20] Our previous in vitro study showed that LPS/5-FU combination significantly induced apoptosis in colorectal cancer cells.[26] Although many studies have been conducted in this area, the effect of LPS on tumor cells remains controversial. The present study aimed to evaluate the cytotoxic effects of5-FU and LPS in breast cancer cells (MCF-7) on the regulation of TLR-4and expression of signaling pathway molecules.

MATERIALS AND METHODS

The study was approved by the Ethical Committee of Golestan University of Medical Sciences (Code: 940216082).

Cell Line and Reagents

The human breast cancer cell line, MCF-7, was purchased from the Pasteur Institute of Iran. The cells were maintained in RPMI-1640 medium (Gibco, UK), supplemented with 10% Fetal bovine serum (FBS) (Gibco, Carlsbad, USA), 100 unit/mL of penicillin (Gibco, Carlsbad, USA),,and 100 µg/mL of streptomycin (Gibco, Carlsbad, USA), at 37°C in a5% CO_2atmosphere.LPS was extracted from *Escherichia coli* O55:B5 (L2880, Sigma Aldrich, USA) and stored in a stock solution of 1 mg/mL at 220uC. Also, 5-FU was purchased from Sigma-Aldrich (03738, UK), and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromideorthiazolyl blue tetrazolium bromide (MTT) powder was obtained from Alfa Aesar (L11939, USA). Primers were synthesized by Arian Gene Gostar Co. (Iran).

Cytotoxicity Assay

MTT assay was applied to evaluate the cytotoxicity of 5-FU, with or without LPS, in MCF-7 cells after 48 hours via setting up in the lab. For this purpose, the MCF-7 cells were seeded at 1×10^4 cells/well in a 96-well plate overnight. Next, they were treated with different concentrations of 5-FUto measure the half-maximal inhibitory concentration (IC_{50}). The tumor cells were exposed to different concentrations of LPS (1, 5, 10, 20, and 100 µg/mL) to evaluate cytotoxicity. In addition, the MCF-7 cells were treated with different concentrations of 5-FU for 24 hours, and then, LPS (1 µg/mL) was added to the wells. The untreated cells were considered the control group. Experiments were performed in triplicate and repeated three times. After 48 and 72 hours, 20 µL of MTT solution (5 mg/mL) was added to each well and incubated for four hours at 37°C in a 5% CO_2 atmosphere.[27] Next, the supernatant was removed, and 100 µL of DMSO was added to each well as a solvent. The cell viability percentage was

assessed by spectrophotometry at 570 nm, using an absorbance microplate reader (ELx800^{TM}, BioTek, USA).

Analysis of Drug Interactions

Twenty-four hours after treating the MCF-7 cells with 5-FU, LPS was added for 48 and 72 hours. Analysis of drug interactions was performed to evaluate the interactions between LPS and 5-FU, using the isobologram and median effect methods, as described by Chou and Talalay.[28-30] The synergistic effects of the drugs were determined by computing the combination index (CI), according to a study by Chou and colleagues. The CI method is a quantitative evaluation of two drugs using Calcusyn software (Biosoft, Cambridge, UK). CI<1 represents synergism, CI=1 represents additively, and CI>1 represents antagonistic effects.[28] Another parameter for quantifying the interaction between two drugs is the dose reduction index (DRI). This index indicates the fold-change of an individual agent when used in synergistic combination to achieve a given effect level in comparison with the dose of each drug alone. Data collected from the cytotoxicity assays were used to determine the values of DRI and CI.

Apoptosis Assay

Apoptosis assay was performed using BD Accuri C6and FITC Annexin V Apoptosis Detection Kit with propidium iodide (PI) (640914; Biolegend, San Diego, CA, USA). Data were analyzed in BD Accuri C6 software. The MCF-7 cells (1×10^6 cells) were seeded in a six-well plate at 37°C in duplicate in a humidified (80%) atmosphere containing 5% CO_2. After 24 hours, the cells were treated with 5-FU (IC_{50}=10 µg/mL), LPS (1 µg/mL), and combination of 5-FU and LPS (LPS added 24 hours after 5-FU) and then incubated for 48 hours. The untreated MCF-7 cells were considered as the control group. After 48 hours, the cells were harvested and stained following the manufacturer's instructions. Viable, apoptotic, and necrotic cells were measured using FL1-H and FL3-H channels. Data were analyzed using BD Accuri C6 software.

The Expression of TLR-4

TLR-4 expression was measured by flowcytometry. The MCF-7 cells (1×10^6) were cultivated in a six-well plate at 37°C in duplicatein a humidified atmosphere containing 5% CO_2. After 24 hours, the cells were treated with 5-FU (IC_{50}=10 µg/mL), LPS (1 µg/mL), and 5-FU plus LPS (LPS added 24 hours after 5-FU). The untreated MCF-7 cells were considered as the control group. After incubation for 48 hours, the cells were harvested and washed three times with phosphate-buffered saline (PBS) plus 0.5% bovine serum albumin (BSA), followed by incubation with 5 µL ofthe anti-human antibody for 30 minutes. The allophycocyanin (APC) anti-human CD284 (TLR-4) antibody (312816; Biolegend, USA), as well as the isotype control, APC Mouse IgG2a, κ Isotype Ctrl (FC) (400222; Biolegend, USA) antibody, was used in this assay.

Gene Expression Analysis by Quantitative Real-time Polymerase Chain Reaction (qRT-PCR) Assay

The MCF-7 cells (1×10^6 cells) were seeded in a six-well plate in duplicate overnight at 37°C in a humidified atmosphere containing 5% CO_2. Next, the cells were treated with 5-FU (IC_{50}=10 µg/mL), LPS (1 µg/mL), and 5-FU plus LPS (LPS added 24 hours after 5-FU), while the untreated MCF-7 cells were used as the control group. After 48 hours, total RNA was extracted from the cells, using the Wizol^{TM} reagent (Wizbiosoultion, Korea), and complementary DNA (cDNA) was synthesized by the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA).

RT-PCR assay was conducted using the target gene primers (Table 1), and mRNA levels were analyzed by qRT-PCR assay, using an Applied Biosystems AB RT-PCR Detection System and an SYBR green-based kit with RealQ Plus Master Mix Green (Ampliqon, Copenhagen, Denmark). There action mixture (20 µL) contained 6.6 mL of nuclease-free water, 2.0 µL of cDNA (1 mg/mL), 0.5 µL (10 µM) of each primer, 0.4 µL of ROX dye, and 10.0 µL of qPCR Master Mix with SYBR green (WizScript RT Master). The thermal cycle profile for PCR was as follows: at 95°C for 5 minutes; 40 cycles of PCR at 95°C for 15 seconds and at 60°C for 30 seconds; and one cycle of PCR at 95°C for15 seconds, at 60°C for 60seconds, and at 95°C for 15 seconds (Table 2). For calculating the relative expression of genes, the ΔΔCT formula was used:

$\Delta\Delta CT=\Delta CT$ (a target sample) $-\Delta CT$ (a reference sample)

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Table 1. The list of primers

Gene	Forward primer	Reverse primer	Amplicon size (bp)
NF-κB*P65*	ATCCCATCTTTGACAATCGTGC	CTGGTCCCGTGAAATACACCTC	**153**
AKT2	TGAAAACCTTCTGTGGGACC	TGGTCCTGGTTGTAGAAGGG	**145**
ERK1	ACCCTGGAAGCCATGAGAGA	GGCGGAGTGGATGTACTTGA	**150**
ERK2	TTCCAACCTGCTGCTCAACA	TCTGTCAGGAACCCTGTGTGAT	**102**
TLR-4	TGGAAGTTGAACGAATGGAATGTG	ACCAGAACTGCTACAACAGATACT	**148**
B-actin	CCTTCCTGGGCATGGAGTCCT	TGGGTGCCAGGGCAGTGAT	**174**

Table 2. The thermal cycle profile for Quantitative Real-time PCR

cycle	Temperature	time
1x	95 °C	5 min
40x	95 °C	15 s
	60 °C	30 s
1x	95 °C	15 s
	60 °C	60 s
	95 °C	15 s

Statistical Analysis

All data are presented as mean±standard deviation (SD). The collected data were analyzed and plotted using GraphPad Prism Version 6.01. Student-test and one-way ANOVA were used for comparing the groups. Spearman's correlation test was also performed for evaluating the correlation between variables. The CI and DRI were calculated by Calcusyn software (Biosoft, Cambridge, UK). All of the experiments were performed in triplicate and repeated three times independently. *p*-value less than 0.05 was considered statistically significant (*p<0.05, **p<0.01, and ***p<0.001).

RESULTS

The main objective of this study was to examine the effect of TLR-4 signaling on the anti-tumor activity of 5-FU in a breast cancer cell line. To explore the effects of LPS and TLR-4 signaling on the anti-tumor activity of 5-FU, cytotoxicity, apoptosis, and gene expression assays were conducted in the MCF7 cell line, exposed to LPS and 5-Fu under different conditions.

Effect of LPS on Cytotoxicity of 5-FU in MCF-7 Cell Line

To explore the effect of LPS and TLR-4 signaling on the cytotoxicity of 5-FU, the MCF-7 cells were cultured and treated with different concentrations of 5-FU (0.625, 1.25, 2.5, 5, 10, and 20 µg/mL), with and without LPS (1 µg/mL). Cell viability was assessed using the MTT assay. The results showed that the IC_{50}values of 5-FUand LPS were 10 µg/mL and 437.93 µg/mL, respectively. We found that different concentrations of LPS (1, 5, 10, 20, and 100 µg/mL) did not have any significant cytotoxic effects on the MCF-7 cells (Figure 1B), while LPS (1 µg/mL),along with different doses of 5-FU, significantly decreased the cell viability of MCF-7 cells within 48 and 72 hours, compared to 5-FU alone (Figure 1A).

Interaction of 5-FU and LPS

The interaction between 5-FUand LPS in the MCF-7 cells was analyzed using the isobologram and median effect methods, as described by Chou and Talalay. The points above, below, and over the isobologram effect line reflect the antagonistic, synergistic, and additive effects, respectively. The isobologram showed that the points accumulated and fell below the line, representing the synergistic interaction between 5-FU and LPS (Figure 2A).

Calculation of CI between 5-FU and LPS showed values<1 (CI values: 0.001 to 0.032); therefore, they had synergistic effects on each other (Figure 2B).

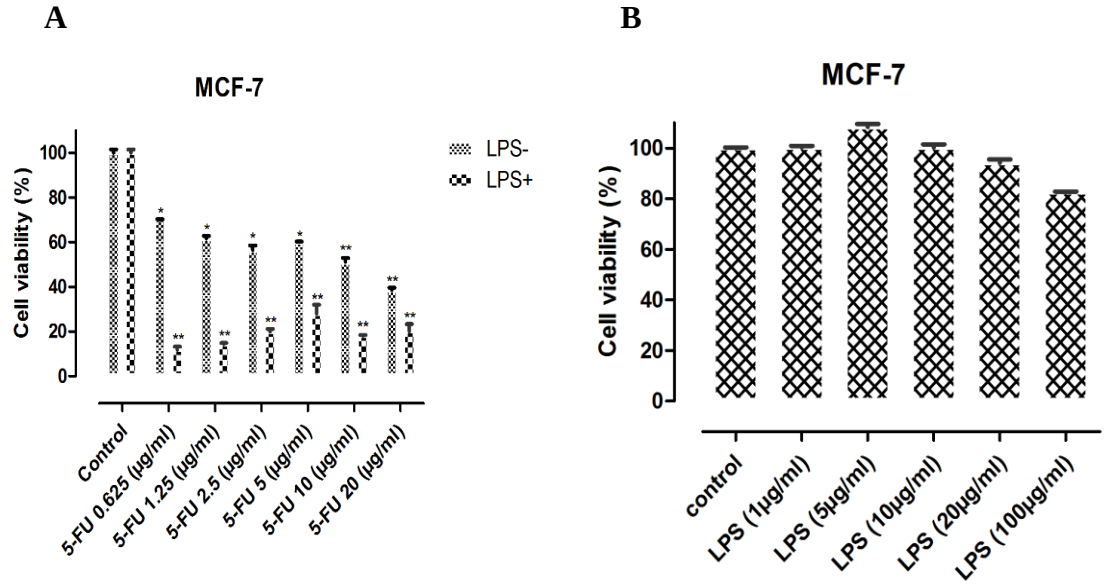


Figure 1. The cell viability of MCF-7 cells by MTT assay. A) To explore the effect of lipopolysaccharide (LPS) and Toll-like receptor 4 (TLR-4) signaling on the cytotoxicity of 5-Fluorouracil(5-FU), The MCF-7 cells were treated by 5-FU (0.625, 1.25, 2.5, 5, 10 and 20 µg/mL) with or without LPS (1 µg/mL) for 48-hours and cell viability was evaluated by MTT assay. One-way ANOVA was used to reveal the statistical difference. B) The cell viability of MCF-7 in exposure to various concentrations of LPS. Different concentrations of LPS did not have any significant cytotoxic effects on the MCF-7 cells, while LPS, along with different doses of 5-FU, significantly decreased the cell viability of MCF-7 cells within 48 compared to 5-FU alone. (*p<0.05, **p<0.01, and ***p<0.001).

Moreover, measurement of DRI indicated that the index value was in the range of 32 to 7052 for 5-FU (0.625, 1.25, 2.5, 5, 10, and 20 µg/mL) and in the range of 894 to 1976 for LPS (1 µg/mL) (Figure 2B). The DRI values in the presence of LPS indicateda 7052-fold reduction of 5-FU at 0.625 µg/mL, 2017-fold reduction of 5-FU at 1.25 µg/mL, 261-fold reduction of 5-FU at 2.5µg/mL, 32-fold reduction of 5-FU at 5 µg/mL, 98-fold reduction of 5-FU at 10 µg/mL, and 32-fold reduction of 5-FU at 10 µg/mL.

Effect of LPS on Apoptosis Induced by 5-FU in MCF-7 Cells

The apoptosis assay, as the pharmacodynamic endpoint of cancer therapy, was performed to investigate whether LPS can affect apoptosis induced by 5-FU. The results of Annexin/PI apoptosis assay showed that the untreated MCF-7 cells as the control group induced 11.4% apoptosis (Figure 3A). In addition, 5-FU (IC_{50}=10 µg/mL) induced apoptosis in the MCF-7 cells (42.7%), as shown in Figure3B. However, LPS (1 µg/mL) significantly increased the rate of apoptosis of 5-FU (59.8%) in the MCF-7 cells (Figure 3C). On the other hand, LPS alone induced a low level of apoptosis, compared to the combination treatment (33.6%) in the breast cancer cell line (Figure 3D). Therefore, apoptosis induced by the combination treatment was 1.4-fold greater than 5-FU alone (Figure 3E).

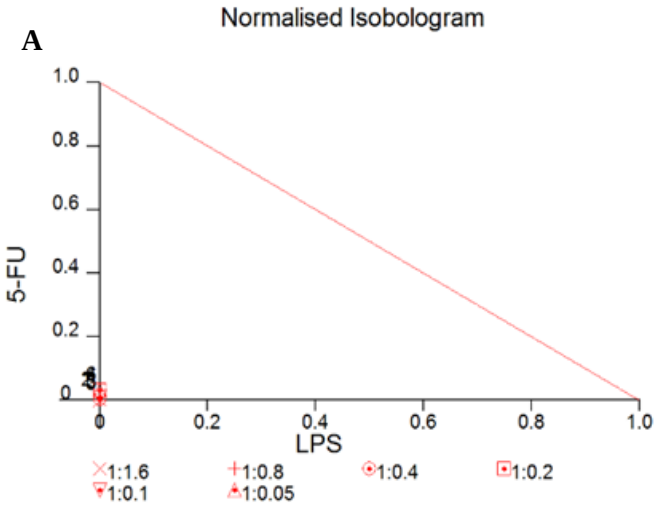
Effect of LPS on TLR-4 Protein Expressionin5-FU-treated MCF-7 Cells

The RT-PCR assay was performed to investigate whether LPS can affect TLR-4 expression in breast cancer cells treated with 5-FU (Figure 4A). The results of RT-PCR revealed that the untreated cells (control group) expressed a low level of TLR-4 (6.4%), compared to the treated cells (Figure 4B). Furthermore, protein expression of TLR-4 in the treated MCF-7 cells with 5-FU and LPS alone was evaluated via flowcytometry and were42% and 33.3%, respectively (Figure 4C and 4E). However, as shown in Figure 4D, the combination treatment with 5-FU and LPS increased the expression of TLR-4 significantly (54%). Therefore, TLR-4 expression induced by the combination treatment was 1.2-fold higher than 5-FU alone.

Effect of LPS on Gene Expression of 5-FU-treated Cells

The RT-PCR assay was performed to evaluate the expression of TLR-4 and several molecules involved in cell signaling of the MCF-7 cell line. NF-κB , ERK1/2, and AKT mRNAs were expressed in the untreated cells (control group). However, when the MCF7 cells were exposed to 5-FU (IC_{50}=10 µg/mL), expression was significantly upregulated. Furthermore, the expression of these genes increased in exposure to LPS (1 µg/mL). However, when the MCF-7 cells were cotreated with 5-FU (10 µg/mL) and LPS (1 µg/mL), the expression of these signaling genes was significantly downregulated (Figure 5).

A



B

Concentration		CI	DRI	
LPS (µg/ml)	**5-FU (µg/ml)**		**LPS**	**5-FU**
		MCF-7		
1	0.625	0.001	1976	7052
1	1.25	0.001	1728	2017
1	2.5	0.005	1249	261
1	5	0.032	894	32
1	10	0.011	1378	98
1	20	0.031	1249	32

Figure 2. A) Isobologram analysis for the interaction of various combinations of 5-Fluorouracil(5-FU) and lipopolysaccharide (LPS) (1 µg/mL) in the MCF-7 cell line. The line indicates the theoretical line of additivity. The points located below, above and near or on the line, represent synergism, antagonistic and additive effects of combination treatments, respectively. The isobologram showed that the points accumulated and fell below the line, representing the synergistic interaction between 5-FU and LPS. B) The combination index (CI) and Dose reduction index (DRI) of LPS and 5-FU combination in MCF-7cell line. Calculation of CI between 5-FU and LPS showed values <1 (CI values: 0.001 to 0.032); therefore, they had synergistic effects on each other. The DRI values in the presence of LPS indicated a 32-fold reduction of 5-FU at 10 µg/mL. Moreover, measurement of DRI indicated that the index value was in the range of 32 to 7052 for 5-FU (0.625, 1.25, 2.5, 5, 10, and 20 µg/mL) and in the range of 894 to 1976 for LPS (1 µg/mL) (Figure 2B). The DRI values in the presence of LPS indicated a 7052-fold reduction of 5-FU at 0.625 µg/mL, 2017-fold reduction of 5-FU at 1.25 µg/mL, 261-fold reduction of 5-FU at 2.5 µg/mL, 32-fold reduction of 5-FU at 5 µg/mL, 98-fold reduction of 5-FU at 10 µg/mL, and 32-fold reduction of 5-FU at 10 µg/mL.

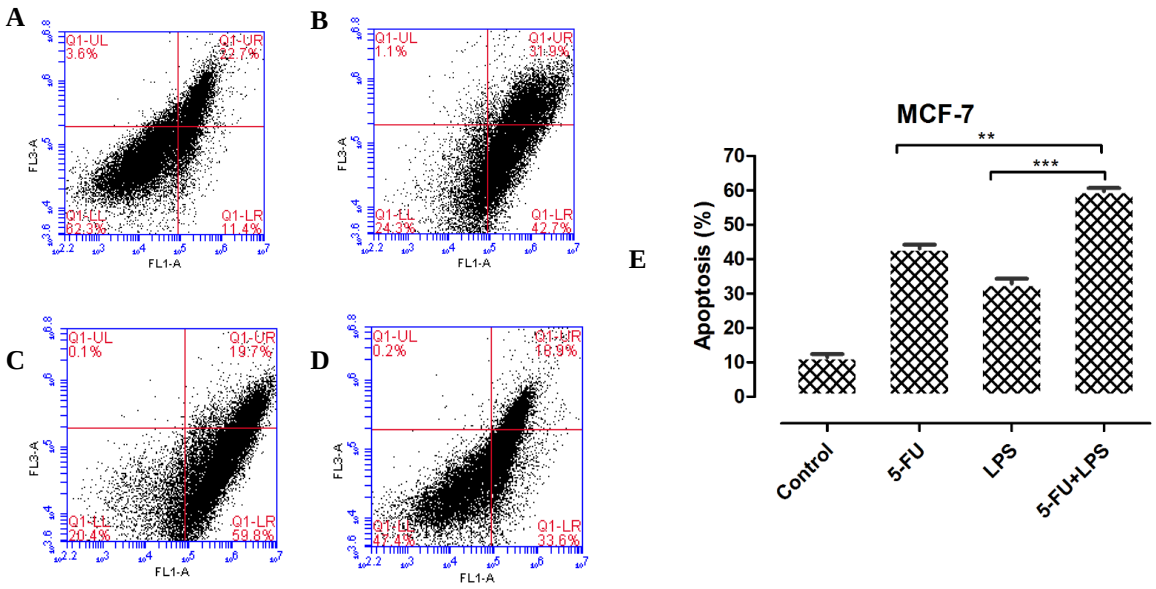


Figure 3. The flowcytometry analysis of MCF-7 with FITC Annexin V/PI Apoptosis. To explore the effect of LPS and Toll-like receptor 4 (TLR-4) signaling on the apoptosis rate of 5-Fluorouracil(5-FU), The MCF-7 cells were treated by 5-FU (10 µg/mL) with or without lipopolysaccharide (LPS) (1 µg/mL) for 48-hours and cell viability was evaluated by Annexin V/PI assay. One-way ANOVA was used to reveal the statistical difference. A) Untreated Control. B) 5-FU (10 µg/mL). C) 5-FU (10 µg/ml) and LPS (1 µg/mL). D) LPS (1 µg/mL). E) The percentage of apoptosis of MCF-7 cells after treatment with 5-fu and LPS. The data showed that the apoptosis rate was remarkably higher in LPS+5-FU combination in comparison to the control, 5-FU alone, and LPS alone. (*p<0.05, **p<0.01, and ***p<0.001).

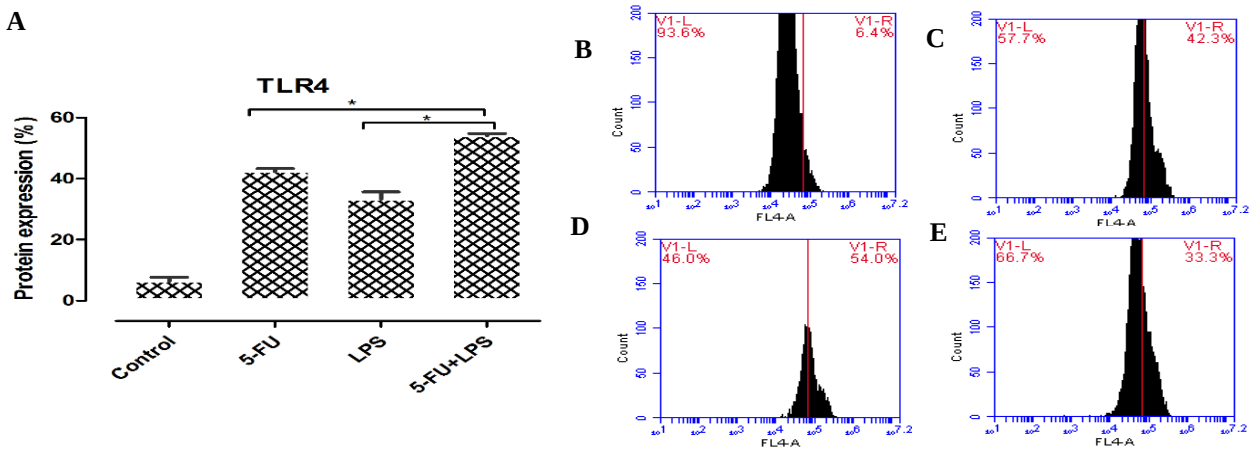


Figure 4. Flowcytometry analysisof Toll-like receptor 4 (TLR-4) expression on the MCF-7 cell line. To determine the effect oflipopolysaccharide (LPS) and 5-Fluorouracil (5-FU) on the TLR-4 expression, the MCF-7 cells were treated by 5-FU (10 µg/mL) with or without LPS (1µg/ml) for 48-hours and TLR-4 gene and protein expression were evaluated by Real-time PCR and Flowcytometry, respectively. One-way ANOVA was used to reveal the statistical difference. A) Toll-like receptor 4 expressions in MCF-7 cells. B) TLR-4 expression in untreated MCF-7 cells as control. C) TLR-4 expression in treated MCF-7 with 5-FU. D) TLR expression in treated MCF-7 with 5-FU and LPS. E) TLR expression in treated MCF-7 with LPS. The combination treatment with 5-FU and LPS increased the expression of TLR-4 significantly (*p< 0.05).

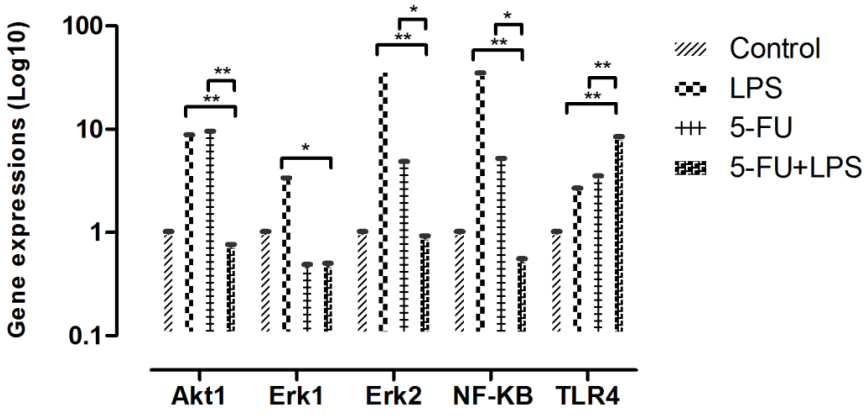


Figure 5. To determine the effect oflipopolysaccharide (LPS) and 5-Fluorouracil (5-FU) on the Toll-like receptor 4 (TLR-4), NF-κB, ERK1/2, and AKT mRNAs expression, the MCF-7 cells were treated by 5-FU (10 µg/mL) with or without LPS (1µg/ml) for 48-hours and the genes expression were evaluated by Real-time PCR. One-way ANOVA was used to reveal the statistical difference. The data showed that TLR-4 was expressed in the untreated MCF-7 cells. LPS significantly increased the expression of TLR-4 and reduced the expression of signaling molecules in MCF-7 cells treated with 5-FU. (*p<0.05, **p<0.01, and ***p<0.001).

Our results showed that TLR-4 was expressed in the untreated MCF-7 cells. It was significantly upregulated when the cells were treated with either LPS or 5-FU or a combination of LPS and 5-FU (Figure 5). The results presented in Figure 5 show that LPS significantly increased the expression of TLR-4 and reduced the expression of signaling molecules in MCF-7 cells treated with 5-FU. TLR-4 protein had a positive correlation with TLR-4 genes (r=1, p=0.01). Furthermore, the expression of NF-κB was positively correlated with ERK2 (r=1, p=0.01).

DISCUSSION

Although 5-FU is a chemotherapeutic agent, which is commonly used for the treatment of different types of solid tumors,[3-7] the response rate of treatment with 5-FU alone is still low. The main problem of long-term treatment with 5-FU is the resistance of tumor cells to this drug.[8] Therefore, there is an urgent need to find better therapeutic options to increase 5-FU sensitivity and reverse resistance to the drug.[9] According to recent studies, LPS, as an effective substance in our immune system, has the potential to modify the immune system in cancer patients. In the present study, the effects of LPS on cytotoxicity of 5-FU, apoptosis, and TLR-4 signaling pathways were assessed in a breast cancer cell line.The most important finding of this study was the synergistic effect of LPS and 5-FU to potentiate cytotoxicity, while LPS alone did not influence the viability of cells significantly (p=0.001).

The idea of a combination of drug and LPS was first presented in our previous study of colon cancer cells. In this study, we showed the synergistic effect of drug and LPS on apoptosis and expression of TLR-4 and molecules involved in cell signaling.[26] In the present study, the synergistic effect of LPS and drug on breast cancer cells was shown. The effect of LPS on the efficacy of 5-fluorouracil in colon and breast cancer cells was almost similar.

Similar to our findings, Edwardson et al (2017) showed that LPS augments docetaxel cytotoxicity in breast tumor cells.[31] A study by Márcia Gonçalves (2016) revealed that LPS at high concentrations induces tumor death in human oral and esophageal cancer cell lines. In addition, in vivo studies on murine models reported that LPS alone has anti-tumor effects in glioblastoma.[32] We also found that LPS in combination with 5-FU can elevate the apoptosis rate of MCF-7 cells. This finding is in line with our previous study on colorectal cancer cells, indicating the synergistic effects of LPS and 5-FU on apoptosis induction.[26] In contrast to our observations, Chung (2016) showed that in human colorectal cancer cells,

combined treatment with 5-FU and LPS can inhibit apoptosis.[33]

Our findings showed that a combination of LPS with 5-FU was far more effective in increasing the expression of TLR-4 in MCF7 cells, compared to individual drugs. Several studies reported that the expression of TLR-4 increased in human breast cancer cells when the cells were stimulated with LPS.[34] Moreover, our previous study demonstrated that LPS remarkably upregulated the expression of TLR-4 and induced apoptosis in 5-FU-treated colorectal cancer cells.[26] TLR-4 signaling induced the expression of pro-inflammatory cytokines and NF-κB production.[35] Moreover, knockdown of TLR-4 inhibits cell proliferation and survival in MDA-MB-231 breast cancer cells.[36] On the other hand, another study showed that TLR-4 over expression after chemotherapy can increase cell viability via activation of GSK3B in colon cancer cells.[33]

To the best of our knowledge, the role of increased TLR-4 expression and LPS-induced TLR-4 signaling after chemotherapy remains controversial. Our findings revealed that treatment with either LPS or 5-FU upregulated mRNA expression of *Akt, Erk1, Erk2,* and NF-κB genes in the breast cancer cell line (MCF-7). However, a combination of LPS with 5-FUdecreased the expression of the mentioned genes, compared to the control groups. In contrast to our findings, Chung et al demonstrated that increased TLR-4 expression in drug-treated colon cancer cells induces the activation of protein kinase B (AKT), NF-κBp65, and ERK after stimulation with LPS; on the other hand, LPS stimulation blocks apoptosis signaling in drug-exposed cancer cells.[33]

In line with our study, Rich et al (2011) reported that LPS-induced TLR-4 signaling activates several kinases, including p38 MAPK, p42/44 (ERK1/2) MAPK, AKT, and β1 integrins in various cell types. LPS-induced TLR-4 signaling contributes to adhesiveness and metastatic capability, and blockage of this signaling pathway may be beneficial for eradicating distal organ metastases. It has been also shown that at different time points, no significant changesoccur as a result of LPS pre-treatment in the cellular proliferation of HT-29 cells.[37]

It has been demonstrated that LPS-activated TLR-4 promotes the migration of breast cancer cells by triggering the PI3K/Akt/GSK3B/B-catenin pathway.[38] LPS can also improve the activation of ERK1/2, P38, and NF-κBin colon carcinoma.[39] Our results showed that among TLR-4 signaling genes, the expression of NF-κB is positively correlated with the level of ERK2. Previous investigations revealed that activation of the MAP signaling pathway, followed by the induction of NF-κB, contributes to tumor invasion and metastasis via MMP-9 in gastric cancer.[40] Furthermore, inhibition of ERK and subsequently NF-κB reversed the epithelial-mesenchymal transition process in breast cancer cells.[41]

In conclusion, our results showed that LPS in combination with 5-FU augmented the anti-tumor effects of the chemotherapy agent and induced cell apoptosis in breast cancer cells. Moreover, LPS/5-FU significantly decreased the mRNA expression of the PI3K/Akt signaling pathway, which is a canonical pathway in breast cancer. Our findings suggest that LPS may be considered a novel complementary therapeutic option and a future target for studies on human cancer cells. Overall, different components of bacteria, such as LPS, may be used as effective agents in combination with other drugs to induce synergistic effects or may be used as immunomodulators to regulate the immune system in response to cancer. Although the role of LPS in tumor inhibition or progression is still controversial, various studies have shown that it is a very important component, affecting the immune system in both health and disease.[42] Since the human body is a natural ecosystem for bacteria to grow and proliferate, the role of bacterial components in different types of cancer should be further considered in future research. One important limitation of this study was its in vitro nature. In vivo studies need to be performed to determine the effect of LPS on the efficacy of 5- Fu in an animal model.

Taken together, our in vitro study demonstrated that activation of TLR4 via LPS in combination with 5-FU inhibited breast cancer cells remarkably. Although the role of LPS in tumor inhibition or progression remains controversial, our findings suggest that LPS can be considered a novel complementary approach in translational oncology research of breast cancer therapy.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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