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Original Article

Combination of Silibinin and Curcumin Reduced Leptin Receptor Expression in MCF-7 Human Breast Cancer Cell Line

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Abstract

Background: Leptin and leptin receptor (Ob-R) are associated with worse prognosis, distant metastasis, and poor survival of breast cancer. We investigated the cytotoxic effect of silibinin and curcumin, individually and combined, on Ob-R expression in MCF-7 cells.

Methods: This study was performed from October 2017 to April 2018 at the Department of Clinical Biochemistry, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. The cytotoxic effect of silibinin and curcumin, individually and combined, and their corresponding half-maximal inhibitory concentration (IC50) values were determined using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. The cells were treated with different concentrations of silibinin (50-400 µM), curcumin (10-35 µM), and their combinations for 24 and 48 hours. The expression of Ob-R was measured using the Western blot analysis by treating the cells with different concentrations of curcumin (10-25 µM), silibinin (50-250 µM), and their respective combinations. The difference in mean cell viability between the groups was calculated using one-way ANOVA followed by Tukey's *post hoc* test.

Results: Silibinin and curcumin exerted time- and dose-dependent cytotoxic effect on MCF-7 cells. After treatment with silibinin, the IC_{50} values were about 250 and 50 µM at 24 and 48 hours, respectively. In terms of treatment with curcumin, the IC_{50} values were about 25 and 15 µM at 24 and 48 hours, respectively. Following treatment with silibinin, the Western blot analysis showed that Ob-R expression significantly decreased at 150 µM (P=0.031) and 200 µM (P=0.023) concentrations. Curcumin did not significantly decrease the Ob-R expression, however, the expression significantly decreased (P=0.004) when it was combined with silibinin.

Conclusion: The combination of silibinin and curcumin significantly reduced Ob-R expression in MCF-7 cells compared with their individual effects.

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Keywords ● Receptors, Leptin ● Silibinin ● Curcumin ● MCF-7 cells

What's Known

- Silibinin and curcumin exhibit significant anti-cancer effects on different types of cancer cell lines. Their combination reduces leptin and leptin receptor (Ob-R) expression in T47-D cell lines.
- The effect of combined silibinin and curcumin on the protein level of Ob-R in MCF-7 cell lines has not been assessed.

What's New

- The combination of silibinin and curcumin synergistically induced cell toxicity and reduced Ob-R expression in MCF-7 cells.
- Combination therapy using natural anti-breast cancer agents is an attractive approach and deserves further research.

Introduction

Breast cancer represents the most prevalent type of malignancy among women and is the most leading cause of cancer-associated

death among women worldwide.[1] Several studies have assessed the effect of obesity on the risk factors, pathogenesis, tumor metastasis phenotype, and poor survival rate of breast cancer.[2-4] Among obesity-related factors having the highest effect on the pathogenesis and progression of breast cancer, the effect of adipose-derived cytokine (leptin) has been widely assessed.[5] Leptin, a circulating peptide hormone, is predominantly secreted by adipose tissue and is produced in non-cancerous breast tissue and breast cancer cell lines.[6] Some studies have reported the importance of leptin in the carcinogenesis, metastasis, and angiogenesis of breast cancer.[7, 8] Leptin receptor (Ob-R), also known as obesity receptor,[9] plays a pivotal role in central and peripheral action of leptin and is commonly involved in the initiation and progression of breast cancer. Among the six leptin receptor isoforms, short leptin receptor (Ob-Ra) and long leptin receptor (Ob-Rb) are predominant. Ob-Rb is predominantly expressed in the hypothalamus. The mitogenic activity of Ob-Ra is expressed in most peripheral tissues.[10] A previous study reported a higher level of both leptin and Ob-R in cancerous epithelial tissues than in non-cancerous epithelium.[9] Leptin and its receptor are expressed in human breast cancer cell lines, MCF-7 and T47-D at the mRNA and/or protein levels.[11] Moreover, a significant positive association between the levels of leptin and Ob-R with breast cancer development has been reported.[12] Interestingly, both leptin and Ob-R appear to be associated with worse prognosis, metastasis, and poor survival of breast cancer.[13] Considering the critical role of leptin and its receptors in the pathogenesis and progression of breast cancer, these molecular markers will provide useful information for a potential therapeutic agent against breast cancer.

Curcumin is the main bioactive compound of *Curcuma longa,* which has been commonly used in most Southeast Asian countries as a traditional medication.[14] Recently, its biological activities (antioxidant, anti-inflammatory, and antimicrobial), chemopreventive, and chemotherapeutic effects on different types of cancer have been reported.[15] Several *in vivo* and *in vitro* studies have also assessed its potential anti-invasive and anti-metastatic activities on breast cancer cells.[16-18] Silibinin is the main bioactive compound of silymarin, which has antioxidant,[19] anticancer, and programmed cell death activity.[20] The results of a clinical trial (phase I and II) showed that silibinin had no toxic effects.[21] The above-mentioned trial investigated the cytotoxicity effect of silybin-phytosome, a commercially available formulation containing silibinin, on prostate cancer. Another study reported the synergistic anti-malignancy effect of silibinin when combined with other chemotherapeutic agents.[22]

The objective of the present study was to investigate the effect of silibinin and curcumin, individually and combined, on the expression of Ob-R in MCF-7 human breast cancer cell lines.

Materials and Methods

This study was performed from October 2017 to April 2018 at the Department of Clinical Biochemistry, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. The study was approved by the Research Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, (IR.AJUMS.REC.1395.570).

Chemicals and Reagents

Silibinin, curcumin, methylthiazolyldiphenyl-tetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA, USA). Anti-leptin receptor antibody and bicinchoninic acid protein assay kits were purchased from Abcam (Cambridge, UK). Monoclonal antibody against Beta-actin and HRP-labeled goat anti-rabbit IgG (H+L) were purchased from Cell Signaling Technology (Danvers, MA, USA). The polyvinylidene difluoride membrane was purchased from PerkinElmer (Waltham, MA, USA). An enhanced chemiluminescence kit (ECL) was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA).

Cell Culture and Drug Treatments

MCF-7 breast cancer cell lines were purchased from the National Cell Bank of Iran (Tehran, Iran), validated and stored according to supplier's guidelines. Cells were cultured in DMEM medium supplemented with 10% heat-inactivated FBS (Invitrogen, USA), and 1% penicillin-streptomycin at 37 °C under 5% CO_2 atmosphere. For the experiments, silibinin (20 mM) and curcumin (50 mM) powder were dissolved in dimethyl sulfoxide (DMSO) as stock solutions, and then diluted with fresh medium containing 10% FBS, such that the amount of DMSO at no time exceeded 0.1%.

Cell Growth Analysis

Cytotoxic effect of silibinin and curcumin on the MCF-7 cells after 24 hours of treatment was investigated using the MTT assay. In brief,

cells were seeded in 96-multiwell plates at a concentration of 3×10^3 cells/well in 100 µL medium. After incubation for 24 hours, the cells were treated with different concentrations of curcumin (10-35 µM) and silibinin (50-400 µM) for 24 and 48 hours; performed in triplicate. Samples of the cells treated with the vehicle served as controls. Then, 20 µl of MTT (1 mg/mL in Phosphate-buffered saline) was added to the wells and incubated at 37 °C for 4.5 hours. The mixture was removed, and 200 µL pure DMSO was added to each well. The optical density (OD) of the culture was measured at 570 nm using a microplate reader (BioTek® ELx800, USA). The cytotoxic effect of silibinin and curcumin on cell viability was assessed and expressed as the percentages of viable cells compared to control cells, assuming that the control cells were 100% viable.

Western Blot Analysis

The cells (5×10^5) were seeded into 6-well plates and incubated overnight at 37 °C with different concentrations of silibinin (50,100,150, and 200 µM) and curcumin (5, 10, 15, and 20 µM). The cells were lysed in ice-cold radioimmunoprecipitation buffer (RIPA) supplemented with the proteases-phosphatase inhibitor. The lysate was centrifuged at 12,000 ×g for 20 minutes at 4 °C to remove cellular debris. The protein concentration was quantified using a bicinchoninic acid protein assay kit. An equal amount of protein (50 µg protein/lane) was subjected to electrophoresis in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then, electrotransferred onto polyvinylidene difluoride membranes. Non-specific binding sites of the membrane were blocked with TBST blocking buffer (Tris-buffered saline with 0.1% Tween 20 containing 5% non-fat milk) for 1 hour at room temperature. The membrane was cut in half for immunoblotting; the upper half with a specific antibody against leptin receptor (ab5593) and the lower half with a monoclonal antibody against actin (4970L) to determine equal loading. The blots were incubated with HRP-labeled goat anti-rabbit IgG (H+L) (7074s) secondary antibody before detection with an enhanced chemiluminescence kit. Finally, the immunoblot bands were visualized using the Bio-Rad ChemiDoc™ system (Hercules, CA, USA) and quantified by densitometry analysis using ImageJ software (Bio-Rad, USA). They were then normalized to the corresponding signal intensity of β-actin.

Statistical Analysis

The data were analyzed using SPSS statistical analysis software and expressed as mean±SEM of three independent experiments. One-way ANOVA followed by Tukey's *post hoc* test was used to compare the mean cell viability differences between the groups. P values<0.05 were considered statistically significant.

Results

Effect of Silibinin and Curcumin on the Viability of MCF-7 Cells

To evaluate the cytotoxic effect of silibinin and curcumin on human breast carcinoma cells, MCF-7 cell lines were cultured and treated with different concentrations of silibinin and curcumin for 24 and 48 hours, respectively, followed by MTT assay. The cells cultured in silibinin and curcumin-free media were used as the control.

After 24 hours of treatment with 250 µM of silibinin, a significant inhibitory effect on cell growth was observed compared with the controls (P=0.007). The inhibitory effect of silibinin on cell growth and its minimum cytotoxicity was observed after 48 hours of treatment at 50 µM concentration. However, at 50 and 250 µM concentrations, the inhibitory effect of silibinin on cell growth at 48 hours was comparable with the 24 hours of treatment (figure 1). The dose-dependent inhibitory effect of silibinin on cell growth was assessed at 24 and 48 hours of treatment based on its IC_{50} value. After 24 hours of treatment with silibinin, the IC_{50} value was estimated at 250 µM (figure 1). However, when the cells were incubated for 48 hours, the IC_{50} value decreased to 50 µM. These results indicated that the inhibitory effect of silibinin on the growth of MCF-7 cells was time- and dose-dependent.

After 24 hours of treatment with 10 µM of curcumin, a significant inhibitory effect on cell growth was observed compared with controls (P=0.035). The inhibitory effect of curcumin on cell growth and its minimum cytotoxicity was observed after 48 hours of treatment at 10 µM concentration. However, the inhibitory effect of curcumin on cell growth at 48 hours with 10 µM concentration was comparable with treatment at 24 hours (figure 2). The dose-dependent inhibitory effect of curcumin on cell growth was assessed at 24 and 48 hours of treatment based on its IC_{50} value. After 24 hours of treatment with curcumin, the IC_{50} value was estimated at 25 µM (figure 2). However, when the cells were incubated for 48 hours, the IC_{50} value decreased to 15 µM. The results showed that curcumin exerted time- and dose-dependent cytotoxic effect on MCF-7 cells.

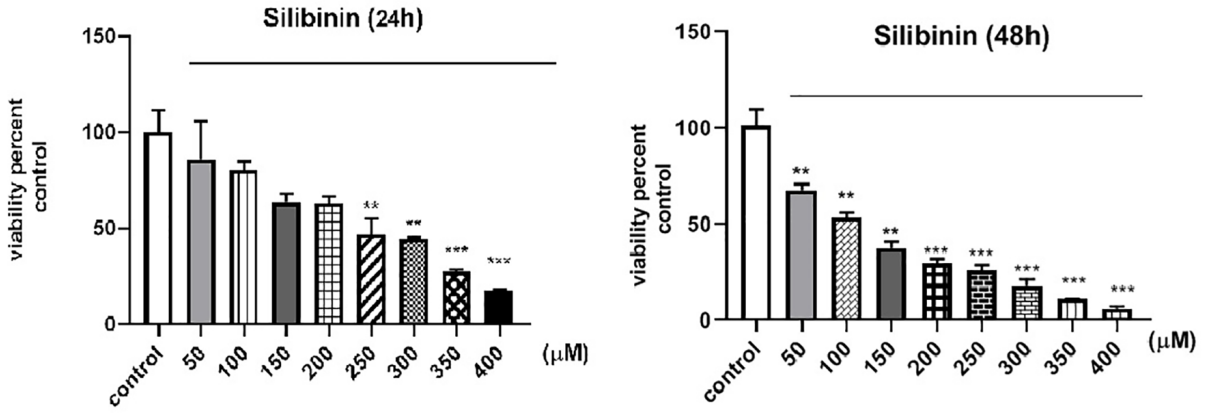

Figure 1: The graphs show the cytotoxic effect of silibinin on the cell viability of MCF-7 cells. The cells were seeded in 96-multiwell plates at a concentration of 3×10^3 cells/well. The cells were incubated in a fresh medium with vehicle alone and with different concentrations of silibinin at 24 and 48 hours, respectively. The cell viability was determined using the MTT assay. The experiments were performed in triplicate and each value is expressed as means±SEM. **P<0.01, ***P<0.001 compared with the control.

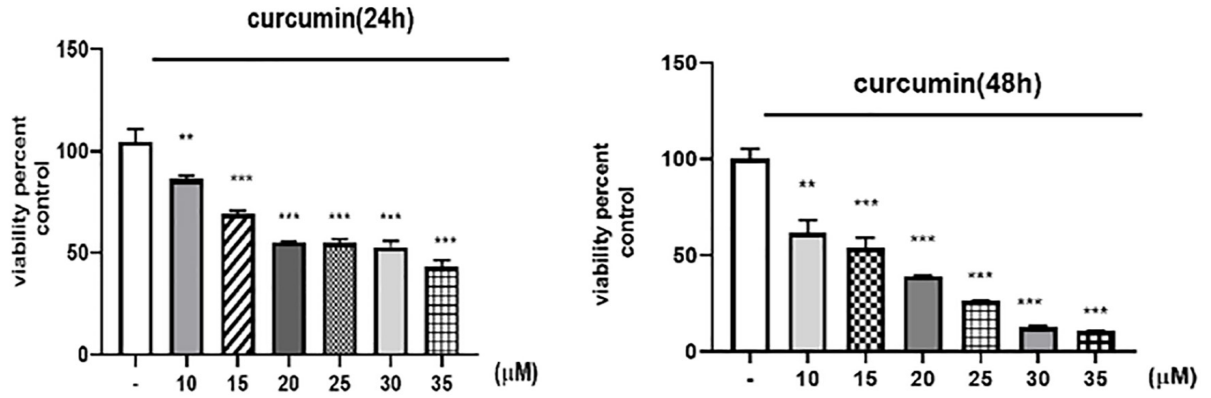

Figure 2: The graphs show the cytotoxic effect of curcumin on the cell viability of MCF-7 cells. The cells were seeded in 96-multiwell plates at a concentration of 3×10^3 cells/well. The cells were incubated in a fresh medium with vehicle alone and with different concentrations of curcumin at 24 and 48 hours, respectively. The cell viability was determined using the MTT assay. The experiments were performed in triplicate and each value is expressed as means±SEM. **P<0.01, ***P<0.001 compared with the control.

The Effect of Combined Silibinin and Curcumin on Leptin Receptor Expression in MCF-7 Cells

The inhibitory effects of silibinin and curcumin (individually or combined) on the Ob-R expression was investigated using the Western blot analysis. The Beta-actin expression level indicated an equal sample loading. After 24 hours of treatment with different concentrations of curcumin (10, 15, 20, and 25 μM), we observed no significant inhibitory effect on the expression level of Ob-R (figure 3a). However, after 24 hours of treatment with different concentrations of silibinin (50, 100, 150, and 200 μM), a reduction in Ob-R expression was observed. This reduction was dose-dependent and significant at 150 and 200 μM concentrations (figure 3b). After 24 hours of treatment with a combination of silibinin and curcumin, a reducing trend in Ob-R expression was observed. However, only the combination of 20 μM curcumin and 200 μM silibinin significantly reduced Ob-R expression (figure 3c).

Discussion

The results showed that silibinin and curcumin could individually inhibit the growth of MCF-7 cells. When combined, a significant reduction of Ob-R expression in MCF-7 cells was observed. These findings indicated that the combination of both, rather than each compound by itself, induced cell cytotoxicity and reduced Ob-R expression in MCF-7 cells more effectively. Some *in vitro* and *in vivo* studies have reported a high potential of this combination on the reduction of cancer cell growth, angiogenesis, induction of apoptosis, and initiation of the Th2 immune response.[16-18] We found that silibinin and curcumin exhibited a cytotoxic effect against cultured MCF-7 cells in a time and dose-dependent manner. These results are in line with previous studies that demonstrated the inhibitory effect of curcumin[14-18] and silibinin[7, 19-22] on the growth of several breast cancer cell

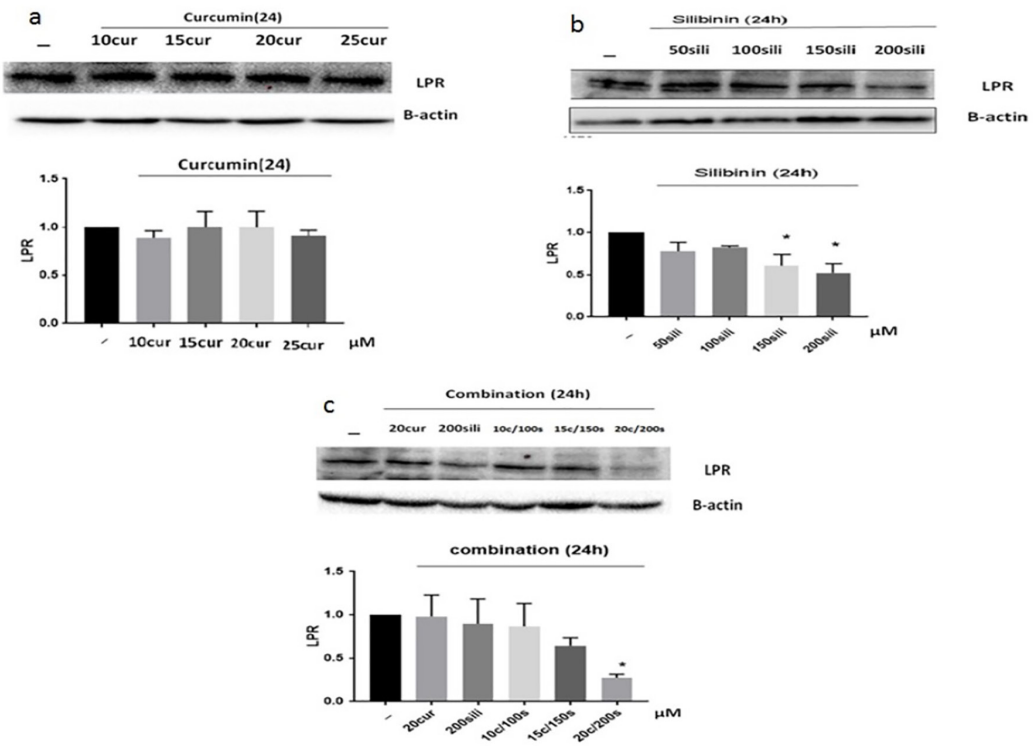

Figure 3: (a) Curcumin did not induce the reduction of leptin receptor expression in MCF-7 cells. (b) Silibinin induced reduction of leptin receptor expression in MCF-7 cells. (c) A combination of silibinin and curcumin-induced reduction of leptin receptor expression in MCF-7 cells. The cells were treated with different concentrations of curcumin, silibinin, and combined silibinin-curcumin for 24 hours. The level of the leptin receptor and Beta-actin expression were analyzed using the Western blot analysis. The data are expressed as mean±SEM of three independent experiments. Cur: Curcumin, LPR: Leptin receptor, Sili: Silibinin, c=Curcumin, s=Silibinin, *P=0.031 and *P=0.023 for treatment with silibinin alone compared with the control. *P=0.004 for treatment with combined silibinin and curcumin compared with the control.

lines. Furthermore, the combination of silibinin with curcumin reduced the growth of MCF-7 cells more strongly than each compound individually.

We found that silibinin and curcumin could reduce Ob-R expression in MCF-7 cells. The results showed that silibinin and curcumin had a significant ability to act on the Ob-R level, which has an important role in the leptin-induced carcinogenesis and rapid growth of breast cancer cells. Considering the pivotal role of leptin in the pathogenesis of breast cancer, several attempts have been made to reduce leptin actions through inhibition of its Ob-Rb isoform. A study conducted on human breast tumor samples showed that almost all (98.7%) of the studied samples co-expressed leptin and its two main isoforms (Ob-Ra and Ob-Rb).[23] Moreover, leptin analog mimics its action,[24] and the use of a monoclonal antibody against Ob-R[25] is a method to attenuate leptin action. However, in the present study, we used herbal compounds to directly assess the inhibition of Ob-R level in MCF-7 cells. Due to the significant anticancer effects of silibinin and curcumin on different types of cancer (colon, skin, prostate, bladder, and breast), these compounds could be potential chemotherapeutic agents for the treatment of breast cancer.

The results of the present study showed that silibinin and curcumin individually decreased the expression of Ob-R in MCF-7 cells. When used in combination, these compounds had a strong effect on reducing the expression of Ob-R. In other words, they complemented each other's inhibitory effect on the Ob-R expression. Nejati-Koshki and colleagues described the mechanism by which silibinin reduced the expression of leptin. They suggested that reduced leptin expression and secretion at the mRNA level in T47-D cell lines were negatively correlated with ERβ gene expression.[26] Note that leptin expression is positively associated with ERα level and negatively associated with ERβ level.[27] Moreover, leptin gene promoter contains response elements for ERα and ERβ binding.[28]

Curcumin inhibits leptin expression by decreasing the estrogen receptor in breast cancer cells.[18] The mechanism for decreasing the estrogen receptor is through reducing ERα gene expression.[18, 26] Besides, considering the autocrine action of leptin and Ob-R in human breast cancer, leptin, and its receptor could directly affect each other's expression.[29]

Therefore, inhibition of either induces a reduction of both through this autocrine system. This could be considered as the second mechanism for reducing the expression of leptin and its receptor. These pathways could be the potential mechanisms for reducing Ob-R expression in MCF-7 cells by combining silibinin and curcumin. Fusco and colleagues reported that inhibition of Ob-R by siRNA resulted in a reduction of ERα gene expression.[30] As a final note, variation in the expression of each gene involved in the autocrine system (i.e., leptin, Ob-R, and ER) may trigger modulation in the entire system, especially in ER+ cells such as MCF-7 cells.

This is the first study to address the effect of combined silibinin and curcumin on the expression of Ob-R at the protein level. Further studies are required to clarify the exact mechanism(s) for the reduction of Ob-R expression by these compounds. As the limitation of our study, we only included one type of human breast cancer cell line. Therefore, the obtained results cannot be generalized to other cell lines, and the clinical outcomes should be interpreted with caution. In addition, since leptin and its receptor could directly affect the expression of each other in an autocrine system, it would be advantageous to measure the expression and secretion of leptin and estrogen receptors.

Conclusion

Silibinin and curcumin significantly inhibited the growth of MCF-7 cells. The combination of these compounds significantly reduced Ob-R expression in MCF-7 cells compared with their individual use. Considering the importance of Ob-R in the pathogenesis of breast cancer, it is recommended to use combined silibinin and curcumin in future studies to demonstrate its strength in reducing the expression of Ob-R.

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Conflicts of Interest: None declared.

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