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Original Article

Curcumin Downregulates the Expression of p44/42 MAPK and Causes Caspase-mediated Cell Inhibition in MCF-7 Breast Cancer Cells

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ABSTRACT: Curcumin is a derivative of the turmeric spice, which is a yellow-pigmented root crop with a resilient sheath and bright orange flesh. It is originally known to be utilized in Asian dishes but, has been discovered to have antioxidant, anti-inflammatory, antiviral, antibacterial and anticancer characteristics. Different researchers have established great possibilities of curcumin's ability to prohibit the growth of cancer cells especially, because of its potentiality to differentiate between normal and cancerous cells. Research questions include understanding the effects of curcumin on the MCF-7 breast cancer cells with regards to the biomolecules of the cells. The results indicated that after attachment of cells for 48 hours, the concentration of curcumin at 15 µM showed more than 90% inhibition of cells within 24 hours. The analysis was carried out on the viability of the cells, western blotting and reverse transcriptase-polymerase chain reaction. Western blot analysis of signaling proteins from curcumin-treated cells showed that the expression level of phosphorylated protein p44/42 in the MAP kinase pathway was significantly decreased and the apoptotic marker cleaved caspase 3 was increased as compared to the curcumin-untreated control cells. Moreover, RT-PCR analysis of the reference genes in the apoptotic pathway (p53, caspase 9, BCL-2 and Bax) demonstrated the upregulation of p53, Bax and caspase 9 genes. The results assembled from this present study suggested that curcumin inhibited the growth and induced caspase-mediated apoptosis of MCF-7 cells via the MAPK signaling pathway. Therefore, breast cancer treatment with curcumin seems to be a promising remedial path in near future.

Keywords: MCF-7, Curcumin, p44/42 MAPK, caspases, cancer

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INTRODUCTION

Curcumin was first characterized in 1815 and is chemically known as 1, 6-heptadiene-3, 5-dione-1,7-bis(4-hydroxy-3-methoxyphenyl) (Ravindran et al. 2009). Although curcumin can be referred to as a non-conventional method for treating cancer, it might just have fewer side effects compared to conventional treatments. Curcumin has been investigated to induce cell cycle arrest and apoptosis *in vivo*, and reduced tumor sizes *in vitro* (Li et al. 2018). Shin et al., (2019) also summarized the effects of phytochemicals, one of which is curcumin, explaining that it has been shown to downregulate the P13K signaling pathway and ERK pathway. Likewise, a number of research studies described over the past years explained the therapeutic effects of naturally occurring compounds such as curcumin on cancer remedy *in vivo* and *in vitro* (Lohse et al. 2018). Cancer is one of the leading causes of death today though, different procedures and methods have been taken and are being taken to either stop or reverse this disorder, but there has not yet been a definite solution. The cancer cell line used in this research is the MCF (Michigan Cancer Foundation) 7 Human Breast Adenocarcinoma Cells. The MCF-7 human breast cancer cell line was first discovered in 1970 from the breast tissue of a 69-year old Caucasian woman and has been established as an immortal cancer cell line (Lee et al. 2015). In this study, the signal transduction pathways are targeted to illustrate how apoptosis may be induced by curcumin.

MATERIALS AND METHODS

Cell culture

The MCF-7 human breast cancer cells were purchased from American Type Culture Collection (ATCC). They

were grown in 100 x 15 mm size of Petri dishes (Fisher Scientific, USA) in RPMI 1640 Medium (Gibco San Francisco CA, USA) which contains 10% FBS (Fetal bovine serum) and 1% Antibiotic/Antimycotic at 37°C with 5% carbon dioxide in a moisturized atmosphere. Cells were counted every 24 hours using a hemocytometer to understand the growth pattern of MCF-7 cells. All cells were tested for mycoplasma and have been found to be mycoplasma negative.

Preparation of Curcumin solution

Curcumin powder was a generous donation from the University Of Texas Health Science Center at Tyler, Texas. Dimethylsulfoxide (Sigma Aldrich, Louis MO USA) was used to dissolve curcumin to form a solution of 50 mM concentration. This solution was stored at 4ºC.

Cell treatment and viability assays

The MCF-7 cells ($3X10^4$) cells/well were cultured in six-well plates in duplicates for three trials. The cells were allowed to attach and then treated with 0, 5, 10, 15, 25 µM of curcumin solution every 24 hours for five consecutive days maintaining the control with DMSO. Each day, the cells were trypsinized (EDTA Trypsin, Gibco) off the surface of the culture vessel and counted using a hemocytometer on an inverted microscope. The viability of cells was expressed by plotting the live cells at different concentrations of curcumin treatment over five consecutive days.

Western blot analysis

The MCF-7 cells (5×10^5) were incubated in petri dishes and allowed to attach for 24 hours. The cells were treated with similar concentrations as stated above of curcumin for three consecutive days, trypsinized using EDTA-Trypsin (Gibco), washed with PBS and stored at -20ºC. Protein was extracted from the cells using M-PER (Mammalian Protein Extraction Reagent) with a protease inhibitor and was quantified using the BCA assay kit (Thermo Scientific). The protein samples (20 µg) to be run were mixed with lysis buffer and loading buffer (dye) and put on 10% SDS-PAGE (Nupage 10% Bis-Tris Gel, Invitrogen). The gel was run at 160V and then transferred to the nitrocellulose membrane (Bio-rad, 0.45µm) at 120V. The membrane was blocked using 5% blotting grade dry milk solution in 1X TBST for an hour and washed in the same buffer. Thereafter, the membrane was soaked overnight in p44/42 MAPK and Caspase 3 on separate blots (1:1000) primary antibody. The membrane was washed three times with 1X TBST and soaked in a secondary antibody (Goat anti-Mouse IgG HRP 1:2000) for an hour. The membrane was then stripped and immersed in GAPDH primary antibody with a corresponding secondary antibody. After repetitive washing, the blot was soaked in chemiluminescence (Clarity Western ECL Substrate, Biorad) solution. Images were visualized using the ChemiDoc Imaging System and Software (Biorad).

Reverse transcription-quantitative polymerase chain reaction

The MCF-7 cells (5×10^5) were plated in petri dishes and allowed to attach for 24 hours. Cells were treated for 72 hours with similar concentrations of curcumin as stated above. RNA was extracted from the cells using the Aurum Total RNA Mini Kit (Biorad). Briefly, the cells were lysed using lysis solution supplemented with β-methcarpenol, washed repetitively with 60% ethanol, and a DNase solution was used to extract the RNA in a binding column. Finally, the elution solution at 70ºC was used to collect the RNA extract for each sample. For the reverse transcription process, the RNA template was incubated for an hour at 42ºC with iScript Reverse Transcription Supermix (Biorad) for cDNA synthesis. Using a SsoAdvanced Universal SYBR Green Supermix (Biorad), and 5 primers for each sample (p53, Caspase 9, Bacl2, Bax, and β-actin). PCR plate (Hard-Shell PCR Plates 96-well, thin-wall, Biorad) was set up and run for 40 cycles at 98ºC (30 sec), 95ºC (15 sec) and 60ºC (25 sec) to obtain Ct values using a CFX96 instrument with appropriate software (Biorad).

Statistical Analysis

Statistical data for the reverse transcription-polymerase chain reaction was obtained in reference to β-actin. The method of calculation was obtained from Kannan (2016). The difference between the Ct values of subtracted and treated samples were obtained for all of the targeted genes (p53, caspase-9, bcl2, bax, and β-actin). The difference between these two newly obtained values was calculated to the negative power of two, for acquiring the relative fold change.

RESULTS AND DISCUSSION

Effects of Curcumin on the viability of MCF-7 cancer cells

A viability assay was carried out to first observe the effects of various concentrations of curcumin on MCF-7 cells for five consecutive days. Figure 1 showed a significant decrease in the proliferation of cells as curcumin concentrations were increased. The ~90% inhibition was found at 15 µM of curcumin concentration within 24h. Several studies reported anticancer activity of curcumin on breast cancer, lung cancer, prostate cancer, and pancreatic cancers (Shoji et. al. 2018; Rivera et. al. 2017). Our data are consistent with previous studies that reported curcumin exerts its anticancer effects via proliferation inhibition and apoptosis induction in breast cancer cells (Simon et. al., 1998). *Effect of Curcumin on the expression of MAPK and cleaved-Caspase 3*

Following treatment of the MCF-7 cells with curcumin at different concentrations for 72 hours, the expression level of phosphorylated proteins p44/42 and cleaved-caspase 3 were analyzed by western blotting using as GAPDH, a housekeeping protein.

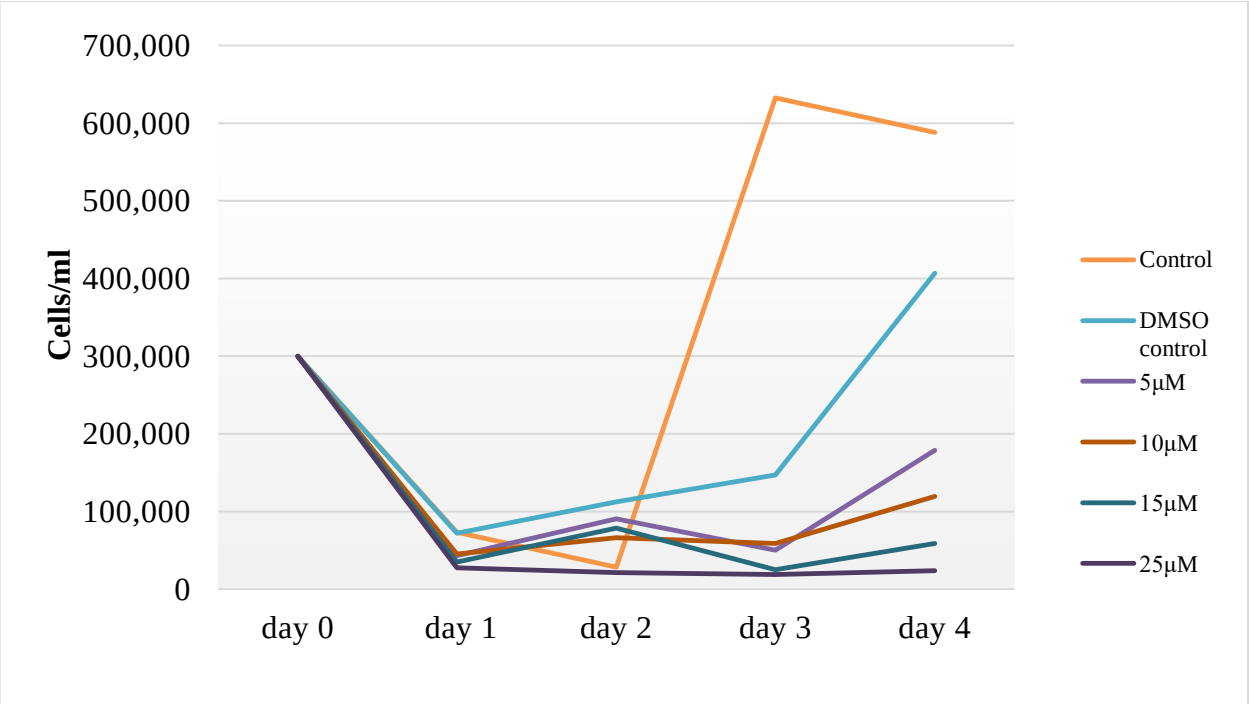

Figure 1. Effects of curcumin on the viability of MCF-7 breast cancer cells. Cells were treated with 5, 10, 15 and 25 µM of curcumin solutions for five days. The graph illustrates data obtained from counting viable cells with a hemocytometer.

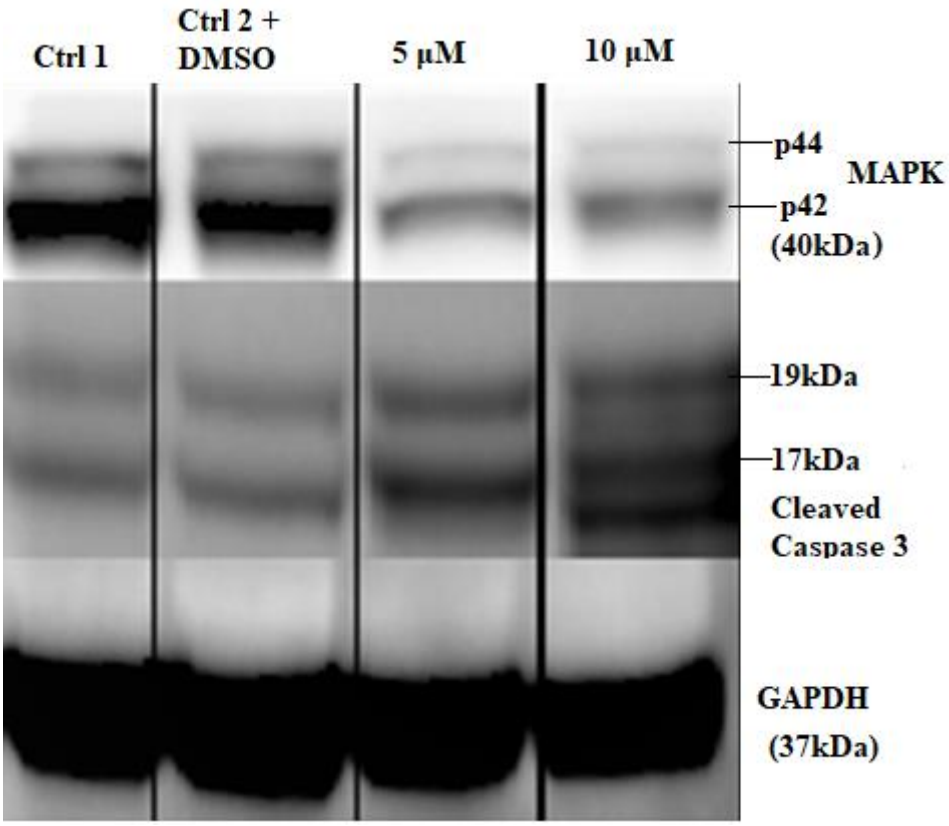

Figure 2. Western blot depicting the effects of curcumin on p44/42 MAPK and cleaved caspase-3. Protein was transferred to a nitrocellulose membrane blot and immersed in antibodies of the targeted protein. The blot showed decreasing p44/42 MAPK, increasing cleaved caspase-3 and consistent GAPDH as house-keeping protein.

As shown in Figure 2, the expression level of phosphorylated protein p44/42 in the MAP kinase pathway was significantly inhibited and the apoptotic marker, cleaved caspase 3, was increased compared to the curcumin-untreated control cells, suggesting curcumin induced caspase-mediated apoptosis in the MCF-7 breast cancer cells. Many reports have already been published in the past few years on how curcumin prohibited proliferation in different cancer cell lines. Zhou et al. (2011), who carried out a study with MCF-7 cells targeting the p38 MAPK pathway *in vitro* and *in vivo*, showed arrest in the cell cycle.

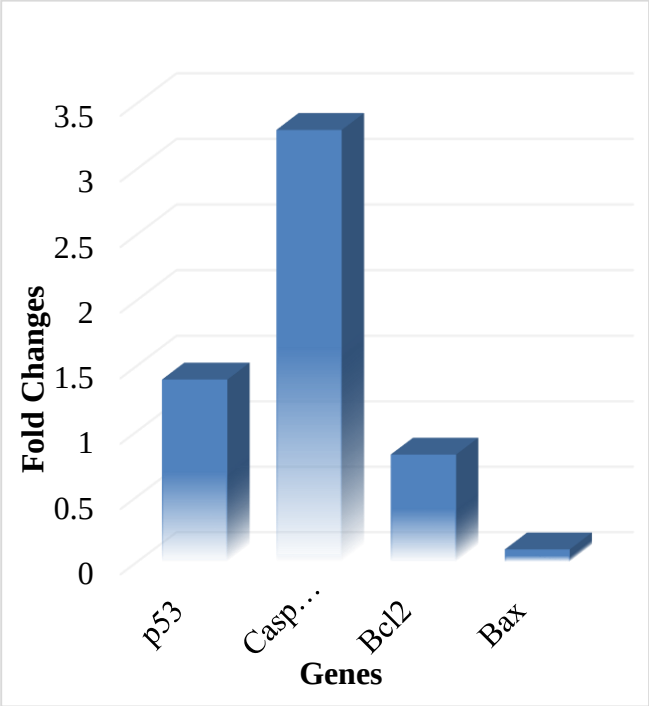

Figure 3. The levels of gene expression after treatment with curcumin. PCR analysis was run targeting p53, caspase-9, bcl2 and bax. The genes showed significant upregulation in p53, caspase-9 and bcl2 to folds of 1.2, 3.1 and 0.7, respectively. Bax levels are seen to be expressed below 0.5 which is considered to be insignificant.

Effects of Curcumin on the expression of apoptotic genes in MCF-7 cells

MCF-7 cells were treated with curcumin at concentrations of 0, 5, 10, 15, 25 µM for 72 hours. The expression level of different genes (p53, caspase 9, BCL-2 and Bax) were examined by RT-qPCR analysis. Figure 3 showed the expression level of the four different genes after curcumin treatment. Analysis of this data was carried out using GAPDH as a housekeeping gene to obtain double delta Ct values and fold change. The figure showed a 1.3-fold change increase in p53, which is a tumor suppressor gene. Also, it illustrated a 3-fold change in caspase 9 gene, about a 0.7-fold change in bcl2 and 0.5-fold change in bax genes, indicating the evidence of curcumin-induced caspase-mediated apoptosis in the MCF-7 cells. The result of the present study was consistent with the previously reported effects of curcumin in the breast cancer cells, shown to activate and regulate p53 tumor suppressor protein, reduce Bcl-2 levels, and increase Bax, Caspase-3, caspase-8 and caspase-9 (Talib et al. 2018 and Zhang et al. 2017). Moreover, Li et al. (2018) reported the induction of apoptosis by curcumin in the SO-Rb50 and Y79 cells. It is also reported by Sohn et al. (2018) that curcumin upregulated caspase-3 and caspase-9 which are proapoptotic enzymes in RT4 schwannoma cells. Similarly, curcumin has also been shown to induce apoptosis in the mitochondria and activate caspases in human papillomavirus positive and negative squamous carcinoma cells and prostate cancer cells (Khan et al. 2018; Rivera et al. 2017). Collins et al. (2013) reported the accumulation of p53 in the cytoplasm of MCF-7 cells after curcumin treatment. Another study suggested that curcumin induced apoptosis via the STAT3 pathway in esophageal squamous cell carcinoma (Liu et al. 2018). Curcumin has been found to arrest the cell cycle in the G1 phase in HCT116 colon cancer cells (Lim et al. 2014) and on lung cancer cells, A549. Also, in an *in vivo* study on mice models, curcumin was found to decrease tumor size and weight (Li et al. 2018).

One of the challenges of curcumin treatment is the low bioavailability of the molecules. Therefore, research has been done enhancing the effects by either combining or modifying the bioactive molecule, for example, Upadhyay et al. (2018) loaded protein microbubbles with curcumin for treatment on cervical cancer cells. Reports showed that the microbubbles enhanced uptake by approximately 250 times and decreased cell viability. Curcumin was combined with tumor necrosis factor-α-related apoptosis-inducing ligand (Ghildiyal et al. 2019). Chen et al. (2018) reported that there was suppression of cell viability and upregulation of caspase-3 and caspase-9 in oral cancer cells when curcumin was combined with cetuximab which is an epidermal growth factor. Over the past few years, synthetic aminocarbonyl analog of curcumin (MACs) have been developed such as UBS109 which was experimented on metastatic cancer to show that there was significantly less cancer metastasis from the breast to the lungs (Shoji et al. 2018). In spite of the tremendous effort to understand the anticancer effects of curcumin, there are still several issues to be addressed in regard to the mechanism of apoptosis in MCF-7 breast cancer cells.

CONCLUSION

The results obtained from this research showed a decline in cell proliferation after treatment by curcumin. The blots in Fig. 2, showed a ~60% decrease in the bandwidth of p44/42 MAPK and a significant increase in bandwidth of cleaved caspase-3. These results suggest that apoptotic activity was induced in the cells as a result of the curcumin treatment. It also confirms this by an increase in p53 tumor suppressor gene. As a result of the expressions of the caspases in both western blotting and RT-PCR, it is therefore implied that inhibition of cell proliferation by curcumin is significantly caspase-mediated. From the results and discussion above, it can be concluded that there is a significant effect on the phosphorylating enzyme (MAPK) and inhibition of the MCF-7 cells by curcumin, is dependent on the caspases which are widely known to be initiators and activators of apoptosis. Given the potential effectiveness of curcumin, it is a promising candidate for the treatment of breast cancer, although more studies are needed.

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