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Original Article

Evaluation of the Anti-Cancer Effect of Curcumin on MCF-7 Cells in 3D Culture Conditions to Increase the Efficacy of Breast Cancer Treatment

Sajedeh Zargan [1], Mehdi Salehi Borough [1*], Jamil Zargan [2], Mohsen Shayesteh [3], Ashkan Haji Noor Mohammadi [2], Mohsen Mousavi [2], Hani Keshavarz Alikhani [4]

[1] Department of Medical Radiation Engineering, Central Tehran Branch, Islamic Azad University, Tehran, Iran
[2] Department of Biology, Faculty of Basic Sciences, Imam Hossein Comprehensive University, Tehran, Iran
[3] Department of Nuclear Physics, Imam Hossein Comprehensive University, Tehran, Iran
[4] Faculty of sciences, Department of biology, Razi University, Kermanshah, Iran

Corresponding Author: Mehdi Salehi Borough, PhD, Professor, Department of Medical Radiation Engineering, Central Tehran Branch, Islamic Azad University, Tehran, Iran. Tel: +98-9391668318, E-mail: m.s.barough@gmail.com

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Abstract

Introduction: Breast cancer is the most common cancer among women. Information published by The Iranian Cancer Research Center in 2019 shows that one of every 10 to 15 women is afflicted with this cancer. As one of the active ingredients of turmeric, curcumin has a wide range of biological properties, such as antioxidant and anti-cancer activity. This study aimed to evaluate the anti-cancer effects of curcumin on breast cancer cells in 3-Dimensional (3D) culture conditions.

Materials and Methods: To achieve a 3D environment, we used encapsulation of cells in alginate hydrogel. The anti-cancer effects of curcumin at concentrations of 20, 40, and 80 µM on MCF-7 breast cancer cells in 3D culture were evaluated by MTT, neutral red, comet assay, cytochrome c, Nitric Oxide (NO), catalase, and glutathione assays. The culture medium was used as the negative control and the cell-containing medium was used as the positive control. Data were analyzed by two-way ANOVA using GraphPad InStat software, and the significance was considered at the level of $p<0.05$.

Results: Curcumin reduces the production of cellular NO and increases the production of catalase and glutathione, which confirms the results of the NO test. In addition, the release of cytochrome c from Mitochondria from cells treated with different concentrations of curcumin compared to control cells are significant. The evaluation of the toxicity effect of curcumin at concentrations of 20, 40, and 80 µM using comet assay showed that this substance induces apoptosis in MCF-7 cells in a dose-dependent manner.

Conclusions: The findings of this study showed that the anti-cancer effect of curcumin on MCF-7 cells under 3D culture conditions could increase the effectiveness of treatment. The Cell survival rate actually depended on curcumin concentration.

Keywords: Curcumin, Breast Cancer, MCF-7 Cell Line, 3D Cell Culture

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Introduction

Breast cancer is the most common cancer among women and the second most common cancer in the world. Despite many advances in early diagnosis and appropriate treatment of this disease, it is still the leading cause of death due to cancer among women.[1] Health centers use a variety of treatment methods for breast cancer. Surgery, radiotherapy, chemotherapy, and hormone therapy are common treatment methods.[2]

Radiotherapy is one of the treatment steps of breast cancer that damages healthy cells in addition to tumor cells.[3] Targeted therapy is used to reduce radiotherapy complications. In this method, sensitive points are determined to cause losses in tumor cells, and as a results, mortality is induced in them.[4] In addition, these combination therapies reduce the systemic toxicity of chemotherapy or radiotherapy. This is due to the fact that by using this strategy, the dose received by the patient will be reduced.[4]

In recent years, various biological materials, such as curcumin, which is an antioxidant ingredient, have been investigated. The structure of curcumin was characterized for the first time in the eighteenth century.[5] The molecular formula of Curcumin is $C_{21}H_{20}O_6$, and its molecular weight is 37.368 grams per mole.[6,7] It was reported that curcumin is one of the intermediates substances that prevent tumor cells from developing and decreases cancer cells' growth rate and development in 2D culture conditions.[8]

It has a bilateral function during dose-dependent radiation. This means that it can protect against damage caused by low-dose radiation (radiation protection) and increases the effect of radiation in a higher dose (radiation sensitivity).[9] As a powerful antioxidant, curcumin exerts its protective mechanism against irradiation free radicals by increasing the expression of antioxidant enzymes. It has pro-apoptotic

properties and has extraordinary therapeutic potential against a variety of cancers.[10,11]

Generally, the cell culture technique is used to investigate the *in vitro* cytotoxicity of different materials. Most studies on biological mechanisms of cell function, such as migration, have been performed in 2D cell culture media. The newer method for cell culture is 3D, in which cells grow and proliferate in a 3D medium to provide the appropriate environment for optimal cell growth, differentiation, and function.[12] The 3D cell cultures now act as a bridge between 2-Dimensional (2D) cell culture and in vivo animal models.[13] Published reports also show that 3D cultures of cancer cells and normal mammalian cells are the best models for understanding the regulation of cancer cell division and assessing various anti-cancer drugs.[12]

The aim of this study was to evaluate the anti-cancer effect of curcumin on MCF-7 breast cancer cell line in 3D culture condition to increase the treatment efficiency.

Materials and Methods

This study was an experimental study. Breast cancer cells (MCF-7 IBRC C10082) were prepared from the cell bank of Imam Hussein Comprehensive University.

Mixing Curcumin in the Suitable buffer and Removing Contamination

Curcumin was obtained with 95% purity from Karen Pharmaceutical Company. At this point, 0.02 mg curcumin with sterile DMEM medium (buffer) containing 1% antibiotic -anti-mycotic volume of 1 ml was incubated overnight in a CO_2 incubator (37 °C and 5% CO_2) to remove biological contaminants. After overnight incubation, media were discarded, and fresh media containing different concentrations of curcumin (5, 10, 20, 40, and 80 µg/ml) was added to each well.

3D Cell Culture, Encapsulation in Alginate and Depolymerization of Capsules

To achieve a 3D environment, we used encapsulation of cells in alginate hydrogel. At first, the alginate solution was produced by dissolving 0.12 g of sodium alginate powder (Sigma-USA) in 10 ml of 0.9% sodium chloride solution. Alginate solution was filtered by a 0.22 µm plastic syringe and added to the cell pellet containing 2×10^4 MCF-7 cells and re-suspended. Cell individualization and suspension of cells in alginate solution were performed using a 22G plastic syringe. Alginate capsules were produced by injection of the cell-alginate mixture by a plastic 22G syringe in a bath of 100 mM calcium chloride (drops were released into the bath from a distance of 5cm above the bath's surface). The capsules were allowed to be polymerized for 10 min. In the next step, after the removal of calcium chloride, the capsules were washed three times with PBS (pH 7.4). The washing solution was replaced with 1 ml DMEM containing 10% FBS. The cell capsules were incubated at 37 °C and humidity of 95% and 5% CO_2. For depolymerization of capsules and releasing cells in GSH, Catalase and Comet assay, after removing the media, the capsules were transferred to sterile tubes and washed three times with PBS. Then, 1 ml of depolymerization solution (50 mM sodium citrate) was added to the cells.[14]

Determination of Cell Viability (MTT Assay)

This method was performed according to the ones conducted by Mossman et al.[15] Briefly, 2×10^4 cells were encapsulated according to the previously described procedure and incubated for 24 h at 37 °C. Then, the old media were discarded and fresh media containing different concentrations of curcumin (5, 10, 20, 40, and 80 µM) was added to each well containing the capsules and incubated for 24 h at 37 °C with 5% CO_2 and 95% humiliation. Then, 5 mg/ml MTT was added to each well and incubated at 37 °C for 3 h under dark conditions. After that, 200 µl DMSO was added to each well and placed in a shaker incubator for 3 h under dark conditions. After completely dissolving the MTT color and its release from the capsules, 100 µl of the solution was removed, and its absorption was measured at 570 nm by a microplate reader (Biotech, USA). In this test, the culture medium was used as the negative control medium, and the cell-containing medium was used as the positive control. This control included cells cultured in alginate capsules that were not incubated with different concentrations of curcumin. This experiment was repeated three times for each concentration of curcumin. Cell viability was calculated after contact with different concentrations of curcumin by the following formula.[15]

The vitality percentage of cells $=100\times(a/b)$

a = Optical Density (OD) of the test sample minus the blank's OD

b = OD of the control minus the blank's OD

Neutral Red (NR) Uptake Assay

NR uptake assay was used to verify the MTT assay results (Winckler 1974).[16] The NR test is based on the ability of live cells to combine and link neutral red to lysosomes. The steps of the NR colorimetric test were similar to the MTT assay, except that after the exposure of cells with curcumin for 24 h, instead of MTT solution, 4 µl of neutral red dye (5 µg/ml) was added to each well and incubated for 1 h at 37 °C. Following the formation of red crystals, the supernatant was discarded and washed twice with PBS. At that point, 200 µl of fixation buffer (formaldehyde 37% (v/v), $CaCl_2$ (10% (w/v) in water) was added to each well and incubated for 1 min, and then, 100 µl of the solubilizing buffer (acetic acid 5%) was added and incubated for 20 min in a shaker incubator

under dark conditions. Finally, the absorbance was measured at 540 nm by a microplate reader (Biotech, USA). Inhibitory percentage of different concentrations of curcumin on cell growth was calculated using the following formula.

Percentage of cell mortality =1-[100×(a/b)]
a = Optical Density (OD) of the test sample minus the blank's OD
b = OD of the control minus the blank's OD

According to the results of the MTT test and very low cell mortality at concentrations of 5 and 10 µM of curcumin, other evaluations, including the type of mortality in cancer cells, studying the effect of apoptosis, and the effect of curcumin on cellular stress, concentrations of 20, 40, and 80 µM of curcumin were used.

Nitric Oxide (NO) Assay

This method was performed according to Zargan et al.[17,18] The steps of the NO assay test are similar to the MTT assay test, except that by following the exposure of cells with curcumin (20, 40, and 80 µM) for 24 h, media from each well was transferred to sterile tubes and centrifuged at 500×*g* for 5 min at 4 °C. A total of 100 µl of the media were transferred to a 96-wells plate and mixed with the equal volume of Griess reagent (Sigma, USA) (0.04 g/ml in PBS, pH 7.4) and incubated for 10 min at room temperature. Absorbance at 520-550 nm was measured by a microplate reader (Biotech, USA). The NO concentration (µM/ml) in treated cells was calculated using the sodium nitrite standard curve

Reduced Glutathione (GSH) Assay

This method was performed according to Sedlak and Linsay.[19] The steps of the GSH test were similar to the NO test, except that encapsulated cells were transferred to a 24-wells plate and incubated overnight in 5% CO_2 at 37 °C. Then, the old media were discarded, and fresh media containing 20, 40, and 80 µM curcumin were added to each well and incubated at 37 °C for 24 h. Then, 200 µl of the lysis buffer was added to each well, and protein concentration was assessed by the Bradford assay. About 40 µl of the obtained solution was removed and transferred to new tubes. Tubes were added to 40 µl of 10% TCA solution and stored at 4 °C for 2 h. The centrifugation was performed at a speed of 1500 rpm for 15 min. The supernatant was transferred into a clean tube. Then, 75 µl of lysis buffer, 55 µl of Tris HCl buffer (pH 8.5), and 25 µl DNTB was added to 20 µl of the supernatant. The Optical Density (OP) of samples was measured at 412 nm.

Catalase Enzyme Activity Assay

Catalase enzyme activity was measured using the method described by Sinha et al.[20] The steps of the catalase test were similar to the GSH test. Briefly, 5 µl of samples were mixed with 50 µl lysis buffer, 20 µl DDW, and 25 µl H_2O_2 (15%). Samples were incubated at 37 °C for 2 min and were mixed with 100 µl of potassium dichromate solution. At this stage, the soluble pink color with the blue color of the upper part of the solution was visible. Next, samples were incubated in boiling water for 10-15 min until the green color was formed, and then, the OD was measured at 570 nm using a plate reader (Biotech, USA).

Cytochrome c Assay

The steps of the cytochrome c test were similar to the GSH test, except that 1×10^6 of the capsulated cells were transferred to 6-wells plates and incubated overnight in 5% CO_2 and 37 °C. The cells were dissolved in 1 ml of cytosol purification buffer and incubated in ice for 10 min. The cells were then homogenized in a dunce tissue grinder. The homogenized samples were transferred to a new 1.5 ml tube and centrifuged at 700 g for 10 min at 4 °C. The soluble samples were transferred to clean 1.5 ml tubes and centrifuged at 10,000 g for 30 min at 4 °C. The soluble solution, which was the cytosolic component, was collected. Protein concentration was measured using the Bradford assay.

Alkaline Comet Assay

Alkaline comet, or single-cell gel electrophoresis assay, is a suitable method for DNA fragmentation analysis in cells.[21] The steps of the alkaline comet test were similar to the GSH method, except that the alginate capsules were dissolved, and their cell depositions were obtained. Then, 200 µl PBS was added to tubes, and cells were singled out using a needle. Slides were covered by normal melting agarose (1% [v/v]) and incubated for 10 min at 4 °C. Cell suspensions were mixed with low melting agarose (1% [v/v]) (1: 2 ratios) and were applied to the slides. To form a cell layer, a cover slip was applied to spread the preparation. To do cell lysis and nucleus distraction, all slides were incubated for 16-18 h in fresh and cold lysis buffer (NaCl 2.5M, EDTA 100 mM, Tris 10 mM, NaOH 0.2 M, and Triton X-100 %1; pH 10) at 4 °C. Then, slides were washed twice with electrophoresis buffer for 20 min at 4 °C and electrophoresed for 45 min at 4 °C (25 V and 300 mA). For neutralization, the slides were incubated for 10 min in the neutralizing buffer (Tris 0.04 M, pH 7.5). Then, the slides were incubated in 100 µl ethidium bromide (20 µg/ml) for 10 min at room temperature. Slides were washed two times (10 min each) with water and analyzed by an inverted fluorescent microscope (Nikon, Japan), and results were statistically analyzed.

Statistical Analysis

Comparison of the anti-cancer effect of curcumin with control and different concentrations on MCF-7 breast cancer

cells was evaluated by GraphPad InStat software using two-way ANOVA. Differences were considered to be significant at $p<0.05$(*), $p<0.01$(**), $p<0.001$(***) and $p<0.0001$(****). All experiments were conducted three times.

Results

MTT Assay

Curcumin decreased cell viability in a dose dependent manner. At a dose of 5, 10, 20, 40, and 80 µM of curcumin cell viability decreased to 85, 81, 67, 73, and 56% respectively. Cell viability of cell line at 5 µM was not significant as compared to the control. However, at a dose of 10 µM and above, the difference of cell viability was significant as compared with each other and the control (Figure 1). The results of the MTT reduction assay showed that IC_{50} of curcumin was 78 µM.

Neutral Red (NR) Uptake Assay

The NR uptake assay was used to confirm the results of the MTT assay and to evaluate the effect of curcumin cytotoxicity in 3D culture. MCF-7 cell line inhibition in 3D culture was 17%, 21%, 37%, 41%, and 32% for 5, 10, 20, 40, and 80 µM of curcumin respectively.

As shown in (Figure 2), curcumin at all concentrations had a significant inhibitory effect on cell growth compared to the control.

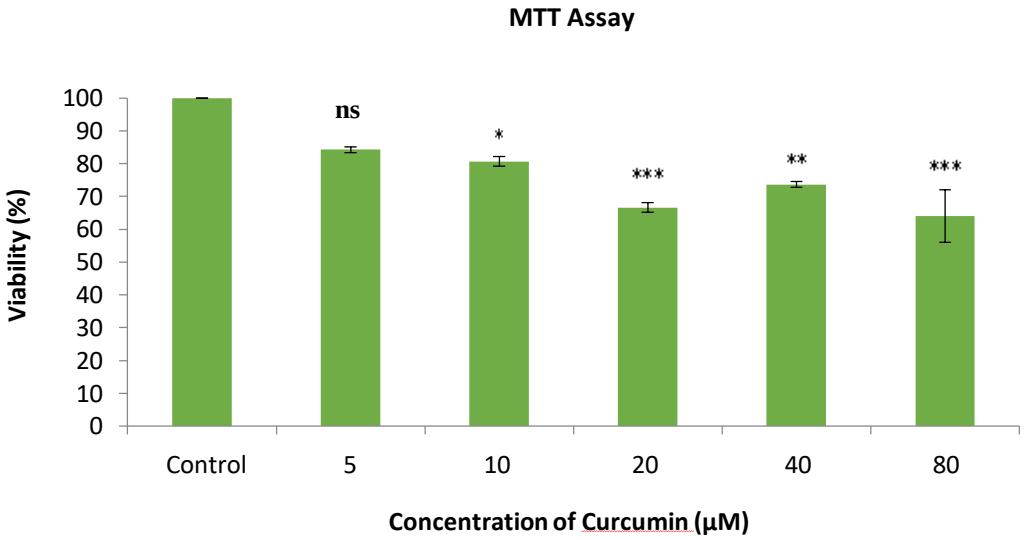

Figure 1. Comparing Cytotoxic Effects of Curcumin on MCF-7 Cells Using MTT Assay in the 3D Culture After 24 Hours Incubation with Concentrations of 5, 10, 20, 40, and 80 µM of Curcumin. This experiment was repeated 3 times for each concentration of curcumin (ns: not significant, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$).

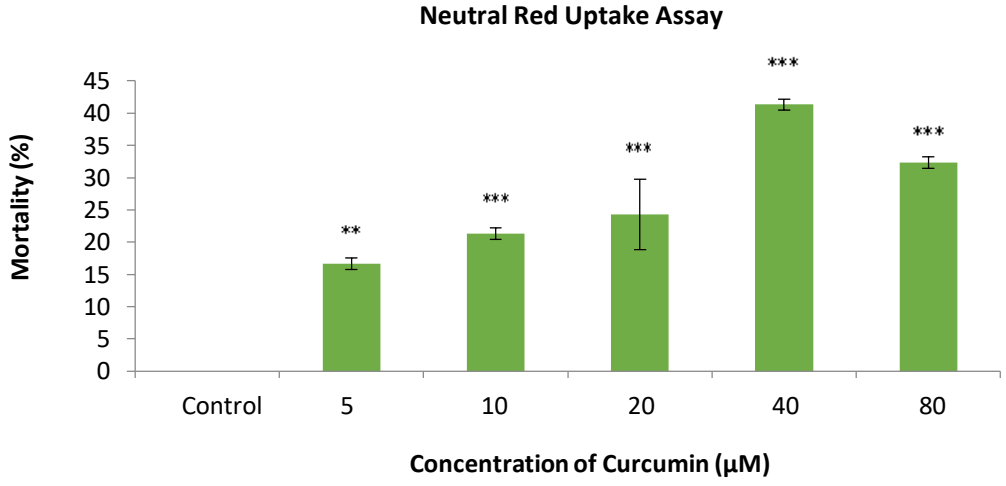

Figure 2. Comparing Cytotoxic Effects of Curcumin on MCF-7 Cells Using Neutral Red Uptake Assay in the 3D Culture After 24 Hours Incubation with Concentrations of 5, 10, 20, 40, and 80 µM. This experiment was repeated 3 times for each concentration of curcumin (**$p<0.01$, ***$p<0.001$).

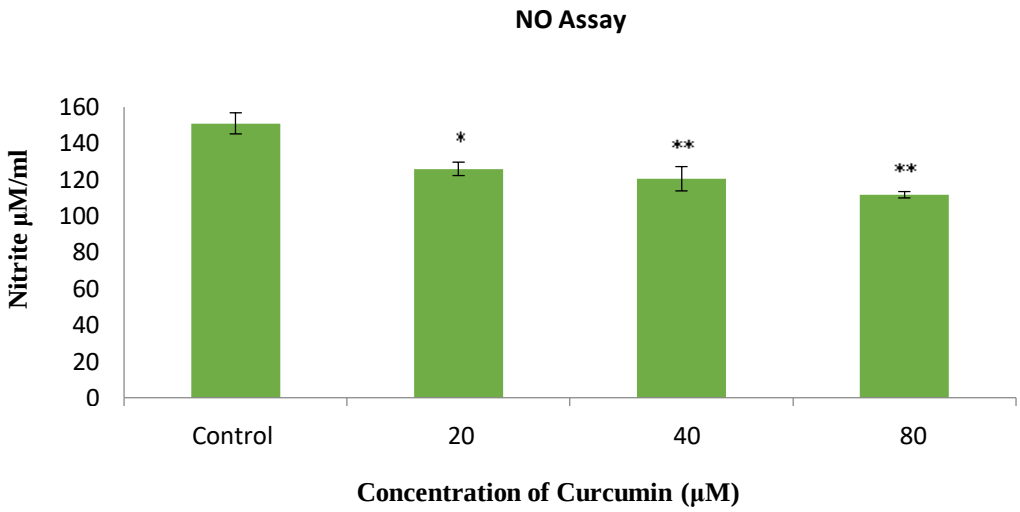

Figure 3. Comparing Nitrite Oxide Released from MCF-7 Cells in 3D Culture After 24 Hours Incubation with Concentrations of 20, 40 and 80 µM of Curcumin Using NO Standard Curve. Concentrations were evaluated compared to the control group. This experiment was repeated 3 times for each concentration of curcumin (*p<0.05, **p<0.01).

NO Assay

The NO released from the control and treated MCF-7 cells with different concentrations of curcumin (20, 40, and 80 µM) were 150.9, 125.8, 120.3, 111.6 µM/ml, respectively (Figure 3). The amount of NO released from the cells due to the effect of curcumin in all concentrations was significant compared to the control. As a result, curcumin significantly decreased the amount of NO in MCF-7 cells in 3D culture compared to the control.

GSH Assay

After treatment with curcumin, the GSH content of the MCF-7 cell line was analyzed. According to the results, curcumin significantly increased the amount of GSH in MCF-7 cells in 3D culture compared to control. The results of this experiment showed that the level of cell glutathione produced in the control sample and at concentrations of 20, 40, and 80 µM of curcumin was 11.13, 13.75, 14.90, and 14.28 µg GSH/mg protein respectively. The statistical analysis also showed that the level of cell glutathione produced by curcumin in all concentrations was significant compared to the control (Figure 4). Furthermore, results showed no significant difference between productions of cell glutathione in three concentrations of 20, 40 and 80 µM.

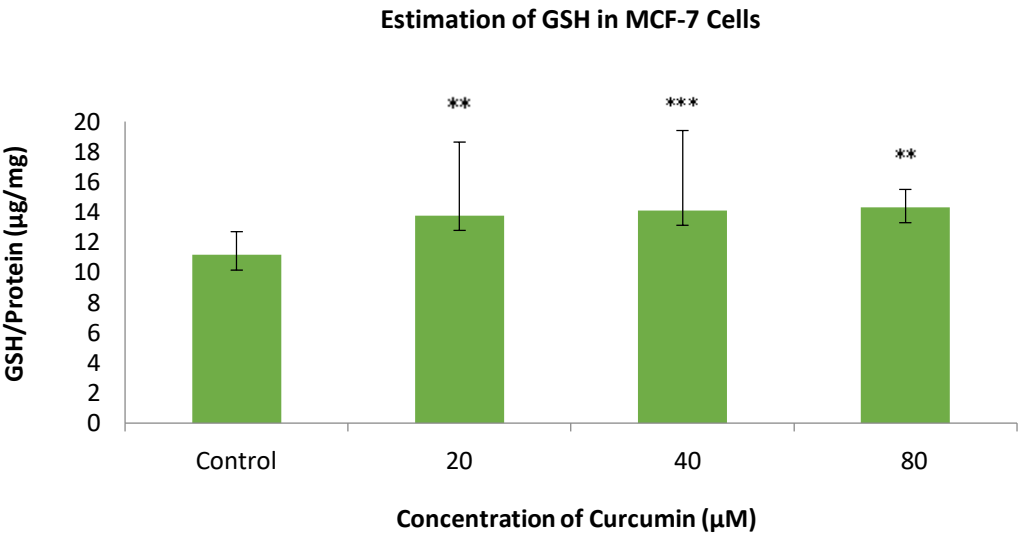

Figure 4. Comparing GSH Content of MCF-7 Cells in 3D Culture Extract Treated with Concentrations of 20, 40, and 80 µM of Curcumin. Concentrations were evaluated compared to the control group. This experiment was repeated 3 times for each concentration of curcumin (**p<0.01, ***p<0.001).

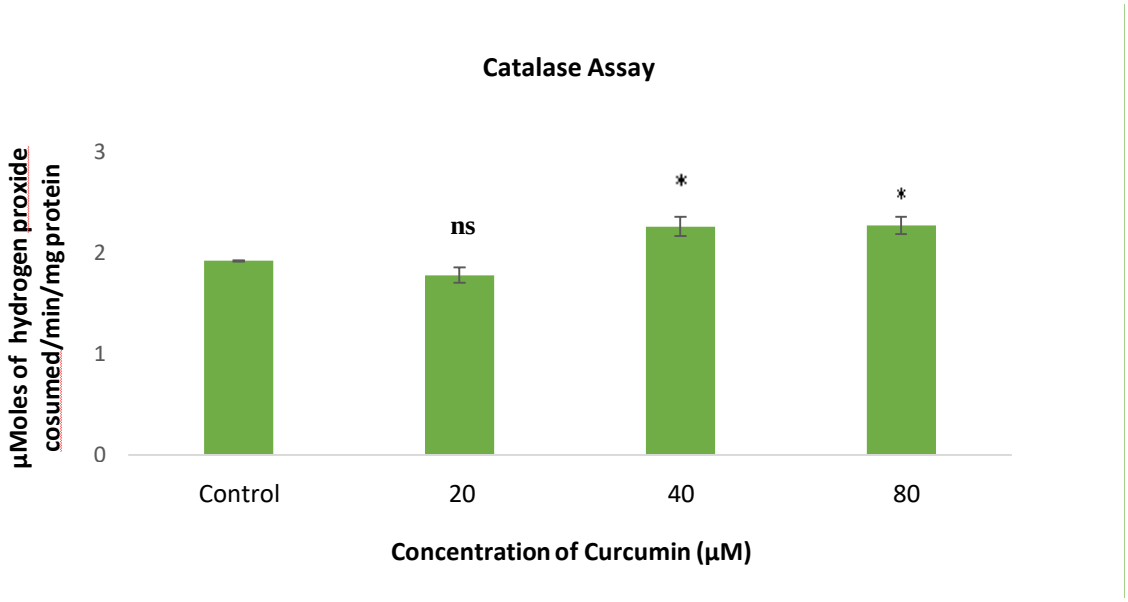

Figure 5. The Amount of Catalase Caused by Curcumin in MCF-7 Cells in 3D Culture After 24 Hours Incubation with Concentrations of 20, 40 and 80 µM of Curcumin. Concentrations were evaluated compared to the control group. This experiment was repeated 3 times for each concentration of curcumin (ns: not significant, *p<0.05).

Catalase Enzyme Activity Assay

Catalase enzyme activity was 1.93, 1.78, 2.26, and 2.27 µmol of hydrogen peroxide consumed/min/mg protein for 0, 20, 40, and 80 µM of curcumin, respectively. In this study, curcumin increased the activity of the catalase enzyme in a dose-dependent manner (Figure 5). According to the results, the amount of catalase produced in this experiment at a concentration of 20 µM was not significant.

Cytochrome c Assay

In 3D culture, cytochrome c inside the MCF-7 cells were 0.138%, 0.138%, 0141%, and 0169% for 0, 20, 40, and 80 µM of curcumin, respectively.

As a result, curcumin significantly induced cytochrome c release in MCF-7 cells in 3D culture compared to control. The results showed that the production rate of this enzyme at a concentration of 80 µM was significant compared to the control (Figure 6).

Alkaline Comet assay

Alkaline comet assay was used for analyzing the potential apoptosis induction effects of the curcumin. In 3D culture, apoptosis induction was 1.5%, 4.5%, 10%, and 10.5% for control and 20, 40, and 80 µM of curcumin, respectively. As a result, curcumin significantly induced apoptosis in MCF-7 cells in 3D culture compared to control (Figure 7).

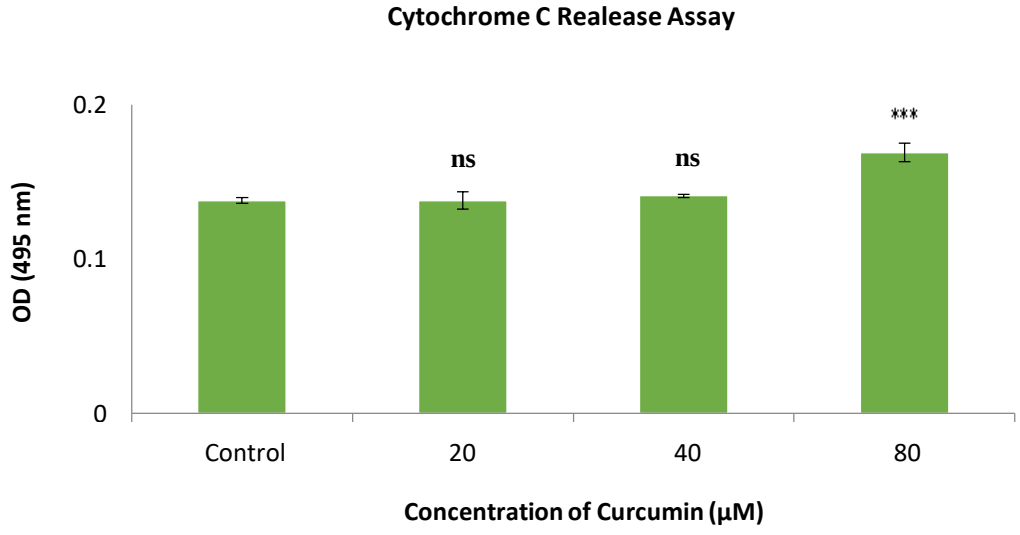

Figure 6. Measurement of Cytochrome c Released From Mitochondria Into Cytosol in MCF-7 cells with Curcumin in 3D Cell Culture. This experiment was repeated 3 times for each concentration of curcumin (ns: not significant،***p<0.001).

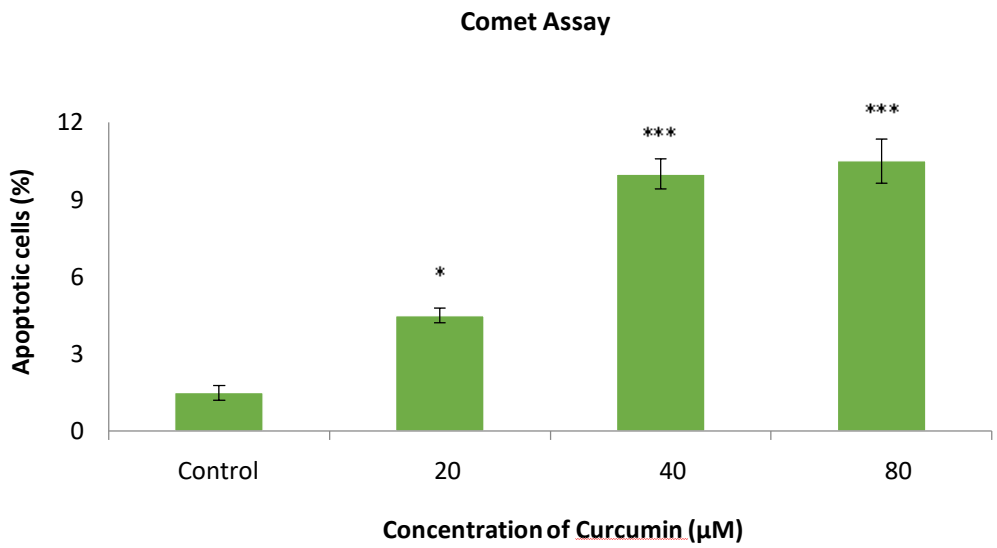

Figure 7. The Amount of Apoptosis Caused by Curcumin in MCF-7 Cells in 3D Culture Using Comet Assay. Concentrations were evaluated compared to the control group. This experiment was repeated 3 times for each concentration of curcumin (*p<0.05, ***p<0.001).

Discussion

It was reported that curcumin can induce mortality on tumor cells in 2D cell cultures.[8] The toxicological results of 2D cell cultures are in many cases not reliable for use in animal experiments and 3D cell cultures now act as a bridge between 2D cell cultures and in vivo animal models.[13]

This research was designed to examine the cytotoxic effects of curcumin on breast cancer cells in 3D culture conditions. In this study, the encapsulating of cells in alginate hydrogel was used to achieve a 3D environment in *in vitro* conditions.[14]

The MTT assay was employed to analyze cytotoxicity effects of curcumin on breast cancer cell (MCF-7) as well as determination of IC 50 in 3D cell culture. Furthermore, neutral red uptake assay was used to confirm the results of the MTT assay.

The results of the MTT test showed that the survival percentage of cells in the presence of different concentrations of curcumin for 24 h had descended in a dose-dependent manner. At a dose of 5, 10, 20, 40, and 80 µM of curcumin in 24 h cell viability decreased to 85, 81, 67, 73 and 56%, respectively.

In a study conducted by Kunwar, the effect of curcumin toxicity on two cancer cell lines (MCF-7 and EL-4) was investigated in 2D culture for 20 h by the spectroscopic fluorescence method. Their results showed that as curcumin concentration increased, the cell mortality rate also increased.[22] In 2009, Mendonça investigated the toxicity effect of curcumin at concentrations of 0.5-128 µg/ml on PC12 cells using the MTT and micronucleus tests in 2D culture conditions. Their results showed that curcumin at 0.8 µg/ml reduced cell growth by 20%, and an increase in the curcumin concentration increased the cell death rate. They have also shown that curcumin is toxic to the cell at high concentrations and causes micronuclei formation.[23] In 2009, Friedman et al., studied the effect of curcumin on pancreatic cancer cell lines at concentrations of 2.5, 5, and 10 µM. Their findings based on the MTT method have shown that curcumin is toxic to the cell and decreases cell viability in a dose-dependent manner.[24] In 2014, Aditya investigated curcumin's anticancer activity in PC3 prostate cancer cells under 2D culture conditions. The results of 24-hour cell treatment with concentrations of 20-60 µM showed that by increasing the curcumin concentration, cell viability decreased so that at a concentration of 60 µM the viability decreased to 83%.[25] Tian-Yu Liu et al., showed that the bladder cancer cells viability decreases with increasing the curcumin dose in a time-dependent manner.[26]

In 2017, Mansoorabadi et al., investigated the toxicity effects of curcumin on mouse breast cancer cell line by the MTT method. In the above mentioned study, concentrations of 5-40 µM of curcumin were added to the cells, and after incubation for 24 and 48 h, the viability of the cells was evaluated by the MTT method. The IC_{50} of the curcumin in 24 and 48 h was 21 ± 0.3 µM and 8.14 ± 0.4 µM, respectively. The cell survival rate depended on curcumin concentration and incubation duration.[27] In 2019, Dalimi et al., investigated the effect of curcumin at concentrations of 10-35 µM for 24 and 48 h on MCF-7 cells under 2D culture conditions using the MTT method. Their results showed that the curcumin was toxic to the cell, and IC_{50} was 25 and 15 µM after 24 and 48 h, respectively.[28] The results of the curcumin cytotoxicity on breast cancer cells in 3D culture conditions was compatible with the reports of studies by Kunwar et al. in 2007,[22] Mendonça et al. in 2009,[23] Friedman et al. in 2009,[24] Tian-Yu Liu et al. in 2013,[26] Mansoorabadi et al. in

2017,[27] and Dalimi et al. in 2019,[28] that investigated the cytotoxicity effect of curcumin in 2D cell culture conditions.

One of the important reasons for using a higher concentration of curcumin in this study is related to the nature of the cell culture in 2D and 3D conditions compared to previous studies. In 2D culture, cultured cells have equal access to the materials in the culture medium, but the concentration of available materials in 3D culture may decrease by increasing the depth of cell placement in the scaffold. Also, the local pH of the cell, which is important in determining the effectiveness of chemotherapy drugs, is related to the local depth of the cell in the cell scaffold and due to the lack of a transport system to remove waste materials from the scaffold center, it may lead to hypoxia regions. Studies have shown that low pH reduces drug uptake and causes cell resistance to drugs.[29] It should be noted that the outer layers of a 3D cell spheroid which are highly exposed to the environment, are mainly composed of living and proliferating cells.[30] Central cells receive lower growth factors and nutrients from the environment and tend to be static or undergone hypoxia.[31] Such cellular heterogeneity is very similar to actual tissues, especially in tumors.[32]

Apoptosis is essential for many physiological and pathological cell processes[33] and may occur via three main mechanisms including the death receptor–dependent (extrinsic inducer) pathway, the death receptor–independent (intrinsic or mitochondrial inducer) pathway[34] and the apoptosis- inducing factor (AIF) pathway.[33]

Apoptotic effects of curcumin were determined by alkaline comet assay and cytochrome c test. Compared to other methods of measuring DNA fragmentation in eukaryote cells, the comet method is highly sensitive, low-cost, and fast.[35,36] Investigating the toxicity effect of curcumin at concentrations of 20, 40, and 80 µM using the comet method showed that the curcumin induces apoptosis in MCF-7 cells in a dose-dependent manner. These results are consistent with Friedman's reports in 2009 and Tian-Yu Liu's report in 2013, who investigated the effect of curcumin on pancreatic and bladder cancer cell lines, respectively. They showed that curcumin caused apoptosis in cancer cells by the induction of caspase-3 expression.[24,26] In addition, in 2014, Aditya reported that curcumin induces apoptosis from the cell receptor-dependent pathway and affects caspase-8 production.[25]

To investigate the effect of curcumin on the release of mitochondrial cytochrome c, breast cancer cells were treated with concentrations of 20-80 µM of curcumin for 24 h. The results showed that in the above concentrations, the released cytochrome c increases with an increase in the curcumin concentration. These results are compatible with a study by Ravindran et al. in 2009, which showed that curcumin increases the mitochondrial membrane permeability and swelling, as well as the loss of membrane potential and release of cytochrome c to cytosol and activation of caspase cascade and induction of apoptosis via mitochondrial-dependent pathway.[37] In addition, in 2004, Damodaran reported that in prostate cancer cells, curcumin releases cytochrome c, activates caspase-9 and caspase-3, and induces apoptosis through the mitochondrial-dependent pathway.[38]

In this study, the effect of curcumin on cellular stress was analyzed using the production of nitric oxide. In order to clarify the data related to nitric oxide, assays two key cellular antioxidants,such as (GSH) and Catalase have been selected.

Nitric oxide is a diffusible toxic molecule and an important effectors molecule in many biological systems[14] that acts in many tissues to regulate a diverse range of physiological functions including inflammation, differentiation, proliferation, and apoptosis.[39] Based on the dose of NO and the kind of cells, No can induce or inhibit the apoptosis through different pathways.[41] Glutathione (GSH) is an antioxidant, preventing damage to important cellular components caused by reactive oxygen species such as free radicals and peroxides[45] and catalase is an enzyme that catalyzes the disintegration of hydrogen peroxide to water and oxygen.[46]

The results of the NO test showed that the production of NO decreased and GSH and catalase increased in the cells after treatment with curcumin in a concentration dependent manner. Our results demonstrated that curcumin induces apoptosis in human breast cancer cells through oxidative stress, induction of mitochondrial membrane potential, cytochrome c and DNA fragmentation which was clearly observed after 24 h of treatment.

This study is in agreement with earlier finding of Hertelano et al. in 1997,[42] Bru et al. in 1999,[43] and Umansky et al. in 2001,[44] who have reported that this molecule effects various activities, including mitochondrial membrane charge and permeability, the release of cytochrome c, activation of caspase cascade, and DNA fragmentation, which causes apoptosis in cells.

Collectively, the results showed that curcumin in 3D culture conditions causes mortality in breast cancer cells (MCF-7). Comparing the MTT and alkaline comet results shows that this molecule reduces cancer cell viability by inducing apoptosis and necrosis, but mainly by inducing apoptosis. On the other hand, although the mortality rate of tumor cells due to the effect of the curcumin was higher in 2D culture reported by other researchers,[27,28] in 3D culture conditions, the necrosis decreased in comparison to 2D culture due to more simulation of 3D culture with natural conditions of living organisms.

Conclusion

The results of this study have shown for the first time that curcumin reduces the production of cellular nitric oxide and increases catalase and glutathione production. It seems that NO as a stimulator, by depolarizing mitochondrial membrane

and enhancing its permeability, releases cytochrome c, the fragmentation of DNA, and finally, cell apoptosis. In addition, due to the significant amount of cytochrome c release from mitochondria in cells treated with different concentrations of curcumin, this molecule has induced apoptosis in cancer cells through the mitochondrial-dependent pathway.

Authors' Contributions

Study concept and design by SZ, MSB, and JZ; Acquisition of data by SZ and MSB; Analysis and interpretation of data by JZ and MS; Drafting of the manuscript by SZ; Critical revision of the manuscript for important intellectual content by MSB, AHNM, MM, and HKA; Statistical analysis by SZ, MSB, and JZ; Administrative, technical, and material support by JZ, AHNM, MM, and HKA.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

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