

Reduction of the clonogenic potential and collapse of the mitochondrial membrane potential in A-431 epidermoid carcinoma cell line induced by curcumin

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Abstract

We examined the ability of curcumin, a component of turmeric isolated from the rhizome of *Curcuma longa*, to display anti-cancer activity in A-431 epidermoid carcinoma cell line. In this study, curcumin strongly reduced the viability and cell density in dose dependent manner after its applications for 48 h. In addition, the natural compound induced the loss of mitochondrial membrane potential ($\Delta\psi_m$) and blocked the cell cycle progression in G2/M phase. The blockage of the cells in G2/M phase was accompanied by the reduction of the number of the cells in G0/G1 phase. Low doses of curcumin (5 μ M) administrated in the cell culture medium for 7-14 days strongly inhibited the ability of A-431 cells to form colonies. Taking together these results support the anti-cancer activity of curcumin in A-431, human epidermoid carcinoma cell line through $\Delta\psi_m$ collapse, inhibition of the clonogenic potential or cell cycle arrest.

Keywords: curcumin, epidermoid cancer cells, clonogenic potential, mitochondrial membrane potential, viability

1. Introduction

Curcumin, a natural polyphenol, is the major constituent of turmeric and represent the active ingredient of *Curcuma longa*, used for its medical properties since ancient time, in traditional medicine (Maheshwari & al. [1]). The main constituents of turmeric are: curcumin, demethoxycurcumin and bisdemethoxycurcumin. Beside these constituents, in turmeric are also found volatile oils, sugars, proteins and resins (Jurenka & al. [2]). Numerous studies reported the anti-cancer, anti-inflammatory and antiproliferative effects and the ability to act as a potential therapeutic agent of curcumin (Priyadarsini & al. [3]). The anti-inflammatory effects of curcumin were related to its ability to down-regulate the activity of several enzymes and to inhibit the production of the inflammatory cytokines (Jurenka & al. [2]). Over the years studies describing the anti-cancer effect of curcumin have increased (Pavan &

al. [4]). Curcumin is proved to possess antitumor activities and the ability to act like an inhibitor of the transcription factor and growth factor receptors, involved in tumor growth and metastasis (Wilken & al. [5]). Reason W. *et al* described the therapeutic activity of curcumin in head and neck cancer. Beside many drugs (cetuximab, etc.) used in therapeutic outcomes for this type of cancer, curcumin was proved to be useful in the treatment of head and neck squamous cell carcinoma (Wilken & al. [5]). The ability of curcumin to have beneficial effects was also shown in many skin diseases, like scleroderma, psoriasis and skin cancer (Thangapazham & al. [6]).

In our study we investigated the anti-neoplastic role of curcumin in A-431 epidermoid carcinoma cell line. Curcumin reduced cell confluence and cell viability in dose-dependent manner. These results were consistent with further investigation of the mitochondrial membrane potential which was collapsed after the administration of curcumin at concentrations higher than 25 μ M. The ability of curcumin to interfere with cell cycle progression was proved by the arrest of the A-431 epidermoid carcinoma cells in G2/M phases. Additionally, low micromolar doses of curcumin inhibited the ability of A-431 cell line to form colonies. Our experimental investigations targeted anti-cancer effects of curcumin to highlight its potential as possible chemopreventive or therapeutic agent.

2. Materials and Methods

Cell culture

The human epidermoid carcinoma cell line A-431 was obtained from the American Type Culture Collection. The cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin and 2 mM L-glutamine in humidified atmosphere at 37°C, 5% CO₂. The cells were passaged 2-3 times per week.

Viability assay

A-431 cells were treated with 5, 10, 50, 100 μ M curcumin for 48 h at a density of 1×10^5 cells/ sample in 6-well flat plate. After 48 h the cells were trypsinized, washed with PBS and stained with 0.05 μ g/ μ l 7-aminoactinomycin (7-AAD), for 15 minutes in the dark. Cell viability after curcumin treatment had been investigated within 1h using Gallios flow cytometer (7-ADD/DNA, excitation wavelength: 488 nm, emission wavelength: 647 nm; for detection was used 695/30 nm BP filter).

Phase contrast and fluorescent microscopy

A-431 cells were treated with 5, 10, 25, 50, 100 μ M curcumin for 48 h. After the treatment with curcumin the cells were stained with nuclear dye Hoechst 33342 and imaged using Zeiss Axiovert 40 for both phase contrast and fluorescent characteristics.

Cell cycle analysis

A-431 cells were treated at 0, 5, 10, 50 and 100 μ M curcumin for 72 h in complete medium. After the incubation with curcumin the cells were fixed in 70% ethanol at -20 °C. After at least 2 h, ethanol was removed by centrifugation, at 1200 rpm for 5 minute at room temperature and washed in PBS. The cells were stained with propidium iodide/RNase for 15 minutes in the dark, at room temperature, according to the manufacturer protocol for cell

cycle analysis. Cell cycle was evaluated after PI/RNase staining by flow cytometry using Gallios flow cytometer; for the excitation the 488 nm laser line was used, while fluorescent emission was detected at 617 nm (620/30 BP filter).

Mitochondrial membrane potential

A-431 cells were treated with 5, 10, 25, 50, 100 μ M curcumin and grown in 6 well plates, for 48 h. Following the treatment, the cells were trypsinized and stained with 5 μ g/ml 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) at 37 °C, for 15 minutes. Gallios flow cytometer with 488 nm laser line was used for the excitation of JC-1 fluorochrome. Detection of the fluorescent signal was performed using 525/40 BP filter for the monomer form (514/529 nm emission maxima) and 575/30 BP filter for the aggregated form (585/595 nm emission maxima).

Clonogenic assay

A-431 cells were seeded in triplicate at a concentration of 500 cells/well in 6 well plates. The cells were treated with 0, 5, 10, 25, 50, 100 μ M curcumin for 48 h at 37°C. After 7-14 days, the cells were fixed with 3.7% formaldehyde, stained with 0.5% crystal violet at room temperature, followed by imaging of the colonies. Plating efficiency (PE) and survival fractions (SF) were calculated after counting the clones with ImageJ software.

Statistical analysis

Data given are mean and standard error of the mean (mean \pm SEM). The comparison between two groups was carried out by student t-test with $P < 0.05$ for differences with statistical significance.

3. Results and discussion

Malignant proliferation of the keratinocytes of the epidermis is a characteristic of the cutaneous squamous cell carcinoma (SCC) (Yan & al. [7]). In addition, this type of non-melanoma malignancy has a high ability to spread and it is considered the second most common form of skin cancer (Alam & al. [8, Kallini & al. [9, Rahimi & al. [10]). In our study, we evaluated the anti-neoplastic effect of curcumin in a cell model for SCC, namely A-431 epidermoid carcinoma cell line. A main feature of curcumin is its pleiotropic activity, characterized by its ability to reduce cell proliferation, cell growth, and to increase the apoptosis in different types of epithelial cancer models (Terlikowska & al. [11]).

Administration of curcumin decreased cell confluence and cell viability in dose dependent manner in A-431 cell line

First observations have been carried out by phase-contrast and fluorescent microscopy. Smaller concentrations of curcumin (10 μ M) decreased cell confluence. At higher concentrations (25 μ M – 100 μ M) the cell growth was almost completely inhibited (Figure 1).

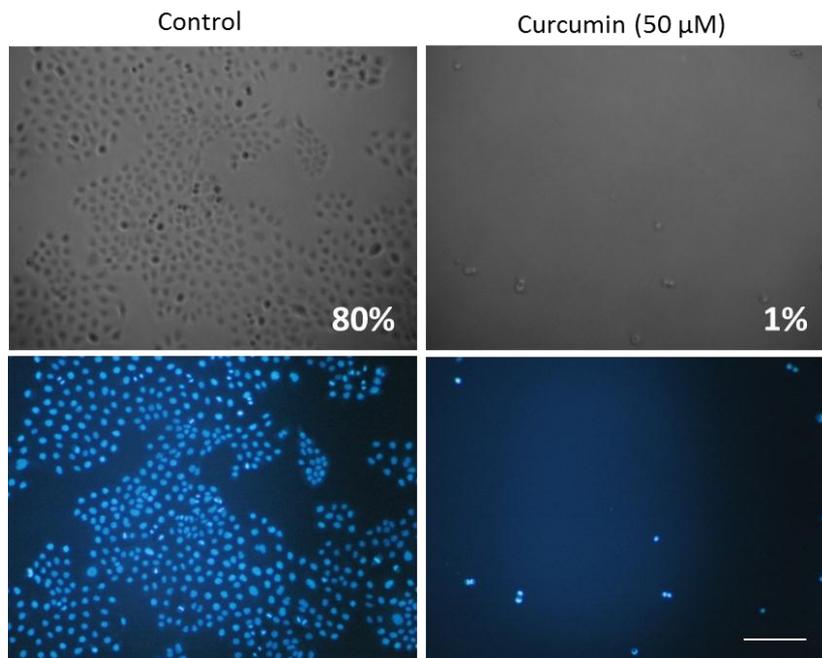


Figure 1. Cell confluence for A-431 epidermoid carcinoma cell line treated with curcumin. A-431 cells were seeded in 6-well plates and treated for 48 h with curcumin (5, 10, 25, 50 and 100 μM). Representative pictures of untreated and treated A-431 cells. Phase contrast and fluorescent (Hoechst 33342) pictures of the control and treated samples. Scale bar: 100 μM .

To evaluate cell viability, the epidermoid carcinoma cell line, A-431 was incubated in the absence (control) or in the presence of different concentrations of curcumin (5, 10, 25, 50 and 100 μM) for 48 h. Representative histograms of the untreated and treated samples are shown in the Figure 2A-C. The presence of curcumin in the cell culture medium reduced the cell viability in dose dependent manner (Figure 2D). Until now the results regarding the role of curcumin on cell viability indicated two modalities of action: low doses were associated with anti-oxidant activity and extended cell survival, while higher doses of curcumin showed cytotoxic effect (Attari & al. [12]). Our results concerning cell viability after curcumin treatment are similar to those reported earlier in an estrogen positive breast cancer cell line (Banerjee & al. [13, Zhou & al. [14]) or colorectal cancer cell lines (Iwuchukwu & al. [15]).

A431 cells were arrested in G₂/M phase after the curcumin treatment

Further, the effect of curcumin in A-431 cells was investigated in different phases of cell cycle: G₀/G₁, S and G₂/M. A-431 cells were incubated for 72 h with smaller concentrations 5, 10 μM or higher concentrations 25 – 100 μM of curcumin. The histograms (Figure 2A) demonstrate that higher concentrations of curcumin (50 μM – 100 μM) induced the blockage of A-431 cells in G₂/M phase of the cell cycle. Concomitant with the increase in the number of the cells in G₂/M phase was revealed the decrease in the number of cells in G₀/G₁ phase (Figure 2B). The effect of curcumin on the cell cycle progression was previous reported in MCF-7, an estrogen receptor positive breast cancer cell line (Zhou & al. [14]) and in several colorectal cancer cell lines (Blakemore & al. [16]). However, to the best of our knowledge, the effect of curcumin on cell cycle in A-431, a model for squamous cell carcinoma was not yet reported.

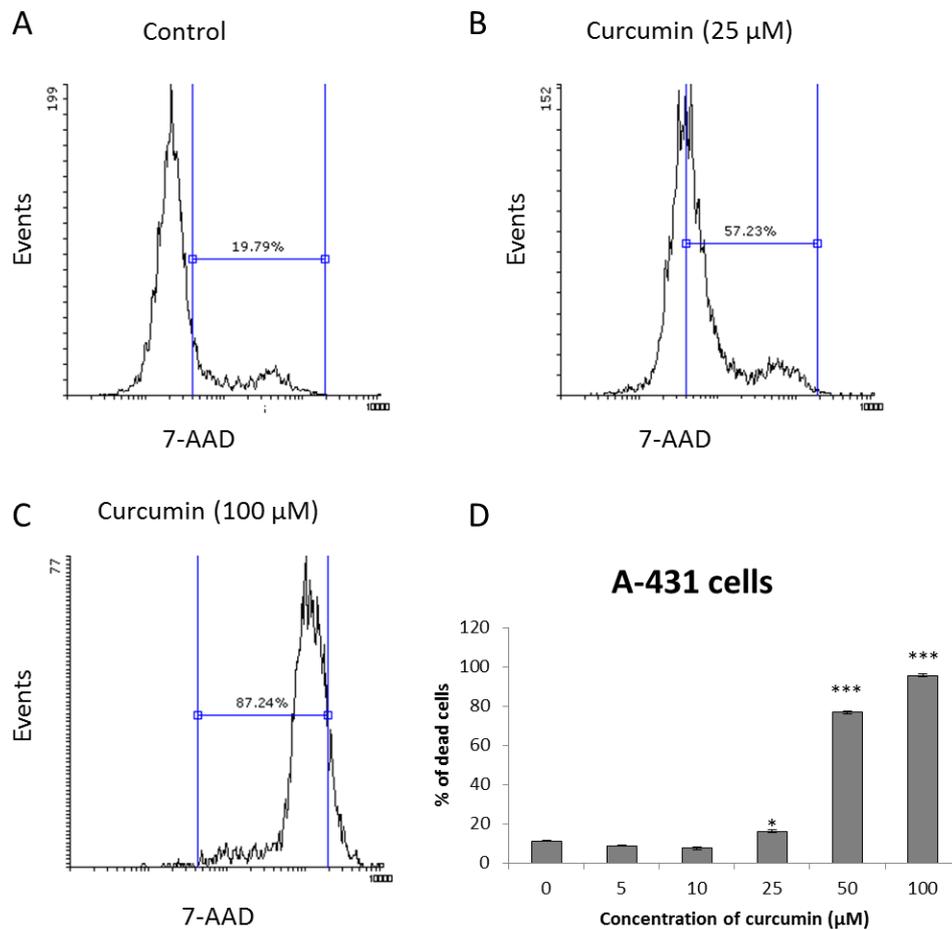


Figure 2. Viability reduction in A-431 cell line after curcumin treatment for 48 h. A-C. Histograms represent the fluorescence intensity of 7-AAD in control and after the treatment with 25 and 100 μM curcumin. D. Administration of curcumin for 48 h reduced cell viability, starting with 25 μM curcumin. Significant changes compared to un-treated samples are visible at doses of 50 and 100 μM curcumin; (*, $P < 0.05$).

Curcumin reduced the mitochondrial membrane potential ($\Delta\psi_m$) in A-431 epidermoid carcinoma cell line

The functional status of the cells was evaluated by monitoring the changes in $\Delta\psi_m$, since the loss of $\Delta\psi_m$ was strongly associated with cell death (Joshi & al. [17, Perry & al. [18]). The alterations induced by curcumin in $\Delta\psi_m$ were evaluated by staining A-431 cells with JC-1 fluorescent probe and flow cytometry. As shown in Figure 4, curcumin induced the loss of $\Delta\psi_m$ quite early, starting with 5-10 μM . In concordance with the previous data regarding the cell viability, the reduction in $\Delta\psi_m$ was dose-dependent. The number of un-polarized cells was highly increased at doses of 25 μM curcumin (Figure 4), suggesting that at these concentrations the mitochondria were completely energy depleted. Similarly, in other cell models for malignant disease, like human hepatocellular carcinoma or lung adenocarcinoma, curcumin disrupted the mitochondrial membrane potential in association with induction of apoptosis (Chen & al. [19, Wang & al. [20]).

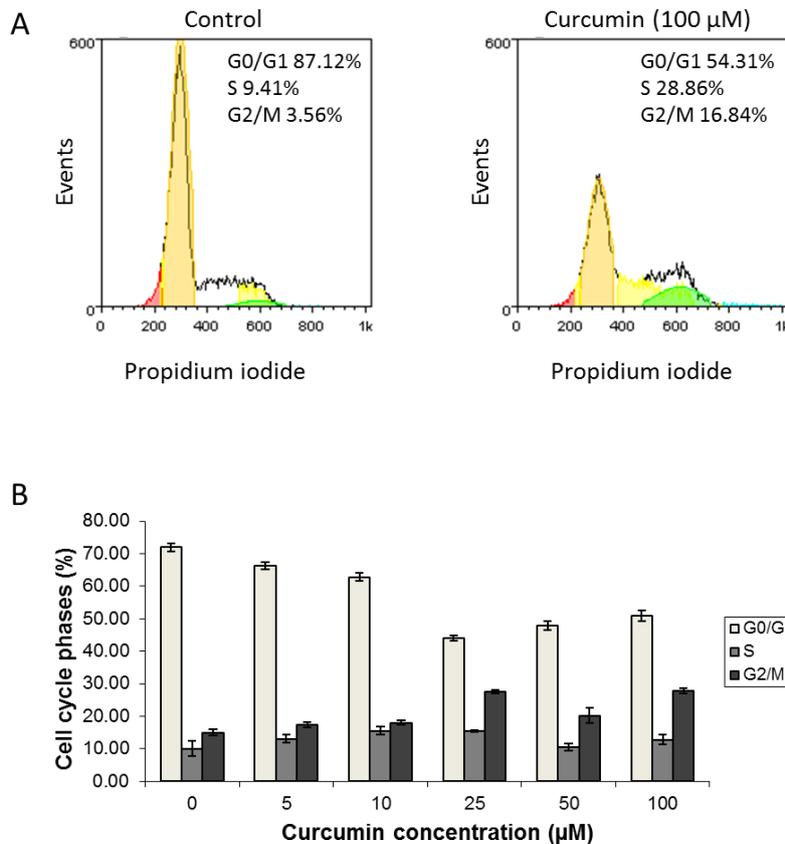


Figure 3. Cell cycle analysis after the treatment with different concentrations of curcumin. A. Representative DNA histograms showing the effect of curcumin on the cell cycle progression in epidermoid carcinoma cells. B. Cell cycle modulation after the incubation of A-431 cells with 5, 10, 25, 50 and 100 μ M curcumin for 72 h.

Administration of curcumin for 48 h decreased the clonogenic capacity of A-431 cells

Since the appearance of the recurrences in cancer depends on the ability of the malignant cells to form new colonies we tested the role of curcumin in inhibition of the new colonies formation. Representative images of the colonies in un-treated and treated samples with curcumin for 48 h are shown in Figure 5. Both the number and the size of the colonies were significantly reduced in the presence of low concentrations of curcumin (5 μ M). Concentrations starting with 10 μ M curcumin almost completely inhibited the colony formation in A-431 cell line (Figure 5), suggesting that longer incubation time with low concentrations of curcumin could be more efficient than high doses applied for a short time. Our results are in line with previous reported experiments, where low micromolar doses of curcumin significantly decreased survival fraction of PC-3 prostate cancer cell line (Chendil & al. [21]).

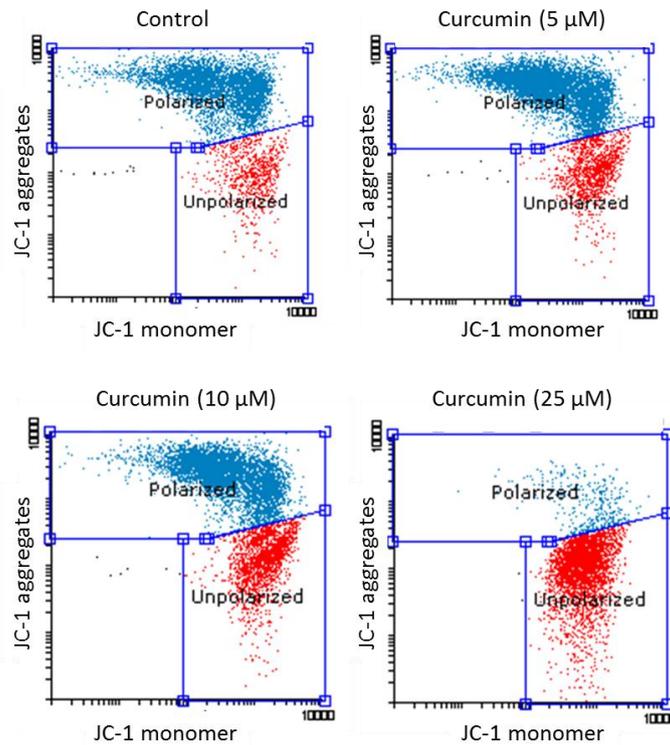


Figure 4. The mitochondrial membrane potential ($\Delta\psi_m$) in A-431 epidermoid carcinoma cell line after curcumin treatment. Representative dot plots of the control and treated samples (5, 10, 25 μM curcumin, 48 h) stained with JC-1, which demonstrate the reduction of the polarized cells after the application of the natural compound.

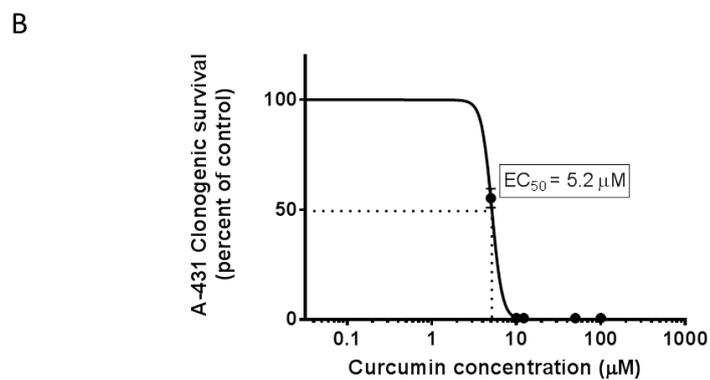
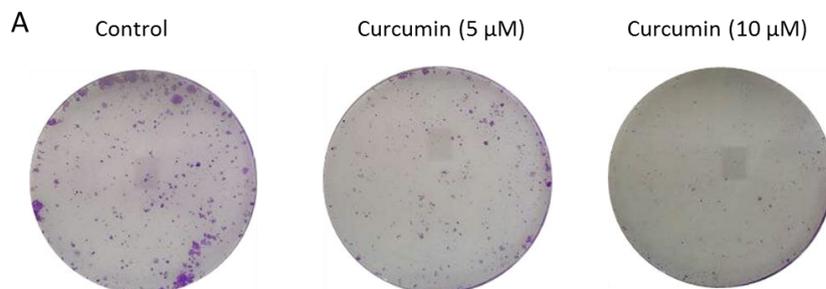


Figure 5. Complete inhibition of the colony formation in A-431 cell line after the administration of curcumin at concentrations higher than 10 μ M. A. Colonies developed in the absence or presence of the curcumin; concentrations of curcumin higher than 10 μ M inhibited the colony formation. B. Low micromolar doses of curcumin inhibited the clonogenic potential of A-431 cells, shown as dose-response curve.

4. Conclusion

In conclusion, our results indicated that curcumin at low micromolar doses applied for longer time interval might be able to reduce the ability of the malignant cells to generate new colonies, while the short treatments with curcumin already demonstrated its capacity to reduce cell growth, and viability, to arrest the cancer cells in G2/M phase and to induce collapse of the mitochondrial membrane potential as preliminary step of the apoptotic process. Although the promising *in vitro* results, the use of the curcumin in clinical applications was hampered by its diminished bioavailability and by the reduced number of studies which focused on the selectivity of the natural compound (Devassy & al. [22]). Considering these challenges, new approaches should be established to avoid the actual issues regarding the clinical applications of curcumin.

5. Acknowledgements

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