

Apoptotic Effect of Capsaicin on Colon Cancer (Caco-2) Cells *via* Bcl-2, P53 and Caspase-3 Pathways

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Abstract: Capsaicin (8-methyl-N-vanillyl-6-nonenamide), possesses anti-proliferative properties that can be used as anticancer intervention as promising cancer chemo-preventive constituent. We investigated capsaicin inhibitory activity against colon cancer (Caco-2) cells viability or proliferation. Cells were incubated with capsaicin at concentrations, 300µM/L, 500µM/L, 700µM/L and 900µM/L for 72h at 37°C and 5%CO₂. Capsaicin induced Caco-2 cell death in a dose-dependent manner. Capsaicin-treated cells showed typical characteristics of apoptosis including inhibited the viability and proliferation of treated Caco-2 cells in vitro even by DNA fragmentation, cytological alterations. Protein over expression of caspase-3, in the capsaicin-treated cells was obviously seen. Also, Bcl-2 expression was down-regulated, whereas p53 expression was increased. Future study will deal with further investigations of capsaicin possible usages as a new alternative or complementary chemotherapeutic agent for human cancer types specially colon cancer type.

Keywords: capsaicin, Caco-2 Cells, p53, Bcl-2, caspase-3.

1. Introduction

A fairly number of studies have focused on anticarcinogenic activity of phytochemicals, particularly those included in human diet. Capsaicin (8-methyl-N-vanillyl-6-nonenamide), the piquant component in red hot pepper of the genus *Capsicum*, has long been used as spices, food additives and drugs¹. Red hot pepper research is now being aimed at osteoarthritis, obesity, and some forms of cancer. Recently, capsaicin has been found to inhibit the growth of various immortalized and malignant cells and induces apoptosis in transformed cells. Capsaicin induces the apoptosis of cancers cells, including myeloid leukemia and human hepatoma².

Apoptosis, a programmed cell death, occurs under a variety of physiological and pathological conditions that control the development and homeostasis of multicellular organisms³. Apoptosis induction was recognized to be the best strategy for agents to kill cancer cells. The major apoptotic pathways can be divided into caspase- and mitochondria- dependent pathways, according to caspase-3 activation which is generally considered to be a key hallmark of apoptosis⁴. It was reported the elevation of caspase-3 activity in cancer cells treated with capsaicin⁵.

In recent findings, capsaicin is seen to show anti-proliferative effects on various human cancer cell lines by apoptosis mediated cell death that was according for its ability to suppressing the inhibitor of caspase-3 activated DNase expression to induce apoptosis specially for human adenocarcinoma cells⁶. Bcl-2 is the founding member of family of genes that either prevents or promotes cellular apoptosis. Bcl-2 itself is an anti-apoptotic gene that prevents initiation steps of apoptosis and programmed cell death⁷. P53 has been shown to be involved in the induction of apoptosis, cell-cycle arrest and differentiation responses that prevent further proliferation of stressed or damaged cells

and so protect from the outgrowth of cells harboring malignant alterations. P53 role in the repair of DNA damage has also been described and the ability of p53 to induce reversible cell-cycle arrest may contribute to the ability of cells to repair and recover from damage before reentering a normal proliferative state⁸.

Over the past decade, there is continuous increase in colorectal carcinoma in the world as the most common malignant diseases. Colorectal cancer is the cause of more than 1/2 million deaths worldwide, and it was ranked as the third leading cause of cancer-related death after lung cancer and stomach cancer⁹. Epidemiological studies have shown strong evidence that diet and lifestyle play an important role in preventing cancer. In particular, an increased consumption of fruits and vegetables is associated with decreasing in cancer onset and mortality¹⁰.

In this study, we focused on capsaicin apoptotic effect by activation of gaspase-3 and mitochondrial pathway on human colorectal carcinoma cell line (Caco-2). That was via participation of caspase-3 and down regulation of Bcl-2 and p53 protein which were involved in the apoptotic process.

2. Materials and Methods

Chemical reagents: Capsaicin, MTT salt or 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide, dimethylsulfoxide (DMSO), commercial methanol, commercial ethanol, commercial acetone, Tris-HCl, edetic acid, Triton-X100, RNase A, proteinase K, NaCl, 2-propanol, phosphate-buffered saline (PBS), ethidium bromide, agarose gel, Peroxidase, trypsin, Hematoxylin and eosin (Hx & E) stain, rabbit polyclonal antibodies against cleaved caspase-3, primary monoclonal antibody against Bcl-2 and against p53, AB reagent, biotinylated immunoglobulin secondary antibody and Tween 20 were purchased from Sigma-Aldrich, Egypt.

Cell line and cell culture: Caco-2 cell line, was obtained from American Type Culture Collection (ATCC, USA). They were sub-cultured as mono-layer according to the instructions provided by ATCC in Dulbecco modified Eagle medium (DMEM) supplemented with 10% heat inactivated (56°C, 30min) fetal bovine serum, 2mmol/L L-glutamine, 100U/mL Penicillin-Streptomycin and 100U/mL Amphotericin B at 37°C in a humidified atmosphere of 5% CO₂. Cells were used when monolayer reached 80% confluence in all experiments. Cell propagation media was purchased from Invitrogen (Carlsbad, CA).

3. Methods

1. Cell Viability Assay: *In vitro* evaluation of antiproliferation effect: growth inhibition was evaluated by MTT assay. MTT salt or 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide was reduced by mitochondrial dehydrogenases to water blue insoluble formazans¹¹. Viable cell number/well is directly proportional to formazans production. 8.25×10^3 cells were seeded into each well of 96-well plate, incubated with culture medium overnight (12h), replaced with fresh medium containing capsaicin at concentrations: 300µM/L, 500µM/L, 700µM/L and 900µM/L for 72h at 37°C in an incubator with 5% CO₂. After incubation, capsaicin modified medium was replaced by 100µL of MTT (0.5mg/mL) medium for incubation (3h at 37°C and 5% CO₂). MTT medium was then replaced with 100µL of DMSO and left for 10min on a platform shaker to solubilize converted formazan. The absorbance values were determined at 570nm test wavelength and 630nm reference wavelength (Spekol 1200 spectrophotometer). Untreated cells were as a positive control cells and all values were correlated with this set of data. The experiment was performed in triplicates. Inhibition Percentage=[1-(net Absorbance of treated well/net Absorbance of control well)]x100%, then was plotted against capsaicin concentrations.

2. Determination of DNA fragmentation by DNA laddering assay: cells were seeded in 60-mm petri dishes at density 4×10^5 cells/plate (treated cells by IC₅₀ concentration of capsaicin or positive control cells). Adherent and floating cells were collected by centrifugation at 1000xg/5min. Cell pellet was suspended in cell lysis buffer (Tris-HCl 10mmol/L pH7.4, edetic acid 10mmol/L pH8.0, Triton-X100 0.5%) and kept at 4°C/10min then, lysate was centrifuged at 25.000xg/20min. Supernatant was incubated with RNase A 40µg/L/1h (37°C), incubated with proteinase K 40µg/L/1h (37°C), mixed with NaCl 0.5mol/L and 50% 2-propanol overnight (-20°C), then centrifuged at 25.000xg/15min. After drying, DNA was dissolved in buffer (Tris-HCl 10mmol/L pH7.4, edetic acid 1mmol/L pH 8.0) and separated by 2% agarose gel electrophoresis at 100V for 50min. DNA was visualized under ultraviolet light after staining with ethidium bromide¹².

3. Cytological changes investigation: detached and trypsinized cells (IC₅₀ concentration of capsaicin treated cells and positive control cells) were collected and centrifuged at 2000 rpm for 5min. Cell pellet was re-suspended with 100µL of PBS (pH7.3). 10µL of the suspension were smeared on a glass slide, allowed to air-dry,

fixed with cool methanol for 5min before proceeding by Hx&E stain and examined under light microscope¹³.

4. Immunocytochemical investigations: by detection of Bcl-2, p53 and Caspase-3 by immunocytochemistry staining kits. The procedure was done according to the manufacturer's instructions, simplified as follows: 1-2 drops of Peroxidase was applied to cells (IC₅₀ concentration of capsaicin treated cells and positive control cells) on the slide (10min), followed by blocking solution (10min). Cells were fixed in ethanol:acetone (9:1) for 30min at -20°C and then rinsed again with cold PBS at room temperature. Cells were incubated overnight with rabbit polyclonal antibodies against cleaved caspase-3 at 4°C, then AB reagent and substrate-chromogen mixture (30min). Between each step, the slide was washed with washing buffer (PBS) with 0.1% Tween 20). Cells were incubated overnight with primary monoclonal antibody against Bcl-2 and against p53 at dilution of 1:75 at 4°C, then in Tris buffer and biotinylated immunoglobulin secondary antibody was used¹⁴. The slides were then mounted and examined under light microscope.

5. Statistical analysis: results were presented as mean±standard deviations (SD). Analysis of variance (ANOVA) for two variables (Two Way-ANOVA) was used together with student t-test. Significant analysis of variance results were subjected to post hoc. Statistical significance was set at P<0.05 and high significance was set at P≤ 0.01¹⁵.

4. Results

1. Cell viability assay: *In vitro* evaluation of antiproliferation effect

Cytotoxic effect of different concentrations of capsaicin (300µM, 500µM, 700µM and 900µM) for 72h on Caco-2 cell line was determined by MTT assay (Figure 1). Cells number started to reduce immediately after treatment with capsaicin concentrations in a dose dependent manner. All concentrations were found to be high significantly different (P≤0.01) in respect to their antiproliferative and apoptotic effects when compared with positive control cells. Cell inhibition percentage was gradually increased with capsaicin concentration increasing and 100% of cell inhibition was observed when cells were treated with 900µM/72h. Cell proliferation reduced about 24% and 35% when cells were treated with 300µM and 500µM for 72h, respectively. Cells proliferation decreased to 55% when treated with concentration of 700µM/72h.

2. Determination of DNA Fragmentation by DNA Laddering Assay

DNA degradation into multiple internucleosomal fragments is a distinct biochemical hallmark for apoptosis. Nuclear DNA isolated from Caco-2 cancer cells was separated by agarose gel electrophoresis and stained with ethidium bromide, and a typical ladder formation was observed upon 72h when treated with capsaicin concentration at 700µM whereas untreated cells did not show typical ladder (Figure 2). Results indicated that capsaicin induced DNA fragmentation which was caused by apoptosis.

3. Cytological Changes Investigation

Positive control cells group had round nuclei, distinct small nucleoli and homogeneous chromatin with an accentuated nuclear membrane (Figure 3a). After Coca-2 cells treatment by capsaicin concentration at 700 μ M/72h, apoptotic cells were identified by a series morphological changes as an important experimental proof of underlying processes alterations appeared as: bleb plasma membrane, cellular shrinkage, chromatin condensation granules, vacuolated cytoplasm, degrading nucleus and apoptotic bodies formation were observed (Figure 3b, 3c and 3d).

4. Immunocytochemical Investigation

After Coca-2 cells treatment by capsaicin concentration at 700 μ M/72h, the reaction of caspase-3 protein was considered positive (over expression of caspase-3 protein) when over 50% of treated tumor cells had a clear brown cytoplasm staining, with slight degrading in the intensity in the same field (Figures 3f). Specially those fields that had necrotic or apoptotic nucleus as sign for capsaicin treatment effect, but fields of positive control cells have negative reaction of caspase-3 (cytoplasm did not show the brownish reaction stain) (Figure 3e). On the other hand regarding to the positive control Coca-2 cells, Bcl-2 protein reaction was considered positive (over expression of Bcl-2 protein) when over 55% of cells had nuclear membrane, mitochondrial outer membrane and endoplasmic reticulum membrane brown staining, with slight intensity degrading in the same field (Figures 3g). After Coca-2 cells treatment by capsaicin concentration at 700 μ M/72h, those fields that had necrotic or apoptotic nucleus as sign for capsaicin apoptotic effect with Bcl-2 negative reaction (faint to non-brown stain) (Figure 3h). Also, when applying p53 stain, p53 protein reaction in the positive control Coca-2 cells, was showed negative reaction (no brown stain) (Figure 3j). After Coca-2 cells treatment by capsaicin concentration at 700 μ M/72h, those fields had necrotic or apoptotic nucleus for capsaicin effect showed p53 positive reaction (over expression of p53 protein) when over 55% of cells had nuclear brown staining, with slight intensity degrading in the same field (Figures 3i).

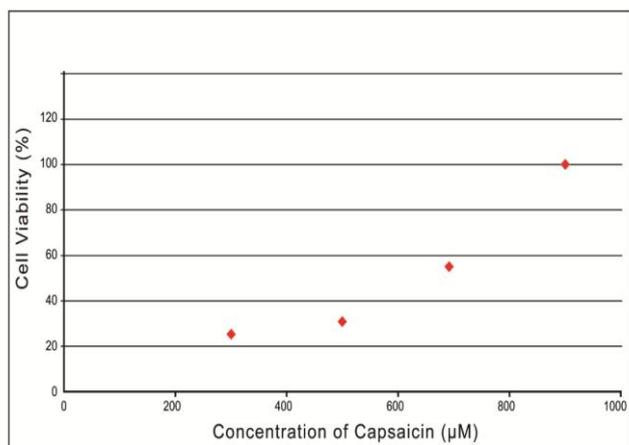


Figure 1: Effect of capsaicin with different concentrations on the cells viability of Caco-2 cells. The experiment was performed in triplicates and values means were calculated [mean \pm SD, n (for each concentration)=4].

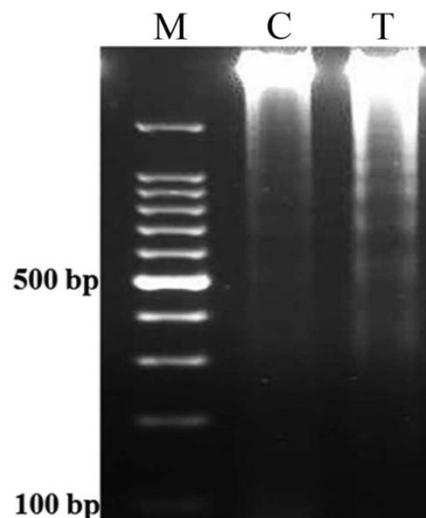


Figure 2: DNA fragmentation by DNA laddering assay of extracted DNA from capsaicin treated cells and positive control cells. DNA laddering, typical for apoptotic cells, which were visible in treated Coca-2 cells (T), and there was no any apoptotic features in the positive untreated cells (C) where M indicating to marker.

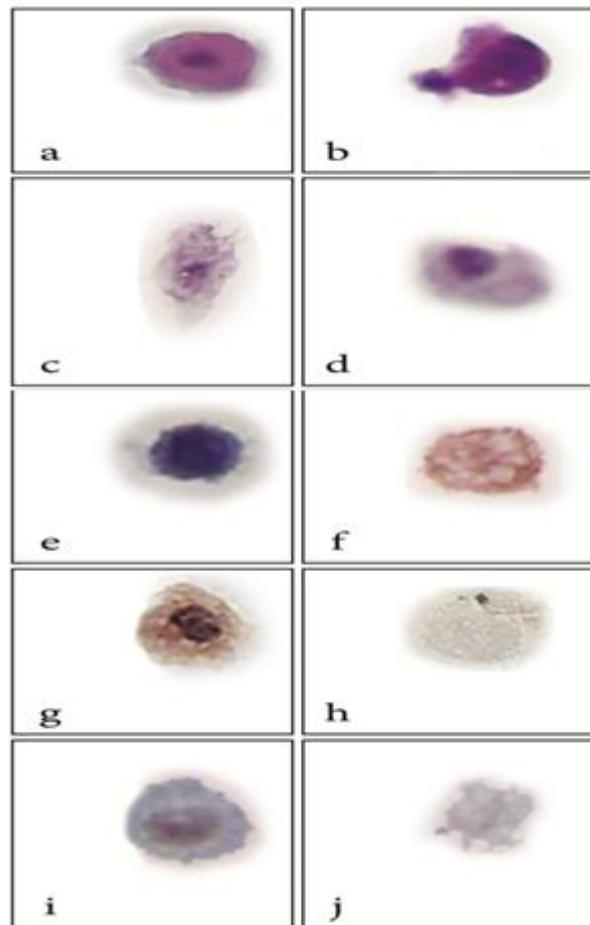


Figure 3: Cells in different stages of apoptosis in treated cells are easily distinguishable. Cell with normal morphology (a). Complete apoptotic cell (b). Degradation of nucleus, vacuolated cytoplasm with apoptotic bodies (c). Nuclear condensation is evident in cells (dark, condensed and irregular rounded nucleus), bleb membrane and cell shrinkage (d). Immunocytochemistry of caspase-3 protein. Control positive cell showing cytoplasm negative reaction for caspase-3 protein (e). Treated cell showing cytoplasm

positive reaction for caspase-3 protein (f). Control positive cell showing Bcl-2 protein nuclear membrane, mitochondrial outer membrane and endoplasmic reticulum membrane showing brownish positive reaction (g) and treated cell showing negative reaction indicating cell apoptosis incidence (h). Treated cell showing p53 protein nuclear positive reaction (i). Control positive cell showing nuclear negative reaction (j).

5. Discussion

Apoptosis, as programmed cell death, is a highly organized cell death process characterized by an early obvious condensation of nuclear chromatin, loss of plasma membrane phospholipid asymmetry, activation of nucleases, enzymatic cleavage of DNA into oligonucleosomal fragments and segmentation of the cells into membrane-bound apoptotic bodies¹⁶. DNA fragmentation, a hallmark of apoptosis, is regulated by a specific nuclease called caspase-activated DNase and its inhibitor¹⁷. Apoptosis has specific signals instructing the cells with specific morphological change as plasma and nuclear membrane blebbings, chromatin condensation, proteases activation and DNA fragmentation that are considered as landmarks of the apoptotic process¹⁸. That was agreed with the results of recent study after treatment by capsaicin. Capsaicin decreased the viable percentage of cell number (dose dependent effect) and induced apoptosis of Caco-2 cells. Therefore, we may presume that as primary mechanism involved in capsaicin growth-inhibitory effects as it considered main apoptotic signals.

It is well known that a family of cysteinyl proteases, caspases, is involved in the apoptotic cell death¹⁹. Caspase-3, one of the active executioners, promotes apoptosis by cleaving cellular substrates such as ICAD²⁰. The protein expression of ICAD is reduced in a time dependent manner, suggesting that caspase-3 is activated in capsaicin-induced apoptosis²¹. That was agreed in the recent study by caspase-3 expression inhibition.

Bcl-2 family of proteins serves as critical regulators of pathways involved in apoptosis. The main protagonists are suggested to be anti-apoptotic and known as Bcl-2²². Caco-2 cells which was treated with capsaicin exhibited reduced levels of Bcl-2 expression. These results suggested that the mitochondrial pathway might be involved in capsaicin-induced Caco-2 cell death. P53 is a tumor suppressor gene product which is very important for cells in multicellular organisms to suppress cancer²³. P53 has been described as 'The Guardian of the genome', referring to its role in conserving stability by preventing genome mutation²⁴. Upon genotoxic and other stress, p53 protein levels increase. Activated p53 releases signal to cells to undergo growth arrest, cell differentiation or apoptosis²⁵. Caco-2 cells which were treated with capsaicin exhibited increased levels of p53 expression at concentration of 700µM/72h, which suggested that p53 involved in capsaicin-induced Caco-2 cell death.

6. Conclusion

In this study, we have demonstrated that capsaicin inhibited proliferation and induced apoptosis in colon cancer (Caco-2)

cells which depended on caspase-3 activation, down-regulation of both Bcl-2, but up-regulation of p53 protein. Future *in vitro* and *in vivo* study will may deal with further investigations of the possible usages of capsaicin as a new alternative chemotherapeutic agent but in limit doses for human colon cancer suggested treatment and other types of cancer.

7. Acknowledgments

We are grateful for all of Nile center for experimental researches team specially Miss. Noha T. Badawy (Department of cell culture) for her kindly support in the cell culture and drug induction stage.

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