In Vitro Studies on Anticancer Activity of Capsaicin a Component of Hot Chili Pepper against Human Hepatocellular Carcinoma Cells

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Abstract

Capsaicin is the major pungent ingredient in red peppers. It exerted antitumor activity in several tumor models. Purpose: To investigate the anti-tumor effect of capsaicin on Human HepG2 cells. Materials and Methods: The MTT assay for cell viability, cell morphology and DNA fragmentation levels andthe expression of apoptosis/cell cycle-related proteins (or genes) was examined by western blotting were as¬sessed and immunocytochemistry. Results: Capsaicin was found to inhibit the growth and proliferation of HepG2 cells in a dose- and time-dependent manner. Apoptotic cell death was confirmed by observing increases in nuclear condensation, nuclear DNA fragmentation, down regulation of cyclin-D1, increased cleaved PARP (p85) levels and increased histone release. Conclusion: Our data suggested that capsaicin, may have a role in the management of HepG2 cells.

Keywords: Capsaicin; Human HepG2 cells; PARP; Cyclin-D1

Introduction

Fruits and vegetables are natural medicines and have been used in our daily diet. Phytochemicals present in the dietary fruits and vegetables have anticancer properties [1]. Capsaicinoids are naturally occurring phenolic compounds commonly present in the genus Capsicum (Solanaceae) and having remarkable anti mutagenic and anti tumour property [2]. Among the chilli varieties C. chinense contains the highest concentration of capsaicinoids. Capsaicin and dihydrocapsaicin are the most abundant capsaicinoids in pepper fruits [3]. In recent findings, capsaicin is seen to show anti-proliferative effects on various human cancer cell lines by apoptosis mediated cell death [4]. Diverse studies have shown that capsaicin has anti proliferative effect on several human cell lines derived from multiple myeloma, gastric cancer, pancreatic cancer, breast cancer [5] and prostate cancer [6], etc. However, the molecular mecha¬nisms underlying capsaicin-induced apoptosis are cell type dependent: capsaicin induces apoptosis in sensory neurons by increasing calcium influx and does so by activating vanil¬loid receptors in some transformed cells [7]. In human colon cancer cells, capsaicin triggers apoptosis through the inhibi¬tion of plasma membrane NADH-oxidoreductase activity and/or NADH:

coenzyme Q oxidoreductase in the mito¬chondrial electron transport system, generating reactive ox¬ygen species [8]. Moreover, capsaicin was found to be as¬sociated with PPARγ during the regulation of cell growth and apoptotic cell death in breast or colon cancer cells. Over the past decade, there is continuous increase in Hepatocellular carcinoma (HCC) in the world as the most common malignant diseases [9].

Materials and Methods

Cell line and reagents

HepG2 Cells were obtained from American Type Culture Collection (ATCC, USA) and cultured in a 37 °C incubator with 5% CO_2 according to ATCC protocols. Capsaicin was purchased from Enzo Life Science Company. Antibodies of PARP-1, Cyclin-D1, β-actin and all secondary antibodies were purchased from Santa Cruz Bio-technology (Santa Cruz, CA, USA).

Growth inhibition

Growth inhibition was assessed via an MTT assay. Briefly, Hep G2 cells were plated at a density of 30,000 cells/well on 24-well plate. After overnight growth, the cells were treated with

various concentrations of capsaicin (10-250µM) for 24h. 10µL of the 12mM MTT stock solution (Loba Chemie Pvt. Laboratory Reagents, India.) was added to each well. A negative control of 10 µL of the prepared MTT stock solution was added to 100µL of medium alone as a control. Cells were incubated at 37°C for 4 hours. 100µL of the prepared SDS-HCl solution were added to each well and mixed thoroughly using the pipette. The micro plate was incubated at 37°C for 18 hours in a humidified chamber. Each sample was mixed again using a pipette, the absorbance was read at 570nm by using a microplate ELISA reader and IC50 was determined.

Cell Morphological changes of HepG2 cell line

The morphological changes of cells were examined before and after the treatment with capsaicin drug (25-250) µM to test its effect on the morphology of HepG2 Cell line. Equal numbers of cells/well were seeded onto 12-well plates with appropriate different concentration of capsaicin drug and incubated for 48 hour. Cells were washed with PBS and fixed with 10% formalin buffer. Cells were viewed by an inverted microscope with magnification 200-X. Digital images were acquired with Codac microscopic digital camera.

DNA fragmentation

Cells were cultured and then treated with different concentration of capsaicin (50-250) µM for 3 days. Adherent cells were detached by 1ml Trypsin/EDTA. Cells were centrifuged for 5 minutes at 15000 rpm and supernatant was discarded and re-suspended in (1-10)ml ice cold PBS. Cells were centrifuged for 5 minutes at 15000rpm, supernatant was discarded. Cells were re-suspended in 1 volume digestion buffer (10 mM EDTA, 50mM Tris-HCl, pH 8.0, 0.5% SDS, 0.5mg/mL proteinase K). For 3×10^7 cells, 0.3ml digestion buffer was used. Samples were incubated, being shacked in tightly capped tubes, 18 hours at 50°C. Samples were extracted with an equal volume of Phenol / Chloroform/ Iso-amyl alcohol, centrifuged for 10 minutes at 15000rpm. The top layer (aqueous) was transferred to a new tube. ½ of the volume of 7.5M ammonium acetate was added. Double of the volume of 100% ethanol was added, centrifuged for 2 minutes at 15000 rpm. Organic solvents were removed by dialysis against 100 volume TE buffer for 24 hours. Samples were washed with 70 % ethanol, left in air to dry and re-suspended in Tris-EDTA buffer at ~1mg/ml prior to electrophoresis on a 1.2 % agarose gel con¬taining ethidium bromide.

Enzyme linked immunosorbent apoptosis assay

Cells were seeded at a density of 2×10^4/ well in a 96 well plate and incubated for 24 hours. Media was changed to media containing the tested drug (add your drug conc µM). Cells then incubated for extra 24 hours. An ELISA assay was performed, using Cell Death Detection ELISAPLUS kit (Roche-Applied Science, Indianapolis, USA) that measures histone release from fragmented DNA in apoptosing cells. Briefly, cells were lysed with 200-µL lysis buffer for 30 min at room temperature.

The lysate was centrifuged for 10min and 20 µL of collected supernatant was incubated with anti-histone biotin and anti-DNA peroxidase at room temperature for 2h. After washing with incubation buffer three times, 100µL of substrate solution (2,2`-azino-di(3-ethylbenzthiazolin-sulphuric acid) was added well and incubated for 15-20min at room temperature. The absorbance was measured using an ELISA reader (Spectra Max Plus) at 405nm. Each assay was done in triplicate and standard deviation determined).

Western blotting

HepG2cells were treated with (50-250) µM capsaicin for 24 hours. The cell culture dishes were placed in ice and the cells were washed with ice-cold PBS. The PBS was drained, and then lysed by the addition of 300µL of ice-cold lysis buffer (150mM Sodium chloride, 1.0%Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH (8.0). Just prior to use add: 10mM beta-mercaptoethanol, 10µg/ml PMSF, 100mM sodium orthovanadate and 15µ/ml Triton X-100). Adherent cells were scraped off thedish using a cold plastic cell scraper. The supernatant from each lysate was collected into new tubes after centrifugation at 20000g for 15 minutes at 4°C. Total protein concentrations were measured using the BCA™ protein assay kit (Pierce, Rock¬ford, IL, USA).Proteins were separated by 10% SDS-PAGE and electrically transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). After blocking in 5% BSA in TBS, the membranes were probed with specific primary antibodies overnight at 4°C, washed three times with TBS-Tween 20, and then incubated with HRP-conjugated secondary antibodies at room temperature for 1h. Then the membranes were washed with TBS-Tween 20 and the protein bands were visualized using 3,3',5,5' tetramethylbenzidine (TMB) is the traditional used kits. Densitometry of bands was performed using Image J analysis software (NIH).

Immunocytochemistry

HepG2 Cells were cultured and then treated with different concentration of capsaicin (150 and 200) µM for 1 day. Adherent cells were detached by 1ml Trypsin/EDTA. Cells were centrifuged for 5 minutes at 1000rpm and supernatant was discarded and washed with 1ml ice cold PBS then transferred to new eppendurf tube and centrifuged again at 1000rpm and supernatant was discarded. About 200µl collagen (Collagen I, bovine: sc-29009 supplied as 30mg in 0.012N HCl at a concentration of 2.9mg/ml.) was added to each eppendurf tube then cooled at 1ºC for 30 minutes to be solidified then transferred to fridge till to be sectioned. Then fixed in 10% buffered formalin overnight, transferred to 70% ethanol, embedded in paraffin, and sectioned at 5µm. Slides were dehydrated, put in a warm sodium citrate (10mM) in the microwave for 20 minutes then left to cool. Slides were incubated in 3% H_2O_2 for 15 minutes to reduce non specific background then blocked with 100-400µl blocking solution for 1 hour.
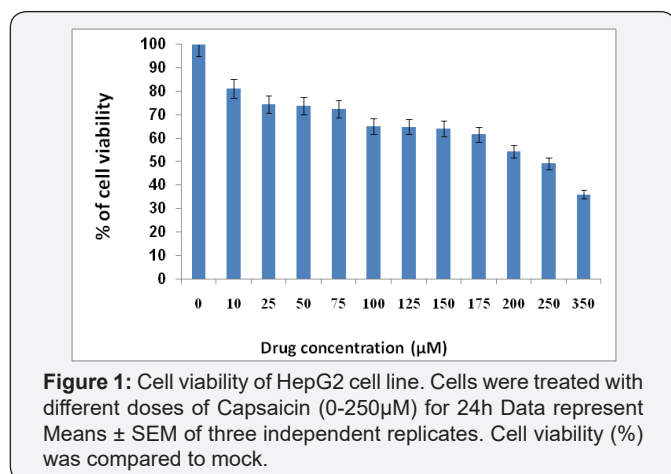
Slides were then incubated with anti-cyclin D1 antibody (1:1000 dilution) overnight and then exposed to the secondary antibody (Biotinylated Goat Anti- polyvalent secondary antibody was applied to the slides and incubated for 10 minutes at room temperature at 1:1000 dilution) from Santa Cruz. The slides were washed four times in PBS buffer. Streptavidin -HRP Peroxidase was applied to the slides and incubated for 10 minutes at room temperature and the slides were rinsed 4 times in PBS buffer. About 100μl DAB or suitable substrate was added to each section and incubated for 15 minutes. As soon as the sections were stained well, slides were immersed in dH$_2$O. The slides were incubated into Hematoxylin for 5-10 minutes and then washed under tap water for 10 minutes and dehydrated by being put in Ethanol 70%, Ethanol 95%, Ethanol 100%, Xylene I and Xylene II for 5 minutes for each. The tissue were stabilized by mounting medium (Canda Palsm), covered by slide cover and examined by microscope.

Statistical analysis: All the statistical analysis was performed by SPSS software (version 14.0). The experiments were performed in triplicate. All the quantitative data are expressed as mean values ± standard deviation, the significant differences between two groups was assessed by a Post-Hoc comparison. A probability value of $P < 0.05$ was considered to represent a statistically significant difference [10].

Results

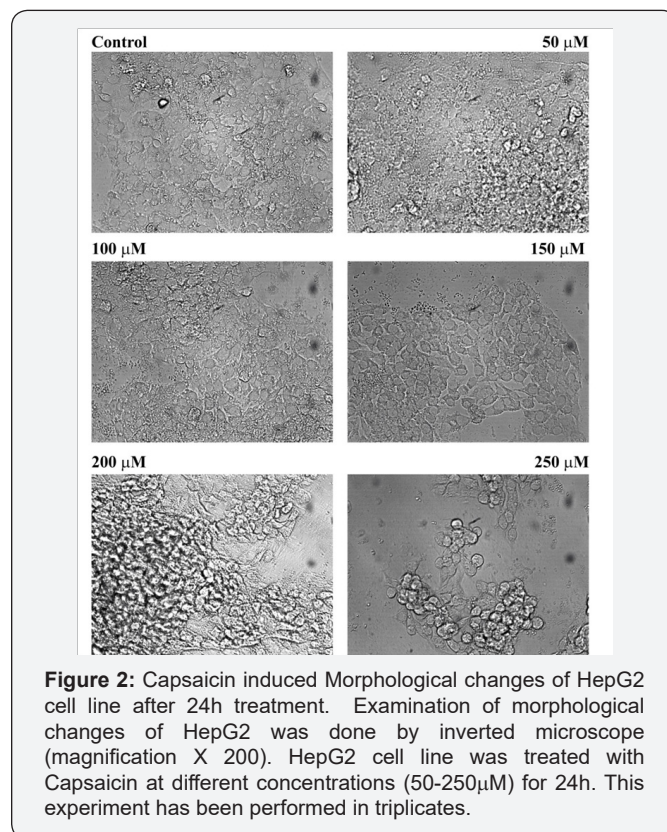### Effect of capsaicin on cell viability of HepG2 cell line

As shown in Figure 1, Capsaicin has significant ($p < 0.001$) inhibitory effect on HepG2 human liver cell line after treatment with 25-250μM for 24h. Collectively Capsaicin significantly decreased the percentage of viable cells in a dose-dependent manner.
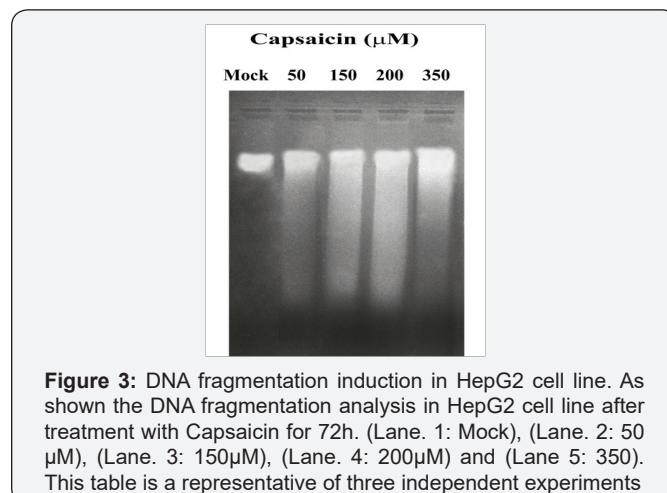

Figure 1: Cell viability of HepG2 cell line. Cells were treated with different doses of Capsaicin (0-250μM) for 24h Data represent Means ± SEM of three independent replicates. Cell viability (%) was compared to mock.

Capsaicin induced morphological changes of HepG2 cell line

Microscopic examination of the treated cells showed that the treatment with 150μM, 200μM and 250μM Capsaicin resulted in dramatic morphological changes in HepG2 cell line after 24h.

We observed morphological changes of cells from epithelial-like shape to spindle and rounded shape. The monolayer cells become rounded up, lost contact with neighboring cells and detached from culture plates. Our data suggested that the morphological changes of HepG2 cell line started after treatment with 150μM. All the treated cells showed cytoplasmic condensation, shrinkage, tendency to float in the medium and reduction in size after Capsaicin treatment for 24h compared to mock. Taken together Capsaicin induced morphological changes in a dose dependent manner (Figure 2).

Figure 2: Capsaicin induced Morphological changes of HepG2 cell line after 24h treatment. Examination of morphological changes of HepG2 was done by inverted microscope (magnification X 200). HepG2 cell line was treated with Capsaicin at different concentrations (50-250μM) for 24h. This experiment has been performed in triplicates.

DNA fragmentation induction in HepG2 cell line

Figure 3: DNA fragmentation induction in HepG2 cell line. As shown the DNA fragmentation analysis in HepG2 cell line after treatment with Capsaicin for 72h. (Lane. 1: Mock), (Lane. 2: 50 μM), (Lane. 3: 150μM), (Lane. 4: 200μM) and (Lane 5: 350). This table is a representative of three independent experiments

One of the biochemical features of apoptosis is the fragmentation of genomic DNA. For analysis of DNA fragmentation

by agarose gel electrophoresis, total DNA was extracted after treatment of the cells with 50 ~ 350 μM of Capsaicin for 72h. Capsaicin was found to induce inter-nucleosomal degradation of DNA in all treatment concentrations, resulting in ladder-shaped nucleosomal DNA fragments compared to mock as shown in Figure 3.

Capsaicin induced apoptosis in HepG2cells by increasing caspase-3 activity and histone release

We selected this time point to determine whether induction of apoptosis in HepG2cells treated with Capsaicin was associated with the activation of caspase-3 and histone release. After the HepG2 cells had been treated with or without various concentrations of Capsaicin for 24h, the cells were harvested and caspase-3 activity was analyzed by ELISA. The data indicated that Capsaicin induced caspase-3 activity in a dose-dependent manner (Table 1) (Figure 4). Our results revealed that caspase-3 activity and histone release, as a mark of apoptosis induction, were significantly increased after HepG2 cells were treated with 50, 100, 150, 200 and 350 μM Capsaicin as compared to the control group ($P < 0.001$).
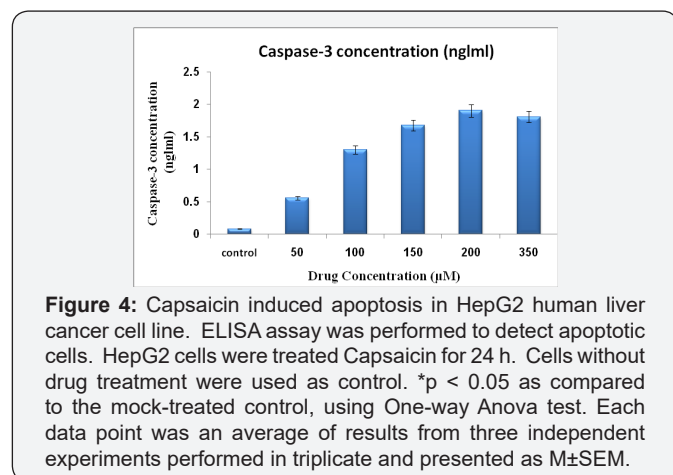
Figure 4: Capsaicin induced apoptosis in HepG2 human liver cancer cell line. ELISA assay was performed to detect apoptotic cells. HepG2 cells were treated Capsaicin for 24 h. Cells without drug treatment were used as control. *p < 0.05 as compared to the mock-treated control, using One-way Anova test. Each data point was an average of results from three independent experiments performed in triplicate and presented as M±SEM.

Table 1: Induction of apoptosis in HepG2cells in response to Capsaicin treatment.

| Capsaicin Concentration	Caspase-3 conc (ng/ml) Means ± SEM
Control	0.0814 ± 0.001
50	0.558± 0.001**
100	1.301± 0.001**
150	1.677± 0.001**
200	1.903± 0.001**
350	1.81± 0.001**

*P< 0.05, **P< 0.001 as compared to control group.

Capsaicin induced PARP-1 cleavage and cell cycle arrest by down-regulation of cyclin D1 inHepG2 cell line

Next, we studied capsaicin's activity on cell cycle and apoptosis. Our data showed that PARP-1 protein expression underwent cleavage due to Capsaicin treatment. PARP-1 significantly decreased in dose dependant manner, moreover, the cleavage of PARP-1 is considered to be an important marker for detection of apoptosis (Figure 5). Also, we examined the protein expression level of cyclin D1, our data revealed that Capsaicin was found to down-regulate expression of cyclin D1 in a dose-dependent manner (Figure 6).
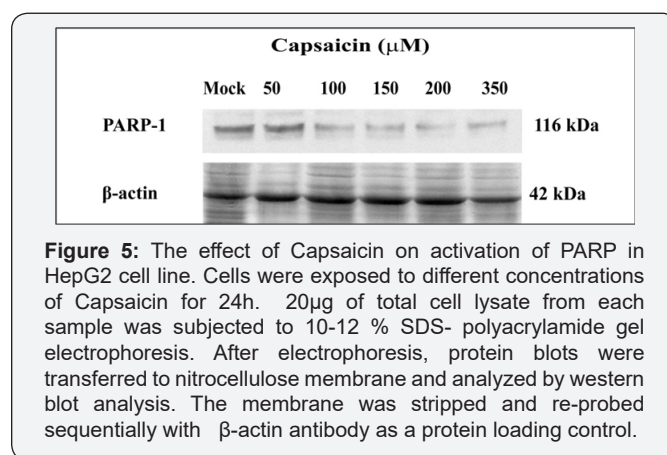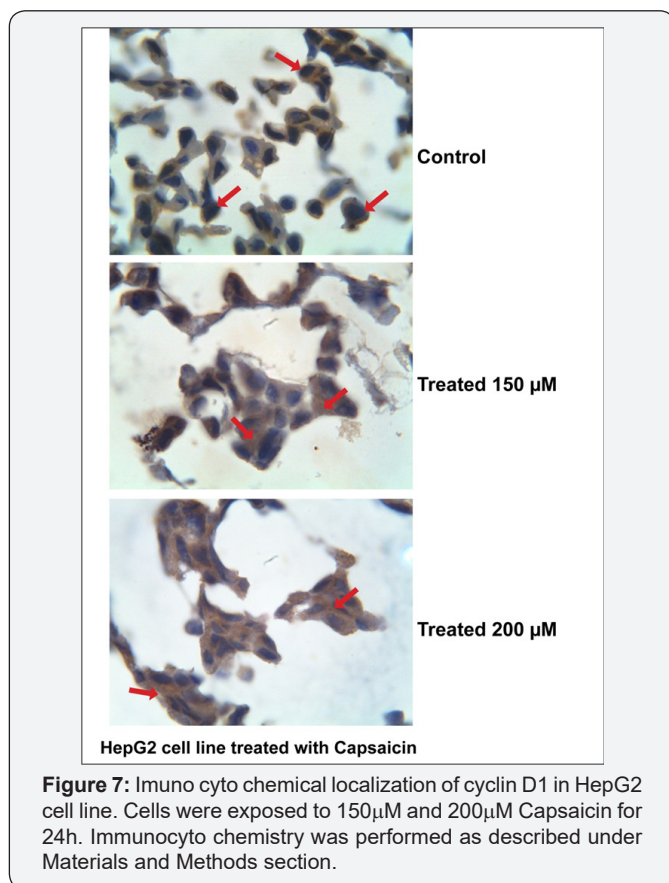

Figure 5: The effect of Capsaicin on activation of PARP in HepG2 cell line. Cells were exposed to different concentrations of Capsaicin for 24h. 20μg of total cell lysate from each sample was subjected to 10-12 % SDS- polyacrylamide gel electrophoresis. After electrophoresis, protein blots were transferred to nitrocellulose membrane and analyzed by western blot analysis. The membrane was stripped and re-probed sequentially with β-actin antibody as a protein loading control.
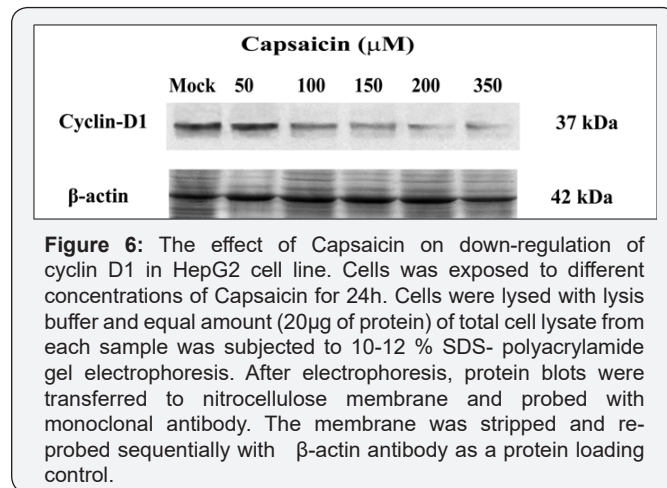
Figure 6: The effect of Capsaicin on down-regulation of cyclin D1 in HepG2 cell line. Cells was exposed to different concentrations of Capsaicin for 24h. Cells were lysed with lysis buffer and equal amount (20μg of protein) of total cell lysate from each sample was subjected to 10-12 % SDS- polyacrylamide gel electrophoresis. After electrophoresis, protein blots were transferred to nitrocellulose membrane and probed with monoclonal antibody. The membrane was stripped and re-probed sequentially with β-actin antibody as a protein loading control.

To confirm the down-regulated of tested cyclin D1 expression after Capsaicin treatment, we determined cyclin D1 localization and expression in nuclei of HepG2 cell line by immunocytochemistry assay (ICC), using specific anti- cyclin D1 monoclonal antibody. Nuclear expression of cyclin D1 was decreased after treatment with 150 ~200μM Capsaicin for 24h and cyclin D1 expression was increased in the cytoplasm of treated cells compared to mock (compare the brown staing of cyclin D1 with the counter staining blue color of the nuclei) as shown in Figure 7.

Figure 7: Imuno cyto chemical localization of cyclin D1 in HepG2 cell line. Cells were exposed to 150µM and 200µM Capsaicin for 24h. Immunocyto chemistry was performed as described under Materials and Methods section.

Discussion

Capsaicin acts as a carcinogen, co-carcinogen, or anti-carcinogen is controversial. Nonetheless, accumulating findings indicate that capsaicin possesses anti-cancer properties in various cancer celllines [11]. The goal of our study was to explore the mechanism underlying capsaicin-induced cell death inhuman HepG2 cancer cells. Here, we report that treatment of HepG2 cells with capsaicin inhibits their proliferation/viability, halts cell cycle progression, and induces apoptosis through increasing PARP-1 cleavage and induce cell cycle arrest by decreasing the expression of cyclin D1. HepG2 liver cancer cell line is widely used due to its relatively high steady-state functioning of the free radical production and antioxidant defenses; therefore, variations of responses at different conditions are more easily detected [12]. To investigate the effect of capsaicin on cell viability of HepG2 cells. Cells were treated with or without different concentrations of capsaicin. Our data found that capsaicin significantly decreased in cell viability of treated HepG2 cells using MTT assay. The proliferation of HepG2 cells showed strong inhibition by capsaicin at 150µM, 200µM and 250 µM, indicating anti-proliferation activity of capsaicin on HepG2 cell line and raising the possibility that capsaicin might be a potential chemo- preventive or therapeutic agent. Those effective doses showed no cytotoxic effects on different cell lines [4]. Our results are in agreement with who stated that treatment of human colon cancer cells HCT116 with capsaicin showed that

at low concentration (0-40µM), capsaicin had shown little effect on the growth inhibition, but at high concentration (80-160µM), long-term (48-72h) treatment with capsaicin substantially inhibited cell proliferation [13]. Amruthra et al. revealed that HepG2 cells showed reduction in the cell viability through MMT assay due to capsaicin exposure in a dose-dependent manner [14].

Apoptosis is an important feature of epithelial tissues with a high turnover and serves to balance the rate of new cell production. Death by apoptosis eliminates specific cells without extensive tissue damage, playing a pivotal role during regeneration and elimination of tumor cells [15]. Tumor cells are known to be resistant to apoptotic stimuli by several mechanisms. Tumors overexpress anti-apoptotic proteins such Bcl-2 and have decreased expression of pro-apoptotic proteins such as Bax or BH3, have impaired signals from death receptors, or activated nuclear factor NF-κB which prevents activation of caspase-mediated cleavage [16]. Large numbers of previous experiments have consolidated capsaicin's activity in cancer prevention, and moreover, it was shown that capsaicin induced apoptosis played an important role in its antitumor activity in a variety of cancer cell lines. The capacity of capsaicin to suppress the growth of cancer cells is primarily mediated through induction of apoptosis. Additionally, the activities associated with capsaicin-induced anti-cancer effects include the arrest of cell cycle progression, regulation of transcription factor expression, and suppression of growth signal transduction pathways [17]. Our study reported that capsaicin induced apoptosis in human HepG2 cells, demonstrated by DNA fragmentation induction and morphological changes of apoptotic cell death characteristics.

Our data revealed that capsaicin induce cell death in a dose-dependent manner in HepG2 cell line. In addition, microscopic examination of the treated cells showed that treatment with capsaicin for 24h resulted in dramatic morphological changes with the following features: monolayer cells become rounded up, shrinkage, with cytoplasmic condensation, lost contact with neighboring cells, nuclear condensation, and tendency to float in the medium. This was consistent with partially DNA fragmentation data.

The fragments were in oligonucleosome size, which could be approve of apoptotic induction by capsaicin treatment in HepG2 cells. Our results are in agreement with Cho et al. [5] stated that MG63 human osteosarcoma cells were cultured for 24h with different concentrations of capsaicin, the cells exhibited the morphological features of apoptosis when treated with 150µM capsaicin for 24h. These morphological changes of the cells represent the apoptotic cell death [18]. Also, Kim stated that A375 human melanoma cells treated with capsaicin induced DNA ladder formation [19]. PARP-1 is a nuclear enzyme, the critical role of PARP1 in DNA repair, PARP1 is involved in the repair of modified bases, ssDB and dsDB, blocking the ADP-ribosylation activity with small molecules, can achieve synthetic lethality

with DNA damaging agents in the treatment of cancer [20]. Besides DNA repair, the importance of PARP1 as a transcriptional regulator is also well established. As an enzyme, PARP1 acts on chromatin remodeling complexes to control DNA accessibility for RNA polymerase. PARP1 also functions as a transcription factor by binding an octamer motif in promoter elements to regulate gene expression [21]. During apoptosis, cleavage of PARP-1 in fragments of 89 and 24 kDa has become a useful hallmark of this type of cell death. This cleavage is well studied and is generated by the caspases 3 and 7, proteases activated during apoptosis [22]. Here, we investigated the possibility to PARP-1 expression by capsaicin treatment. Protein extracts were prepared from treated cells and analyzed by immuno blotting, using an antibody specific for PARP-1.

Our data showed that expression level of PARP-1 significantly decreased after treatment of HepG2 cell line with different concentrations of capsaicin for 24h compared to mock cells, indicating that PARP-1 underwent cleavage, thereby confirming apoptosis. The appearance of cleaved PARP fragment indicates that the DNA repair system in the cell has been damaged. Our results are in line with who found that treatment of KB cancer cells with capsaicin induced disruption of the mitochondrial membrane potential as well as activation of caspase 9, 3 and poly-(ADP-ribose)polymerase in KB cells [13]. Also, Jin et al. reported that capsaicin had a profound anti-proliferative effect on human colon cancer cells via inducing cell cycle G0/G1 phase arrest and apoptosis, which was associated with an increase of p21, Bax and cleaved PARP [14]. Zhang et al. indicated that capsaicin induced apoptosis in human pancreatic cancer cells is initiated by the generation of ROS, which was followed by increased protein expression of Bax, disruption of the mitochondrial membrane potential and release of cytochrome c and AIF into the cytosol leading to the activation of caspase 9/3cascade. Mitochondria play a pivotal role in the signal transduction of apoptosis. Disruption of mitochondrial membrane potential results in the modulation of Bcl-2family of pro- and anti-apoptotic proteins and release of cytochrome c and/or apoptosis inducing factor (AIF) from the mitochondria into the cytosol. Once cytochrome cis released into the cytosol, it forms an apoptosome withApaf-1 and procaspase-9. This causes the activation ofcaspase-9, which further activates caspase-3 and PARP as evident by their cleavage. Results suggest the activation of intrinsic mitochondrial death pathway by capsaicin [23].

We additionally examined the effect of capsaicin on cell cycle progression; we examined cyclic D1 expression by capsaicin treatment. Protein extracts were prepared from treated cells and analyzed by immunoblotting, using an antibody specific for cyclin D1. Our data showed that expression level of cyclin D1significantly decreased after treatment of HepG2 cell line with different concentrations of capsaicin for 24h compared to mock cells, indicating that cyclin D1 down-regulated due to capsaicin treatment. The cyclin D1 is required for the activity of cyclin-dependent kinase 4, which phosphorylates Rb, thus releasing E2F to mediate the G1to S transition, inturn leading to DNA synthesis and cell cycle progression [24]. Our results are in aggrement with Le et al. [25] revealed that capsaicin suppressed the cell cycle progression at the G1/S phase in FaDu cells by decreasing the expression of the regulators of cyclin B1 and D1, as well as cyclin-dependent protein kinases cdk-1, cdk-2 and cdk-4 [25]. Results revealed that antiproliferative effect of capsaicin correlateswith G1arrest of endothelial cells. It is possible that this effect is mediated through either inhibition of cyclin D1 expression or induction of a cyclin-dependent kinase inhibitor, p21. Our results of western blotting are confirmed by cyclin D1 localization in the nucleus of HepG2 cell line. Immunocytochemistry assay (ICC) was performed using ant-cyclin D1 antibody. Results revealed nuclear expression of cyclin D1 showed weak expression after treatment with 150µM and 200µM capsaicin for 24h.

Conclusion

It is suggested that capsaicin inhibit the growth and proliferation of HepG2 cells by increasing nuclear condensation, nuclear DNA fragmentation, down regulation of cyclin-D1, increased cleaved PARP (p85) levels and increased histone release. Capsaicin could be developed as a promising chemopreventive natural supplement for liver cancer.

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