The Molecular Effect of Curcumin Subsidized by Radiation on HepG2 cell lines

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ABSTRACT

Cancer is a major health problem around the world. Cancer treatment depends on radiation therapy sometimes in combination with chemotherapy or hormonal therapy. The treatment of cancer with radiation and chemotherapy cause several unwanted harmful side effects. Turmeric (Curcuma longa), is a type of herb widely used as a spice. Recent researches have shown that curcumin, the active component of turmeric possesses anticancer properties. This study aimed to evaluate the effect of γ-radiation and curcumin each of them alone or combined on HepG2 cell line proliferation, p53, Her2/neu, and MMP9 gene expression, and total antioxidant capacity. HepG2 cells were exposed to γ-radiation (R), or treated with different concentrations of curcumin alone or combined with each other. The cell viability was examined by MTT assay. Gene expression of p53, Her2/neu, and MMP9 were evaluated by using real-time QPCR. The total antioxidant capacity (TAC) was also measured. Curcumin or R alone or combined decreased cell viability and induced cell death in a dose-and time-dependent manner when compared to untreated group. Curcumin and R induced p53 gene expression, while reduced Her2/neu, and MMP9 expression. Furthermore, the TAC was decreased in the treated groups compared to the untreated. Conclusion: Curcumin can sensitize HepG2 cells to radiation and would potentiate the effect of radiotherapy in cancer.

KEYWORDS
Curcumin; Hepatocellular Carcinoma; Gamma-Radiation; p53; Her2/neu; MMP9.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world, leading to more than 500,000 deaths every year (Fung and Lok, 2005). Most HCC cases (> 80%) occur in either sub-Saharan Africa or Eastern Asia. HCC cases in China alone account for more than 40% of all cases in the world (El-Serag and Rudolph, 2007). Treatment of HCC is still a great challenge for oncologists because most HCC patients are diagnosed at its advanced stage with metastasis (Rivenbark and Coleman, 2007). In Egypt, HCC is ranked as the 6th most common cancer in women and second in men (Ziada et al., 2016). Cancer is a genetically rooted disease, some genes were found to play critical roles in HCC (Badra et al., 2010).

p53 gene is the tumor suppressor gene most frequently mutated in human tumors and are the most common in HCC tumor (Li et al., 2015). p53 has been described as “the guardian of the genome”, referring to its role in conserving stability by preventing genome mutation (Li et al., 2015). The cellular concentration of p53 must be regulated. While it can suppress tumors, high level of p53 may accelerate the aging process by excessive apoptosis. The major regulator of p53 is Mdm2 (Mdm2 is an important negative regulator of the p53 tumor suppressor), which can trigger the degradation of p53 by the ubiquitin system (Wade et al., 2006).

Her2/neu is a member of the human epidermal growth factor receptor (HER/EGFR/ERBB) family. Amplification or over-expression of this oncogene has been shown to play an important role in the development and progression of certain aggressive types of breast cancer (Mitri et al., 2012). The over-expression of the Her2/neu gene occurs in approximately 15-30% of breast cancers (Mitri et al., 2012). It is strongly associated with increased disease recurrence and a poor prognosis (Tan and Yu 2007).

Matrix metalloproteinase 9 (MMP9) is involved in several human cancer. MMP9 can cause degradation of collagen of the extracellular matrix (Apoorv et al., 2015). Metalloproteinases (MMPs) are a family of zinc-dependent enzymes. These enzymes regulate many physiological events such as cell motility, and cell differentiation in tumour progression (Estella et al., 2012). MMP9 also can regulate embryonic development and wound healing (Vandooren et al., 2013).

Radiotherapy is used for cancer treatment because it efficiently eradicate cancer cells with minimal damages to the surroundings. Meanwhile, ionizing radiation cause biological alterations, which can cause cellular damage. The cellular damage may lead to arrest of cell division, modified cell that carries some damage in its molecular structure which cannot cause cell death, loss tissue function, or cell death (El-Naggar, 2009).

Curcumin, a polyphenol derived from the plant Curcuma longa, is considered a promising anticancer drug due to its efficient induction of proliferation arrest and apoptosis in a variety of tumor cells. Curcumin also exhibits pro-oxidant properties under certain conditions, such as high concentrations (e.g., 50 mM), which were found to promote reactive oxygen species (ROS) generation (Aggarwal et al., 2003), while a low concentration of curcumin (e.g., 10 mM) reduced ROS generation (Chan et al., 2003). Both the antioxidant and pro-oxidant activities of curcumin are considered to be involved in anticancer activity (Aggarwal et al., 2003).

MATERIAL AND METHODS:

Cell culture and reagents

HepG2 cells (Hepatocellular carcinoma, human) cell line provided by Dr. Ali M (Center of Scientific Excellence for Influenza Viruses, National Research Centre, Egypt), was cultured in Dulbecco’s Modified Eagles Medium (DMEM) (Lonza, Walkersville, MD,
USA), containing 10% fetal bovine serum (Gibco, Grand Island, NY), penicillin (100 units/ml, Gibco), and streptomycin (100 µg/ml, Gibco). Dimethyl sulfoxide (DMSO) was used as solvent. The cell cultures were incubated at Class II biological safety cabinet, Incubator, 37°C, 5% CO₂, and checked daily.

**Cell culture treatment:**

The cell culture was treated with curcumin purchased from Bio Basic, Canada INC.). Curcumin treatment was prepared by dissolving curcumin powder in dimethyl sulfoxide (DMSO) to the concentration of 10, 20, 40, 80, 160, 320 µM, and or Gamma irradiation which was performed at the National Center for Radiation Research and Technology (NCRRT), Atomic Energy Authority, Cairo, Egypt, using an Atomic Energy of Canada Limited Commercial products (AECL) Gamma Cell-40 (137Cs) biological irradiator. The plates were submitted to 2Gy and 5Gy in a single dose with a dose rate of ~ 0.758 rad/ Sec, and incubated at 37°C, 5% CO₂ for 24, 48, and 72h.

**Cell viability assay (MTT):**

Cell viability was assessed by the methyl thiazol tetrazolium bromide (MTT) assay as described previously (Mosmann, 1983). HepG2 cells were plated in a 96-well microtiter plate at a density of 5 × 10⁴ cells /well. The cells were treated with curcumin (10, 20, 40, 80, 160, and 320µM) alone or with γ-irradiation then incubated for 24, 48, and 72 h. After treatment, the cells were incubated with MTT solution (5 mg/ml) for 4 h at 37°C. The formazan crystals formed were dissolved in DMSO at 37°C for 1 h in the dark, and the absorbance was read at λmax 540 nm with 620 nm as reference wavelength by 96-well microtiter plates (Greiner Bio-One, Germany).

**RNA extraction:**

RNA was extracted from harvested cells (HepG2 cell) using SV total RNA isolation Promega products with quantification of RNA concentrations with no-dropper. cDNA was synthesized then according to the instructions of cDNA synthesis kit (Fermentus, Thermo Fisher Scientific Inc, UK). Polymerase Chain Reaction (PCR) amplifications for cDNAs were accomplished in Rotor Gene 2000 real-time fluorescence thermal cycler (Corbett Ltd., Australia) with a heated lid (105°C) based on the PCR programs. PCR conditions were 15 seconds (s) denaturation at 95°C, 30 s annealing at 55, 58 or 60°C for each gene and 1min of extension at 72°C for 45 cycles. Each cDNA fragment was amplified in duplicate for all target genes as well as GAPDH gene. All primers were purchased from (Jena Bioscience, Germany). Her2/neu and GAPDH primers were designed according to Cuadros et al. (2010), P53 (Li et al., 2015) and MMP9 (Estella et al., 2012).

For verifying the amplification specificity and distinguishing any artifacts from the specific amplicons, melting curves were generated by denaturing the PCR products by slowly increasing the temperature from (65-99°C) with the rate 0.1°C/sec). PCR products also were run on 1.5% agarose gel to confirm that correct molecular sizes were present.

**Estimation of Total Antioxidant Capacity (TAC):**

Total antioxidant capacity was determined by using a commercially available kit (Biodiagnostic, Egypt) according to Koracevic et al. (2001).

**Statistical analysis:**

All experiments were repeated three and four times. Analysis of data was performed using analysis of variance (One-Way ANOVA) processed by SPSS software (Statistical Package for Social Science), followed by Less significant difference (LSD) with P≤0.05 considered statistically significant.
RESULTS

Figure (1) shows the effect of curcumin with different concentrations on HepG2 cells at 24, 48, and 72 h. There was a significant decrease in cell viability of cells treated with Curcumin in a dose- and time-dependent manner. The mean values at 24h were 75.59%, 65.837%, 65.8%, 46.98%, 46.78% and 41.265% with the concentrations of 10, 20, 40, 80, 160 and 320 µM respectively. This effect increases slightly at 48 h the values were 71.91%, 58.88%, 55.98%, 46.33%, 34.46%, 33.78% in concentrations of 10, 20, 40, 80, 160 and 320 µM respectively. But the highest cytotoxicity of curcumin was at 72 h and 320 µM that was 11.46%.

In figure (2) the results show the cytotoxicity effect of 2Gy γ-irradiation alone or with previous concentrations of curcumin. In case of 2Gy alone there is a significant decrease in cell viability by the time, where at 24h the cell viability was 89.86%, at 48h was 86.96%, and 87.4% at 72h, but when 2Gy was combined with curcumin there was a significant decrease in cell viability especially at doses above 40 µM and in 72 h. The IC₅₀ (inhibitory concentration 50%) values at 72 h in 80 µM was 55.3%, in 160 µM was 47.09%, and in 320 µM was 46.685%.

Figure (3) illustrates the cell viability of 5Gy alone or after treatment with different concentrations of curcumin, which was significantly lower than in case of 2Gy, and 5Gy irradiation and recorded 57.015%, 14.113%, and 10.59% in 24, 48, and 72h respectively. The cell death was also dramatically increased in HepG2 cells exposed to 5Gy combined with curcumin also by the time and concentration. In 24 h IC₅₀ was in 160, and 320 µM 46.13%, and 46.04% respectively. The cell death increase significantly in 48 h, and 72 h with all curcumin concentrations, and recorded 5.53% with 320 µM at 72 h.

**Table (1)**: Primers sequences for amplified genes using real-time QPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Strand</th>
<th>Sequence 5’-3’</th>
<th>Product length (bp)</th>
<th>Anneling (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P53</td>
<td>Forward</td>
<td>5’-TCAACAAGATGTTTGGCAACTG-3’ MW: 1337</td>
<td>118</td>
<td>60 °C</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-ATGTGCTGTGACTGTTGTAGATG-3’ MW: 1454</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP9</td>
<td>Forward</td>
<td>5’-TGGGGGGCAACTCGGC-3’ MW: 634</td>
<td>224</td>
<td>58 °C</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-GGAATGATCTAAGCCGCCAG-3’ MW: 857</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Her2/neu</td>
<td>Forward</td>
<td>5’-CCAGGACCTGCTGAACCTGGT-3’ MW: 2631</td>
<td>72</td>
<td>55 °C</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-TGTACCGAGCCGCACATCC-3’ MW: 2702</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>5’-GAAGATGGTGATGGGGATTTC-3’ MW 194</td>
<td>226</td>
<td>55 °C</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-GAAGGTGAAGGTTCGGAGTC-3’ MW 419</td>
<td></td>
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was 47.09%, and in 320 µM was 46.685%.

Fig. (1): The effect of curcumin on HepG2 Cell cytotoxicity (MTT assay IC50) (± SD), P <0.05.
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Figure (4) illustrates the effect of curcumin (10, 20, 40, and 80µM) and γ-radiation (5Gy) each of them alone or combined with each other on p53 expression levels. 5Gy γ-irradiation alone increases the expression of p53 gene directly by the time. The highest expression was at 72h and the mean was 2.64 ± 0.38 fold relative to control. In the same direction, in case of treatment with curcumin alone there is a direct relation between expression of p53 gene time and concentrations. The largest increase was in 72h and 80µM and the mean value was 5.2±5.17. The combination of curcumin and 5Gy γ-irradiation revealed a significant increase in p53 expression, in case of 80µM concentration and 72h.

Her2/neu gene expression Figure (5) was down regulated by treatments of curcumin and this effect increase by concentrations and time. The expression after exposure to 5Gy alone was downregulated to 0.57±0.18, 0.52±0.16, 0.328±0.09 fold change in 24, 48, 72h respectively. In combined treatment of curcumin and 5Gy the reduction increase and the highest reduction was 0.078±0.023 in 80µM concentration and 72h.

MMP9 gene expression decreased in a dose- and time-dependent manner when treated with curcumin, and/or 5Gy γ-radiation figure (6). The expressions after exposure to 5Gy were 0.69 ± 0.003, 0.44 ± 0.05, 0.36 ± 0.036 fold decrease at 24, 48, and 72h respectively compared to control group (HepG2 cell not treated). In case of curcumin alone MMP9 gene expression was down regulated at 48, and 72h more than at 24h. With high concentrations the effects in increase. At 48h the expressions were 0.312 ± 0.07, 0.22 ± 0.05 and 0.208 ± 0.045 fold decrease with 20, 40 and 80 µM respectively. At 72h the effects were the lowest. The values were 0.44 ± 0.02, 0.179 ±
0.013, 0.179 ± 0.02 and 0.138 ± 0.005 fold decrease with 10, 20, 40 and 80 µM respectively.

In combination between 5Gy and curcumin the expressions of MMP9 were dramatically down regulated with all curcumin concentrations at 72h, and was 0.059 ± 0.002 and 0.0236 ± 0.001 fold decrease with 40, 80 µM respectively.

DISCUSSION

The inhibitory effects were increased in high doses of curcumin treatment and by time especially above 40µM and after 72h of incubation. These results are in agreement with the results of Bharti et al. (2003) and Li et al. (2015). The previous studies referred that the proliferation and survival of almost types of tumor cells has been inhibited by curcumin. They suggest that the action of curcumin-induced cell death is by the inhibition of growth and proliferation of cancer cells, and the activation of cell death pathways.

2Gy γ-radiation induces cell toxicity on HepG2 cell lines, but the effect was lower than for 5Gy, which may be attributed to the mechanism of cell recovery due to the low dose. Furthermore, the results revealed that 5Gy γ-radiation dose cytotoxicity is time dependent. These results are in agreement with Chung et al. (2015) and Girdhani et al. (2009), who reported that γ-radiation can induce cell death in a dose-and time-dependent manner. Moreover, Chung et al. (2015) stated that radiation-induced apoptosis
is due to the generation of reactive oxygen species (ROS) in tumor cells, which causes oxidative damage to the cells.

**The effect of curcumin combined with γ-radiations:**

The cell viability was decreased significantly when treated with curcumin then exposed to γ radiations than in case of radiation only. This effect was in a dose-and time-dependent manner. These results are in agreement with Girdhani et al. (2009), who found that the combination treatment of cancer cells with curcumin followed by radiation significantly enhanced the cell death, and this effect was increased with dose and time of incubation.

**The effect of curcumin and/or radiation on gene expression:**

The tumor suppressor gene p53 becomes activated in response to stress, such as DNA damage, oxidative stress, but in normal condition p53 was at low level. This study demonstrated that HepG2 cells when feeding with curcumin the viability was decreased in a dose- and time-dependent manner, which was agreed with Bharti et al. (2003) and Li et al. (2015). In case of curcumin alone with 10, 20, 40, and 80 µM concentrations, p53 gene expression increase with increase of curcumin concentration and time of treatment. P53 expression at 72h was 5.2 ± 0.38 fold relative to control with 80µM curcumin treatment. This result is in agreement with Li et al., 2015 and Gallardo & Calaf, 2016. Who reported that curcumin down regulate MDM2 which is considered the negative inhibitor of p53 expression, and this lead to increase in p53 gene expression. The p53 expression when exposed to 5Gy γ-radiation was increased in a dose and time dependent manner which is in agreement with Veeraraghavan et al. (2010). This effect was increased when 5Gy was combined with curcumin by time and concentrations (Veeraraghavan et al., 2010).

Her2/neu gene expressions decreased on HepG2 cells when treated with curcumin and this effect increased by the increase in dose and time. These results agree with Lai et al. (2012), who reported that cancer cells treated with curcumin, the phosphorylation of Akt and MAPK was decreased, combined with the down regulation of HER-2 oncoprotein, in a dose-dependent manner (Lai et al., 2012). The MMP9 decreased in a dose and time dependent manner when treated with curcumin leading to inhibitory effects on HepG2 cell adhesion and invasion. These results agree with Tong et al. (2016) who indicated that curcumin probably can inhibit MMP9 transcription by suppressing NF-κB DNA binding activity to MMP9 promoter region. So, the anti-metastatic effects of curcumin in cancer cells may be mediated by inhibition of the NF-κB signaling pathway (Killeen et al., 2009 and Tong et al., 2016).

**Total antioxidant capacity (TAC):**

Reactive oxygen species (ROS) play an important role in many clinical diseases including cancer (Koren et al., 2008). The antioxidants play a crucial role in the defence against oxidative stress. TAC includes enzymatic and non-enzymatic constituents. Our results revealed that when HepG2 cells were treated with curcumin, this cause a decrease in TAC, which is in line with the results of Satson et al. (2017) who reported that there is a reduction of SOD activity (The main antioxidant enzyme) in D. discoideum strain after treatment by curcumin for 24 hours and this was accompanied by a decrease in the expression of sodA, sodB and sod2 mRNA and suggested that curcumin is not acting as an antioxidant in D. discoideum cells. In the same direction, previous studies suggest that curcumin can act as a pro-oxidant in some situations (Khan et al., 2012 and Aggeli et al., 2013). Swatson et al., 2017 try a variety of existing mutant strains with deleted or over-expressed genes in knowing regulatory/signaling pathways or genes involved in generating or scavenging ROS were tested for their response to curcumin. They explain the negative effect of curcumin on antioxidant enzymes through the relation
between curcumin and Protein Kinase A (PKA). The cAMP-depend protein Kinase A is known to regulate the activity of a wide number of proteins.

Moreover it has important regulatory functions in human, zebrafish, drosophila, yeast, and D. discoideum. Swatson et al., 2017 reported that cells with an inactive form of PKA (pkaCnull or pkaR-OE) showed increased resistance to curcumin than the parent strain and curcumin had little to no effect on catalase A and SOD activity in pkaC null cells suggesting that ROS may not be up-regulated as observed with the wild-type strain.

Radiation exposure induced generation of reactive oxygen species (ROS) on HepG2 cells, which consequently reduces the TAC in a time-dependent manner at 24 h, and 48 h, but at 72 h there was unexpected elevation which may be due to cell repair mechanism. This result agrees with (Nada 2013). When radiation was combined with curcumin the TAC was higher than in the case of radiation alone, but still lower than its respective value in the untreated group.

Conclusion

Curcumin can effect on viability, apoptosis, and invasion of cancer cell. Curcumin can sensitize HepG2 cells to radiation. Curcumin combined with radiation probably have therapeutic effect on cancer.

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The Molecular Effect of Curcumin Subsidized by Radiation on HepG2 cell lines


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التأثير الجزيئي للكركم مدعوم بالإشعاع على خلايا سرطان الكبد

تعتبر زيادة معدلات الاصابة بسرطان الكبد في بعض المناطق من مصر والعالم من المشكلات التي تحظى بالإهتمام على المستوى العالمي. وهذا قد يرجع إلى تقدم وسائل التشخيص على المستوى الطبي، وبالرغم من هذا التقدم في تقنيات التشخيص إلا أن هناك انحرافات في معدلات الوفيات حيث لا يتم اكتشاف المرض إلا في مراحل متاخرة. ونظراً لأن العلاجات الكيميائية والإشعاعيه لها أثار جانبية شديدة من الصعب تجنبها فإن ذلك أعطى أهميةً قصوى لاستخدام المواد الطبيعية ذات القدرة العالية مع معدلات الإصابة بالسرطان على رأسها البوليفين. وغذاً، المجموعة الحالية حيث قسمت إلى مجموعات ض Registered HepG2 cell line على تأثير الكركم على خلايا سرطان الكبد غير معالجة ومجملة تغذت على الكركم بتركيزات مختلفة ومجموعات تعرضت للإشعاع الجامى جراى ومعجوبة ومجملة تعرضت للإشعاع الجامى جراى ومعجوبة تحتوى الكركم مع جرعة 5 جراى وتدريب الكركم ذات العلاقة بالبتركم الأوروم في خلايا سرطان الكبد. 2

الكربون 10 و 20 و 40 و 80 ميكرومول بالإضافة إلى دراسة محتوى الخلايا المعالجة في كل المجموعات من مضادات الأكسدة. وربما كنت النتائج أن الكركم ادى إلى فقد الخلايا لحيويتها وخاصة مع التركيزات العالية 20 و 30 ميكرومول وخاصة بعد اليوم الثالث مقارنة بالمجموعة الضابطة. وربما أن الخلايا التي تعرضت للإشعاع وخاصة المعرضة لجرعة 5 جراى اظهرت فقدانها لحيويتها ولكن بنسبة اقل من المعالجة بالكركم. ومع ذلك يمكن الكركم والإشعاعcontinuous مع الجرعات العاليه تشير إلى فقد في حيوية الخلايا بعد 22 ساعة من تغذية ولكن المكانت الكركم الأكثر تأثيراً من الفعالية لجرعة 2 جراى واقل تأثيراً من المجموعات الفرصة له جراي وعلى مستوى التعبير الجيني الكركم والإشعاع Her2/neu MMP9 لادى إلى زيادة في التعبير الجيني لجين p53 ونقصاً في التعبير الجيني لكل من9 مقارناتً بالمجموعات الضابطة. وكذلك انخفضت نسبة مضادات الأكسدة في الخلايا في المجموعات المعالجة مقارنةً مع غير المعالجة. وفي النهاية يتضح أن الكركم يزيد من حساسية الخلايا السرطانية للإشعاع ويسهل ذلك زيادة في فاعلية العلاج بالإشعاع.

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