ASSESSING THE OXIDATIVE THERAPY OF CURCUMIN (IN-VITRO STUDY)

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ABSTRACT

Objectives: Cell structure changes due to oxidative stress, and the response of cells to this change depends on the amount of oxidative stress. Generally, severe oxidative stress causes cell death. This study evaluates the oxidative therapy of curcumin on Hep-2 cell line.

Materials and Methods: Laryngeal squamous cell carcinoma cell line (Hep-2) cells were treated with curcumin for 48 h. Then, cell viability was tested using MTT assay, and the amount of Cox-2 and ROS (reactive oxygen species) was measured by enzyme-linked immunosorbent assay (ELISA) assay; the amount of MDA was measured by colorimetric/fluorometric assay. Finally, slides were prepared for hematoxylin and eosin (H & E) staining for microscopy.

Results: The data revealed that curcumin had a cytotoxic effect on Hep-2-treated cells. Furthermore, curcumin downregulates Cox-2 and increases the accumulation of intracellular ROS and MDA levels compared with control untreated cells. Additionally, Hep-2-treated cells showed apoptosis.

Conclusions: Our findings suggest that curcumin can be an oxidative therapy for laryngeal squamous cell carcinoma and induce apoptosis. Also, curcumin has anti-inflammatory activity in Hep-2 cells as it can decrease Cox-2 levels in cells. Thus, curcumin is a potential antiproliferative and therapeutic agent.

KEYWORDS: Oxidative therapy; Curcumin; Cyclooxygenase-2; Malondialdehyde; Reactive oxygen species;

INTRODUCTION

Squamous cell carcinomas of the head and neck are the sixth most common cancer. They are developed from the epithelium of the mucosal lining of the oral cavity, pharynx, and larynx (Johnson et al., 2020), with about a 50% mortality rate (Chen et al., 2012).

Chronic inflammatory cells and their chemical mediators in the neoplastic environment play a role in neoplasm progression (Bonomi, Patsias, Posner, & Sikora, 2014). An important cancer hallmark is cancer-related inflammation (Colotta, Allavena, Sica, Garlanda, & Mantovani, 2009). However,

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inflammatory cells in the tumor environment can promote tumor cell multiplication, survival, and movement (Coussens & Werb, 2002).

The inflammatory mediator, cyclooxygenase-2 (Cox-2), and its product, Prostaglandin E2 (PGE2), are overexpressed in head and neck squamous cell carcinoma (HNSCC) (Buchanan, Wang, Bargiacchi, & DuBois, 2003; Cooper et al., 2004; Bonomi et al., 2014). PGE2 induces epithelial–mesenchymal transition (EMT) (Dohadwala et al., 2006), cell proliferation, movement, and angiogenesis (Buchanan et al., 2003; Cooper et al., 2004). Furthermore, an increasing level of Cox-2 in HNSCC is associated with poor prognosis and outcome (Chung et al., 2011; Wilson, Anderson, Murray, & Hughes, 2011).

Oxidative stress is defined as an excess of reactive oxygen species (ROS) due to an imbalance between ROS production and removal rates. Excess may be caused by ROS overproduction, the reduction of antioxidants that reduce ROS, or both conditions (McMichael, 2007). Malondialdehyde (MDA), an end product of lipid peroxidation, has been widely used as a biomarker of oxidative stress (Giera, Lingeman, & Niessen, 2012). In addition, it plays a significant role in toxicology associated with oxidative stress (Grotto et al., 2009).

Suitable ROS levels are required for physiological functions, such as tissue regeneration and wound healing (Datta et al., 2014). However, ROS can damage proteins, lipids, and nucleic acid to facilitate cell apoptosis (Schieber & Chandel, 2014). Consequently, scientists rely on ROS production to induce cell death in chemotherapy, radiotherapy, and phototherapy of cancer cells (Ma et al., 2017; Zhou, Song, Nie, & Chen, 2016). Therefore, a new method for managing malignancy, called oxidation therapy, is based on increasing the amount of ROS in cancer cells to initiate ROS-mediated cell death (Lin et al., 2018; B. Yang, Chen, & Shi, 2019).

Curcumin is considered an herbal medicine with anti-inflammatory, antioxidant, and anticancer activities (Zia, Farkhondeh, Pourbagher-Shahri, & Samarghandian, 2021). Phenolic groups in the chemical structure of curcumin are responsible for their activities through the potent donating property of hydrogen atoms (Amalraj, Pius, Gopi, & Gopi, 2017; Farkhondeh, Samarghandian, Pourbagher-Shahri, & Sedaghat, 2019). Additionally, curcumin can inhibit telomerase function (Zia et al., 2021).

Curcumin has been studied in multiple human carcinomas, including melanoma, head and neck, breast, colon, pancreatic, prostate, and ovarian cancers (Mukhopadhyay, Bueso-Ramos, Chatterjee, Pantazis, & Aggarwal, 2001; LoTempio et al., 2005; D. Wang et al., 2008). Additionally, epidemiological studies attribute the low incidence of colon cancer in India to the chemopreventive and antioxidant properties of diets rich in curcumin (Mohandas & Desai, 1999).

Therefore, this study assesses curcumin as an oxidative therapy for laryngeal squamous cell carcinoma by measuring ROS and MDA levels. Furthermore, the anti-inflammatory properties of curcumin on laryngeal squamous cell carcinoma by measuring Cox-2 levels were assessed.

MATERIAL AND METHODS

- **Cell line:** Head and neck squamous cell carcinoma (Hep-2) cells were obtained from VACSERA-EGYPT. They were bought from the "American Type Culture Collection."

- **Herbal medicine:** Curcumin was obtained from Alex Biotechnology Company.

- **MTT assay:**

Hep-2 cells were seeded in 96-well plates and treated with curcumin for 48 h. Then, 10-μL MTT solution was added to the wells and incubated at 37°C. Next, dimethyl sulfoxide (DMSO) was gently added to each well, and the number of viable cells in each well was recorded from its absorbance by spectrophotometry. Finally, the data were analyzed using Master Plex Reader Fit software system to obtain the IC50. (van de Loosdrecht, Beelen, Ossenkoppele, Broekhoven, & Langenhuijsen, 1994)

- **Human Cox-2 ELISA Kit**

All reagents, samples, and standards were prepared as instructed. First, 100 μL standard or sample was added to each well and incubated for 2.5 h at room temperature. Next, 100 μL of biotin antibody was prepared, added to each well, and incubated for 1 h at room temperature. Also, 100 μL of streptavidin solution was added and incubated for 45 min at room temperature; 100-μL One-Step Substrate Reagent was added to each well and incubated for 30 min at room temperature. Then, 50 μL stop solution was added to each well and read at 450 nm immediately. The standard curve was plotted on log-log to calculate results, with standard concentration on the x-axis and absorbance on the y-axis (Human COX2 ELISA Kit (ab267646)).

- **Human ROS ELISA Kit**

First, plates were washed twice before adding standard, sample, and control to the wells; then 100 μL standard or sample was added to each well for 90 min at 37°C. Next, 100 μL biotin-detection antibody working solution was added to each well for 60 min at 37°C, which was then aspirated and washed three times. Also, 100 μL HRP-streptavidin conjugate (SABC) working solution was added to each well, incubated for 30 min at 37°C, aspirated, and washed five times. Next, 90 μL TMB substrate was incubated for 15–30 min at 37°C, and 50 μL stop solution was added, then read at 450 nm immediately. To calculate results, the standard curve can be plotted as the relative OD450 of each standard solution (Y) vs. the respective concentrations of the standard solution (X). Finally, the human ROS concentration of the samples can be interpolated from the standard curve. (Human ROS ELISA Kit Catalog #: BG-HUM20964 (96 wells)).

- **MDA Quantification Assay:**

For tissues or cells, 10 mg (1×106) can be homogenized on ice in 300 μL of MDA lysis buffer with 3 μL BHT (100 ×), and then centrifuged (13,000 × g, 10 min.) to remove insoluble materials. Alternatively, protein can be precipitated by homogenizing 10 mg sample in 150 μL dH2O + 3 μL BHT and adding 1 vol of 2 N perchloric acid, vertexing, and centrifuging to remove precipitated protein. Finally, place 200 μL of the supernatant from each sample into a microcentrifuge tube. Lipid Peroxidation (MDA) Colorimetric/Fluorometric Assay Kit (Catalog # K739-100; 100 assays; Store kit at –20°C).

- **Microscopic Examination:**

I. Slide Preparation: The pelleted cells of control and treated groups were resuspended in phosphate-buffered saline (PBS) and dispensed on clean ethanol-washed glass slide, air dried, and fixed using methanol as a preparatory step for cytological examination.

II. Hematoxylin and Eosin Staining: The fixed slides were rehydrated in descending alcohol concentrations and washed in distilled water. The slides were immersed in filtered hematoxylin stain for 3 min. and washed with distilled water. Finally, the slides were immersed in eosin stain, and dried slides were immersed in xylene and mounted with Canada balsam. (Llewellyn, 2009).

III. Photomicrography and Cytological Evaluation: the slides were photomicrographed (100×, oil immersion).

- **Morphometric analysis:**

All the steps performed for immunohistochemical evaluation were carried using image analysis software (ImageJ, version 1.41) in x4, x10, x20 and x40 magnifications. Phase analysis was calculated automatically to give the percentage of immunopositivity area circularity of nucleus to calculate nuclear area fraction. Image analysis was performed at the Oral Pathology Department, Minia University Dental Hospital.

Statistical analysis:

The collected data were tabulated using Microsoft Excel (Microsoft Office 2019). The mean nuclear area fraction (NAF) for each case was then calculated and used for statistical analysis. The data was stored and analyzed by SPSS 20 for windows. For immunostaining data, independent sample t-test

RESULTS

I.   MTT assay:

The data revealed that curcumin showed a decrease in cell viability of Hep-2-treated cells with an increase in the concentrations of curcumin (Figure 1). The IC_{50} was 48 μgm after 48 h.

II. Cox-2 ELISA:

The data revealed that the concentration of Cox-2 in Hep-2 cells decreased after 48 h treated with 48 μgm of curcumin compared with control untreated

Hep-2 cells (Figure 1). The concentration of Cox-2 in treated cells was 15.99 nmol/mg and 45.21 nmol/mg in control untreated cells.

III.ROS ELISA:

The data revealed that ROS concentration in Hep-2 cells increased after 48 h treatment with curcumin compared with control untreated Hep-2 cells (Figure 1). The concentration of ROS in treated cells was 491.3 pg/ml and 267.2 pg/ml in control untreated cells.

IV. Lipid Peroxidation (MDA) Colorimetric/Fluorometric assay:

The data revealed that the concentration of MDA in Hep-2 cells increased after 48 h treatment with curcumin compared with control untreated Hep-2 cells (Figure 1). The concentration of MDA in treated cells was 6.526 nmol/mg and 2.467 nmol/mg in control untreated cells.
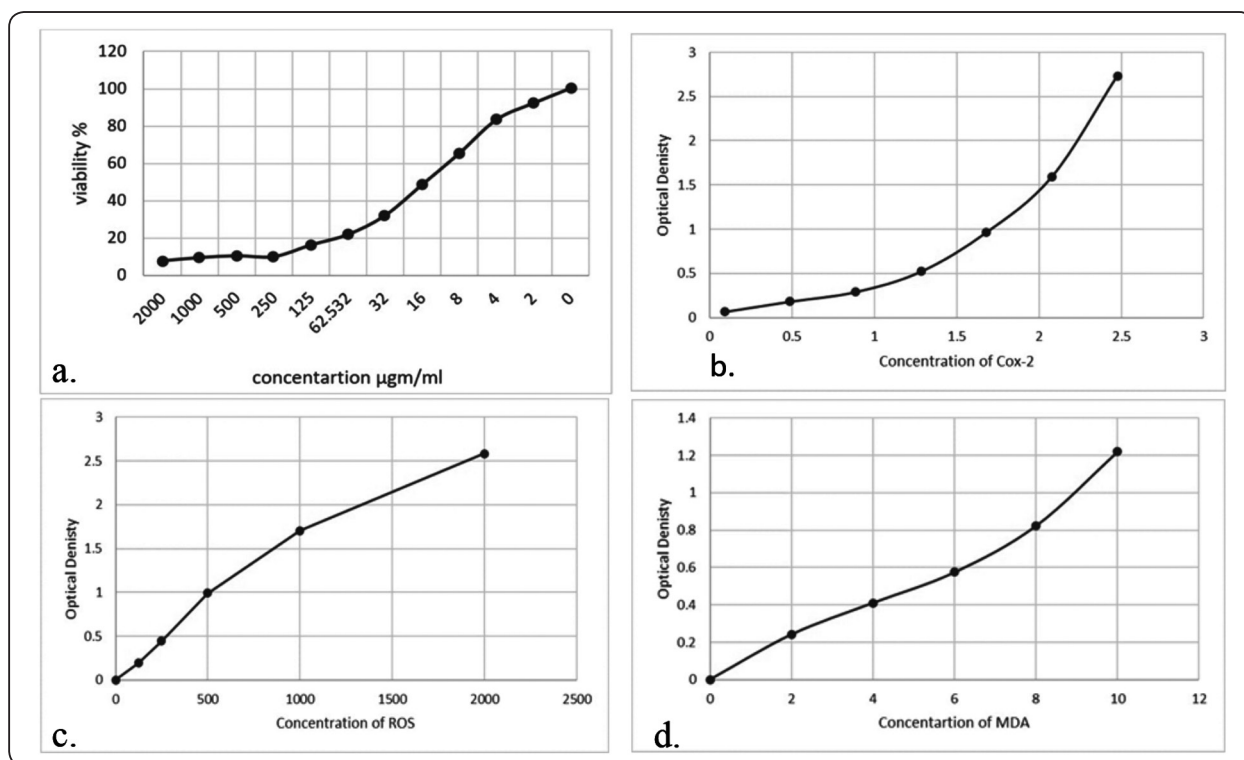

Fig. (1) **a)** Graph showing cell viability of Hep-2 cells after 48 h treated with curcumin**.** Graph showing a reverse relationship between optical density and concentration of Cox-2 **(b.),** concentration of ROS **(c.),** concentration of MDA **(d.).**

V. Histopathology feature:

a) Control untreated cells:

The untreated Hep-2 cells showed malignancy properties, such as hyperchromatic and pleomorphic nucleus with increased nuclear-cytoplasmic ratio (Figure 2).

b) Treated cells:

The Hep-2 cells treated with IC_{50} of curcumin for 48 h showed features of apoptosis, such as shrunken cells with an irregular cell membrane, membrane blebbing, peripheral condensation of chromatin, nuclear fragmentation, and apoptotic bodies. Furthermore, necrotic features occurred in some cells (Figure 3).
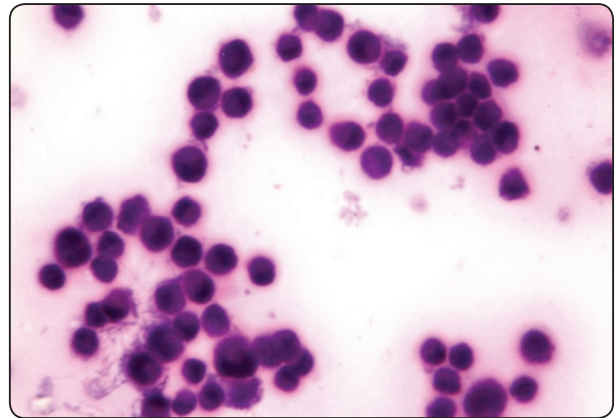

Fig. (2) A photomicrograph showing regular cells with hyperchromatic nuclei and nuclear pleomorphism of control cells (H & E, original magnification 100×, oil immersion).
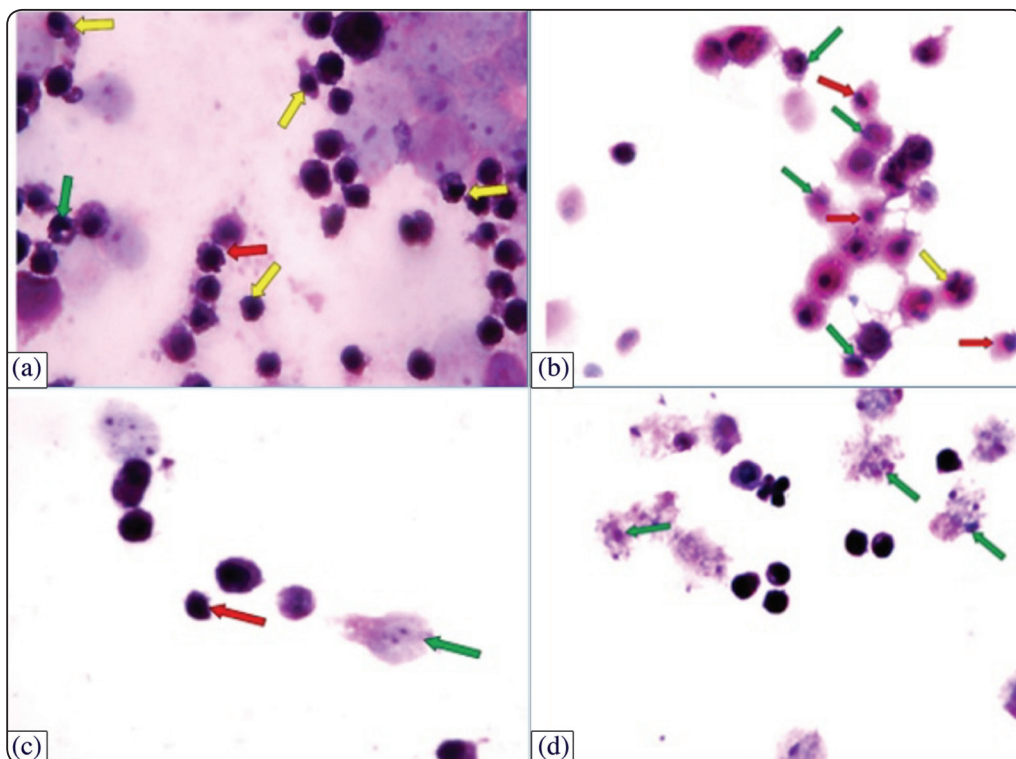

Fig. (3) Photomicrographs showing treated cells. **a)** Shrunken apoptotic cells with shrunken nuclei (yellow arrows), irregular cell membranes (red arrows), and peripheral condensation of chromatin (green arrows). **b)** Shrunken apoptotic cells with shrunken nuclei (red arrows), peripheral condensation of chromatin (green arrows), and nuclear fragmentation (yellow arrow). **c)** Shrunken apoptotic cells with shrunken nuclei (red arrow), necrotic cells (Green arrow), and **d)** apoptotic bodies (green arrows) (H & E, original magnification 100×, oil immersion).

VI. Statistical Analysis

The independent samples t-test was applied to compare between nuclear area factors of control untreated Hep-2 cells and curcumin treated Hep-2 cells and there was a highly significant difference between the NAFs of the two groups of cells. (p=0.0001)

DISCUSSION

This study suggests that curcumin induces cell death in the Hep-2 cell line due to oxidative stress. Also, it acts as an antiproliferative agent by its anti-inflammatory property. Giordano & Tommonaro, 2019 stated that curcumin has anticancer properties by targeting different cell signaling pathways, including growth factors, cytokines, and genes modulating cellular proliferation and apoptosis.

Furthermore, this study found that curcumin reduces the viability in Hep-2-treated cells dose-dependently. This agrees with a study by Khafif et al., 2009, who compared the effects of curcumin and single-dose radiation alone and with HNSCC cell lines, SCC-1, SCC-9, A431, and KB. In vitro growth suppression with curcumin or radiation was observed in all four cell lines, and the combination of both therapies resulted in an additive growth-suppressive effect.

Treatment with curcumin reduces Cox-2 concentration in Hep-2 cells, consequently reducing inflammation. Many cancers arise from chronic irritation, infection, or inflammation; much data have expanded the concept that inflammation is a critical component of tumor progression (Xiao & Yang, 2008; Balkwill, 2009; Berasain et al., 2009). These results agree with Khafif et al., 2009, who found that curcumin decreased Cox-2 expression and inhibited epidermal growth factor receptor (EGFR) phosphorylation in SCC-1 cells. Sharma, Kaur, Shishodia, Aggarwal, & Ralhan, 2006, stated that curcumin downregulates Cox-2 expression in human oral premalignant and cancer cells.

This was also supported by a study by Jurenka, 2009, who stated that curcumin suppresses the activation of NF-kB, an inducible transcription factor that regulates the expression of a host of genes involved in inflammation, including Cox-2. Additionally, curcumin regulates various pro-angiogenic growth factors, enzymes, and transcription factors, including Cox-2, proving its inhibitory effect on tumors (Sharma et al., 2006), angiogenesis, and metastasis (Yoysungnoen, Wirachwong, Bhattarakosol, Niimi, & Patumraj, 2006).

As a polyphenol, curcumin can induce abnormal energy metabolism and ROS accumulation in cells. Accordingly, our results showed a marked increased ROS production in curcumin-treated cells than in control cells. This finding agrees with our finding that curcumin-treated cells have less viability than those of control cells. Additionally, low doses of hydrogen peroxide and superoxide stimulate cell proliferation in many cancers cell types (Burdon, Gill, & Rice-Evans, 1990).

The disproportional increase in intracellular ROS can induce cancer cell cycle arrest, senescence, and apoptosis. This can be achieved by cancer chemotherapy, cell depletion from antioxidant proteins, or ROS generation by immune cells. Additionally, apoptosis is linked to increased mitochondrial oxidative stress that causes cytochrome C release, an irrevocable event that leads to caspase activation and cell death (Cadenas, 2004; Simon, Haj-Yehia, & Levi-Schaffer, 2000). Similarly, (Burdon, Gill, & Rice-Evans, 1990), T. Wang, Wu, Al Rudaisat, Song, & Cheng, 2020 investigated the effect of different doses of curcumin on human cervical cancer cells. They found that curcumin-induced cellular senescence in those cells. Moreover, they observed that this process was preceded and accompanied by apoptosis, autophagy, and ROS accumulation.

Regarding MDA, cells treated with curcumin showed a high amount of MDA compared with control untreated cells. It was established that there is a direct lipid damage at high ROS concentrations

(Ayala, Muñoz, & Argüelles, 2014). This led to the peroxidation of the lipid membrane of cells, a chain reaction that produces multiple new compounds, such as MDA.

The cell response to this molecule depends on the amount of lipid peroxidation. In the case of a subtoxic state in which the concentrations of lipid peroxidation (MDA) are low, the cell survives by enhancing the antioxidant defense mechanisms. However, the cell induces cell death at a toxic state (Ayala et al., 2014). Therefore, MDA is the most reliable indicator of oxidative damage (Grotto et al., 2009).

Histopathological pictures of this study revealed that there were criteria for apoptosis in cells treated with curcumin. **Yang et al**., 2012 stated that an increase in intracellular ROS level accompanied curcumin-induced apoptosis. These results indicated that a ROS-mediated mitochondrial pathway played an important role in curcumin-induced apoptosis of human SCLC NCI-H446 cells. According to **Afifi NS et al., 2012** the nuclear area factor is a profound predictor of the early apoptotic changes of anti-cancer drugs. Our statistical results showed a highly significant decreasing in NAFs of curcumin treated cells when compared to untreated Hep-2 cells, which means that the criteria of early apoptosis were increased after treatment of cells with curcumin.

CONCLUSIONS

Curcumin has antiproliferative activity as it can decrease intracellular Cox-2 levels in Hep-2 cells. In contrast, it can increase ROS levels. As a result, MDA levels are increased. Thus, curcumin induces oxidative damage to cells and can be used as an oxidative therapy. Our study has proved that curcumin can be used as an adjuvant agent with other chemotherapy drugs. However, further studies on the details of its underlying molecular mechanisms are required.

RECOMMENDATION

From our study, we recommend to use curcumin as adjunctive chemotherapy in treatment of laryngeal squamous cell carcinoma. Moreover, we recommend curcumin as anti-inflammatory agent.

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