Journal of Research in Pharmacy

Research Article

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Forty Micromole Hydroxychloroquine Enhanced Cytotoxic Effect of Doxorubicin Against Laryngeal Cancer Cell Line HEp-2

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Received: 18 November 2021 / Revised: 07 March 2022 / Accepted: 11 March 2022

ABSTRACT: Autophagy processes are essential biological mechanisms control human cell survival and induction of cell death. Many researches indicate that cancer cells growth is affected by inhibition or induction of autophagy processes and laryngeal cancer "the most common type of head and neck cancer" is one of various types of tumors which effected by autophagy process. Numerous articles studied impact of adding autophagy inhibiter to cancer treatment protocol, and the current work study the in-vitro anticancer effect of doxorubicin alone and in combination with an autophagy inhibitor agent hydroxychloroquine against HEp-2 (laryngeal cancer) cell line. The present study suggested valuable effect of doxorubicin anticancer activity against HEp-2 cell line when used after hydroxychloroquine pretreatment, which may play promising role in treatment of laryngeal cancer.

KEYWORDS: Autophagy; autophagy inhibitor; doxorubicin; head and neck cancer; HEp-2; hydroxychloroquine; laryngeal cancer.

1. INTRODUCTION

Eight million patients dead every year due to many forms of cancers [1]. The most common type of head and neck cancer is carcinoma of larynx or voice box cancer which comprise around 25% of all cases [2]. Smoking and alcoholism are the common causes of glottis cancer. Environmental carcinogens play a critical role in glottic tumor. Men over 40 years old are the most affected group of patients [3]. In Iraq 77.5% of voice box cancer patients are males and 42.5% of all patients at the age range 61-70 years old then 32.5% of all patients at the age range 51-60 years old [4] The five years survival of laryngeal carcinoma depend on site and stage of cancer where the localized larynx cancer shows high survival rate reach to 64.3% and the 5-years survival rate may decrease to 37.6% for hypopharynx [5]

Doxorubicin is strong anticancer drug fellow anthracyclines group, effectively used in treatment of many types of cancer [6]. Doxorubicin has following Mechanisms of Action:

1- Insert between the nucleotides of the RNA and DNA strands to block them. As a result, protein synthesis is inhibited [7].
2- Inhibition of DNA replication by topoisomerase inhibition- II [8].
3- Cancerous cells produce more intercellular free radicals, which ultimately bind to DNA, causing double-strand DNA breaks [9].
4- Inducing antigen expression on the surface of tumor cells, which makes them targets for macrophages (eat me signal) [10].

Although doxorubicin good anticancer treatment it is faced resistance, chemoresistance is the ability of cancer cells to withstand the effects of chemotherapeutic drugs that cause cell death. Chemoresistance is a significant obstacle for cancer patients. Many research lines set to overcome chemoresistance, one of them is autophagy process [11].

How to cite this article: Owadh H, Ghaleb R, Alzubaidi F. Forty Micromole Hydroxychloroquine Enhanced Cytotoxic Effect of Doxorubicin Against Laryngeal Cancer Cell Line HEp-2. J Res Pharm. 2022; 26(4): 714-721.

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http://dx.doi.org/10.29228/jrp.169
ISSN: 2630-6344

Autophagy is an important intracellular energy supplier system during stressful or fasting condition. It is lysosomal degradation and recycling system [12]. Autophagy, on the other hand, is frequently dysregulated in cancer cells and plays a role in carcinogenesis. Furthermore, autophagy is involved in a number of cancer hallmarks, including cell survival, cell death, metabolic dysregulation, immune response modulation, epithelial–to–mesenchymal transition, cancer stem cell activation, and multidrug resistance [13] Autophagy may play role in laryngeal cancer resistance to chemotherapy, like cisplatin, so the inhibition of autophagy process may enhance efficacy of anticancer drugs [14]. At present time laryngeal carcinoma consider as an aggressive tumor and the classical treatment need to be modified or combined with new enhancer agents [15].

Hydroxychloroquine (HCQ) is a chloroquine derivative and its a famous autophagy inhibitor enhances antitumor agents activity [16]. HCQ has been used for sixty years in treatment of malaria, systemic lupus erythematous and inflammatory arthritis [17]. Hydroxychloroquine is used to treat a variety of cancers, and HCQ is combined with chemotherapy drugs to boost their anti-cancer effects by inhibite tumor cells autophagic flux [18].

2. RESULTS

2.1. Cell viability of HEp-2 after exposure to doxorubicin alone

The data of figure 1 reveals that the cytotoxic effects of serial concentrations of Dox (100, 50, 25, 12.5, 6.25 and 3.125 µg/ml) (after 24 hours incubation) on the laryngeal cancer HEp-2 cell line were significantly increased versus the control group (*p < 0.05) for first three concentrations and (***p < 0.001) for last three concentrations.

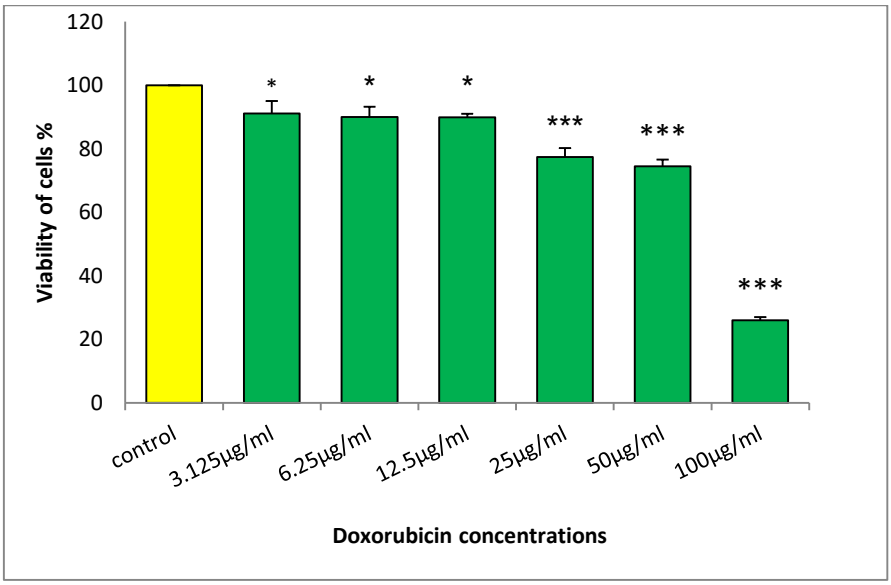

Figure 1. The effect of different concentrations of Doxorubicin on HEp-2 cell line after incubation for 24 hours. versus control group represented by mean± SEM. (*p< 0.05 and ***p < 0.001).

2.2. Cell viability of HEp-2 after exposure to HCQ and doxorubicin treatment

The data of present study that represented by Figures 2, 3, 4, and 5 reveal the good role of autophagy inhibitor HCQ in enhancement of anticancer activity of Dox against HEp-2 cell line. Where Figure 2 shows that there was a significant decrease (p < 0.001) in percentage of cell viability of laryngeal cancer HEp-2 cell line treated with serial concentrations of Dox (100, 50, 25, 12.5, 6.25 and 3.125 µg/ml) (after 24hours incubation) at all concentrations with comparison to control group (p < 0.001). HEp-2 that was already pretreated with 40 µm HCQ for 3 hours. the data reveals the cytotoxicity of treatment related linearly to concentration of Dox. 40 µm HCQ alone had no cytotoxic effect against HEp-2 cell line with comparison to control group.

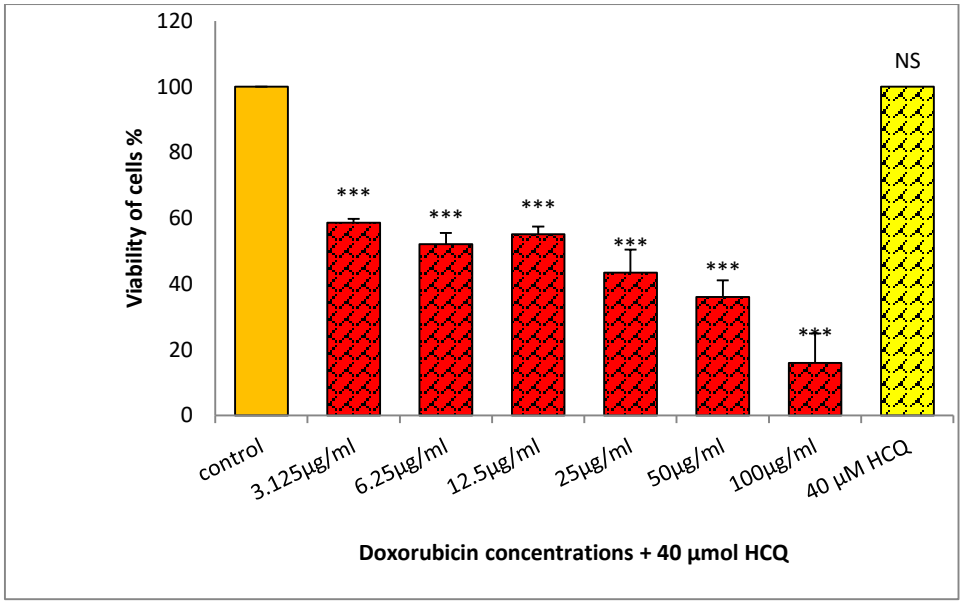

Figure 2. The effect of different concentrations of doxorubicin on 40 µm HCQ -pre-treated HEp-2 laryngeal cancer cell line (after incubation for 24 hours) versus control group represented by mean± SEM. (***P < 0.001).

Figure 3 shows that there was a significant decrease in percentage of cell viability of laryngeal cancer HEp-2 cell line treated with of serial concentrations of Dox (100, 50, 25, 12.5, 6.25 and 3.125 µg/ml) (after 24hours incubation) at all concentrations with comparison to control group ($p < 0.001$) with except at 25 µg/ml ($p < 0.05$). HEp-2 that was already pretreated with 100 µm HCQ for 3 hours. Further it significantly showed the best effect at high concentration. 100 µm HCQ alone showed significant cytotoxic effect against HEp-2 cell line with comparison to control group ($p < 0.05$).

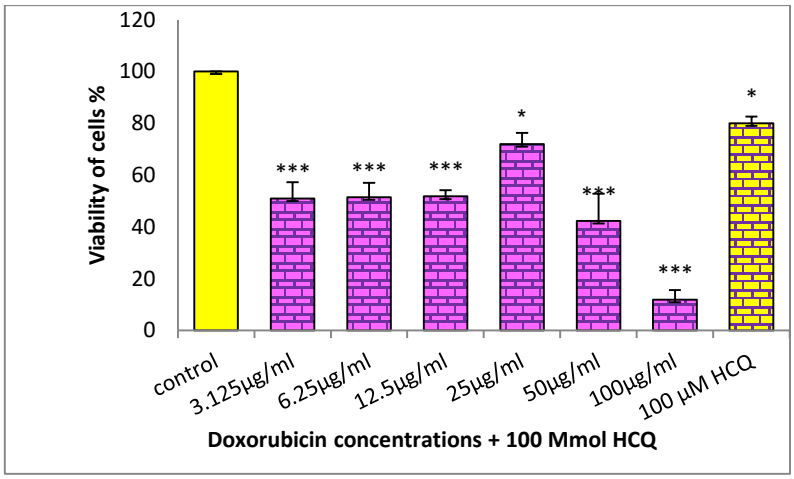

Figure 3. The effect of different concentrations of doxorubicin on 100 µm HCQ -pre-treated HEp-2 laryngeal cancer cell line (after incubation for 24 hours) versus control group represented by mean± SEM. (*P < 0.05 and ***P < 0.001) NS= non-significant difference.

Figure 4 shows that there was a significant decrease in percentage of viability of cells of HCQ-pre-treated HEp-2 cell line at concentrations of Dox (50, 25, 12.5, 6.25 and 3.125 µg/ml) in comparison with non HCQ pre-pretreated HEp-2 cell line ($p < 0.001$) and non-significant differences at 100 µg/ml.

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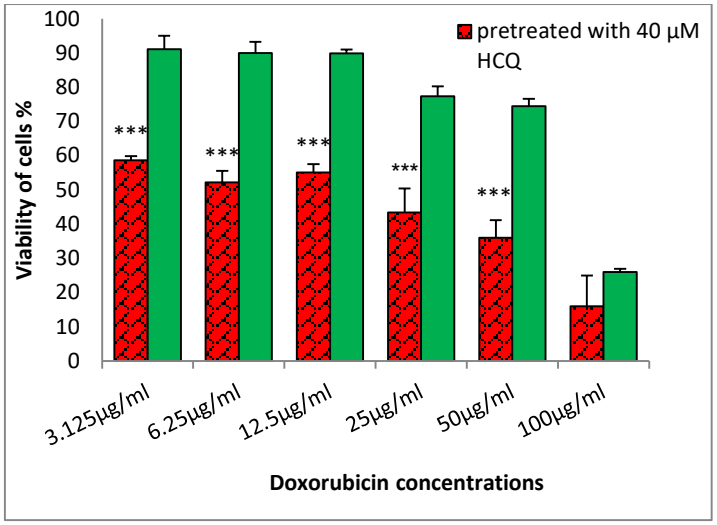

Figure 4. Comparison the effect of serial concentrations of doxorubicin on 40 µm HCQ -pre-treated HEp-2 laryngeal cancer cell line versus without HCQ represented by mean± SEM. (***p < 0.001).

Figure 5 shows that there was a significant decrease in percentage of viability of cells of 100 µM HCQ-pre-treated HEp-2 cell line ($p < 0.001$) at concentrations of Dox (50, 25, 12.5, 6.25 and 3.125 µg/ml) and ($p < 0.05$) at concentrations of Dox (100 µg/ml) in comparison with non HCQ pre-pretreated HEp-2 cell line ($p < 0.001$) and non-significant differences at 25 µg/ml.

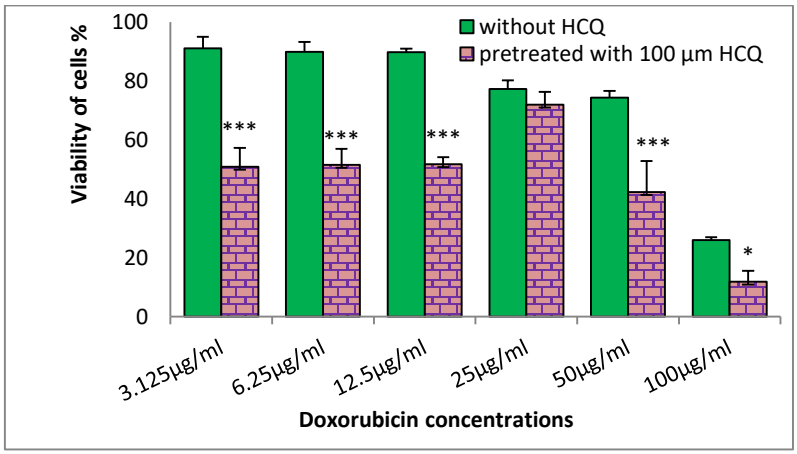

Figure 5. Comparison the effect of serial concentrations of doxorubicin on 100 µm HCQ -pre-treated HEp-2 laryngeal cancer cell line versus without HCQ represented by mean± SEM. (*p < 0.05) and (***p < 0.001).

2.3. Comparison between the effect of (40 µM and 100 µM) HCQ-pre-treatment on doxorubicin anticancer activity against laryngeal cancer (HEp-2) cell line proliferation:

The results show that there was a non-significant difference in percentage of viability of cells of 40 µM HCQ-pre-treated HEp-2 cell line at concentrations of Dox (100, 50, 12.5, 6.25 and 3.125 µg/ml) and ($p < 0.001$) significant decrease at concentrations of Dox (25 µg/ml) in comparison with 100 µM HCQ pre-pretreated HEp-2 cell line (Figure 6).

http://dx.doi.org/10.29228/jrp.169
J Res Pharm 2022; 26(4): 714-721

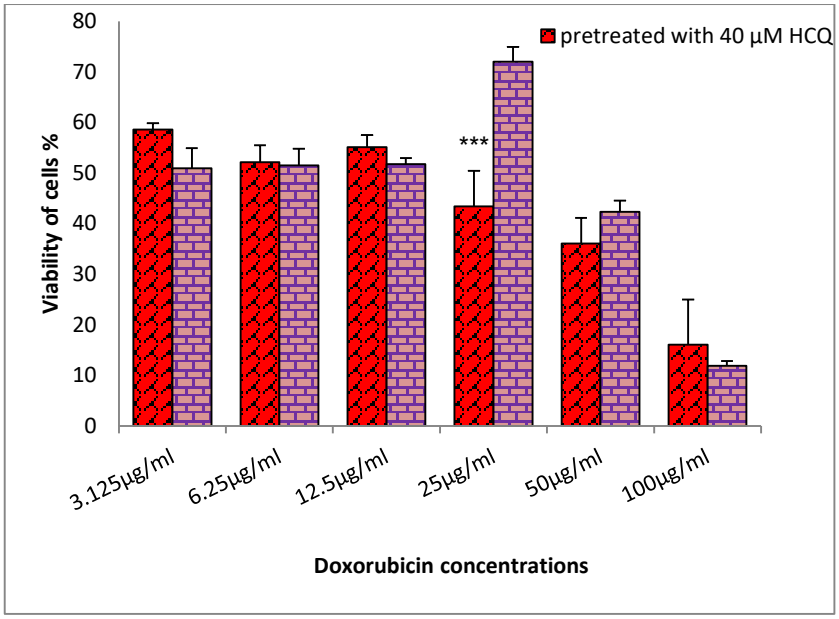

Figure 6. Comparison the effect of serial concentrations of doxorubicin on 100 µm HCQ -pre-treated HEp-2 laryngeal cancer cell line versus effect on 40 µm HCQ -pre-treated HEp-2 laryngeal cancer cell line represented by mean± SEM. (***p < 0.001).

3. DİSCUSSİON

Many studies demonstrated that inhibiting of autophagy process leads to enhancement of anticancer activity [12]. HCQ is an autophagy inhibitor was studied in present article and following results was found:

3.1. The effect of doxorubicin on HEp-2 proliferation

The present study tested the cytotoxic effect of Dox against HEp-2 cell line, the finding indicated that Dox inhibited Hep-2 proliferation and showed the best effect at high concentration or in other word the percentage of cells viability of laryngeal cancer HEp-2 cell line decreases with increasing of Dox concentration. These results coming with fact of Dox is one of the most effective anticancer agents [19]. Dox Kills tumor cell through causing DNA damage, inhibit topoisomerase II, and induce free radical formation [16].

3.2. HCQ enhances sensitivity of laryngeal cancer (HEp-2) cell line to doxorubicin

The incidence of resistance of cancer cells to Dox anticancer activity was increased [20]. Our previous study showed that autophagy process plays important role in reducing Dox anticancer activity and using of autophagy inhibitor HCQ; enhanced Dox anticancer activity against osteosarcoma [16]. In present study we evaluate the impact of using pretreatment autophagy inhibitor HCQ with Dox in treatment of laryngeal cancer HEp-2 cell line. The pretreatment 40 µm HCQ with serial concentrations of Dox didn't attenuate the cytotoxicity of Dox but induces death of HEp-2 cells and successes in increasing cytotoxicity of Dox against glottis tumor cells. Similarly, the second protocol of pretreatment 100 µm HCQ killed tumor cell. These finding agree with work of Barbeau team, they found autophagy was induced in hypoxic condition, then autophagy helps laryngeal cancer cells to overcome low oxygen condition and survival, additionally when they knocked out autophagy related gene, the hypoxic cell death was induced [12].

Additionally, this study found that HCQ doses (40 µm and 100 µm) increase tumoricidal effect of Dox that consistent with Shippey article which mentioned mechanism of HCQ in helping antineoplastic therapy including: 1- HCQ inhibits multidrug resistant pump. 2-Inhibiting of autophagy process. 3-HCQ improves penetration of anticancer agent inside tumor cell. 4- Improvement of immunity [17].

In either HCQ dose (40 µm and 100 µm) and Dox there is liner relation-ship between Dox dose and cytotoxic effect of combination, the highest Dox concentration produce highest cytotoxic effect, these finding were compatible with Małek study observation that found increasing concentration of Dox leads to decreasing the viability of all cell lines included in study in a dose-dependent manner [21].

Although, the enhancement effect of pretreatment 40 µm HCQ similar to effect of 100 µm HCQ but 40 didn't produce cytotoxic effect on tumor cell while 100 µm HCQ alone reduce percentage of cell viability which agree with Li *et al*. study, that observed 40 µm HCQ alone didn't cause lung cancer cell death while 100 µm did. And when they used low dose HCQ pre- Dox treatment the cytotoxicity of Dox was increased [22]. This study observed that 40 µm pretreatment-HCQ is better than 100 µm pretreatment-HCQ for enhancement Dox cytotoxicity against HEp-2 laryngeal cancer cell line.

4. CONCLUSİON

The HCQ contain protocol produced the best anticancer effect against HEp-2 cell line, while 40 µm HCQ containing protocol produce some better effect than 100 µm HCQ containing issue. The pretreatment-HCQ + Dox had a promising potential for overcome of laryngeal cancer treatment resistance.

5. MATERIALS and METHODS

5.1. Cell culture

HEp-2 human larynx epithelioma cancer cells were kindly provided by Cell Culture Unit of Babylon Medical College / University of Babylon. This line was seeded in rosswell park memorial institute (RPMI-1640) medium (Gibco, UK) supplemented with 10% fetal bovine serum (Gibco, UK) with different concentrations of Dox (Ebewe, Austria), and/or hydroxychloroquine (Bristol lab, UK), at 37°C in a humidified chamber with 5% carbon dioxide.

5.2. Cell viability assay

To test the cytotoxic effect of study drugs, the cell viability was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells of laryngeal cancer were seeded in 96-well plates and grown to 70% confluence. Dox was added to culture medium at the indicated concentration (laryngeal cancer HEp-2 cell line was already pretreated with 100µm or 40 µm HCQ for 3 hours). After 24 hours twenty microliters of MTT (5mg/ml) were added to each well, then the 96 well plates were incubated at 37°C, then after 4 hours 150 µl of DMSO was added to adherent cells of each well for dissolving of crystal. Then the luminescence of each well was measured using a microplate reader (Human, Germany) at a wavelength (570 nm).

5.3. Statistical Analysis

Statistical package for social sciences (SPSS) version 23 was used for statistical analysis. Results were analyzed using one-way analysis of variance (ANOVA) for multiple comparisons and LSD post-hoc test. The results variability was expressed as mean± standard error of mean (SEM). P value of 0.05 was considered statistically significant.

Acknowledgements: The authors would like to thank Al-Mustaqbal University College and University of Babylon for their support.

Author contributions: Concept – H.O.; Design – H.O., R.G.; Supervision –R.G., F.A.; Resources – H.O., R.G.; Materials – H.O.; Data Collection and/or Processing – H.O., R.G.; Analysis and/or Interpretation – H.O., R.G., F.A.; Literature Search – H.O.; Writing – H.O., R.G., F.A.; Critical Reviews – H.O., R.G., F.A.

Conflict of interest statement: "The authors declared no conflict of interest" in the manuscript.

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