IOSR Journal Of Pharmacy
(e)-ISSN: 2250-3013, (p)-ISSN: 2319-4219
www.iosrphr.org Volume 5, Issue 4 (April 2015), PP. 40-43

The Radiosensitivity Effect of Hydroxyurea on HT29 Cell Line

Alireza Doroudi [1*] , Mostafa Erfani [2]and Seyyed Mohammad Mousavinia[1]

[1]*School of Pharmacy , Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.*
[2] *Radiation Application Research School, Nuclear Science and Technology Research Institute [NSTRI], Tehran, Iran*

ABSTRACT: *The radiosensitizer compounds are used in order to increase the efficacy of radiotherapy. Hydroxyurea is one of the antineoplastic agents and used orally for treatment of melanoma, metastatic or inoperable ovarian cancer, and chronic myelocytic leukemia and as an adjunct to radiation in the treatment of squamous cell carcinoma and cancer of the head and neck. This approach was launched to evaluate the efficiency of combination of hydroxyurea and gamma ionization irradiation on HT29 cell line. After the proliferation of HT29 cell line and cells were in the exponential phase, the density of 10^5 of HT29 cell line was seeded in every well of 96-well dishes; different doses of hydroxyurea were added to each well. The ionizing irradiation has been performed 4 hours after drug treatment. Then the viability of cells was determined by MTT assay. Hydroxyurea at 2.5 μM concentration has not shown significant cytotoxicity on HT29 cell line without ionizing irradiation but the viability of cells have been decreased significantly to about 55 % of control with irradiation combination. The use of low dose of hydroxyurea in conjunction with tumor radiotherapy might results in much greater toxicity to tumor than normal tissues.*

Key words: *Hydroxyurea, MTT assay, Radiosensitizer*

I. INTRODUCTION

While the results of radiotherapy have substantially improved over the years, one-third of patients with solid tumors receiving curative treatment will suffer local recurrence due to residual tumor (1).Treatment failures can be attributed to factors associated with the treatment delivery and with the biological response of the tumor cells to ionizing radiation. Attempts to improve the efficacy of radiotherapy have focused on improved methods to deliver the dose or on studies to modulate the biological response to ionizing radiation (2). The combinations of radiotherapy and chemotherapy have improved survival in patients with tumors known to be intrinsically radio resistant. The combination of radiotherapy and chemotherapy is advocated primarily because of the independent effects of each modality. Radiotherapy is aimed at controlling the primary tumor, while chemotherapeutic drugs destroy tumor cells by their own cytotoxic action and additionally enhance the effects of radiotherapy, chemotherapeutic drugs that have the potential to produce substantial sensitization of tumor cells to radiation treatment are defined as radiosensitizer and the process is called radiosensitization. Improved treatment results have been demonstrated in several in vitro and in vivo studies and in clinical trials in patients with locally advanced solid tumor (3, 4). Additive or synergistic effects against a tumor without substantial increase in toxicity to normal tissue may then lead to a therapeutic advantage. Hydroxyurea is an antineoplastic chemotherapeutic agent. It has been synthesized in 1869 and used orally for treatment of melanoma, metastatic or inoperable ovarian cancer, and chronic myelocytic leukemia and as an adjunct to radiation in the treatment of squamous cell carcinoma and cancer of the head and neck (5-8). It is also used to relief the rate of painful attacks in sickle-cell disease (9). Hydroxyurea increases the effectiveness of radiation therapy (10, 11). According to the literature, HT29 colon cancer cell line has been reported to be resistant to ionization irradiation (12, 13).Therefore, HT29 cell line is suitable model for investigation the radiosensitizer effect of hydroxyurea. This study was conducted to assess the radiosensitizer effect of hydroxyurea on HT29 cell line.

II. MATERIALS AND METHODS

All chemical materials have been purchased from Sigma or Fluka. The chemical and solvents were of the highest purity and analytical grade and used without further purification. HT 29 colon cancer cell line has been provided by Institute of Pasteur Tehran, Iran.

2.1. Cell Culture

HT 29 cells were cultured in 25 t-flask in medium containing Dulbecco's Modified Eagle's Medium (DMEM), 10% Fetal Bovine Serum (FBS) , 100 U/ml penicillin and 100 μ/ml streptomycin at 37 ºC with 5% CO_2 , 95% air and complete humidity. When these cells reached approximately 90% confluency, they were

detached using 0.05% trypsin/ EDTA and counted by means of tryptan blue and hemocytometer. The freshly stock solutions of hydroxyurea in ethanol were prepared in every experiment .Then stock solution was diluted with media and added into each well of plate. The experiments for cytotoxicity determination of hydroxyurea and ionization irradiation were carried out in 96 well culture dishes. The cells were seeded in 100 µl complete medium per well of 96-multiwell plates at the density of 10^5 cells/well. The cells were incubated for 24 hours at 37 ºC with 5% CO_2, 95% air for cells to adhere. After 24 hours , when the monolayer HT 29 cell line was formed the supernatant decanted and 100 µl of different doses of hydroxyurea were added to the cells in microtitre plates and kept for incubation at 37 ºC with 5% CO_2, 95% air for 4 hours. Then the cells were irradiated with 4 Gy ionizing irradiation. The cells were periodically checked for shrinkage, swelling and granularity. After 24 hours, the test supernatant solution in each well was decanted and then 50 µl of 3-(4,5-dimethyl thiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) dye at a final concentration of 0.5 mg /ml was added to each well. The plates were gently shaken and incubated for 1 hour at 37 ºC with 5% CO_2, 95% air in incubator. The supernatant was removed, 125 µl of DMSO was added .Then the plates were gently shaken to solubilize the formed formazon for 10 minutes. The plates were read on an ELISA reader (Bio-Tek Instruments Inc., USA) using a wavelength 570 nm, reader using DMSO as a blank with reference reading at 690nm. The control HT 29 cell line were underwent the same procedure but without any modalities. The percentages of cell survival were expressed by the ratio of the absorbance of treated cells to the absorbance of control cells multiple 100.Eight wells were allocated for each treatment and control cells. All experiments were carried out three times.

2.2. Ionization Irradiation

The cells were irradiated for 4Gy ionization irradiation at room temperature, using a Co-60 gamma ray at 1.65 Gy/Sec.

2.3. Statistical Analysis

Statistical analysis was performed using SPSS 11.0 for window (SPSS Inc, Chicago IL, USA) and descriptive statistics are shown as arithmetic mean ± standard deviation. Independent samples t-test was used to investigate the differences between irradiated and p value smaller than 0.05 was considered statistically significant.

III. RESULTS

The MTT colorimetric assay is depended on the activity of succinate dehydrogenase which is present in mitochondria of HT29 cell line. When the cells are live, the yellow water soluble substrate 3-(4, 5-dimethyl thiazol-2-yl)-2, 5 diphenyl tetrazolium bromide can be reduced into an insoluble colored formazon product which is measured spectrophotometrically by ELIZA reader. The reduction of MTT compound can only occur in metabolically active cells. The production of resultant formazon appears to be proportional to the level of energy metabolism in the cells. Therefore, it is possible to measure the metabolically activated cells even in the absence of cell proliferation. By using the following formula, the percentage growth inhibition was calculated. Cell inhibition % = $[(Ab_t - Ab_b)/ (Ab_c - Ab_b)] \times 100$. Where Ab_t , Ab_b and Ab_c are Absorbance value of test, Absorbance value of blank and Absorbance value of control respectively. Absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Conversely, a higher absorbance values indicate an increase in cell proliferation. The preliminary experiments with different density of HT29 cells showed that there was a linear relation between the number of the cells and absorbance. Therefore, the above mentioned formula has been used to evaluate the cell viability.

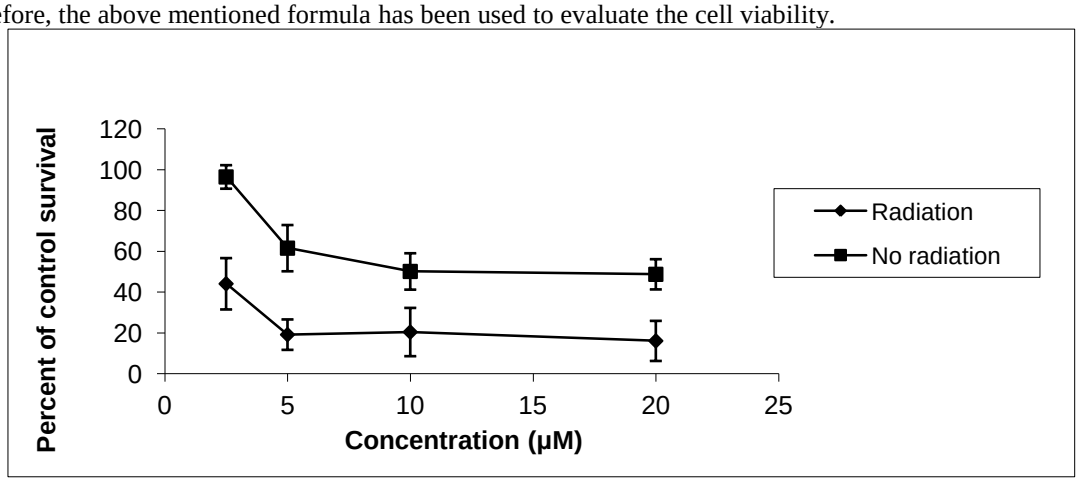

Figure1. The effect of different concentrations of hydroxyurea on the HT29 survival with or without 4 Gy ionization irradiation by using MTT assay.

The effect of different concentrations (2.5, 5, 10 and 20 μM) of hydroxyurea on the lethality induced by4 Gy ionization irradiation has been examined. Hydroxyurea at 2.5 μM concentration has not shown significant cytotoxicity on HT29 cell line without ionizing irradiation (Fig1), but the viability of cells have been decreased significantly to about 55 % of control with irradiation combination . The cytotoxicity of hydroxyurea has been increased significantly when the dose of drug increased from 2.5 μM to 20 μM without irradiation. The combination of gamma irradiation with hydroxyurea at 5 to 20 μM could increase the cytotoxicity by this intervention. The viability of cells has been decreased to 20 % of control cells. The combination of gamma irradiation and hydroxyurea with concentrations of 5 to 20 μM has not increased further cytotoxicity. The remaining cells might be part of subpopulation which by some mechanisms was extremely resistant to treatment or drug could not enter to these cells and subsequently gamma irradiation could not increase the cytotoxicity of hydroxyurea. Although hydroxyurea at the dose of 5 to 20 μM with gamma irradiation has shown to decrease the viability cells but the drug was very high cytotoxic at the above mentioned concentrations. The radiosensitizer effect on HT29 cell line has been observed when the dose of hydroxyurea was 2.5 μM without demonstrated any obvious cytotoxicity effect.

IV. DISCUSSION

The precise mechanism action by which hydroxyurea produces its antineoplastic effect is not elucidated completely. However, various investigations support the hypothesis that this drug causes an immediate inhibition of DNA synthesis by acting as a ribonucleotide reductase inhibitor without interfering with the synthesis of protein and ribonucleic acid. Three mechanisms have been suggested for the enhanced effectiveness of concomitant use of hydroxyurea with gamma irradiation. First hydroxyurea is lethal to naturally radioresistant stage cells. Second hydroxyurea can hold other cells of the cell cycle in the G1 or pre-S DNA synthesis stage where they are most susceptible to the damage effects of gamma irradiation. Third in addition to the above mentioned mechanisms, hydroxyurea by inhibition of DNA synthesis hinders the normal repair process of cells damaged but not killed by ionizing irradiation (3). Ionization irradiation is clinically administered either by an external source (gamma irradiation or linear accelerator) directed toward the tumor or an internal source, radioactive decay from within the tumor. The following mechanisms have been suggested for the interaction of ionizing irradiation with biological matter. These mechanisms are the photoelectric effect, the Compton effect, the pair production and finally the photodisintegration. The Compton effect is widely considered as the mode of interaction most relevant for the range of energies used in clinical ionizing radiation therapy. The observed biological effect results from photons creating multiple ionizations by ejection of electrons from the target biomolecule by the Compton effect. The extent of biological responses in the target cells after exposure to ionization radiation is largely due to oxygen with the subsequent production of free radicals. These free radicals are responsible to break chemical bonds present in target cell structures and biomolecules, basically cellular DNA. DNA is by the most critical target for the biological effects of ionization radiation. Cell death is associated with the extent of DNA damage (14). Cell death occurs at a higher rate when ionizing radiation is focused on the nucleus as opposed to the cytoplasm (15). A signal is transmitted to the regulators of the cell cycle machinery and the sensors of DNA damage cells. Cells with damage DNA undergo cell cycle arrest. During the arrest phase, the damaged cells can repair and proceed through the cell cycle, not repair and remain arrested or not repair and undergo apoptosis. The irradiated cells with DNA damage, which eventually activate the mechanisms for DNA repair. Different processes are activated according to the lesion types, with double-strand breaks being the most lethal lesion to the cell as comparison to single-strand breaks. Repair of these lesions can be performed either through homologous recombination or non-homologous end-joining (16, 17). In the former, either the intact chromosome or the sister chromatid serve as a template to reconstruct the damage DNA. Homologous recombination is most effective in late S or G2 phase, when the sister chromatids have replicated but still attached to division spindle. Non-homologous end-joining is more important in G1 and early S phase, but it essentially occurs throughout the cell cycle. The mechanism of adaptive response for resistant HT29 cell line to gamma irradiation is not completely understood, but it widely suggested that inducible DNA repair mechanisms have pivotal role (18-20). Other mechanisms such as decreased damage fixation, apoptosis pathways, and DNA conformation changes are probably involved. Protein synthesis appears to be essential for the induction of an adaptive response. Several genes have been identified that play a crucial role in this phenomenon (21-24). Our approach indicated that the response of HT29 cell line to gamma irradiation was varied when different doses of hydroxyurea applied before ionizing irradiation. Hydroxyurea has not demonstrated any obvious cytotoxicity on the HT29 cell line when the low dose of drug was used alone. The dose of drug was not sufficient to induce cytotoxicity without irradiation. But the concentration of hydroxyurea was enough to inhibit DNA repairing processes in order to repair the damages induced and created due to ionizing irradiation. The cytotoxicity of hydroxyurea without the combination of irradiation has been observed when the dose of drug enhanced. The high concentration of hydroxyurea was sufficient to induce cytotoxicity. The combination of hydroxyurea and gamma irradiation has demonstrated synergistic effect when the doses of drug have increased from 5 to 20 μM. The use of low dose of hydroxyurea in conjunction with tumor radiotherapy might results in much greater toxicity to tumor than normal tissues.

V. CONCLUSION

In conclusion, we demonstrated that cell line such as HT29 known to be relative radioresistant exhibited radiosensitive reaction when the low dose of hydroxyurea was applied before gamma irradiation. If the results of our approach prove to be true by in-vivo investigations as well it could be confirmed this hypothesis that the pretreatment of HT29 cell line with low dose of hydroxyurea may be optimized the balance between local tumor control and injury to normal tissue in modern radiotherapy.

Acknowledgement

This research work was supported by Ahvaz Jundishapur University of Medical Sciences of Iran.

Competing of interests

The authors declare that they have no competing interests.

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