The Study of Anticancer Effect of Magnetic Chitosan-Hydroxyurea Nanodrug on HeLa Cell Line: A Laboratory Study

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Abstract- Background and Objectives: Drug nanocarriers have usually optimized features which facilitate cellular uptake and increase drug efficacy, prevent drug degradation against enzymatic factors and decrease drug complications by targeted drug delivery to cancer cell. This study aimed to determine the anti-cancer nature of the synthesized magnetic Chitosan-hydroxyurea nanodrug on HeLa cell line, cervical cancer, and to determine the effective dose of the nanoparticle in order to remove cancerous cells.

Materials and Methods: In this laboratory study, after analyzing the structure of synthesized magnetic nanoparticles and culture of HeLa cell line, cells were incubated with different concentrations of nanodrug for 48 h. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) method was used in order to evaluate the vital activity of the cells. For analyzing induction of apoptosis, the Annexin-PI kit was used by flowcytometry. Data were analyzed using one-way ANOVA and independent t-test.

Results: The viability of the cells decreased in a dose dependent manner by increasing the concentration of nanodrug hydroxyurea. So, at the concentrations of 1000 (p<0.001) and 2000 (p<0.05) μg/ml, a significant difference was observed compared to the control group. The results also demonstrated that the nanodrug significantly increased apoptosis induction 2.48 times in the treated HeLa cells in comparison with the control group (p=0.0003).

Conclusion: Hydroxyurea nanodrug has a cytotoxic enhancement effect on the Hela cancer cell line and can induce apoptosis.

Keywords- Hydroxyurea, Chitosan, HeLa Cells, Antineoplastic Agents, Apoptosis

I. INTRODUCTION

Cervical cancer is one of the most common causes of death and the second most common malignant neoplasm in women [1]. Common treatments for cervical cancer include chemotherapy, surgery and radiation, which are unfortunately not completely effective [2-3]. Hydroxyurea is one of the important chemotherapy drugs that inhibit the conversion of ribonucleotide to deoxyribonucleotide by inhibiting the ribonucleotide reductase enzyme. It may also directly damage the DNA strand and stop the cell in the G1 phase through inhibition of thymidine binding in deoxyribonucleotide (DNA) [4-5]. Hydroxyurea is associated with various side effects along with its therapeutic properties. Targeted drug delivery system can reduce undesirable side effects and improve drug efficacy by targeted drug delivery to the tumor site [5-6]. An optimal drug delivery system is a system capable of transferring effective drug level to target tissue, high capacity for drug loading, biocompatibility, safe from accidental release, easy synthesis, ability to control and easy removal [7]. Chitosan is the most abundant natural polymer after cellulose, today chitosan is one of the most popular bio-carriers that known in drug field [8]. The chitosan polymer nanocarrier properties can be noted to natural positive charge, selective adsorption power and neutralization of surface charge effect present in tumor cells. The negative charge on the surface of the plasma membrane results in high adsorption and adhesion strength of the nanocarrier to the cells. As a result, it is a good option in the drug delivery system to the solid tumor tissue. Chitosans also form more disulfide bind with the mucosal glycoprotein in the mucosal gel layer, resulting in greater adhesion of the polymer to the mucosa, thereby leading to continued release of the drug into the body [9-11]. Another advantage of chitosan is its lower toxicity than other cationic polymers [12]. The most important disadvantage to chitosan is its poor solubility at physiological pH due to the presence of a minor proton in the acetyl amine group. One of the major strategies for eliminating weak chitosan solubility is the use of water-soluble derivatives such as PEG (Poly ethylene glycol) groups [9]. Advantages of medicinal compounds containing PEG include high drug shelf-life in the blood, reduced rate of drug degradation by metabolic enzymes, and reduced immunogenicity [11]. The use of magnetic iron oxide nanoparticles in the structure of chemotherapeutic drugs dramatically reduces the side effects of drugs by targeted drug delivery to the tumor site (under magnetic field), without any cytotoxic effect [13]. In this study, we aimed to increase the cytotoxic nature of hydroxyurea and target it in cancer chemotherapy utilized hydroxyurea loaded on a magnetic PEGylated chitosan nanocarrier that was first synthesized and reported. Therefore, in the present study, we evaluated the anticancer effect of this new hydroxyurea
nanoparticle on Hela cell line (cervical cancer) at different concentrations and times by MTT assay and its effect on induction of apoptosis and necrosis cell death by staining Dual Annexin V and PI were required studied[13].

II. MATERIALS AND METHODS

The present study is a laboratory type which was carried out from October 2016 to September 2016 at Islamic Azad University, East Tehran Branch and Yadegar Imam Branch of Shahr Rey. For this purpose, Hela cervical cancer cell line (C155) was purchased from the Institute Pastor Cell Bank of Iran. DMEM (Dulbecco’s Modified Eagle Medium or DMEM High Glucose) culture medium, (FBS or Fetal bovine serum) bovine serum, trypsin / streptomycin and L-glutamine were purchased from Gibco Company (Scotland). Other materials such as trypsin blue, phosphate salt buffered (PBS), Phosphate buffered saline, dimethyl sulfoxide (DMSO), 3-2.5-diphenyl tetrazolium bromide (MTT; 3-2.5-diphenyl tetrazolium bromide), were purchased from Sigma Aldrich company (Germany) and the annexin / propidium iodide (Annexin V-FITC / PI) staining kit from Affymetrix company (America). In this study, Eliza reader Teco and FACS Calibur BD flow cytometry (made in America), Nicon inverted and light microscope (made in Japan), Elma S80H ultrasound (Italy), Biocenter co2 incubator (made in America) and Ultra Vercion 1.6.7.

Cell staining and detection of early and late apoptosis by necrosis of cells were stained with Annexin V / PI according to the instructions of the Annexin/propiudm iodide Kit (Affymetrix). The cultured Hela cells were treated with IC50 concentration of hydroxyurea nanoparticles for 24 h and then trypsinized and then washed with saline phosphate buffer for 5 min and centrifuged at 1500 rpm for 5 min. The resulting precipitate was suspended in 500 μL of binding buffer and incubated with Annexin V-FITC for 3 min at room temperature and dark for 15 min. The cells were then washed with 1 ml of binding solution and centrifuged. After adding 250 μl of binding buffer to the cell precipitate, 5 μl of PI dye was added to the cell suspension. The cell suspension was then immediately stained using a 488 nm excitation flow cytometer and a 5 nm readout filter (in green FL1 channel) for fluorescein isothiocyanate (FITC) and a 600 nm filter (in FL2 red channel) for propidium iodide. The data were analyzed using the Flowjo software version 10.5. The areas of green and red fluorescence were the main parameters. The results were analyzed using Graph Pad Prism version 1.6. The calculation of the mean optical absorption of the control/sample optical absorption of the test * 100 Cell viability was calculated according to the following formula: Biopotency: (mean optical absorption of the control/sample optical absorption of the test) * 100
III. RESULTS

By studying synthesised nanoparticle FTIR spectrum, the chemical structure of the nanodrugs was confirmed. The absorption frequency of tensile vibration in 531, 674, 3439, 1020, 2920 cm$^{-1}$ regarding to CH$_2$, C-O-C, OH, NH and Fe-O groups in nano-drug structure, respectively. Increase of absorbance intensity of CH$_2$ group confirmed polyethylene glycol bind and eliminate absorbance of glutaraldehyde C=O group, shows PEGylated chitosan chemical binding to magnetic nanoparticles. C=N absorption peak in 1586 cm$^{-1}$ confirmed PEGylation of chitosan and C=O tensile vibration of Hydroxyurea loaded in 1674 cm$^{-1}$ can be seen. Chemical schematic structure of PEGylated chitosan magnetic nanodrug loaded with anticancer drug is shown in fig.1. Drug loading rate obtained 4.88% by spectrophotometry UV method.

![Figure 1. Magnetic nanoparticle structure contain Hydroxyurea.](image)

SEM electron microscope image with 1µm scale it has been shown in 2-a figure. Being spherical and surface morphology and of synthesised nanoparticle is obvious in SEM image. Nanoparticle size is in the range of 43 to 67 nm. TEM electron microscope image with 50 nm scale in fig.2 confirms the formation of core –cortex structure with dark magnetic core and PEGylated chitosan cortex conation drug.

![Figure 2. Electronic microscope images of drug-carrier nanoparticles (a)SEM (x4000) and (b) TEM (x100000)](image)

Statistical analysis of the present study showed that increasing concentration of nano-drugs are reduce cell viability. According to the table 1 viability of treated cell in 1000, 2000 μg/ml hydroxyurea nanodrug concentration, 72/433% (p<0/001) and 68/171% (p<0/05) has significant biodegradation than control group, repectively. There was no significant difference observed between viability of treated cell with low concentration such as 62,5,125,200,500 μg/ml and control group (p> 0/05). IC50 concentration of hydroxyurea nanodrug was calculated to be 3017 μg / ml for 48 hours treatment. The morphological changes of Hela cell lines after treatment with IC50 concentration of magnetic chitosan-hydroxyurea nanodrug in comparison of untreated cells by light microscope are shown in Fig.3.

<table>
<thead>
<tr>
<th>Time</th>
<th>control</th>
<th>62.5 μg/ml</th>
<th>125 μg/ml</th>
<th>250 μg/ml</th>
<th>500 μg/ml</th>
<th>1000 μg/ml</th>
<th>2000 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>48h</td>
<td>19/1± 100</td>
<td>12/5± 95/09</td>
<td>5/43± 86/49</td>
<td>7/39± 99/81</td>
<td>4/06± 80/97</td>
<td>5/59± 72/43</td>
<td>2/09± 68/17</td>
</tr>
</tbody>
</table>

P value: 0/055 0/061 0/059 0/062 0/0009 0/024

The obtained values are significant according to the standard deviation ± the mean and mean difference at P< 0/05. The type of statistical test used is one-way ANOVA.

![Figure 3. Evaluation of morphological changes of HeLa cell line before and after treatment with magnetic chitosan-hydroxyurea nanodrug by light microscope ×10, a) control group b) after 48 h treatment with nanodrug](image)

During programmed cell death, the appearance of anionic phospholipids (phosphatidylserine) on the outer surface of the membrane, which is transmitted by Flip Flap movement from the inner membrane, is the most important membrane change in the early phase of apoptosis. Phosphatidylserine binds to Annexin V-FITC conjugates during staining. This binding also occurs during the late phase of apoptosis. In the early phase of apoptosis, cell is impermeable to the PI, but in the necrotic cellular population or late apoptosis, the integrity of the cell membrane is disappears, resulting in cell permeability to PI. After PI entrance into the cell, it is attached to the fragmented DNA of the dead cell nucleus and detected by flow cytometry. Well living cells are impermeable to both Annexin V-FITC and PI.

In Figure 4, histograms a show the selection of cell ranges for cell death assessment in the control (no drug treatment) and
treatment groups, respectively. Colored points in the Q1 region (top left square) of histograms B, show necrotic cells, Q2 region (top right square) indicates late apoptosis, and Q3 region (downright square) indicate early apoptosis. The colored points in the Q4 region represent living cells. The mean percentage of apoptotic and necrotic Hela cells in each control and treated IC50 (3017 µg/ml) hydroxyurea nanodrug after repeated two times is shown in Table 2.

### Table II. Mean Percentage of Induced Apoptosis in Cells Treated with Nano-drug compared to Control Group after Two Times Experiments

<table>
<thead>
<tr>
<th>Cell death</th>
<th>Treated group with IC50 concentration of nanodrug</th>
<th>Control group</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>early apoptosis</td>
<td>2/09 ± 9/42</td>
<td>1/12 ± 4/84</td>
<td>0/111</td>
</tr>
<tr>
<td>late apoptosis</td>
<td>1/98 ± 20/20</td>
<td>0/66 ± 7/06</td>
<td>0/012</td>
</tr>
<tr>
<td>apoptosis</td>
<td>29/62 ± 0/11</td>
<td>1/90 ± 0/45</td>
<td>0/0003</td>
</tr>
<tr>
<td>necrosis</td>
<td>1/70 ± 14/20</td>
<td>1/20 ± 6/13</td>
<td>0/032</td>
</tr>
<tr>
<td>Living cell</td>
<td>5/67 ± 1/59</td>
<td>1/65 ± 8/19</td>
<td>0/004</td>
</tr>
</tbody>
</table>

The obtained values are significant according to the standard deviation ± the mean and mean difference at P< 0.05. The type of statistical test used is independent t-test.

According to the results of Table 2, 2/09 ± 9/42% of Hela cells treated with hydroxyurea nanodrug have early apoptosis and about 1/98 ± 20/20% have late apoptosis and about 1/70 ± 14/20% have necrosis. These results clearly show that the percentage of apoptotic cells in cells treated with hydroxyurea nanodrug was higher than the control group. Increased late apoptosis with a significant level of 0/111 did not show a significant difference between the two groups. Whereas the frequency percentage of total apoptosis) early and late significant at 0/0003 level. Apoptosis and necrosis of treated cells were analyzed by flow cytometry software version 7/6/1.

![Histogram a](image1.png)  
![Histogram b](image2.png)

**Figure 4.** Results of histogram Annexin/PI in flow cytometric test of apoptosis in control and nano-drug treated groups

Cancer nanotherapy is growing rapidly due to some of the limitations of the (traditional medicine) drug delivery system, such as lack of biodegradability, lack of water solubility, and low therapeutic indexes and the use of nanoparticles in modern drug delivery is to improve the quality of drug delivery and distribution, increase blood circulation system viability, increase drug uptake, reduce immunogenicity, targeted therapy, and reduce drug side effects. In the present study, for the first time, synthetic magnetic chitosan-hydroxyurea nanodrug was used to enhance the cytotoxic properties of hydroxyurea and more effective drug delivery and reduce the side effects of hydroxyurea. The schematic structure is shown in Figure 1. Since the chitosan nanocarrier has poor solubility in physiological pH, the PEG's composition has been used in the nanocarrier structure. It has also been used paramagnetic iron oxide particles in nanodrug structure to allow targeted nanodrug delivery to tumor tissue and reduce its side effects on healthy tissues. The presence of iron oxide nanoparticles (Fe3O4) in nanodrug structure allows the nanoparticles to be directed to the tumor site under an external magnetic field. The structure of the nanodrug was confirmed by FTIR, SEM and TEM spectroscopy before the experiments (Fig. 2). The results of this study showed that magnetic chitosan-hydroxyurea nanodrug can decrease the cell viability of Hela cervical cancer cell line and also induce apoptosis in them. Hydroxyurea is one of the common drugs in chemotherapy [19]. Studies have shown that hydroxyurea also inhibits DNA replication in a wide range of cells, including Saccharomyces cerevisiae [20]. In addition to its therapeutic properties, hydroxyurea has several side effects, such that hematological toxicity due to treatment is very common in patients with cervical cancer [19].

Natural chitosan polysaccharide is one of the most popular drug delivery nanocarriers due to its remarkable biological and structural properties such as cationic properties, aqueous solubility, biocompatibility, biodegradability and high adhesion to the nanocarrier, found many supporters in the field of medicine [9-11]. Chitosan has poor solubility in physiological pH. This intrinsic defect of chitosan has been resolved by changes such as pegylation, carboxylation, various conjugates, acetylation and thiolation, and has significantly increased the biological viability and increased drug circulation time and improved nanocarrier solubility [21]. Polyethylene glycol (PEG) is a non-toxic, non-antigenic, water soluble polymer, approved by the US Food and Drug Administration (FDA). Pharmaceutical compounds containing PEG have several benefits, such as increasing drug Persistence time, reducing the rate of nanocarrier degradation by metabolic enzymes or eliminating it by the immune system [22-23]. Therefore PEGylated chitosan is digested later by proteolytic enzymes of intestinal and gastric fluids [21]. In the present study, the cytotoxic effect of magnetic chitosan-hydroxyurea nanodrug...
on Hela cervical cancer cells was evaluated by MTT assay. As the results of this study showed (table 1), the viability of Hela cell line under the influence of hydroxyurea nanodrug decreased significantly with increasing concentration from 62/5 to 2000 μg/ml. The cellular cytotoxicity of the nanodrug was dose-dependent and there was a significant difference in the high concentrations (1000 and 2000 μg/ml) in the 48 h treatment with hydroxyurea nanodrug Compared to the control group.

The cytotoxicity of the nanodrug was dose-dependent and there was a significant difference in the high concentrations (1000 and 2000 μg/ml) in the 48 h treatment with hydroxyurea nanodrug compared to the control group. The IC50 of magnetic PEGylated hydroxyurea nanodrug 3017 μg/ml resulted in 4.88% loading of hydroxyurea on the magnetic PEGylated chitosan nanocarrier, the amount of hydroxyurea present in the nanodrug structure at IC50 concentration in Hela cell line. 7/14 μg/ml was calculated. Whereas the IC50 reported amount of hydroxyurea standard drug was 428 μg/ml in the Hela cell line [24]. As such, the cytotoxicity of the synthesized nanodrug on the Hela cell line was increased 1.29-fold compared to the standard hydroxyurea drug, which could significantly reduce the side effects of hydroxyurea. Figure 3, shows the morphology and mortality change of Hela cancer cells after the effect of nanodrug from spindle to spherical shape and granular cytoplasm. In a study consistent with the present study, Hou et al. showed that mPEG-FA-CNP nanocarrier toward mPEG-CNP and CNP nanoparticles were more strongly absorbed by tumor cells and increased mitomycin C toxicity. The shelf life of the nanodrug in the blood also increased [25]. In another study, Xiaodaon et al. showed that chitosan nanoparticles protected the enzymes at 37 °C and effectively bind the nanoparticle to the surface of human breast Bcap37 cancer cells [26], which is confirmed the role of chitosan nanocarriers in enhancing the cytotoxic nature of anticancer drugs similar to the results of this study. Chitosan is also a good candidate for siRNA transfer, as Ragelle et al. have shown that PEGylated chitosan along with polyethylene imine causes high levels of in vitro gene silencing, low cytotoxicity, and stability in blood plasma [27].

In a study consistent with the present study by Feng et al. the ability of polyelectrolyte chitosan complex and carboxymethyl chitosan sensitive to pH for drug oral doxorubicin hydrochloride was investigated. Their results showed that the administration of doxorubicin hydrochloride increased the stability of the drug in the blood. Also, biopsy studies on rat tissue treated with synthetic nanoparticles showed that the use of these nanocarriers significantly reduced the toxicity of the drug to the kidney and heart tissue [28]. In addition, the results of Annexin-V-FITC/PI apoptosis flow cytometry (table 2) test also showed that the IC50 concentration of magnetic PEGylated chitosan-hydroxyurea nanodrug for 24 hours significantly affected cell death by increasing apoptosis and necrosis induction by 2.48 and 3.2 times, respectively in nanotreated Hela cells than the control group, confirming the results obtained from the evaluation of nanodrug cytotoxicity. Induction of planned cell death or apoptosis was also higher than the incidence of cell necrosis, which was also statistically significant. These findings, which confirm other studies, suggest that synthesized hydroxyurea nanodrug such as hydroxyurea are able to induce apoptosis and can inhibit the growth of cancer cells. For example, Iglesias-Guimaraes et al. showed that the accumulation of DNA fragments treated with hydroxyurea was associated with cytotoxicity. During the study, the researchers studied and reported fragmented DNA, DNA fragments as regular fragments of oligonucleosomes, which is a prominent feature of apoptotic cell death [29]. Also, another study by Hakan et al. found that hydroxyurea can induce apoptosis in the human Hela cell line. In this study, the induced response under the influence of hydroxyurea, the regular accumulation of chromatin on the cell wall along with the formation of apoptotic membrane bodies was quite evident [30]. Singh et al. reported that the hydroxyurea drug, in addition to inducing apoptosis, inhibited DNA synthesis by fragmented DNA and stopping the cell cycle in the S phase [31]. Also, Yeo et al. showed that treatment of human diploid fibroblast cells with hydroxyurea could induce cellular aging through induction of P53 and p21(Waf1) genes and inhibit cell growth. Increased P53 that results from induction of hydroxyurea can also induce apoptosis and cell death [32], which confirms the results of this study. Since this is a laboratory study, it has some limitations and its results cannot be generalized to human society. Therefore, it is recommended to investigate the adverse effects of nanodrug on cells as well as in vivo in animal models. It is also recommended that in future studies evaluate the effect of this nanodrug on other cancers and diseases such as anemia, where hydroxyurea is commonly used for treatment.

V. CONCLUSION

The findings of this study showed that the magnetic chitosan-hydroxyurea nanodrug decreases cervical cell viability in a dose dependent manner. It also significantly induces apoptotic death in these cells. These findings indicate that hydroxyurea nanodrug as an anticancer drug is likely to have a lower dose efficacy than the hydroxyurea drug in the treatment of cancer and can be used in vitro to reduce side effects of the drug and replaces the standard anti-cancer hydroxyurea drug.

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