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# The Apoptotic and Necrotic Effects of Cisplatin Loaded Chitosan Nanoparticles on Hela Cell Lines

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**Abstract:** This study was designed to fabricate chitosan nanoparticles and to evaluate expression profiles of apoptotic and necrotic markers as well as cytotoxicity on HeLa cell lines following interaction with cisplatin loaded chitosan nanoparticles. Nanoparticles were synthesized and determined by scanning electron microscopy and atomic force microscopy, the size of chitosan nanoparticles varied from 100 to 200 nm. The amount of cisplain released from cisplatin loaded nanoparticles was measured at different time points by Graphite Furnace-Atomic Adsorption Spectrometer. Almost whole amount of loaded cisplatin was relased within 12 hours. The effective cisplatin dose was achieved by incubating HeLa cells onto cisplatin loaded chitosan nanoparticles.HeLa cells were incubated with chitosan, cisplatin and cisplatin loaded nanoparticles. While chitosan nanoparticles exerted no noticable cytotoxicity, cisplatin and cisplatin loaded chitosan nanoparticles exerted cytotoxicity in a dose-dependent manner. Cytotoxicity in cells seeded into nanopaticles was slightly lower compared to those treated with cisplatin alone, which was, however, statistically insignificant. The apoptotic indexes generated from caspase-3 staining and double staining was compatible and found as 60 ± 5%, 53 ± 4% for cisplatin and cisplatin loaded nanoparticles, respectively. The necrotic index was 64.2 ± 3%, 60.3 ± 2% for cisplatin and cisplatin loaded nanoparticles. Cisplatin and cisplatin loaded chitosan nanoparticles exert cytotoxic, apoptotic and necrotic effects on HeLa cells in a dose dependent manner. The cytotoxic effect of cisplatin loaded nanoparticles is comparable to that of cisplatin alone. Thus, chitosan nanoencapsulation of cisplatin possibly provides a prolonged effect.

**Key words:** Nanoparticle · Chitosan · Cisplatin · SEM · Cytotoxicity · Caspase 3 · Apoptosis · Necrosis

## INTRODUCTION

Cisplatin (cis-diammine-dichloroplatinum (II)) is one of the most widely used anticancer agents. It has been found effective in the treatment of a variety of cancers, including testicular, bladder, ovarian, head and neck, cervical, lung and colorectal cancer [1-4]. The efficacy of cisplatin in cancer treatment is limited due to resistance, which can be intrinsic (such as observed in patients with colorectal, lung and prostate cancer) or acquired following cisplatin chemotherapy (such as often seen in patients with ovarian cancer). Moreover, the cisplatin effect in its conventional form is very short lasting. Thus, frequent doses are required to maintain theurapautic concentrations. Due to repeated doses, the body develops drug resistance against cisplatin. Consequently, the cumulative toxicity of cisplatin may often overcome itstherapautic effect [5].

Biodegradable nanoparticles are frequently used to enhance the therapeutic value of various water soluble/insoluble medicinal drugs and bioactive molecules by improving bioavailability, solubility and retention time [6]. These nanoparticle–drug formulationsare found to reduce the risks of toxicity [6]. Chitosan has been largely

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suggested as a potential nanoparticle carrier due to its favourable properties. It is a mucoadhesive polymer that has the ability to enhance drug absorption by re-arranging the tight junction proteins [7]. Chitosan nanoparticles provide protection against enzymatic degradation, ensuring that encapsulated drugs or genes are delivered [8]. Chitosan nanoparticles have been shown to be able to control the release of genes or drugs in a controlled, sustained manner [9]. Chitosan nanoparticles are able to enter the nuclear membrane and deliver the treatment agent directly into the nucleus [10]. Chitosan on its own demonstrated growth inhibitory effects on cancer cells to a certain extent apoptosis of bladder tumour cells via caspase-3 activation or other yet unknown mechanisms [11]. As mentioned above, chitosan derivates and copolymers with different crosslinkers have been used as cisplatin carriers, but pure chitosan has not been used as a cisplatin carrier in HeLa cancer cell line.

In this study, we aimed to fabricate chitosan nanoparticles and to evaluate expression profiles of apoptotic and necrotic markers. We also evaluated cytotoxicity on HeLa cell lines following their interaction with cisplatin loaded chitosan nanoparticles. We investigated the apoptotic and necrotic effects in HeLa cancer cell line of the release of cisplatin, loaded on chitosan nanoparticles and applied with the appropriate dose and duration.

## MATERIALS AND METHODS

**Materials:** Human cervix epithelioid carcinoma cell line (HeLa) was obtained from the Tissue and Cell Culture Bank of the Foot and Mouth Disease Reseach Institute (Ankara, Turkey). Cell culture flasks and other plastic material were purchased from Corning (USA). The growth medium, Dulbecco Modified Medium (DMEM) without L-glutamine supplemented with fetal calf serum (FCS) and Trypsin-EDTA were purchased from Biological Industries (USA). Chitosan (MW: 50-190.000 DA and Deacetylation degree 75-85%) and tripolifosfat (TPP) were purchased from Sigma-Aldrich(USA). Caspase 3 antibody was purchased from Neomarkers (Fremont, CA). 2-(4-iodophenyl)- 3-(4-nitrophenyl)-5-(2, 4-disulfo-phenyl)-2H-tetrazolium, monosodium salt (WST-1) was purchased from Roche (Germany). Hoeshct 33342 and propodium iodide were purchased from Serva (Israel). Phosphate buffer solution (PBS) was purchased from Sigma-Aldrich (USA). Cisplatin solution (1mg/ml) was purchased from Mayne Pharma Plc (UK).

**Synthesis of Chitosan Nanoparticles:** A 5% chitosan solution was prepared as follows: 500 mg chitosan was dissolved in 1% v/v acetic acid and its pH was adjusted to 4.6-4.8 by 10 MnaOH. The prepared chitosan solution was then mixed with 0.5 % TPP (tripolyphosphate) (3:1 (v/v) using a sonicator while stirring. After being kept for an hour at room temperature, the mix solution was centrifuged at 9.000 rpmfor 15 minutes. Chitosan particles were collected in the form of pellets[12]. The basis of the mix is based on an electrostatic bonding between the positive amine groups of chitosan and the negative phospahate groups of TPP [13] (Figure 1).

**Characterization of Chitosan Nanoparticles:** The shape and surface characteristics of the nanoparticles were observed by a scanning electron microscope (SEM, JEOL JSM 6060, Japan) and atomic force microscope (AFM) (Nanomagnetics, Turkey). The zeta potential of the nanoparticles in PBS was measured by a zeta potential analyzer (Malvern, Nano-ZS). The nanosizer measures size and zeta potential with DLS (dynamic light scattering) technique.

**Prepation of Cisplatin Loaded Chitosan Nanoparticles:** 100 mg of chitosan nanoparticles was poured into a glass beaker containing 50 ml of cisplatin solution (1mg/ml). Upon sonicating for 15 min to prevent aggregation, the mixture was then stirred at 4.000 rpm for 30 min at room temperature, using a magnetic stirrer. The mixture was then ultracentrifuged at 10°C and 40.000 rpmfor an hour. The supernatant was filtered through a 0.1 μm syringe filter (Millipore). The amount of unloaded cisplatin was determined with a graphite furnace- atomic absorption spectrometer (GF-AAS) based on a standard graph prepared from a commercially bought cisplatin. The amount of cisplatin loaded into chitosan nanoparticles was determined by subtracting the amount of unloaded cisplatin from the total amount used (50 mg). Then the cisplatin loaded chitosan nanoparticles were then lyophilized at -58°C and 0.5 bar for 24 hours (HETO, Denmark) and kept in lidded small glass bottles at room temperature until assayed. The % loaded cisplatin was calculated using the following formula:

$$cisplatin\ loading\ ratio\% = \frac{cisplatin\ loaded\ amount - unloaded\ cisplatin\ amount}{cisplatin\ loaded\ amount} \times 100$$

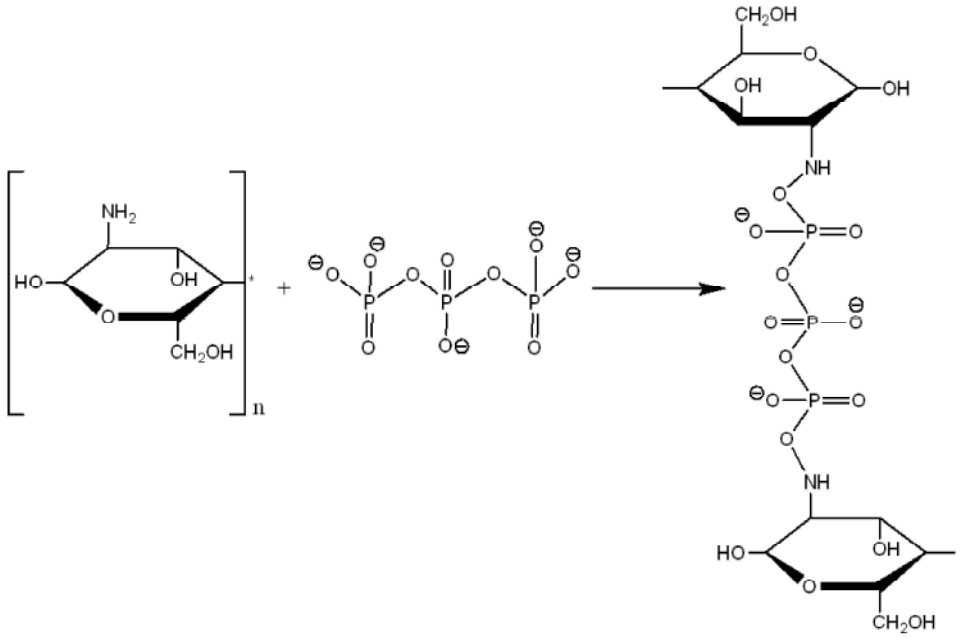

Fig. 1: Polyanionicinteraction of tripolyphosphatewithchitosan

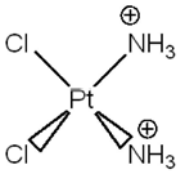

Fig. 2: Molecularstructure of cisplatin

The molecular structure of cisplatin is presented in Figure 2.

**Cisplatin Release from Chitosan Nanoparticles:** 100 mg of cisplatin loaded chitosan nanoparticles were dispersed in 20 ml of distilled water and sonicated for 15 minutes. The mixture was divided into 5 different beakers, each containing 4 ml of the mixture. The mixture in the beaker was stirred at room temperature for the duration of different time periods: 12, 24, 48, 72 and 96 hours. At the end of the stirring periods, the mixture was centrifuged at 13.000 rpm for 15 min. Then the supernatants were filtered through a 0.1 μm pore filter. The cisplatin released was measured in the filtered solution using a GF-AAS [14]. For GF-AAS analysis, a Perkin Elmer Analyst 100 Atomic Absorption spectrophotometer with HGA 800 Electrothermal Atomization unit (Boston, USA) was used. The machine was equipped with deuterium lamp. Background correction was applied. Hallow cathode lamp of platinum was used and pyrolitic coated graphite furnaces were used for analyses.

**Scanning Electron Microscopy for Cell Surface Integrity and Apoptotic Figures of Hela Cells Seeded on Chitosan Nanoparticles:** HeLa cells ($25x10^3$ cells per well) were seeded into6-well plates containingDMEM solution. After incubating with chitosan nanoparticles loaded with various amount of cisplatin (50, 100, 250, 500, 1000 μg/ml)for 24 hours period, cells were washed with PBS (pH 7.2) and kept in % 0.3 gluteraldehyde at +4°C for 30 minutes. Then, HeLa cells were washed 2 times with cold PBS (pH 7.2 and 4°C).PBS was then replaced with 70% alcohol. Upon critical point dry and gold sputter coating (15–20 nm), cells were examined by SEM (JEOL JSM 6060, Japan) for presence of apoptotic figures.

**Cytotoxicity:** The WST-1 assay was used to evaluate cytotoxicity exerted by chitosan nanoparticles, cisplatin and cisplatin loaded nanoparticles. The WST-1 assay is a simple colorimetric assay to measure cell cytotoxicity, proliferation and viability [15].

HeLa cells were seeded into 96-well microassay plates at a density of $5 \times 10^3$ cells/well and incubated overnight. The chitosan nanoparticles (7.5, 15, 30, 50, 75, 100 μg/ml) and cisplatin (7.5, 15, 30, 50, 75, 100 μg/ml) and cisplatin loaded chitosan nanoparticles loaded with various amount of cisplatin (7.5, 15, 30, 50, 75, 100 μg/ml) were diluted with cell culture medium and inoculated into the wells and incubated for 48 h. The targeted concentration of cisplatin loaded in nanoparticles was calculated based

on the loading ratio obtained (46%). To obtain above mentioned cisplatin concentrations in nanoparticles, the following cisplatin loaded chitosan concentrations were used: 32.6, 65.2, 130.4, 217.3, 326.08 and 434.7 µg/ml. Nanoparticles were UV sterilized for 30 min prior to use. The cell culture medium in each well was then replaced with 100 µl of fresh medium and 15 µl of WST-1 solution. After incubating for another 4 h at 37°C in a dark condition, the wells were read at 420-480 nm using an ELISA plate reader (Biotek) and then the percentage of viable cells was calculated. For WST-1 assay, the control HeLa cell viability was defined as 100?. The assay was validated with positive and negative controls, for which the cell culture medium with or without $H_2O_2$, respectively, were applied to the cells. The samples were evaluated for each group.

**Analysis of Apoptotic and Necrotic Cells**

**Double Staining:** Double staining of Hoechst dye 3342 (2 µg/mL$^{-1}$) and propodium iodide (PI) was performed to quantify the number of apoptotic cells HeLa cells ($25x10^3$ cells per well) were seeded into 24-well plates containing DMEM solution. After treating with different concentrations of cisplatin and cisplatin loaded nanoparticles for 24 hours, attached and detached cells were harvested. Upon PBS wash, cells were incubated with Hoechst dye 3342 (2 µg/mL$^{-1}$), PI (1 µg/mL$^{-1}$) and DNAse free-RNAse (100 µg/mL$^{-1}$) for 15 min at room temperature. Then, 10-50 µL of cell supension was smeared on a glas slide and coverslipped for examination by fluorescence microscopy (Leika, DMI 6000) [15]. In doublestaing method of Hoechst dye and PI, the nuclei of normal cells are stained light blue while apoptotic cells are stained dark blue [15]. The apoptotic cells were identified by their nuclear morphology based on nuclear fragmentation or chromatin condensation. Necrotic cells were stained red by PI. As the necrotic cells lack of plasma membrane integrity, PI dye can crossthe cell membrane. However, it cannot cross an intact cell membrane [15]. The number of apoptotic and necrotic cells in 10 random microscopic fields were counted. The number of apoptotic and necrotic cells were determined with DAPI and FITC filters of a Fluorescence Inverted Microscope (Leica, Germany). Data were expressed as the ratio of apoptotic or necrotic cells to normal cells.Three samples were run for each group.

**Caspase 3 Immunostaining for Detection of Apoptotic Cells:** Caspase-3 immunostaining wasused as previously described with minor modifications[16]. In brief, harvested HeLa cells were incubated with 3% $H_2O_2$ for 5 min and rinsed with PBS for 15 min. The cells were incubated with a blocking solution prepared in PBS and then incubated with primer antibodies caspase-3 (at a dilution rate of 1:300) for 45 min at room temperature. The cells then were incubated with a biotin-conjugated secondary antibody (at a dilution rate of 1:300) for an hour at room temperature and then with avidin-biotin-peroxidase complex (Santa Cruz Biotechnology, Inc. USA) for an hour. For coloring reaction, 3, 3 diaminobenzidine (DAB) as substrate. Sections were counterstained with hematoxylin. For negative controls, primary antibody was omitted in a section. Using the 10X microscope objective, caspase 3-positive and negative cells were counted. At least 100 cells per field were evaluated in three randomly selected microscopic fields. Three samples were run for each group.

**Statistical Analysis:** Cytotoxicity, apoptosis and necrosis studies were performed in triplicate. Data are represented as mean ± standard deviation(std) and analyzed by the dependent samples t-test (for two groups). Statistical significance was set at $P<0.05$. Caspase-3 immunostaing data were evaluated separately by Mann-whitney U test.

## RESULTS

**Characterization of Chitosan Nanoparticles:** SEM and AFM analyses revealed that chitosan nanoparticles were spherical in shape and 100-200 nm in size (Figure 3). In DLS analysis, the average size and average potential were 174.5 nm and +37.5mV, respectively (Figure 4).

**Release of Cisplatin from Chitosan Nanoparticles:** Figure 5 illustrates the release of cisplatin from chitosan nanoparticles. The GF-AAS results indicated that 46% of the used cisplatin was loaded into chitosan nanoparticles. At the end of a 12 hour period, 60.9% of the loaded cisplatin was released. At the end of a 24 hour period, nanoparticles were disintegrated.

**Relase of -Cisplatin from Chitosan Nanoparticles:** Figure 5 presents the release of cisplatin from chitosan nanoparticles. The GF-AAS results indicated that 46% of the used cisplatin was loaded into chitosan nanoparticles. At the end of a 12 hour period, 60.9% of the loaded cisplatin was released. At the end of a 24 hour period, nanoparticles were dispersed.

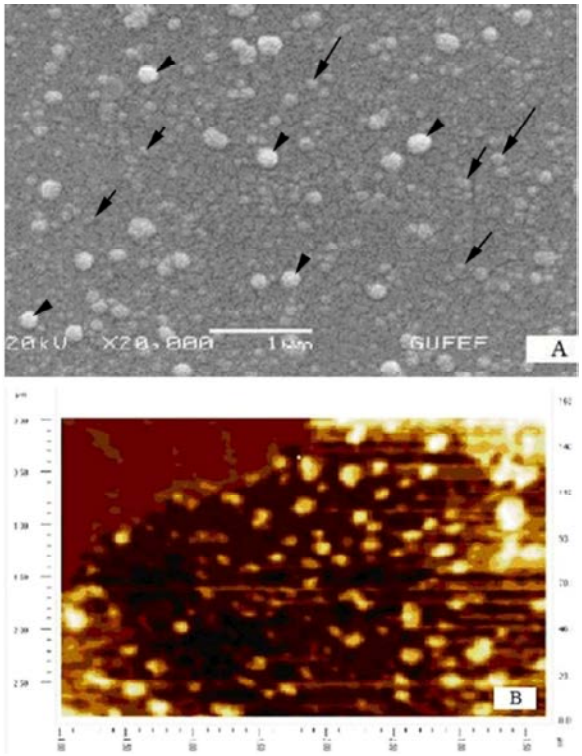


Fig. 3: Morphological features of chitosan nanoparticles A) scanning electron microscopy (SEM) photograph B) Atomic force microscopy (AFM) photograph

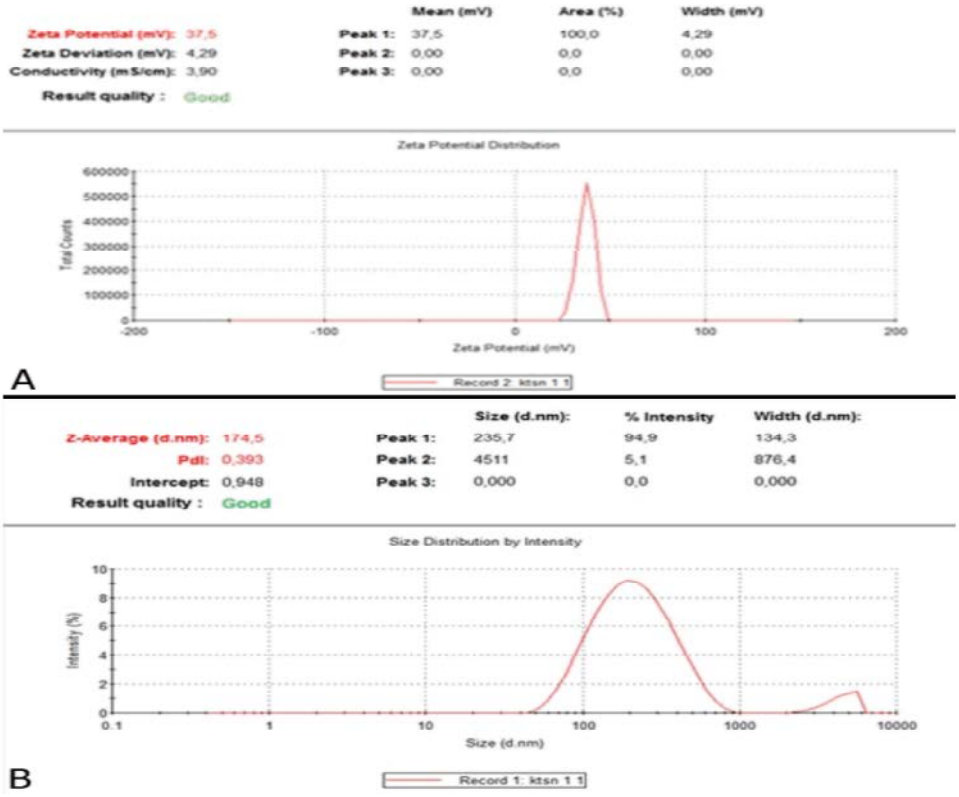


Fig. 4: Zeta-potential (A) and zeta-size (B) analyses of chitosan nanoparticles

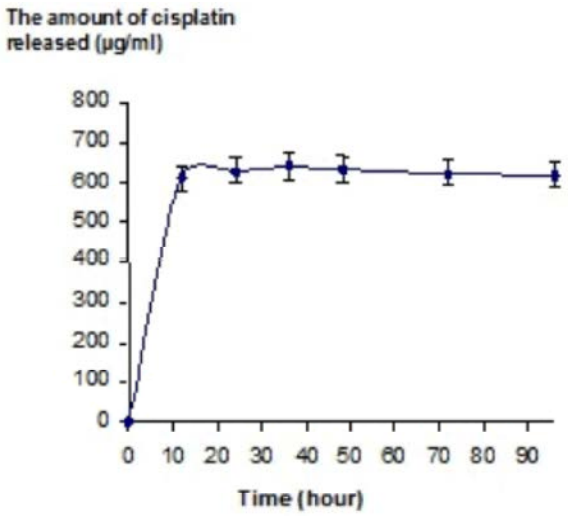


Fig. 5: Cisplatin releasing profile from chitosan nanoparticles

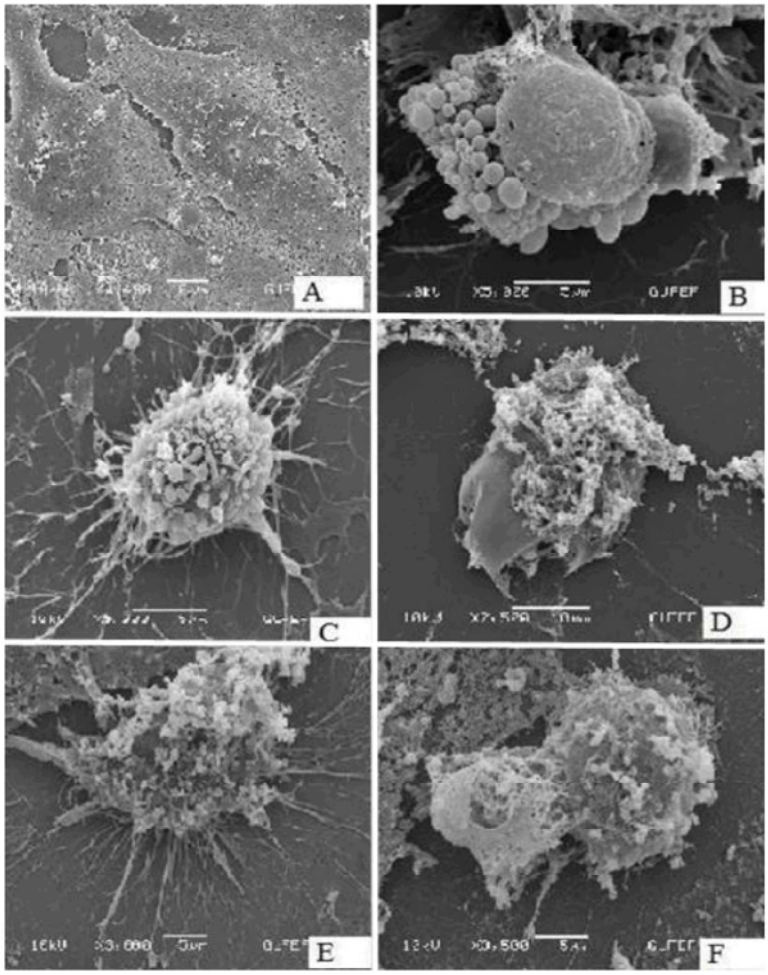


Fig. 6: Scanning electron microscopy photographs of transfected HeLa cells.(A) scanning microscope image of untreated HeLa cells as a control; (B) 50 µg.mL concentration of cisplatin/HeLa cells conjugate indicates cytoplasmic blebs of apoptotic cells; (C) 100 µg.mL concentration (D) 250 µg.mL (E) 500 µg.mL (F) 1000 µg/ml of cisplatin loaded chitosan nanoparticles/HeLa cells conjugate

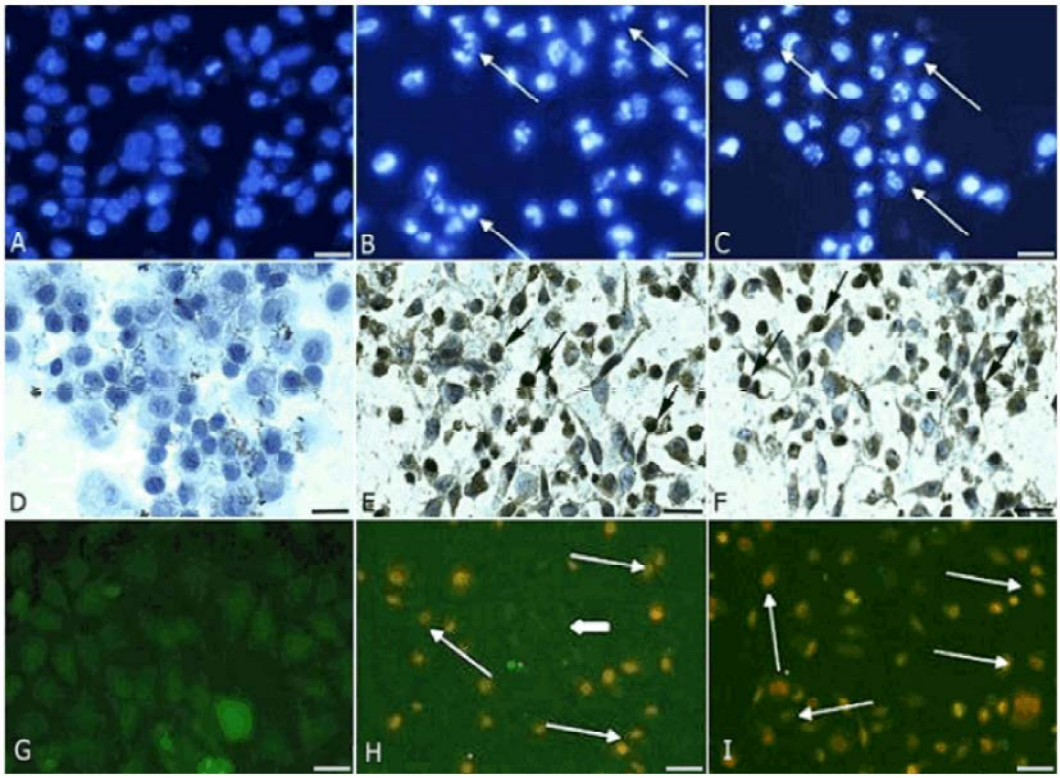


Fig. 7: Apoptotic an necrotic effects of chitosan, free cisplatin and cisplatin loaded chitosan nanoparticles. (A) Fluorescent microscope image of nucleus of untreated HeLa cells as a control, where formation of lifeless spots demostrates nucleus of non-apoptotic cells; (B) 30 µg.mL concentration of cisplatin/HeLa cells conjugate (stained with Hoechst 33342), where bright spots and dispersed nuclei(long arrow ) indicates nucleus of apoptotic cells; (C) 30 µg concentration of cisplatin loaded chitosan nanoparticles/HeLa cells conjugate (stained with Hoechst 33342), where bright spots and dispersed nuclei(long arrow ) indicates nucleus of apoptotic cells. (D) under visibileligth (stained with caspas-3 immunostaining kit), where blue cytoplasma of cell image indicates non-apoptotic cells; (E) 50 µg.mL concentration of Cisplatin/HeLa cells (stained with caspas-3 immunostaining kit), where brown cytoplasma of cells image indicates the formation of apoptotic cells; (F) 50 µg concentration of cisplatin loaded chitosan nanoparticles/HeLa cells conjugate (stained with caspase-3). (G), Control(H)30 µg.mL concentration of cisplatin/HeLa cells conjugate (stained with Propodiumiyodide (PI)) (I), 30 µg concentration of cisplatin loaded chitosan nanoparticles/HeLa cells conjugate indicated necrotic cell as red nucleus(long arrow). All images were recorded with x400 magnification. Scale bar is 10 µm.

Table 1: The percentage of live HeLa cells in toxicity test after interaction with chitosan, cisplatin and cisplatin *loaded nanoparticles on HeLa cells*

| The amount of test material (µg/ml) | Live cells (%) Exposed to Chitosan nanoparticles | Live cells (%) Exposed to cisplatin | Live cells (%) Exposed to cisplatin loaded Chitosan nanoparticles* |
|---|---|---|---|
| Control | 99.96±1 | 98.78±1 | 99.54±1 |
| 7, 5 | 99.60±1 | 69.2±2 | 74.61±3 |
| 15 | 98.3±2 | 42.32±4 | 49.3±4 |
| 30 | 93.4±2 | 31.23±4 | 37.2±2 |
| 50 | 90.7±2 | 20.64±5 | 28.34±3 |
| 75 | 88.12±2 | 9.85±6 | 21.67±2 |
| 100 | 85.67±2 | 3.19±3 | 13.86±2 |

For control, cell culture medium was used instead of effective substance

*The targeted concentration of cisplatin in nanoparticles was calculated based on the loading ratio obtained (46%). To obtain above mentioned cisplatin concentrations in nanoparticles, the following cisplatin loaded chitosan concentrations were used: 32.6, 65.2, 130.4, 217.3, 326.08 and 434.7 µg/ml

Table 2: Apoptotic index in HeLa cancer cells resulting from cisplatin alone and cisplatin and chitosan nanoparticles loaded various concentrations of cisplatin. Data generated by caspase-3 methods. The percent data were given as mean±std

| Cisplatin concentration (µg/ml) | Apoptotic index by only nanoparticles (%) | Apoptotic index by cisplatin alone (%) | Apoptotic index cisplatin loaded chitosan nanoparticles* (%) |
|---|---|---|---|
| Control | 3±1 | 5±1 | 5±1 |
| 7.5 | 5±1 | 29±3 | 21±1 |
| 15 | 6±2 | 42±2 | 30±2 |
| 30 | 6±4 | 45±2 | 38±3 |
| 50 | 8±3 | 58±4 | 52±5 |
| 75 | 9±2 | 60±5 | 53±4 |

*The targeted concentration of cisplatin in nanoparticles was calculated based on the loading ratio obtained (46%). To obtain above mentioned cisplatin concentrations in nanoparticles, the following cisplatin loaded chitosan concentrations were used: 32.6, 65.2, 130.4, 217.3, 326.08 and 434.7 µg/ml

Table 3: Necrotic index in HeLa cancer cells resulting from cisplatin alone and cisplatin and chitosan nanoparticles loaded various concentrations of cisplatin. Data generated in double staining of Hoechst dye 33342 and propodium iodide. The percent data were given as mean±std

| Concentration of the test material (µg/ml) | Necrotic index induced by chitosan (%) | Necrotic index induced by cisplatin alone (%) | Necrotic index induced by cisplatin loaded chitosan nanoparticles* (%) |
|---|---|---|---|
| Control | 1±1 | 2±1 | 3±1 |
| 7.5 | 2±1 | 21.8±2 | 16.4±2 |
| 15 | 3.7±2 | 33.3±2 | 28.5±2 |
| 30 | 5.2±2 | 44.6±2 | 39.8±2 |
| 50 | 8.4±2 | 56.5±3 | 52.2±2 |
| 75 | 11.3±2 | 64.2±3 | 60.3±2 |

*The targeted concentration of cisplatin loaded in nanoparticles was calculated based on the loading ratio obtained (46%). To obtain above mentioned cisplatin concentrations in nanoparticles, the following cisplatin loaded chitosan concentrations were used: 32.6, 65.2, 130.4, 217.3, 326.08 and 434.7 µg/ml

**Scanning Electron Microscopy for Determination of Surface Integrity and Apoptotic Figures of Hela Cells Seeded in Chitosan Nanoparticles:** In absence of cisplatin or cisplatin loaded nanoparticle treatment, HeLa cells were observed quite adherent (Figure 6A). In Cytoplasmic blebs and apoptotic bodies were visible in cells treated cisplatin (50 µg/mL) and nanoparticles loaded with the same amount of cisplatin. The severity of apoptotic figures increased with increasing cisplatin concentration 100 µg/ml, 250 µg/ml, 500 µg/ml and 1000 µg/ml (Figure 6).

**Cytotoxicity:** The cytotoxicity data are given in Table 1 Chitosan nanoparticles exerted a minimum cytotoxicity on cells. Cisplatin and cisplatin loaded nanoparticles exibited a marked cytotoxicity in a dose-dependent manner. When used at a concentration of 7.5 µg/ml, cisplatin either alone or loaded on nanparticles exerted a cytotoxicity rate of 1.22±1and 0.46±1, respectively. When cisplatin concentration inreasedupto 100µg/ml, cisplatin either alone or loaded on nanparticles exerted a cytotoxicity rate of 96.81±3 and 86.14±2, respectively.

**Analysis of Apoptotic and Necrotic Index**

**Apoptotic Effect:** Two different methods were used to determine the apoptotic effects of cisplatin and cisplatin loaded chitosan nanoparticles: caspase 3 immunocytochemistry and double staining of hoechst (33342) and PI in HeLa cells (Figure 7). The results of double-staining methods (Figure 7A, 7B, 7C) and caspase-3 immunocytochemistry (Figure 7D, 7E, 7F) are quite competible. The apoptotic index generetared in caspase-3 immunostaining is presented in Table 2. In the double staining, hoechst (33342) fluorescent dye binds to DNA that results in a blue color in the cell nucleus visible under the blue fluorescent light. Apoptotic cell nuclei are distinguished from other blue stained nuclei by their distorted borders and brighter appearance (Figure 7A, 7B, 7C). In caspase- 3 immunostaining, the cytoplasm of non-apoptotic cells appears blue in color (Figure 6D) while the cytoplasm of apoptotic cells appears brown in color due to DAB chromogen (Figure 7E, 7F).

The use of cisplatin alone resulted in a high apoptotic index in HeLa cell compared to cisplatin loaded nanoparticles (Table 2). Cisplatin alone and cisplatin loaded nanoparticles exibited a marked apoptotic effects in a dose-dependent manner. Cisplatin at a concentration of 7.5 µg/ml either alone or loaded in nanoparticles caused apoptosis in 29±3% and 21±1% of cells, respectively. When cisplatin concentration inreased upto 75µg/ml, the percentage of apoptotic cells also incerased up to 60±5 and 53±4 in cells treated either with cisplatin or cisplatin loaded nanoparticles, respectively. Cisplatin loaded chitosan nanoparticles had lower apoptotic index in general; but no statistical significant difference was obtained at any concentrations.

**Necrotic Effect:** When HeLa cells are stained with propodium iodide fluorescent dye in double-staining solution, the nuclei of necrosed cells appeared red in color under red and green fluorescent lights (Figure 7G, 7H, 7I). The non-necrotic appeared green due to Hoecsht staining under red filter (Figure7G). On other hand, nectrotic cells appeared red dur to PI nuclear staining (Figure 7H, 7I). The results of the necrotic index study are presented in Table 3. Chitosan nanoparticles without cisplatin had low necrotic effects, approximately 11% even at the highest concentration (75 µg/ml). However, cisplatin and cisplatin loaded nanoparticles exibited a marked necrotic effects in a dose-dependent manner. Cisplatin at a concentration of 7.5 µg/ml either free or loaded in nanoparticles caused necrotic effects in 21.8±2% and 16.4±2% of cells, respectively. When cisplatin concentration increased upto 75µg/ml, the percentage of necrotic cells incerased up to 64.2±3 and 60.3±2 in cells treated cisplatin alone and cisplatin loaded nanoparticles, respectively. Cisplatin loaded chitosan nanoparticles had a relatively lower necrotic index, but no statistically significant differences were obtained at any concentrations.

## DISCUSSION

Cisplatin is an effective chemotheraupatic agent employed in the treatment of various cancers including cervical cancer. Despite impressive anti-neoplastic activity of cisplatin, side effects and drug resistance limits its use in cancer therapy. Cisplatin is often used in high doses to obtain a more effective treatment. Unfortunately, several severe side effects, notably nephrotoxicity and ototoxicity limit the dose that can be given to patients. Moreover, repeated use of cisplatin promotes resistance to cisplatin-induced apoptosis in cancer cells. Thus, drugs that sensitize cancer cells to cisplatin could increase the clinical efficacy of cisplatin. Several attempts have been made for a more selective cisplatin administration, including its systemic administration in the form of a) soluble drug–polymer conjugates, such as the complexes with polycarboxylates, poly(amidoamines), polyamidoamine dendrimers and the complexes with N-(2-hydroxypropyl) methacrylamide, or b) colloidal carriers, such as pegylated liposomes, poly(aspartic) acid-poly(ethylene glycol) micelles and poly(caprolactone)-poly(ethylene glycol), or c) poly(caprolactone)–poly[2-(N, N-dimethylamino)ethyl methacrylate] micelles [17]. The association of cisplatin with long-circulating carriers alters drug pharmacokinetics and results in increased drug accumulation in tumors, based on the ‘‘enhanced permeability and retention’’ (EPR) effect. The EPR effect is a result of leaky capillaries adjacent to solid tumors and a lack of a lymphatic system for the drainage of drugs back to the systemic circulation [18]. The halflife of cisplatin is 12 hours, thus a sustained cisplatin presence is needed to obtain maximum effect. With this study, we aimed to prolong cisplatin effects through its controlled release from chitosan nanoparticles.

Tseng and coworkers [19] employed gelatin nanoparticles (GPs) as carriers of cisplatin (CDDP) with anticipated improved therapeutic effect and reduced side effects. They studied the anticancer activities of CDDP-incorporated in GPs (GP–Pt) with biotinylated-EGF (bEGF) modification (GP–Pt–bEGF). GP–Pt–bEGF with EGFR affinity produced much higher Pt concentrations in A549 cells (high EGFR expression) than in HFL1 cells (low EGFR expression). The GP–Pt–bEGF-treated group at 24 h was necrotic due to cisplatin toxicity. Gryparis and coworkers [20] studied the cytotoxicity of blank PLGAmPEG nanoparticles and the in vitro anticancer activity of cisplatin-loaded PLGA-mPEG nanoparticles on human prostate LNCaP cells. Blank PLGA-mPEGnanoparticles exhibited low cytotoxicity, which increased PLGA/PEG ratio in the PLGA-mPEG copolymer used to prepare the nanoparticles. PLGA-mPEG nanoparticles loaded with cisplatin entered the cells and exerted in vitro anticancer activity against LNCaP human prostate cancer cells that was comparable to the activity of free (non-entrapped in nanoparticles) cisplatin. Moreno and coworkers [21] developed biodegradable lactic acid-glycolic acid copolymer (PLGA) formulations incorporating cisplatin to evaluate its cytotoxicity in cultured cells. The cytotoxic effect caused by encapsulated cisplatin was lower than for the free-agent at most of the doses and exposure times used in Moreno’s work. In our study, cisplatin loaded chitosan nanoparticles exerted a cytotoxic effect in a dose dependent manner that was comparable to that of free cisplatin. Thus, we think that the release rate of cisplatin from chitosan nanoparticles can prolong the cisplatin effects.

Chitosan has been used as a drug delivery system in various studies. According to Hu F-Q’ study [22], chitosan released doxorubicin, another cancer drug, for 12 hours. In our study, chitosan nanoparticles also released cisplatin effectively for 12 hours. We fabricated chitosan nanoparticles which are 100-200 nm in size for cisplatin delivery. In our release study, 60.9% of the loaded cisplatin was releasedat the end of the 12 hour period and nanoparticles were dispersed at the end of a

24 hour period.We think that more functional groups in chitosan became available upon its dispersion of chitosan; thus, the relase of cisplatin was interfered after this point due to an increase in electrostatic bonding.

As previously mentioned that chitosan has many advantages as compared to other drug delivery systems such as cationic liposomes [8]. As a raw material, it is easy to find and cheaper to modify for various applications Due to its physical properties, chitosan, as drug delivery system, has some advantages. Grenha and coworkers [23] prepared chitosan nanoparticles cross-linked with various concentrations of tripolyphosphate (TPP) and characterized with zeta size and potential analyses. They found zeta size and potential of chitosan as 300-390 nm and +34 - +45 mV, respectively, at the chitosan: TPP ratios of 3.6:1, 4:1, 5:1 and 6:1 (w/w). In our studytheaverage size and average potential were 174.5 nm and +37.5mV, respectively. Such data indicated that chitosan nanoparticles are stable and has a high wear resistance. In AFM and SEM analyses in our study, the particle sizes change between 80nm-200nm. In SEM analyses, some particles were larger than 200 nm due to aggregation of the particles. We think this aggregation is also reflected in zeta analyses as the first data showed particles size smaller than 200nm, but later data showed particle sizes larger than 200nm as seen in Figure 4.

Chitosan itself has been reported to have some anticarcinogeniceffects. Hasegawa and coworkers [11] showed thatthe cytotoxic effect of chitosan itself on human bladder cell line. They determined cytotoxicity with WST-1 test, DNA fragmentation and upregulation of caspase-3, an apoptotic marker. In our study, chitosan nanoparticles exerted minimal cytotoxicity on HeLa cells especially at lower concentrations. When the cells were exposed to chitosan at a concentration of 7.5 µg/ml, the live cell percentage was 99.60±1, which was quite comparable to that of control (99.96±1). We detected some cytotoxicity at very high concentrations of chitosan. For instance, the live cell percentage dropped down to 85.67±2 at a concentration of 100 µg/ml. In Hasegawa's [11] study, however, the live cell percentage detected by WST-1 test in human bladder cancer cells was approximately 20% when chitosan concentration was 100µg/ml. We think that the low molecular chitosan had minimal cytotoxicity in HeLa cells. Parallel with our study, Campos and coworkers [24] claimed a minimumcytotoxicity on changconjuctival cells exerted by chitosan nanoparticles. At 0.25, 0.5, 1.0 and 2.0 mg/ml concentrations, chitosan nanoparticles exerted 95.2%, 98.4%, 91.25% and 96.15%, respectively.

In conclusion, chitosan nanoparticles effectively retain and release cisplatin for 12 hours. Cisplatin loaded chitosan nanoparticles exert a cytotoxic effect comparable to that of free cisplatin. Further study should focus on possible *in vivo* use of cisplatin-loaded chitosan nanoparticles.

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