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Original Article

MODULATION OF CELL-CYCLE GENE EXPRESSION BY CHITOSAN COATED SILVER NANOPARTICES ON HUMAN EPIDERMOID CARCINOMA CELLS

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ABSTRACT

Objective: To detect the variability in the expression of cell cycle genes such as Cyclin *D1,CDK4 p21,p27* and *p57* associated with cytotoxicity of Human epidermoid carcinoma cells using qPCR.

Methods: The synthesis silver nanoparticles and chitosan coated silver nanoparticles was done by chemical reduction method using polyvinyalpyrolidone (PVP) as surfactant and glucose as reducing agent. The cell proliferation assay was performed using MTS assay. Gene expression analysis was performed using Eppendorf Realplex 2 PCR systems.

Results: The lowest concentration of 1mg of Chitosan-AgNPs with 25µg/ml concentration showed 35% reduction in the A431 cell line growth. Significantly increased expression of *p21, p27, p57, CCND1* and *CD4* gene was observed among 1mg of Chitosan-AgNPs with 25µg/ml concentrations indicating potential apoptotic activity at the lowest concentration of Chitosan-AgNPs on A431 cells.

Conclusions: The effect of AgNPs inducing apoptosis/growth arrest at low concentrations (1mg-25µg/ml) has been more effective while coating the nanoparticle with chitosan. The potential apoptotic properties of Chitosan-AgNPs are evident by the significant up regulation of the cell cycle genes when treated with low concentration of Chitosan-AgNPs.

Keywords: Silver nanoparticles, Cell Cycle genes, Apoptosis, Chitosan, Gene expression.

INTRODUCTION

Nanoparticles are widely used in biological and industrial applications. In medicine, it has been widely used in tissue engineering, cancer therapy, drug and gene delivery[1]. Among several nanoparticles, Silver nanoparticle (AgNP) are used in medicine, textiles, sensors, nanocomposites, agriculture and waste-water treatment[2]. Silver nanoparticle has unique optical, thermal and electrical properties which are used in industrial and medical applications. AgNPs are most commonly used for cancer detection, diagnosis and therapy which helps to overcome the side effects and toxicity to healthy cells [3]. AgNPs along with some tumor specific bio markers can help in identification of cancers and can permit selective uptake into cells or accumulation in tumor micro environment [3]. Chitosan has been a promising biomaterial in the world because of its various properties and unique biological such as antibacterial activity, antitumor activity and immune enhancing activity [4]. Chitosan consists of glucosamine and N-acetyl glucosamine units linked together by β -1,4-glucosidic bonds. Chitosan exhibits number of interesting biological properties such as biocompatibility, biodegradability, non-toxicity, non-antigenicity and adsorption properties [5]. Chitosan when coated to silver nanoparticles with different shapes especially triangular shaped nanoparticles with the help of photo thermal reduction the particles go to the tumor site and kill the tumor cells[6]. The cytotoxicity and genotoxicity of silver nanoparticles were conducted in different cancer cell lines such as Lungs (A549) [1], breast (MCF-7) [8], Osteoblast [9], Human bronchial epithelial cell lines (BEAS-2).[10], however studies on A431(Human Epidermoid carcinoma) are minimal.

Earlier studies indicated that the AgNPs, apart from disrupting normal cellular function and affecting the membrane integrity, induced various apoptotic signaling genes of mammalian cells leading to programmed cell death [11]. Products coated with AgNPs have been widely used and have been profoundly effective in several dermal applications, however, the effect of AgNPs and Chitosan coated AgNPs on Human epidermoid cancer cells (A431) has been scarcely understood. Cell cycle being a pivotal phenomenon for any given cell, the study was conducted to evaluate how, AgNPs and Chitosan Coated AgNPs are effective in inducing apoptotic signaling during the cell cycle. Hence in the present study we aimed to evaluate the effect of Silver nanoparticles(AgNPs) and Chitosan coated silver nanoparticles(Ch-AgNPs) and their possible mechanism of action in inducing cytotoxicity when treated on Human Epidermoid carcinoma cells (A431).

MATERIALS AND METHODS

Synthesis of Chitosan coated Silver nanoparticles

Silver nanoparticles (AgNPs) were prepared by reducing the silver nitrate in polyvinylpyrolidone (PVP) aqueous solution. Glucose acts as a reducing and sodium hydroxide to accelerate the reaction [12]. Three different concentrations of AgNPs were formulated namely 1mg,5mg and 8mg. The Chitosan nano carriers preparation is a 72 hrs process which involve low molecular weight chitosan (1mg/ml) prepared in 1% acetic acid and kept for overnight stirring for the chitosan to dissolve completely in acetic acid. On day 2,the synthesized AgNPs were then dispersed in the distilled water and dissolved in the prepared Chitosan and kept for overnight stirring and on the third day the solution is centrifuged and the pellets is kept in -80◦c overnight and lyophilized to form powdered Chitosan-AgNPs.

Physicochemical characterization of the synthesized nanoparticles

The characterization techniques were performed to analyze the presence, shape, stability, crystal groups present in the nanoparticles. The prepared silver nanoparticles and chitosan coated silver nanoparticles were characterized under UV-Visible spectroscopy to check the absorbance of AgNPs and Chitosan Coated AgNPs. Particle analyzer to check the particle size distribution, Zeta potential to check the stability of the nanoparticles. The particle size was calculated using Debye-Scherer formula where

$$D = 0.9\lambda \div W\cos\theta$$

Where 'λ' refers to wave length of X-Ray (0.1541 nm), 'W' refers to FWHM (full width at half minimum) which was 0.356 obtained from

the instrument 'θ' was the diffraction angle and 'D' is particle diameter (size). Fourier transform infrared spectroscopy (FTIR) was done to check the functional groups present in both the materials, X-ray diffraction spectroscopy (XRD) to check the crystalline lattice of the nanoparticles and also the particle size. Scanning electron microscopy is performed to check the morphology of the silver and chitosan coated silver nanoparticle.

Cytotoxicity studies

The Human Epidermoid carcinoma A431 cell lines were procured from National Centre for Cell Science (NCCS), Pune for the study. Cell lines were cultured in Dulbecco's modified Eagle's medium(DMEM) supplemented with l-glutamine (4 mM), penicillin (100 units/mL), streptomycin (100µg/mL) and 10% (v/v) heat inactivated fetal bovine serum (growth medium). Cells were maintained in 5% CO2 humidified incubator at 37 ◦C. Growth medium was changed every 3 days.

Cell proliferation assay

To evaluate the effect of the Silver nanoparticles (AgNPs) and Chitsan-coated Silver nanoparticles (Ch-AgNPs) on the Human Epidermoid Carcinoma A431 cell line proliferation, the MTS assay was performed. To evaluate the toxicity of AgNPs and Ch-AgNPs from the the 3 different stock concentrations ie (1mg, 5 mg and 8mg) were formulated. The MTS assay kit was from CellTiter 96 AQueous one solution, Promega. The assay was performed based on the manufacturer's instructions with appropriate controls. Cells were seeded in 96 well microtitre plates with 2 x 10^4cells/200µl growth medium/well followed by overnight incubation. Supernatants from the wells were aspirated out and fresh aliquots of growth medium (containing AgNPs and Ch-AgNPs in the formulated concentrations were added. Two time points were ascertained for the study, 24 hours and 48 hours. At the end of each time point, the samples were washed with PBS solution to remove the non adherent cells. MTS reagent (200 µL) and 1 mL of serum-free media were added to each of the samples and incubated at 37 _C for 2 h. The reaction was stopped by the addition of 250 µL of Sodium Dodecyl Sulfate (SDS) solution. The absorbance was measured at 490 nm using a Multiplate Reader (Infinite 200M, Tecan, Durham, North Carolina, USA).

Quantification of gene expression

Qualitative assessment of effect of AgNPs and Ch-AgNPs on A431 cell lines was done using Real time PCR. The cytotoxicity of cells is governed by several factors, of which genes play a major role in triggering the process of apoptosis or cell death. In the present study five genes have been chosen namely, *p21,p27, p57, CCND1* and *CD4*. The groups which showed cytoxic effect on A431 cell lines namely 1mg AgNPs, 1mg AgNP-Chitosan, 5mg AgNPs, 5mg AgNP-Chitosan, 8mg AgNPs and 8mg AgNPs-Chitosan were taken.

Trizol digestion

At 48 hour time point cellular contents were washed with PBS and the total RNA was isolated using Trizol (Gibco BRL, USA) following the procedure described by the manufacturer (1). Briefly, 1 mL of Trizol was added to the samples and kept for half an hour at room temperature. The solution was collected and RNA was extracted with 0.2 mL of chloroform (Merck, India).

RNA isolation

The solution was centrifuged at 12 000 rpm for 15 min at 4 ◦C and extracted RNA were stabilized using 70% ethanol prepared with nuclease-free water (Qiagen, USA). The RNA was centrifuged using a QIA Shredder Spin Column (Qiagen, USA), and dissolved in RNAse-free water (Qiagen, USA). The isolated RNA was quantified using NanoDrop (Thermo Fisher Scientific, USA).

cDNA synthesis

cDNA was obtained after a two-step reaction and subjected to a real-time RT–PCR (Eppendorf AG22331, Germany). Reverse transcription was performed with Quantitect Reverse transcription kit (Qiagen, USA) and random hexamers as per the manufacturer's protocol. Then, the cDNA samples were subjected to amplification using specific primers (table 1) and quantified in real time RT-PCR (Eppendorf AG22331, Germany) using SYBR-Green as probe (Qiagen, USA). Quantitative values were determined by the delta–delta method and normalized with the reference gene β-actin.

RESULTS

Physicochemical characterizations of the AgNPs and Chitosan coated AgNPs

Particle size determination The average particle size for silver nanoparticle was around 14 nm. In the case of Chitosan coated Silver nanoparticle, where 'λ' Is wave length o f X-Ray (0.1541 nm), 'W' is FWHM (full width at half minimum) which is 0.656 obtained from the instrument 'θ' is the diffraction angle and 'D' is particle diameter (size). The average particle size was around 27 nm**.**

To check the absorbance of silver nanoparticles and chitosan coated silver nanoparticles 2 ml of solution is taken and de ionized water is used as blank. The wavelength was set from 200-800 nm. The peak absorbance for all the three concentration mentioned above showed absorbance in-between 400 nm-420 nm range (fig. not shown). The zeta potential was taken for the samples prepared from three different concentrations of silver nitrate (8mg, 5mg, 1mg) and the results obtained from the instrument have been mentioned below in fig. 1 (A, B) shows the zeta potential for 1 mg concentration for both AgNPs and Ch-AgNPs, however, the Zeta potential for 8mg and 5 mg for AgNPs and Ch-AgNPs were -8.02mv,+8.04 mv and -27.7 mv, +37.7mv respectively. The 1 mg and 5 mg concentrations of Ch-AgNPs showed significantly higher stability when compared to AgNP alone.

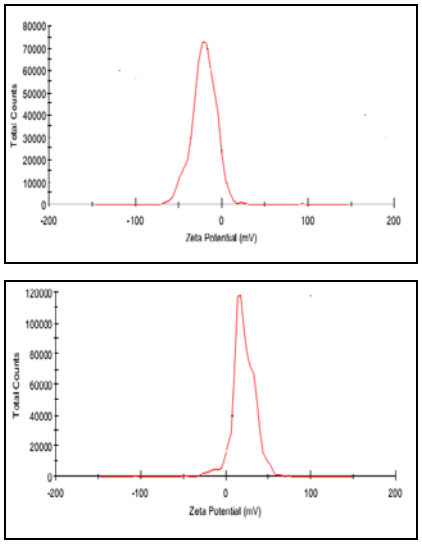

Fig. 1: Zeta Potential of AgNPs and Chitosan-AgNPs at 1mg concentration

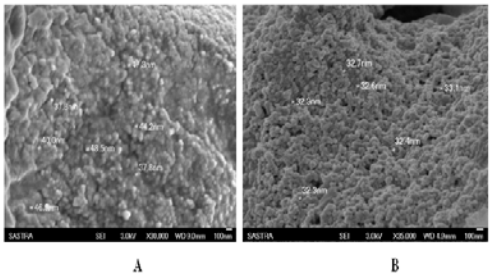

Fig. 2: The SEM image of silver nanoparticles in the magnification of X30000 which stands for examining the area of 100 nm surface.(B)) The SEM image of chitosan-AgNPs in the magnification of X35000 which stands for examining the area of 100 nm surface

The fig. 3 shows the XRD showing the peak indices and 2-Theta positions of silver nanoparticles. table 1 gives the peak indexing from d-spacing and the lattice planes have been calculated. A number of bragg reflections [111], [311], [222], [200] corresponding to the sets of lattice plane are found. These peaks are matched with face entered cubic structure o f silver (JCPDS file no: 04-0783).

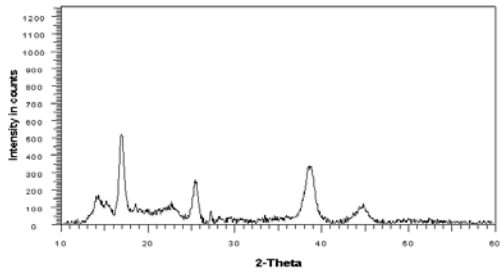

Fig. 3: The XRD showing peak indices and 2-Theta positions in AgNPs

Table 1: Peak indexing from d-spacing in silver nanoparticles

2Θ	D	$1000/d^2$	$(1000/d^2)/60.62$	Hkl
16.76	5.28	35.8	0	311
25.35	3.51	81.1	1	222
38.53	2.33	184.1	3	111
44.67	2.02	243.6	4	200
13.95	6.34	24.8	0	311

Figure-4 represents the XRD showing the peak indices and 2-Theta positions of chitosan coated silver nanoparticles. The particle size was calculated using the Debye-Scherer formula mentioned in equation1.

Where 'λ' Is wave length o f X-Ray (0.1541 nm), 'W' is FWHM (full width at half minimum) which is 0.656 obtained from the instrument 'θ' is the diffraction angle and 'D' is particle diameter (size). The average particle size is calculated to be around 27 nm. table 2 gives the peak indexing from d-spacing and the lattice planes are been calculated. A number of Bragg reflections [111], [222], [311], [200] corresponding to the sets of lattice plane are found. These peaks are matched with face entered cubic structure of silver (JCPDS file no: 04-0783).

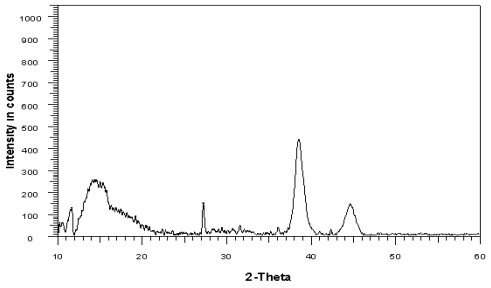

Fig. 4: The XRD showing peak indices and 2-Theta positions in Ch –AgNPs

Table 2: Peak indexing from d-spacing in Chitosan coated Silver nanoparticles

2θ	**D**	**$1000/d^2$**	**$(1000/d^2)/60.62$**	**Hkl**
11.537	7.66418	17.0	0	311
27.174	3.27893	81.4	1	222
38.523	2.33508	184.1	3	111
44.622	2.02906	243.6	4	200

Effect of AgNPs and Ch-AgNPs on A431 cell lines

The fig. 5 shows the various trends of both AgNPs and Ch-AgNPs on A431 cells at 24 hour time points. No significant cytotoxic effect was observed, however, a moderate trend of cytotoxicity was observed among 1mg Ch-AgNPs at 20µg/ml concentration and 5mg Ch-AgNPs at 20 µg/ml. Also similar effect was observed among 1mg Ch-AgNPs at 10µg/ml concentration.

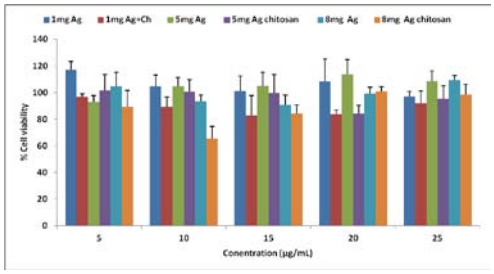

Fig. 5: The cytotoxic effect of AgNPs and Ch-AgNPs on A431 cells after 24 hours

The fig. 5A shows the various trends of both AgNPs and Ch-AgNPs on A431 cells at 48 hour time point. The 1mg Ch-AgNPs at 25µg/ml concentration showed cytotoxic effect on the A431 cells by reducing the cell proliferation by 35% compared to other groups. Similar reduction in cell proliferation was observed among 20 µg/ml concentration of 1mg Ch-AgNPs. Though marked reduction in cell proliferation has been seen among all the concentrations of AgNPs and Ch-AgNPs, none showed 50% reduction in cell proliferation.

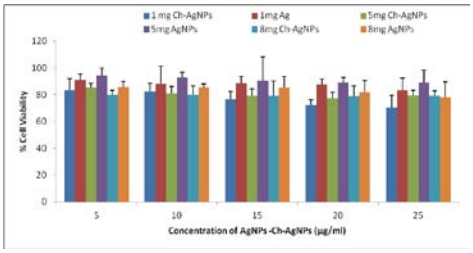

Fig. 5A: The Cytotoxicity effect of AgNPs and Ch-AgNPs on A431 cells after 48hours

Altered gene expression profiles of Cell-Cycle associated genes in A431 cell lines

The fig. 6 A,B, C,D and E shows the altered gene expression profiles of five gene predominantly associated with cell cycle the *p21,p27, p57, CCND1*, and *CD4*. The *p21* gene expression was significantly up regulated by 2.38 fold in the 1mg-Chitosan-AgNPs (25µg/ml), when compared to the control group. The *p27* gene showed increased expression by 4.68 fold among the 1mg-Chitosan-AgNP (25µg/ml) group when compared to the control group (TCPS). It was also observed that the 1mg-AgNPs (25µg/ml) and 1mg-AgNPs (5µg/ml) groups also showed increased *p27* expression by 2.36 and 3.6 fold respectively compared to the control (TCPS) group. The 1mg Chitosan-AgNPs (25µg/ml) showed 1.74 times increased *p57* gene expression in the present study compared to control (TCPS) group. We observed a significant increase in the expression of CCND1 gene ie, by 1.64 folds when compared to the other groups. In the present study, the *CD4* gene expression has been found to be significantly higher among the 1mg-Chitosan-AgNPs (25µg/ml) group when compared to the control group (TCPS).

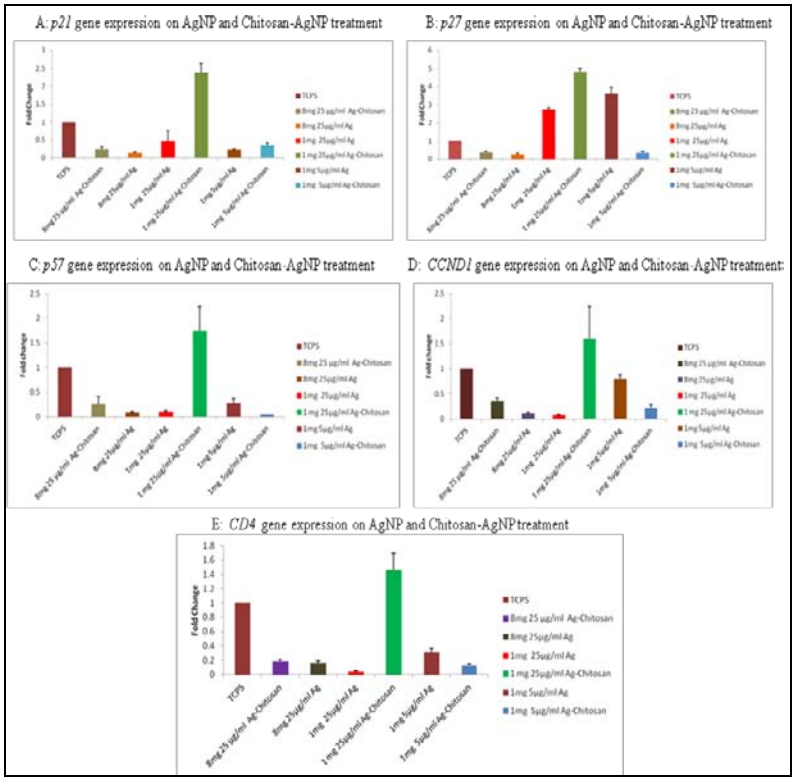

Fig. 6: A-E The altered gene expression of *p21*, *p27* ,*p57*, *CCND1* and *CD4* gene on 48 hour incuabtion with AgNPs and Chitosan-AgNPs in A431 cell lines

DISCUSSION

Silver Nanoparticles (AgNPs) possess proven anti-bacterial properties, and are currently been used in variety of dermal applications. However, the potential ani-cancer effect of AgNPs has been very minimal studied, and the mode of action still unclear. Moreover, the direct delivery of AgNPs does not provide efficiency of the nanoparticle, the usage of Chitosan, a non-toxic, biocompatible and biodegradable compound, encapsulating the AgNPs and aiding in effecive delivery of the NPs into the cellular membrane has greater importance. Hence the present study was aimed to decipher how AgNPs are able to induce apoptosis in A431 human epidermoid cancer cells.

The cytoxicity studies have shown marked reduction in the A431 cells on treating with Chitosan-coated AgNPs at the lowest concentration group of 1 mg-25µg/ml by 37%. This shows the potential of the low concentration AgNPs coatd with chitosan effectively initiating apoptosis in the cells leading to significant reduction in the cell proliferation.

The gene expression studies on genetic markers regulating the cell cycle such as the *p21*, *CCND1*,*p27*,*p57* and *CD4* shed more light on the possible pathway, Chitosan-AgNPs act through to initiate apoptosis or cell cycle arrest. The *p21* gene has been reported to be specifically cleaved by CASP3-like caspases, which thus leads to a dramatic activation of CDK2, and may be instrumental in the execution of apoptosis following caspase activation. The Caspase mediated apoptosis by Chitosan-AgNPs was evident among HT29 cells underlying the increased expression by p21 in these cells [11]. This hints the possible activation of *p21* gene by Chitosan-AgNPs, which thereby triggers apoptosis among the A431 cell lines.

The *p27* gene similar to *p21* binds to several classes of Cyclin and CDk molecules. It has also been observed that increased levels of p27 cause cells to arrest in the G1 phase of the cell cycle. However in the case of cancer cells, the inactivation of *p27* gene results in oncogenic activation of several receptor tyrosine kinases (RTK), phosphatilidylinositol 3-kinase (PI3K), SRC, or Ras-mitogen activated protein kinase(MAPK) [13]. The present observation of increased *p27* expression by Chitosan-AgNPs could explain the activation of *p27* gene leading to growth arrest in the G1 phase among the A431 cell lines.

Similarly the *p57* gene, a potent inhibitor of several G1 cyclin/CDK complexes, and over expression leads to cell-cycle arrest in G1 phase [14]. Over expression of *p57* gene leads to blocked growth of Astrocytomas and cell senescence [15]. The observation in the present study has been in agreement with the previous studies with respect to the activation of *p57* gene in inducing cell senescence and apoptosis among A431 cell lines. However, it has been observed in the present study that this activation of *p57* is by the action of low concentration of Chitosan-AgNPs (1mg-25µg/ml) on the A431 cell lines.

The *CCND1* gene expression pattern was significantly higher fold increase at lowest concentration of 1mg (25ug/ml) of Chitosan-AgNPs. The G1/S-specific cyclin-D1 encoded by the *CCND1* gene, functions as a regulator of CDKs (Cyclin-dependent kinase). This cyclin forms a complex and functions as a regulatory subunit of CDK4 or CDK6, which is required for cell cycle G1/S transition. The increased expression of *CCND1* gene among 1mg-Chitosan-AgNPs (25µg/ml) group has indicated the potential activation of this gene thereby increasing it binding affinity with *CD4* leading to arrest of cell cycle at G1 phase in the A431 cell lines.

The *CD4* gene expression has been found to be significantly higher among the 1mg-Chitosan-AgNPs (25µg/ml) group when compared to the control group (TCPS or Non-treated). *CD4*,a co-receptor that assists the T cell receptor (TCR) in communicating with an antigen-presenting cell. Using its intracelluar domain, *CD4* amplifies the signal generated by the TCR by recruiting an enzyme, the tyrosine kinase Lck, which is essential for activating many molecular components of the signaling cascade of an activated T cell [16]. This shows that the Chitosan-AgNPs could possibly induce an immune response among the A431 cells leading to the activation of *CD4* gene leading to a cascade of events resulting in the apoptosis of A431 cell lines. The Chitosan-AgNPs activated the *CCND1* gene leading to cascade of events leading to the activation of *p21, p27, p57,* and *CD4* genes leading to growth arrest (fig. 7).

CONCLUSION

The effect of AgNPs inducing apoptosis/growth arrest at low concentrations (1mg-25µg/ml) has been more effective while coating the nanoparticle with chitosan. The potential apoptotic property of Chitosan-AgNPs is evident by the significant up regulation of the cell cycle genes when treated with low concentration of Chitosan-AgNPs.

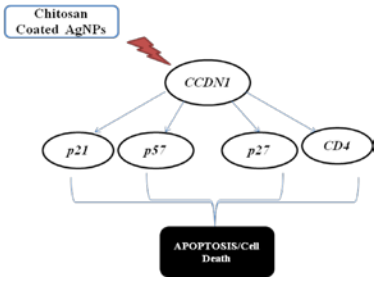

Fig. 7: Schematic outline showing the proposed mechanism of action of Chitosan-AgNPs

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CONFLICT OF INTERESTS

Declared none

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