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CYTOTOXIC EFFECT OF SILVER NANOPARTICLES ON L_{20} B CELLS

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ABSTRACT

Pure silver colloids nanoparticles were synthesized using laser ablation in liquid environment. A transmission electron microscope (TEM) was employed to take the electron micrographs of the solutions.The UV–VIS absorption spectra of Ag nanoparticles exhibit a characteristic peak around 400 nm, indicating the formation of Ag nanocolloids. The Ag colloids nanoparticles have an average diameter of 17 nm were produced. Amplitude parameters was: Sa (Roughness Average) 4.98 nm, Sq (Root Mean Square) 6.53 nm, Ss (Surface Skewness) -0.0738, Sku (Surface kurtosis) 3.55, Sy (Peak-Peak) 43.2 nm, Sz (Ten Point Height) 42.4 nm.The concentration of produced Ag nanoparticles of 100 µg/ml was estimated by AAS.

This study was carried out to evaluate the cytotoxic effects of silver nanoparticles and silver non- nanoparticles on L20B cell line.There were non-significance changing in cell viability treating with all concentrations of silver nanoparticles. But the higher concentrations of silver non-nanoparticles (10 µg/ml) reduced cell growth significantly. The inhibitory rate was: 40.54%. All lower concentrations (0.01 ng/ml-1 µg/ml) show non-significance changing on cell viability.

There were significant differences between means of cell viability treating with (10 µg/ml)silver nanoparticles compared with silver non- nanoparticles concentrations.It was more reduction and cytological study of higher concentrationshowed that there were the cells were very small with vacuolated cytoplasm with few dead cells and pyknosis with cellular

debris. There was no important difference in morphological changes between cell lines exposed to (0.01 ng/ml - 1 µg/ml) of silver non- nanoparticles and control negative.The cytological study of silver nanoparticles showed no cytotoxic effect of low concentration (0.01 ng/ml), while at a medium concentration (0.1 ng/ml - 0.1 µg/ml), formation of multinucleated giant cells, few dead cells and earlier stages of cytolysis could be seen. At higher concentration (1 µg/ml - 10 µg/ml), cytolysis appeared to be more severe.The results suggest that silver colloids nanoparticles, synthesized by laser ablation in liquid environment, showed less cytotoxic effect on L20B cell linecompared with silver non- nanoparticles.

Keywords: Silver nanoparticles, Synthesis, Cytotoxic, L_{20}B Cell Line.

INTRODUCTION

Nanotechnology has been expanding rapidly in recent years, impacting on diverse areas such as the economy and the environment. In this context, the number of commercial products comprising nanomaterials is increasing because of their unique physicochemical and high specific surface properties, leading to their application in electronics, optics, textiles, household devices, medical devices, drug delivery systems, in addition to environmental remediation (1-3).

Among the commercially available nano-sized materials, silver nanoparticles are by far the most used nanocompounds(4).Several physical, chemical and Biological methods have been used for synthesizing and stabilizing silver nanoparticles (5,6).Silver nanoparticles are of interest because of the unique properties (e.g., size and shape depending optical, electrical, and magnetic properties) which can be incorporated into, biosensor materials, composite fibers, cryogenic superconducting materials, cosmetic products, and electronic components, antimicrobial applications (7), food packaging, cancer therapy (8,9).

With the widespread application of engineered nanoparticles (NPs), numerous nano-scale compounds might consequently be released into the aquatic environments and elicit an impact not only on the ecosystems but also on the human health (10, 11), and would directly interfere with calcium-based signaling processes in neural tissue once reaching the brain (12).

Toxicological information on nanoparticles remains insufficient; thus frameworks for future toxicological assessments on nano-sized materials have been promoted (2).In this study,

silver nanoparticles were synthesized using *laser ablation in liquid environment* and its cytotoxicity was assessed against $L_{20}B$ cell line.

MATERIALS AND METHODS

1. Synthesis and Characterizationof silver nanoparticles.

Synthesis and Characterization of silver nanoparticles was prepared in the Center of Nano-Technology and Advanced Material at the University of Technology using laser ablation in liquid environment. The experimental setup for it is perfectly simple.

Pure metal plate of silver (ounce: 99.999%) placed inquartz cell containing 1ml of duple distilled demonized water DDDW. The Nd-YAG laser (type HUAFEI) of 1064 nm at energy set in the range of 1 J per pulse, with a lens having a focal length of 110 mm, was utilized as an ablation source.The pulse duration and the repetition rate of the laser pulse were 10 ns and 10 Hz respectively; andthe laser energy of 600 mJ/pulses.Silver atoms as suspension were prepared by electric discharge in pure water (the electric current and discharge voltage of 0.5 mA and 20 V, respectively). A transmission electron microscope TEM (CM10 pw6020, Philips-Germany) was employed to take the electron micrographs of the solutions. Practically, a drop of the sample solution of interest was placed on a copper mesh, and then dried by heating up to 320 K. Absorbance spectra of the nanoparticles solution were measured by UV-visible double beam spectrophotometer CECIL, C. 7200 (France). Atomic absorption spectroscopy AASmodel GBS 933,Australia, was carried out for the estimate the concentration of prepared silver nanoparticles(13). The silver nanoparticles was taken and sterilized by Millipore filter (0.02 µm).

2. Cytotoxic Effect of silver nanoparticles on L20 B Cells.

Preparation of L_{20} B Cells

$L_{20}B$ cell line (Pass 68/16), genetically engineered non malegnant mouse cell line expressing the human poliovirus receptor (CD155), was used as cell model. It was maintained at the Viral laboratory / Central Laboratory of General Health/ Baghdad –Iraq.

Secondary culture was prepared under sterilized conditions according to (14).The cell culture medium was Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma/U.S.A) supplemented with: 10% foetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/ml streptomycin. The cells were seededat a population of 1.5X104 cells perwell in Flat bottomed

96-well polystyrene coated plate. The cellswere incubated for 24 hours at 37°Cunder a humidified atmosphere containing 5% CO2.

Cytotoxic Effect of silver nanoparticles and silver non- nanoparticles on $L_{20}B$ Cells in vitro

Cytotoxicity testing was performedwith a neutral red (NR) cytotoxicity assay with some modifications (15). After 24 hours incubation, the cells reached 90-95% confluence. The spent media was removed and thecells were washed with PBS (0.01 M phosphate buffer, 0.0027 M KCl and 0.137 M NaCl) and 0.2 ml fresh media was added. Series of dilution (0.01 ng/ml, 0.1 ng/ml, 1 ng/ml, 10 ng/ml, 0.1 µg/ml, 1 µg/ml, 10 µg/ml) of concentrated silver nanoparticles or silver non- nanoparticles, diluted with (RPMI) 1640 medium, was added to the plate. Inaddition, a negative control (PBS), were included for each experiment to ensure validity of the assay. There were three replicates for each tested concentration. The cells were incubated for 24 hours at 37°C under a humidified atmosphere containing 5% CO_2. Then, cells were washed twice with PBS and the supernatant was discarded. A 50 µl of 50 µg/ml NR solution (dissolved in PBS) was added to each well and incubated at37°C for two hour. The wells were washed three times with PBS. Then 50 µl of (PBS and absolute ethanol 1:1 V/V) were added to each well to extract the dye from the viable cells. The optical density of each well was read by using a micro-ELISA reader at atransmitting wavelength on 492 nm.

The cell viability was calculated bythe following formula:

% Cell Viability = [Mean absorbance in test wells/ Meanabsorbance in control wells] x 100.

The percentage of cytotoxicity compared to the untreated cells was determined with the equation givenbelow. A plot of % cytotoxicity versus plant concentrations was used to calculate the concentration which showed 50% cytotoxicity (IC_{50}).

Inhibitory Rate (%) = [100×(Absorbance of untreated group−Absorban of treated group]/Absorbance of untreated group.

- **Cytological study**

Hematoxylen and Eosin stain was used to examine the cell after exposure to silver nanoparticles.

Statistical Analysis

Statistical analysis was conducted using the software SPSS (Statistical package social sciences V. (11.5)) Differences were considered significant if the *P* value was($P \leq 0.05$) and ($P \leq 0.01$) .T test was used.

RESULT AND DISCUSSION

1. Synthesis and Characterization of silver nanoparticles.

(Fig. 1) shows the UV-VIS absorption spectra of the pale yellow silver colloids nanoparticles, prepared by PLAL of a metal plate immersed in pure water. The ablating energy of 600 mJ was employed to ablate a target for 15 pulses. The products formed in the ambient liquid were transparent just after ablation, and then changed to contaminated ones after more application of nanoparticles. The UV–VIS absorption spectra of Ag nanoparticles exhibit a characteristic peak around 400 nm, indicating the formation of Ag nanocolloids(16). The concentration of produced Ag nanoparticles of 100 µg/ml was estimated by AAS (Fig. 3). The optical absorption spectra of metal nanoparticles are dominated by surface plasmon resonances (SPR), which shift to longer wavelengths with increasing particle size (17). The position and shape of plasmon absorption of silver nanoclustersare strongly dependent on the particle size, dielectric medium (17), (18). The presence of the single surface plasmon peak implied that the formed nanoparticles were nearly spherical. In the case of ellipsoidal particles the absorption spectrum would have two Plasmon's peaks (13).

Fig. 2 shows a typical TEM images and the particle size distributionsof silver nanoparticles produced by laser ablation (λ=1064 nm and laser energy of 600 mJ/pulse) of a silver plate immersed pure water. The Ag nanoparticles have an average diameter of 17 nm were produced.

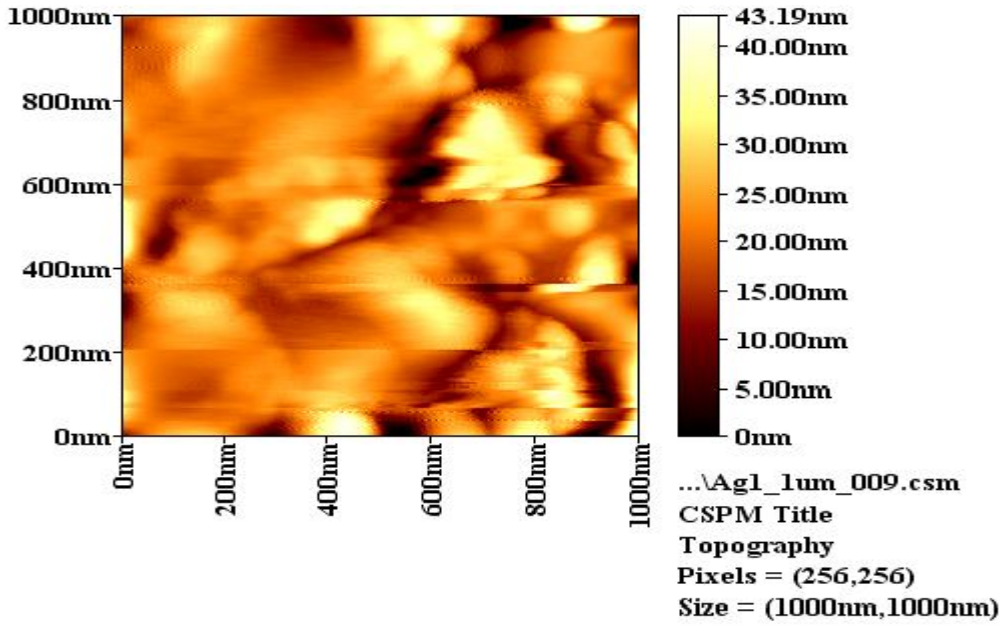


Fig.(1): CSPM Imager Surface Roughness Analysis, Image size: 1000.00nm

Amplitude parameters:

Sa(Roughness Average) 4.98 nm

Sq (Root Mean Square) 6.53 nm

Ss (Surface Skewness) -0.0738

Sku (Surface kurtosis) 3.55

Sy (Peak-Peak) 43.2 nm

Sz (Ten Point Height) 42.4 nm

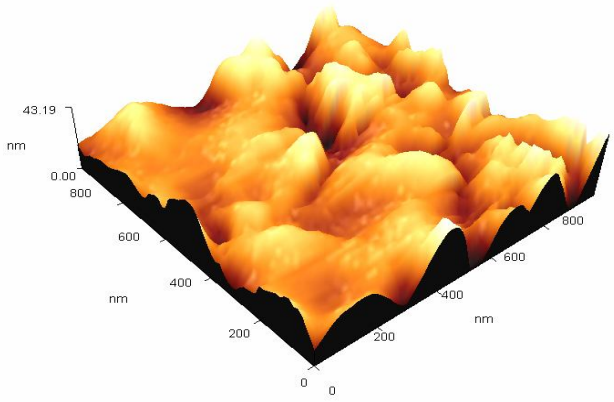
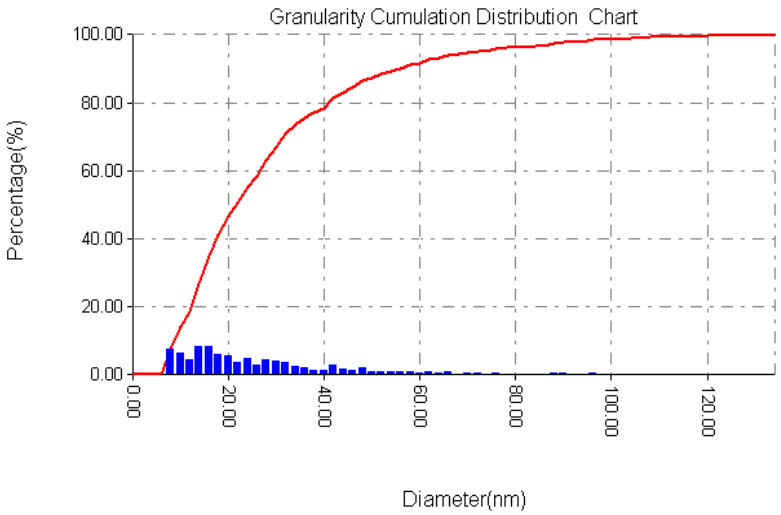


Fig.(2): TEM Image for silver nanoparticles.



(Fig. 3): Granularity cumulation distribution chart.

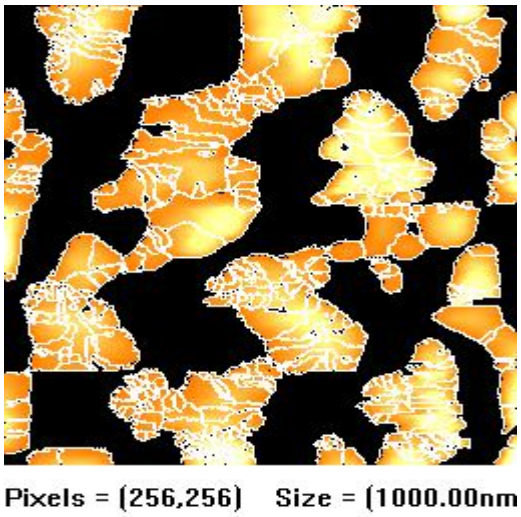


Fig.(4): Image of silver nanoparticles.

2. Cytotoxic Effect of silver nanoparticles and silver non- nanoparticles on L20 B Cells.

- **cell viability.**

The toxicity of silver nanoparticles and silver non- nanoparticles with different capping agents was studied using non malegnant mouse cell line (L20B). The level of toxicity was evaluated using changes incell viability and cell morphology studies. Cell lines were examined after 24 hr. of exposure tosilver nanoparticles and silver non- nanoparticles.

There were non-significance changing ($P<0.05$) in cell viability treating with all concentrations of silver nanoparticles, Table (1).On the other hand, the higher concentrations of silver non- nanoparticles (10 µg/ml) reduced cell growth significantly ($P \leq 0.05$). The inhibitory rate was: 40.54%. While all lower concentrations (0.01 ng/ml-1 µg/ml)show non-

significance changing on cell viability, Table (2). However, there were significant differences ($P \leq 0.01$) between means of cell viability treating with silver nanoparticles compared with silver non- nanoparticles concentrations (10 µg/ml), (Fig. 5).

Results showed that silver nanoparticles had no cytotoxic effect on $L_{20}B$ cell line.

Table (1): Cytotoxicity assay of silver nanoparticles on L_{20} B cells.

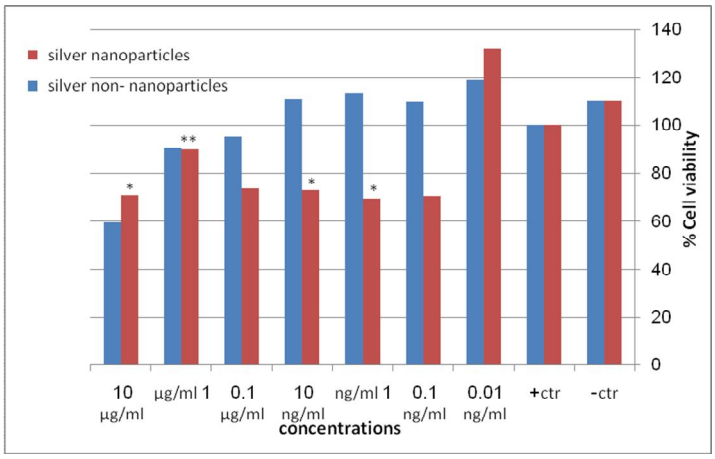
concentrations	% Cell viability ± S.D	% inhibition or induction
10 µg/ml	70.811 ± .0077 BC	29.189
1 µg/ml	89.720 ± .0136 BC	10.27
0.1 µg/ml	73.514 ± .1038 BC	26.486
10 ng/ml	72.946 ± .0144 ABC	27.054
1 ng/ml	69.189 ± .0812 C	30.81
0.1 ng/ml	70.27 ± .0943 BC	29.728
0.01 ng/ml	131.892 ± .1158 A	-31.891
ctr+	100 ± .0155 ABC	0
ctr-	110.277 ± . 0127 AB	-10.27

NOTE: Ctr- negative control, Ctr+: Positive Control, S.D: Standard Deviation; Different Letters:Significant Differences ($P \leq 0.05$) Between Means; Similar Letters: Non significant ($P \leq 0.05$); Negative sign :%induction.

Table (2): Cytotoxicity assay of silver non- nanoparticles on L_{20} B cells.

concentrations	% Cell viability ± S.D	% inhibition
10 µg/ml	59.459 ± .0166 C	40.54
1 µg/ml	90.243 ± .0139 AB	9.757
0.1 µg/ml	95.135 ± .024 B	4.864
10 ng/ml	110.811 ± .0185 AB	-10.81
1 ng/ml	113.514 ± .0189 AB	-13.513
0.1 ng/ml	109.73 ± .0149 AB	-9.728
0.01 ng/ml	118.919 ± .0797 A	-18.9189
ctr+	100 ± .0155 AB	0
ctr-	110.27 ± .0127 AB	-10.27

NOTE: Ctr- negative control, Ctr+: Positive Control, S.D: Standard Deviation; Different Letters: Significant Differences ($P \leq 0.05$) Between Means; Similar Letters: Non significant ($P \leq 0.05$); Negative sign : % induction.



(Fig. 5) Compared the cytotoxic effect of silver nanoparticles and silver non-nanoparticles on L20 B Cells.NOTE: Ctr- negative control, Ctr+: Positive Control, S.D: Standard Deviation; ** Significant Differences ($P \leq 0.01$) Between Means; *: Significant ($P \leq 0.05$).

- **Cytological study**

In the present study, cell lines were examined microscopically after 24 hr. of exposure to the silver nanoparticles and silver non- nanoparticles. The patterns of growth seem to be similar for both control positive and control negative group cell line. It showed complete confluent monolayer of cohesive cells (Fig. 6), which were pleomorphic, hyperchromatic, well differentiated with high nucleus/cytoplasm ratio. Some of these monolayers showed an evidence of overlapping of small dark proliferating cells.

The results revealed some cytological changes. These changes were diverse depending on the concentration of silver nanoparticles. There was no cytotoxic effect of low concentration of silver nanoparticles (0.01 ng/ml) on cell line. At a medium concentration of of silver nanoparticles (0.1 ng/ml - 0.1 µg/ml), Formation of multinucleated giant cells could be seen. There is cellular growth inhibition and earlier stages of cytolysis were noticed. The dead cells became more prominent (Fig. 7).

At higher concentration of silver nanoparticles (1 µg/ml - 10 µg/ml), the cells appeared to be more severe and the morphological features regarding to lysis of cells with bare nuclei were observed. Large numbers of cells were retaining their normal features and the examination revealed only the presence of few cells of dead cells and pyknosis with cellular debris (Fig. 8).

Depending on the daily microscopical examination of cell lines, it was obvious that there was no important difference in morphological changes between cell lines exposed to (0.01 ng/ml - 1 µg/ml) of silver non- nanoparticles and control negative. While at higher concentration (10 µg/ml), the cells was very small with vacuolated cytoplasm. There were few dead cells and pyknosis with cellular debris.

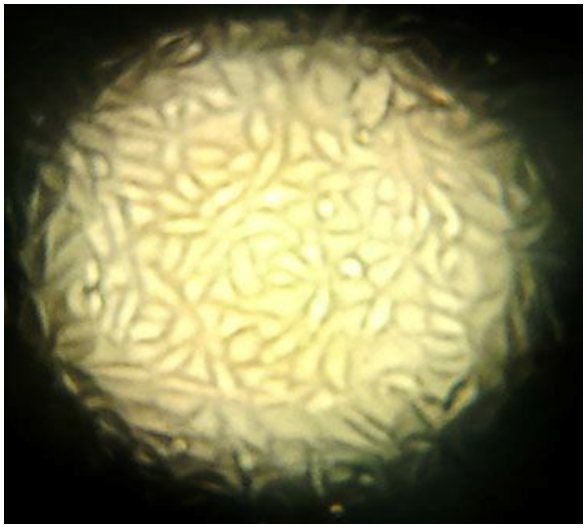


Fig. (6): The positive control group of $L_{20}B$ cell line reveals complete confluent monolayer of cohesive cells (400X).

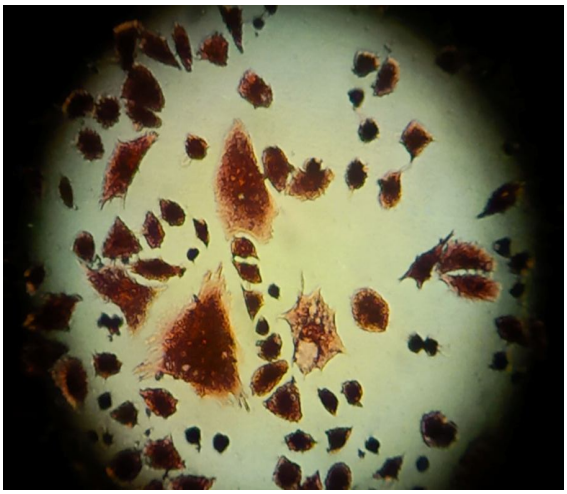


Fig.(7): Formation of multinucleated giant cells, dead cells, vacuolated cytoplasm in other cells in $L_{20}B$ cell line after exposure to 0.1 µg/mlof silver nanoparticles (400X,H&E stain).

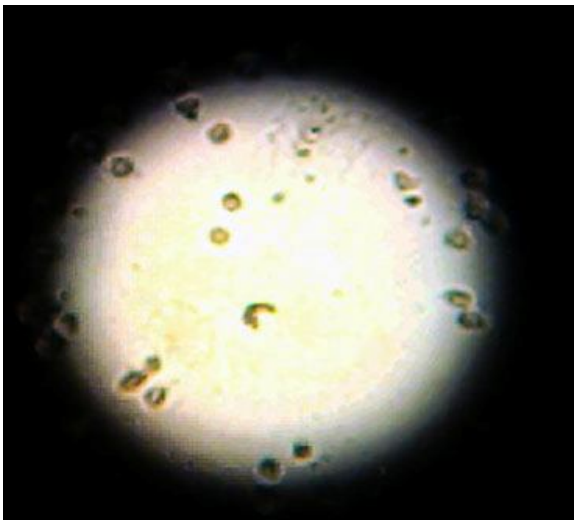


Fig. (8): Numbers of dead cells of L20B cell line after exposure to 10 µg/mlof silver nanoparticles (400X).

At present, silver nanoparticles are the most commonly applied nanomaterial to clothing, foot wear, textiles, medical devices, home appliances, and cosmetics (19, 20).

Despite their enormous benefits, their health and environmental effects are not obvious and many researchers have studied the genotoxicity of silver nanoparticles.

There have been several in vitro genotoxicity experiments for silver nanoparticles. Yet the results conflicted with each other due to the use of various test methods, test materials, and other conditions, such as capping agents, particle aggregation/agglomeration and the size of the nanosilver dictates its mode of cytotoxicity (3). It is known that silver nanoparticles can cause primary DNA damage and cytotoxicity but not mutagenicity in cultured mammalian cells (21).Furthermore, Erythrocytes exhibit significant lysis, haemagglutination, membrane damage, detrimental morphological variation, and cytoskeletal distortions following exposure to Ag NPs at a concentration of 100 µg mL−1, The haemolyzed erythrocyte fraction has the ability to induce DNA damage in nucleated cells (22) (63).

On the contrary, human cells were found to have a greater resistance to the toxic effects of silver nanoparticles in comparison with other organisms (23).In animal models, the genotoxicity effects of silver nanoparticles are less effective compared with the genotoxicity effects in cell cultures. The genotoxicity of silver nanoparticles after 28 days of oral administration was negative for the in vivo micronucleus test (24). In addition, there is a safe range of 7–20 nm spherical silver nanoparticles (SNP) for the intended application as a topical antimicrobial agent after appropriate in vivo studies (7).

The results of (25) suggest that exposure to silver nanoparticles by inhalation for 90 days does not induce genetic toxicity in male and female rat bone marrow in vivo.

CONCLUSIONS AND RECOMMENDATIONS

In conclusion, the silver nanoparticles synthesized by laser ablation in liquid environment, were less cytotoxic effect on $L_{20}B$ cell line compared with silver non- nanoparticles.

Some studies demonstrated that the Biogenic silver nanoparticles are generally less cyto/genotoxic compared with chemically synthesized nanoparticles (23). As future experiments, Biosynthesis of silver nanoparticles using various Bacteria (6), Fungi (26) and Plant (27) could be used. The exact cellular pathway of silver nanoparticles in normal cells must be elucidated and if there are any potential complications for the silver nanoparticles would surface after prolonged clinical use.

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