Iraqi Journal of Cancer and Medical Genetics

Diluted concentrations of large (above one hundred nanometer) silver nanoparticles inhibited the growth of different types and origin of cancer cells

Amer T. Tawfeeq

Iraqi Center of cancer and medical genetic research, Al-Mustansiriya University.

Abstract:

This study describes utilization of large silver nanoparticles (above one hundred nanometer in diameter) synthesized by plus laser ablation method in liquid (PLAL) in growth inhibition and apoptosis induction of cancer cells. Two different types of cancer cell lines and two different types of transformed cell lines were used. These cell lines were Rabdomyosarcoma (RD), glioblastoma (AMGM), rat embryo fibroblast transformed cell line (REF) and green monkey kidney transformed cell line (VERO). Cells were challenged with diluted concentration of silver nanoparticles they were 62.5µg/ml, 30.25µg/ml, and 15.12µg/ml. The synthesized silver nanoparticles was characterized it had surface plasmon resonance peak at 411nm and particle size distribution average diameter of 193.19 nm. Growth inhibition assay revealed that these relatively large nanoparticles are lethal toward cells under investigation; this lethality was concentration dependant during 24 hours of incubation at 37°C. According to acridine orange/propidium iodide mixed fluorescent staining assay, the used concentrations were able to induce apoptosis in all cells tested. However, the highest concentration was able to induce highest apoptosis percentage in the treated cells. This was confirmed by DNA fragmentation assay as well. In conclusion this study determined that above-one hundred nanometer size silver nanoparticle is capable of inducing apoptosis in cancer cell lines.

Keyword: *silver nanoparticles, nanotechnology, apoptosis, cancer cell, growth inhibition*

Introduction:

Nanotechnology and nanobiotechnology are now almost an equal terms in the sense of utilizing nanomaterial synthesized by physical or chemical procedures in biological applications. Inorganic nanoparticles demonstrate substances that possess suitable characteristics to apply them in many biological applications (1). Silver nanoparticles as well as gold, zinc oxide, and titanium nanoparticles conceders to be one of most important inorganic nanomaterials that have been investigated in the field of biological applications. Their antibacterial, antifungal, and anticancer activity was postulated thoroughly and proved to be suitable as potential therapies in this regard (2-4). The toxicity issue have been raised in the face of this potentiality, since these materials are inorganic and not biodegradable they may accumulates in different body organs such as liver spleen and kidney, hens may cause an unexpected undesirable complications. Addressing the toxicity issue researches stated that silver nanoparticles toxicity was highly correlated with its exposure concentration, nanoparticle shape, and nanoparticle size (5). The higher the exposed concentrations were the higher toxicity they induces both in vitro and in vivo experiments, furthermore the smallest the particles were the higher the toxicity they could induce (6). The direct correlation between toxicity and elevated concentrations of silver nanoparticles was supported by the theory of nanoparticle ability to induce reactive oxygen species (ROS) in the exposed cells. These radicals can cause wholesome cellular damage to active proteins molecules and genetic material of the exposed cell (7). At the same time the correlation between nanoparticle size and toxicity stemmed from the ability of smaller particles to penetrate deeper and faster into the cellular compartment mainly into the nucleus and other compartments which results in wholesome destruction of the cellular functions (7). The smallest the nanoparticles are the greater surface area they contain the more ROS they induce and the more metallic ions they produce. This was true for silver nanoparticles with size below one hundred nanometer in diameter. Accordingly the use of above one hundred nanometer in diameter of silver nanoparticles

Corresponding Address:
Amer T. Tawfeeq
Iraqi Center of cancer and medical genetic research, Al-Mustansiriya University.
Email: amer.tawfeeq@iccmgr.org

may reduce the potential toxicity since large diameter will reduce surface area (8). In Iraq different attempts were made to penetrate this new field and brought the academic researches attention to it. Synthesis of silver nanoparticles by up-down method was first carried out in the University of Technology. Laser ablation in liquid environment of metallic silver was successfully conducted to produce sub-one hundred nanometer silver nanoparticles. Particle size distribution of the produced nanoparticles was precisely characterized with electron microscope (9). The antibacterial activity of silver nanoparticles produced and characterized by the same method was described for the first time in Iraq at the same University (10). Later on number of studies appeared in the Iraq Academic scientific journals describe syntheses of silver nanoparticles with different methods to demonstrate their antibacterial activity (11, 12, 13). Another study carried out by a group in Al-Mustansiriyah University demonstrates the cytotoxic effect of silver nanoparticles toward L20B cell line. Unfortunately this study was limited to the cytotoxic effect only and did not detect the induction of apoptosis or necrosis in the tested cell line (14). In detailed previous research, our laboratory have tested the toxicity of silver nanoparticles ranged in size between 8 to 66 nm with particle size average of 26.77 nm in the prepared solution toward glioblastoma cell line (type of brain tumor). That study showed the ability of the synthesized nano silver to induce apoptosis in the tested cell line. At that time, the toxicity of the prepared silver nanoparticles toward normal cell was assessed in vivo. Mice were injected with silver nanoparticles in concentrations which were found to be lethal to brain tumor cells. Mice brains histopathological assessment revealed that the prepared silver nanoparticles were unable to induce brain damage (15). In concluding to that work we have prepared silver nanoparticles by the same method and almost with the same concentration but with larger particle size and assessed its toxicity toward two cancer cell lines and two normal cell lines (transformed cell line adapted to in vitro cultivation). The aim was to determine the effect of larger particle size (above one hundred nanometer) on growth inhibition and apoptosis induction in these cells.

Materials and Methods:

Synthesis and characterization of silver nanoparticles

The syntheses and characterization of silver nanoparticles was carried out according to Albakri etal. (10) and Tawfeeq (15). The only modification was a less laser energy (400 mJ/pulse) used. Time between pulses shooting was elongated to allow the formation of larger particles and to let the aggregation of large particles.

Cancer cell line

Four cell lines were use d in this study all of them were provided by the Iraqi Center for Cancer and Medical Genetic Research (ICCMGR). These cell lines were rhabdomyosarcoma (RD) originally brought from General Public Health Laboratory (GPHL) of the Ministry of Health in Iraq. This cell line establishment and characterization was described by Johnston and Siegel (1990) (16) human glioblastoma (AMGM) which is cell line originally derived from Iraqi patient with brain tumor, it was adapted to in vitro cultivation as a stable cell line by Dr. Ahmed Majeed Al-Shimmari in the department of experimental therapy ICCMGR., rat embryo fibroblast (REF) is an embryonic transformed cell line adapted to in vitro cultivation in the same department at ICCMGR and kindly provided by Dr.Ahmed Al-Shimmary, and African green monkey kidney cell line (VERO) (17). Rabdomyosarcoma cells (RD) were maintained in MEM with L-glutamine and HEPES buffer (USBiological, USA) supplemented 100 Uml–1 penicillin, and 100 µg ml–1 streptomycin (SDI, Iraq) 10 ml and 10% fetal calf serum. The rest of the cell line (AMGM, REF, and VERO) were maintained in RPMI-1064 medium with 10% fetal calf serum and supplemented with 2 mM glutamine, 100 Uml–1 penicillin, and 100 µg ml–1 streptomycin (SDI, Iraq). All cells were cultivated and exposed to silver nanoparticles in a humidified atmosphere of 95 % air and 5 % CO2 at 37 °C.

MTT assay

Cell viability was determined with Cell Viability QMTT Assay kit (USBiological, USA) as described elsewhere in details (Tawfeeq, (15)). Three concentrations of silver nanoparticles were used in this study; they were 60.5, 31.25, and 15.12µg/ml. Suitable growth media free of silver nanoparticles was used in untreated control cells treatments. The treated cells were incubated fro 24 hr at 37°C.

Acridine orange and Propidium iodide (AO/PI)

The method described by Martin and Lenardo (2001) was conducted (18).

DNA fragmentation assay

Conducted exactly as described by Tawfeeq (15).

Statistical analysis

One way analysis of variance (ANOVA) was determined and the significant differences were calculated on probability level of 0.05 and 0.01(19).

Results:

The peak absorbance of UV-Vis spectra for the prepared silver nanoparticle was at 411.46 nanometer and its value was 0.3440. This is typically representing the surface plasmon resonance for the synthesized silver nanoparticles (Figure 1). The synthesized nanoparticles shape as imaged by atomic force microscope was almost spherical irregularly and aggregated in the liquid which induces the formation of large particles size (Figure 2). The average diameter of these nanoparticles was 193.91 nm as

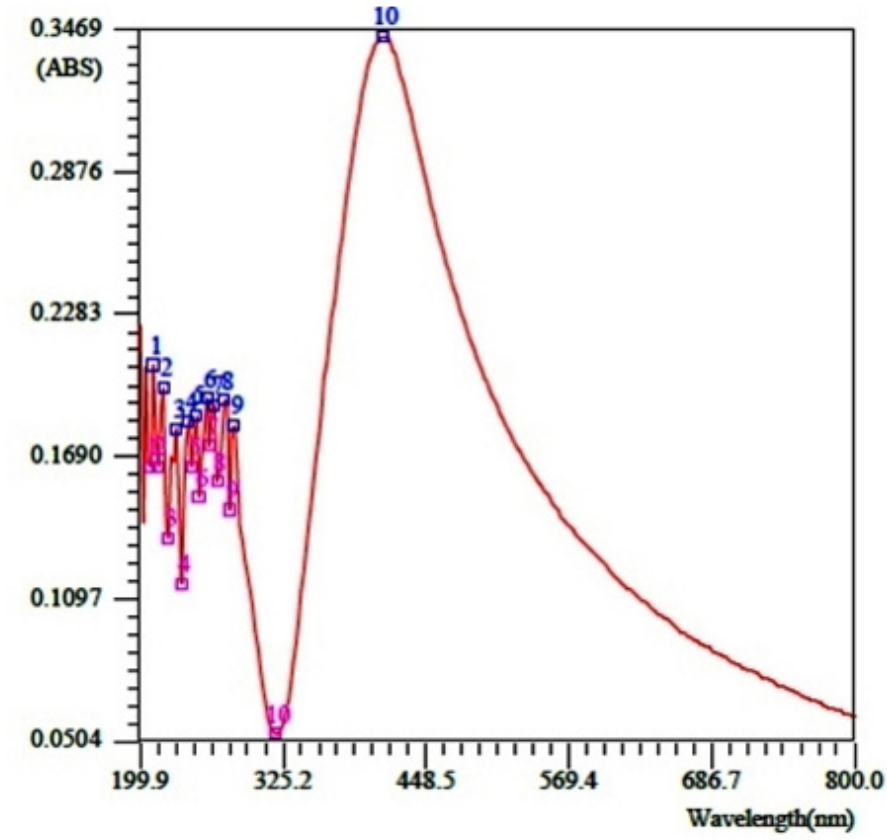


Figure 1: Surface plasmon resonance of the prepared silver nanoparticles

Determined by atomic force microscope (AFM) device software (Table 1). These results refer to the formation of above one hundred nanoparticle size in diameter in the conducted method of silver nanoparticle formation.

Cytotoxic assay revealed that the prepared silver nanoparticles were able to inhibit the growth and propagation of all cell lines under investigation with different percentages (Figure 4). The three concentrations used of silver nanoparticle inhibition these cell lines in concentration dependant manner. Among cancer cells AMGM was the most effected cells they inhibited significantly ($P< 0.01$) by 51.96% when treated with 61.5µg/ml. inhibition of these cells were less and did not differ significantly ($P<0.01$) when 31.25 and 15.12 µg/ml of nanosilver was used (14.41% and 13.49% respectively). Rabdomyosarcoma cells were less effected cells by this treatment not among cancer cells but also among normal transformed cells (REF and VERO). It inhibited only by 24.89% when the highest concentration used. That inhibition significantly differed ($P<0.01$) from the treatment of these cells with the two remaining concentrations 31.25 and 15.12µg/ml of silver nanoparticles which was 8.75% and 10.46% respectively. The normal transformed cells REF was inhibited by these large silver nanoparticles in similar way as cancer cells. The three concentrations used 60.5, 31.25 and 15.12µg/ml inhibited REF cells by 32.80%, 28.91%, and 14.39% respectively.

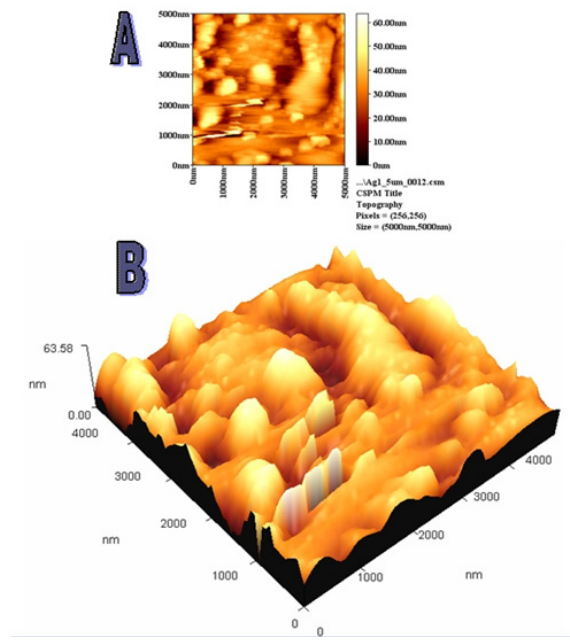


Figure 2: Atomic force microscopy analysis for the prepared silver nanoparticles deposited over the surface of glass slide, A; a topography image, B; three dimension image.

In this cell line the lower concentration inhibition differed significantly (P<0.01) than the two highest concentration. The inhibition of VERO cells differed significantly (P<0.01) among silver nanoparticles concentrations used and in relation with REF cells. The three concentrations inhibitions were 51.69%, 35.41%, and 18.21% respectively

Table 1: size distribution chart for the prepared silver nanoparticles as determined with atomic force microscope software.

Avg. Diameter:193.91 nm **<=10% Diameter:80.00 nm**

<=50% Diameter:160.00 nm **<=90% Diameter:340.00 nm**

Diameter(nm)	Volume(%)	Cumulation(%)	Diameter(nm)	Volume(%)	Cumulation(%)	Diameter(nm)	Volume(%)	Cumulation(%)
40.00	0.31	0.31	220.00	4.72	66.98	400.00	0.63	94.03
60.00	1.89	2.20	240.00	6.92	73.90	420.00	0.94	94.97
80.00	7.55	9.75	260.00	4.72	78.62	440.00	0.63	95.60
100.00	8.49	18.24	280.00	3.77	82.39	460.00	1.57	97.17
120.00	7.23	25.47	300.00	2.52	84.91	480.00	1.57	98.74
140.00	10.38	35.85	320.00	3.14	88.05	520.00	0.31	99.06
160.00	9.43	45.28	340.00	1.57	89.62	540.00	0.31	99.37
180.00	11.32	56.60	360.00	1.89	91.51	580.00	0.31	99.69
200.00	5.66	62.26	380.00	1.89	93.40	600.00	0.31	100.00

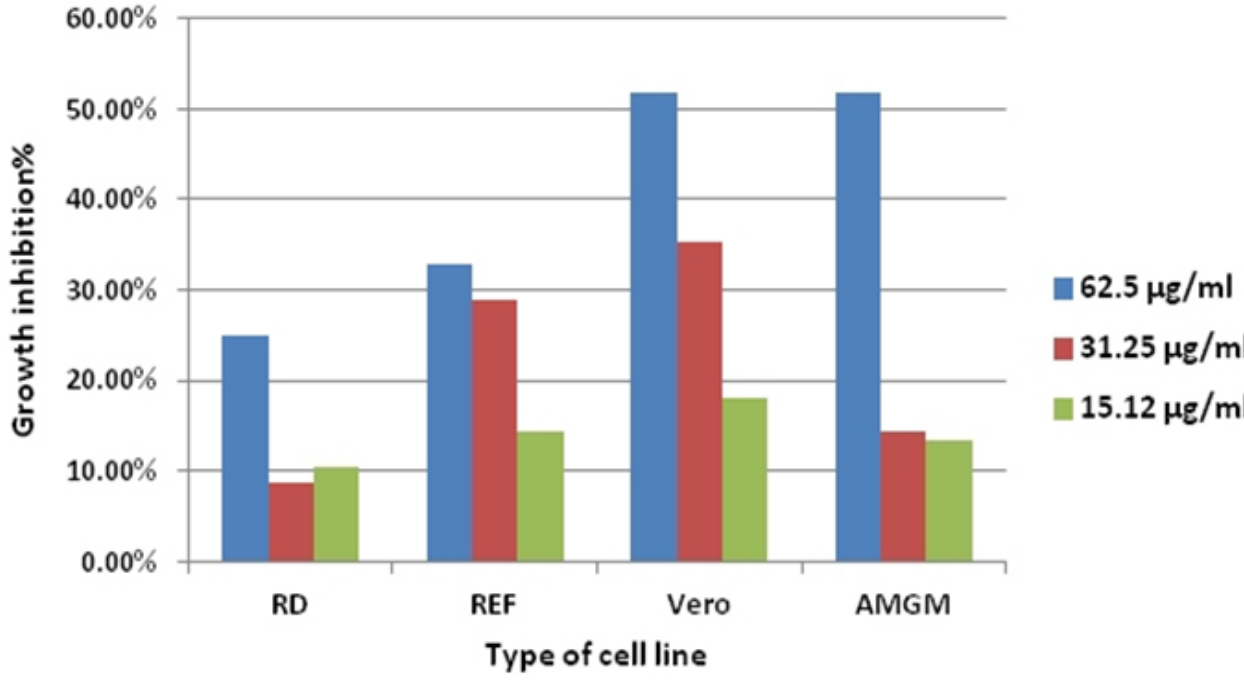


Figure 3: Growth inhibition induced by silver nanoparticles in four different types of cell lines when incubated at 37°C for 24 hr.

The apoptosis induction was followed in the treated cells for all concentrations of silver nanoparticles conducted in this study. Figures (4 to 7) demonstrate that all cell types were venerable to the induction of apoptosis with different percentages when treated with the three concentrations of silver nanoparticles for 24 hr. Whoever, These apoptotic percentages did not differ significantly among cell lines types

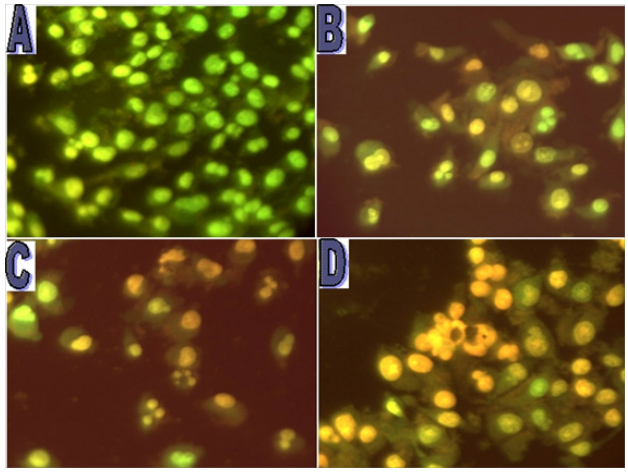


Figure 4: Rabdomyosaecoma cells exposed to three different concentrations of silver nanoparticles for 24 hours. A, control untreated cells; B, cells treated with 15.12 µg/ml; C, cells treated with 30.25 µg/ml; D cells treated with 62.5 µg/ml.

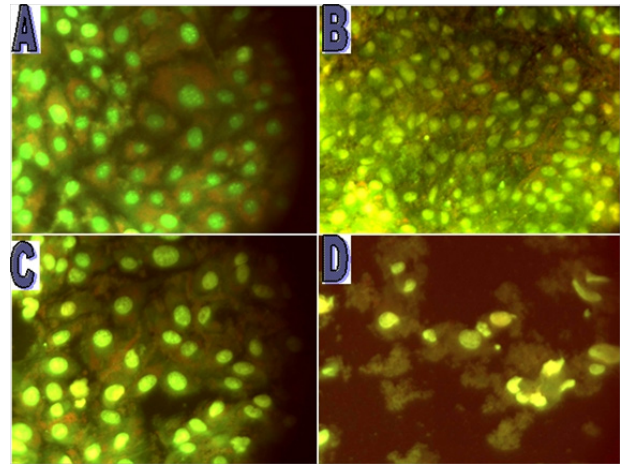


Figure 5: Glioblastoma cells exposed to three different concentrations of silver nanoparticles for 24 hours at 37 C. A, control untreated cells; B, cells treated with 15.12 µg/ml; C, cells treated with 30.25 µg/ml; D cells treated with 62.5 µg/ml.

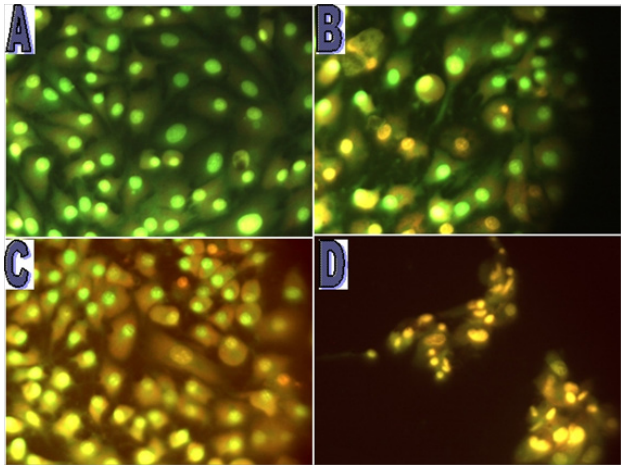


Figure 6: Rat embryo fibroblast transformed cells (REF) exposed to three different concentrations of silver nanoparticles for 24 hours. A, control untreated cells; B, cells treated with 15.12 µg/ml; C, cells treated with 30.25 µg/ml; D cells treated with 62.5 µg/ml.

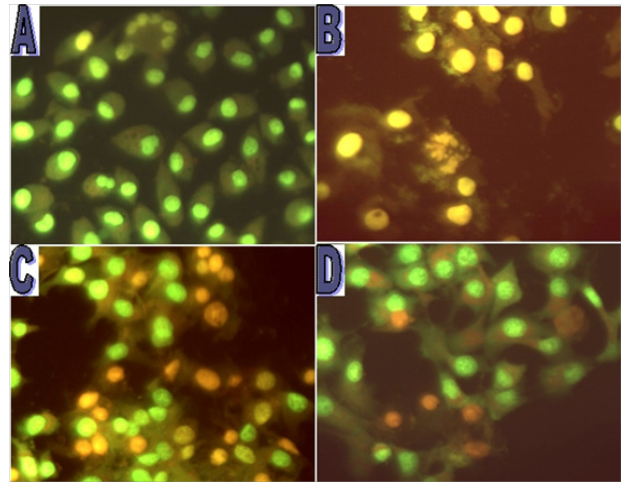
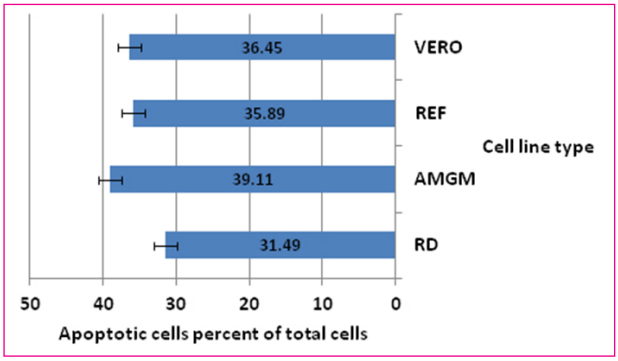


Figure 7: African green monkey kidney transformed cells exposed to three different concentrations of silver nanoparticles for 24 hours. A, control untreated cells; B, cells treated with 15.12 µg/ml; C, cells treated with 30.25 µg/ml; D cells treated with 62.5 µg/ml.



◀ Figure 8: percentage of apoptotic cells induced in the cell lines under investigation after treatment with silver nanoparticles at 62.5µg/ml for 24 hours at 37C.

At the highest concentration of silver nanoparticles used the apoptotic cells percent of RD, AMGM, REF, and VERO cells 31.49%, 39.11%, 35.89%, and 36.45% respectively. In order to further emphasize the apoptotic induction status in the treated cells, DNA fragmentation assay was conducted for the cells treated with the highest concentration of silver nanoparticles for 24 hr (Figure 8). The ladder patrons were evident in all treated cells. That clearly indicates the capability of the prepared large silver nanoparticles to the indices of apoptosis in both cancer cell (RD and AMGM) and normal transformed cells (REF and VERO).

Discussion:

Many researchers emphasized that inorganic nanoparticles may induce health problems as a result of its toxicity (20, 21, 22). The mechanism of toxicity induced by silver nanoparticles was described to be as Trojan-horse type mechanism. Their presence in the cytosol of the activated cells, but were not in the dead cells indicate its ionization in the cells and cause cyto-toxicity (23). The released ions interact with several vital macromolecules such as enzymes, DNA and RNA inducing oxidative damage, mitochondrial damage resulting in cellular toxicity (24).

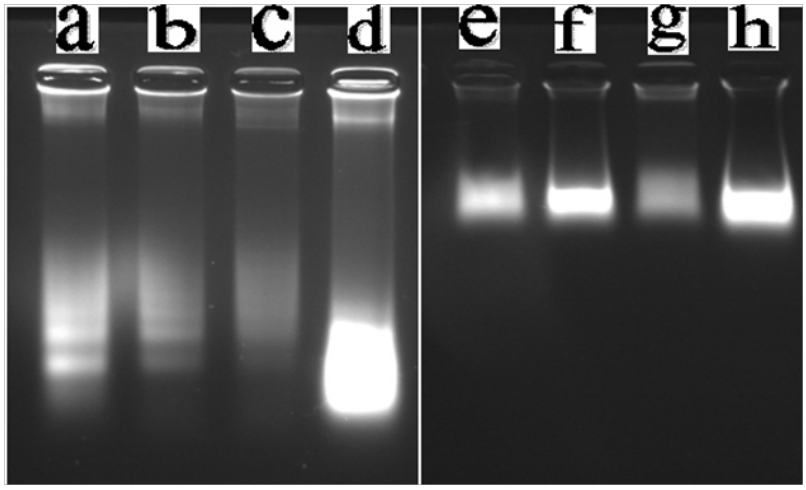


Figure 8: Apoptosis induced in cell lines under investigation treated with 60.5 µg/ml silver nanoparticles indicated by DNA fragmentation assay. a and e lanes, treated and control untreated RD cells; b and f lanes, treated and control untreated AMGM cells; d and g lanes; treated and untreated control cells; c and g lanes, treated and control untreated REF cells; d and h lanes, treated and control untreated VERO cells.

In previous study our lab has proved that below one hundred nanometer particle size of silver nanoparticles induced apoptosis in human glioblastoma cancer cell line (brain tumor). The concentrations of silver nanoparticles used at that study did not harm mice brains as investigated in vivo (15). Therefore we tried to have more understanding for this toxicity issue as related to particle size, since the below one hundred nanometer silver nanoparticles did not affect normal cells of mice brain. Although we undertake in vitro assays in this study, the results indicated that above one hundred nanometer particle size of silver nanoparticles still capable of inhibit cancer cells growth and induce apoptosis. Both normal transformed cell lines (Vero and REF) and cancer cells effected almost equally in all concentrations of silver nanoparticles used. These results urge the testing of these nanoparticles in vivo.

References:

1. Salata, O. V. (2004). Applications of nanoparticles in biology and medicine. Journal of Nanobiotechnol. 2:3.1477-1482.
2. Lima, E.; Guerra, R.; Lara, V. and Guzmán A. (2013). Gold nanoparticles as efficient antimicrobial agents for Escherichia coli and Salmonella typhi. Chem. Central J. 7:11. 153-159.
3. Zhou, Y.; Kong, Y.; Kundu, S.; Cirillo, J. D and Liang, H. (2012). Antibacterial activities of gold and silver nanoparticles against Escherichia coli and bacillus Calmette-Guérin. Journal of Nanobiotechnology. 10:19.
4. Geethalakshmi, R.. and Sarada D.V.L. (2013). Characterization and antimicrobial activity of gold and silver nanoparticles synthesized using saponin isolated from Trianthema decandra L. Biomaterial. 51: 107–115.
5. Yu, J.; Chung, H. E. and Choi, S. J. (2013). Acute Oral Toxicity and Kinetic Behaviors of Inorganic Layered Nanoparticles. J. Nanomaterials Volume 2013: 628-636.
6. Stefaan J. Soenen, Pilar Rivera-Gil, José-María Montenegro,Wolfgang J. Parak, Stefaan C. De Smedt, Kevin Braeckmans. (2011). Cellular toxicity of inorganic nanoparticles: Common aspects and guidelines for improved nanotoxicity evaluation. Nanotody. 6: 446–465.
7. Soo-Jin Choi, Jong Kwon Lee, Jayoung Jeong, Jin-Ho

Choy (2013).Toxicity evaluation of inorganic nanoparticles: considerations and challenges. Mol. Cellular. Ther. 9: 205-210.
8. Adjei, I. M.; Sharma, B. and Labhasetwar V. 2014. Nanoparticles: cellular uptake and cytotoxicity. Adv Exp Med Biol. 811:73-91.
9. Ali, A. K. and Raouf, D. N. (2011). Preparation of Silver Nanoparticles by Pulsed Laser Ablation in Liquid Medium. Eng. Tech. J. 29: 3058-3066.
10. Al-Bakri, S. A.; Ali, A. K. Tawfeeq, A. T. (2011). Effects of Ag Nanoparticles on Escherichia coli Species. Proceedings of the Second international conference of nanotechnology and advanced materials and their applications. University of Technology. Iraq, Baghdad.
11. Mohammed, N. A. (2012). Antibacterial activity of Silver Nanoparticles Using Stem bark of Juglans regia against, Streptococcus mutans , Streptococcus. sanguis and ,Porphyromonas gingivalis. M. D. J. 9: 184-190.
12. Ghassan M. Sulaiman; Hamssa E. Abd-ul-Wahed; Afnan Ismail Abd-ul-Wahab and Abbas Abdullah Mohammad. (2013). Antimicrobial Activity of Silver Nanoparticles Synthesized by Myrtus Communis Extract. Eng.Tachno. J. 31:400-410.
13. Salman, J. A. S. (2013). Synthesis of silver nanoparticles by some locallyLactobacillus spp. and detection of their antibacterial activity. A. J.P.S.13:164-173.
14. Abdul-Jalill, R. D. H; Al-Bakri, S. A.; Shafiq, S. A. ; Tawfeeq, A. T. (2013). Cytotoxic effect of silver nanoparticles on l20 b cells. World J. Pharm. Res. 2:792-742.
15. Amer Talib Tawfeeq. (2013). Pulsed laser ablation synthesized silver nanoparticles induce apoptosis in human glioblastoma cell line and possess minimal defect in mice brains. Iraqi J. Biotech.12: 92-106.
16. Johnston, S. L. G. and Siegel, C. S. (1990). Presumptive Identification of Enteroviruses with RD, HEP-2, and RMK Cell lines. Journal of Clinical Microbiology, 28(5): 1049-1050.
17. American public health Association . (1996) compendium of the methods for the microbial examination fro foods. American Association of Public Health. Washington DC, P:43.
18. Martin, D and Lenardo, M. (2001). Morphological, biochemical, and flow cytometric assays of apoptosis. Curr Protoc Mol Biol. Chapter 14: Unit 14.13.
19. Susan, G. B.; Voelki, K. E; Anderson, T. W. and Finn, J. (1997): SPSS Guid to the New Statistical Analysis of Data. Springer.New York. USA.
20. Casals, E.; Vazquez-Campos, S. ; Bastus, N.G. ; Puntes, V. (2008). Distribution and potential toxicity of engineered inorganic nanoparticles and carbon nanostructures in biological systems . J. TrAC Trends in Analytical Chemi. 27: 672-683.
21. Soenen, S.J.; Rivera-Gil, P.; Montenegro, J.M.; Parak, W.J.; De Smedt, S.C.; Braeckmans, K. (2011). Cellular toxicity of inorganic nanoparticles: Common aspects and guidelines for improved nanotoxicity evaluation. J. Nano. Today. 6: 446-465.
22. Clement, L.; Zenerino, A.; Hurel, C.; Amigoni, S.; Taffin de Givenchy, E.; Guittard, F.; Marmier, N. (2013). Toxicity assessment of silica nanoparticles, functionalised silica nanoparticles, and HASE-grafted silica nanoparticles. J. Sci. Total Environ. 450-451: 120-128.
23. Park, E.J. ; Yi, J.; Kim, Y. ; Choi, K.; Park, K. (2010). Silver nanoparticles induce cytotoxicity by a Trojan-horse type mechanism. Toxicol in Vitro. 24 : 872-878.).
24. Ahn, J.M.; Eom, H.J.; Yang, X.; Meyer, J.N.; Choi, J. (2014). Comparative toxicity of silver nanoparticles on oxidative stress and DNA damage in the nematode, Caenorhabditis elegans. Chemosphere. 108: 343-352.

التراكيز المخففة من عالق جسيمات الفضة النانوية الكبيرة الحجم تعمل على تثبيط نمو أنواع من الخلايا السرطانية مختلفة المنشأ

عامر طالب توفيق
المركز العراقي لبحوث السرطان والوراثة الطبية / الجامعة المستنصرية

الخلاصة:

تصف هذه الدراسة إستخدام جسيمات الفضة النانوية الكبيرة الحجم (أكبر من 100 نانومتر) المصنعة بطريقة الإزالة بالليزر النبضي في تثبيط نمو وحث الموت المبرمج لنوعين من الخطوط الخلوية السرطانية ونوعين من الخلايا الطبيعية المتحولة . هذه الخطوط شملت سرطان العضلات الهيكلية البشري RD وسرطان الأرومة الدبقية المتعددة الأشكال البشري (سرطان الدماغ) AMGM وخط خلايا جنين الجرذ المتحولة REF وخط خلايا الكلى للقرد الأفريقي لاخضر VERO . عوملت هذه الخطوط بتراكيز مخففه من محلول الفضة النانوية , تلك التراكيز شملت 60,5 مايكروغرام / مل و 30,25 مايكروغرام / مل و 15,12 مايكروغرام /مل . إمتلكت الفضة النانوية المصنعة رنين بلازمون سطحي أعلى في طول موجي 411 نانومتر وكان معدل أقطار الجسيمات النانوية المصنعة بمقدار 193,19 نانومتر . كما بينت النتائج القدرة المهلكة للجسيمات النانوية الكبيرة المصنعة إتجاه الخطوط الخلوية تحت الدراسة وكان تثبيط نمو الخلايا متناسب بشكل طردي مع التراكيز المستخدمة خلال 24 ساعة من وقت الحضن بدرجة 37 م . وإستنادا إلى نتائج التصبيغ بمزيج الصبغات المتفلورة أكردين البرتقالي والأيوديد البروبيديوم فإن كل التراكيز المستخدمة من جسيمات الفضة النانوية كانت قادرة على إحداث الموت المبرمج في كل الخطوط الخلوية تحت الدراسة وقد تفوق التركيز الاعلى في إحداث أعلى نسبة ما ظاهرة الموت المبرمج في كل الخطوط المدروسة وقد تم تأكيد وجود هذه الظاهرة من خلال فحص تقطع المادة الوراثية DNA . يمكن الاستنتاج من هذه الدراسة أن جسيمات الفضة النانوية التي تمتلك حجم أكبر من 100 نانو متر قادرة على حث الموت المبرمج في الخلايا السرطانية .