Citotoxicidad de nanopartículas metálicas en cultivos de células cerebrales

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Resumen

Las nanopartículas (NP) se han investigado por su tamaño diminuto y su posibilidad de llegar a sitios específicos. Se estudian materiales para diseñar nanovectores inocuos. Son pocos los estudios de la exposición a diferentes NP que atraviesan la barrera hematoencefálica (BHE) y de los efectos que tienen sobre células en este tejido.

Objetivo: determinar el deterioro celular en presencia de tres diferentes nanopartículas de origen metálico en cultivo de células cerebrales.

Material y métodos: Realizamos un estudio de viabilidad con MTT y morfológico de cultivos cerebrales (glía y neurona) al contacto con concentraciones de: 10, 50 100 y 250 µg/ml de NP de óxido de silicio (NPSiO$_2$), oro recubierto con silicio (NPAu@Si) y plata (NPAg)

Resultados: las células cerebrales se ven afectadas en su viabilidad y morfología, siendo más aparente la muerte celular con NPAg, con NPSiO$_2$ se observa un descenso en la viabilidad de las mismas pero no tan severo como con NPAg, y para las NPAu@Si, se nota un poco más de resistencia incluso a la concentración más alta.

***Palabras claves:** glía, nanopartículas, neuronas,citotoxicidad.*

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Cytotoxicity of metal nanoparticles in brain cell cultures

Abstract

Nanoparticles (NP) have been investigated for their miniscule size and their remarkable to reach specific sites; these materials have been studied to design harmless nano-vectors. However, there are insufficient studies about the effects of the exposure of different NP are able to cross the blood-brain barrier (BBB), over the cells in this tissue.
Aim: To determine the cellular toxicity in the presence of three different nanoparticles of metallic origin in brain in vitro.
Materials and methods: MTT assay and morphological analysis were performed on cultured glia and neurons after treatment with Silica ($NPSiO_2$), gold-coated with Silicon (NPAu@Si) and silver (NPAg) NP at 10, 50 100 and 250 µg/ml.
Results: Cerebral cells were affected in their viability and morphology after treatment with NPAg. $NPSiO_2$ treated cells showed a decrease in their viability but not as severe as with NPAg. For NPAu@Si, a slightly higher resistance was observed even at the highest concentration.

Key words: glia, nanoparticles, neurons, cytotoxicity.

Introduction

In recent years, nanoparticles (NP) have been studied from various scientific approaches. The characteristics derived from their size, give them particular physicochemical properties different from those presented by the crystalline or bulk material. Particles at the molecular level in a nanomaterial have a maximum size of 100 nm in diameter, they are between one hundred and one thousand times smaller than particles than those found in an amorphous material[1-3]. NP can access sites of action as they were synthesized. In addition, they can be functionalized so that they can reach sites with complex environments, as it is the case of cancerous cells, where traditional treatments fail to target exclusively the intended tissue[4-8]. Nano sciences have become of great importance in the field of health sciences; specifically in the cellular and molecular treatment of diseases, where research is being directed to design and synthesize NP that are able to encapsulate drugs and deliver them to a particular site, across physiological barriers without being metabolized[9-11].

The physicochemical properties of certain materials used in different industries, for example metallurgy, metal mechanics or even electronics have served as a basis for devising some mechanisms to target particular diseases. For this purpose, some nanomaterials have been tested in the brain in order to observe their behavior, distribution and specially their toxicity[12-18].

The blood-brain barrier (BBB) is an interface that separates the brain from the circulatory system and protects the central nervous system (CNS) from potentially dangerous chemicals and pathological agents; while regulating the transport of essential molecules. Few substances manage to cross the BBB; however, it has been reported that some NP can cross that barrier. Nevertheless, some NP tested both in vivo and in vitro have caused more damage than cellular benefit[19-22]. The aim of this work is to evaluate changes in morphology and cell viability of primary cultures of brain cells after being treated with three types of NP: SiO_2, Au@Si and Ag.

Materials and methods

i. Synthesis of nanomaterials:

a. Silica Nanoparticles: To obtain the $NPSiO_2$, a monocrystalline type p wafer doped with boron (Silicon Prime wafers: Part 89), with a resistance of 0.001-0.005 Ω·cm, was carefully fragmented and washed with a 2% HF aqueous solution(Fermont: 01291) followed by an ethanol wash (Golden Bell: HY18101000). The fragments were dried with gaseous nitrogen and placed in a teflon cell with a silver electrode and a platinum counter-electrode. The superficial layer was removed with a HF:Ethanol solution (3:1) in a current density of 350 mA/cm^2, with an electrical potential of 20 V during 5 seconds. Posteriorly, the waffer was cleaned with a 96% ethanol solution. Following this wash, 1 M KOH was added (Golden Bell: PQ36841) during 5 min in order to remove the superficial porous layer. Finally the waffer was cleaned with 96 % ethanol. For the formation of porous layers, an electrochemical anodizing with alternating pulse intensities was used to create a multilayer structure in a HF:ethanol (3:1) solution. The porous layer of low density was made in the following conditions: 350 mA/cm^2, 20 V, 150 s. The porous layer of high density was made in the following conditions: 700 mA/cm^2, 20V, 0.5 s. Three cycles of alternated densities were repeated. The wafer was washed as previously mentioned. The prying of the porous layers was done with 3% HF in ethanol at 70mA/cm^2, 50 V, 120 s. To carry out the anodizing, power sources connected to a computer with the software Labview were used. The layers recovered were sonicated until particles of the desired size were obtained; they were separated by centrifugation at 14,000 rpm and decantation. The nanoparticles obtained were oxidized with deionized water to obtain $NPSiO_2$.

b. Silicon-coated gold nanoparticles: 200 ml of 0.5 mM $AuCl_4$, were poured in a 250 ml round-bottomed flask heated under reflux until boiling while maintaining vigorous stirring. 10 ml of a 38.8 mM sodium citrate solution (pH = 10.5) were rapidly added. After the appearance of a deep red color it was left to boil and stirring for about 1 hour. Subsequently, the temperature was gradually lowered until reaching 25-30 °C. 50 ml of this colloid were taken and 3 ml of 1 mM APTMS were added drop by drop and allowed to stir for 30 minutes. After this, the solution was heated to 90-95 °C and 6 mL of 0.54% sodium silicate was added and maintained at

constant temperature and stirring for 4 hours. The sample was centrifuged at 12,000 rpm for 30 minutes and the precipitate was collected.

c.Silver nanoparticles: 18 mg of Silver Nitrate were added to 100 ml of deionized water in a 250 ml round-bottomed flask, the mixture was let to boil for 20 minutes. After boiling, 2 ml of 0.01 M sodium citrate were added, and let to react for one hour.

ii. Particle size determination

To determine the hydrodynamic diameter and the size distribution of the nanoparticles collected, a light scattering apparatus (Malvern Instruments) was used, performing the dispersion of 100 µl of NP in 1 ml of distilled water.

iii. Primary cultures of cerebral cells

Cells were extracted from 5-7 days old mice brains, by mechanical and enzymatic dissociation of the tissue with trypsin (Gibco: 25300-054). The obtained cells were resuspended in DMEM (Sigma: D5796) enriched with KCl 25 mM (Golden Bell: PI304005), 1 % of streptomycin-penicillin (Sigma: P4333), and 10 % fetal bovine serum (FBS) (Gibco: 16000-044). They were seeded in 35 mm culture plates treated with 2 mM poly-L-lysine (Sigma:4707). The cells were incubated for 24 hours at 37 ºC in 5% CO_2.

Subsenquently, the obtained cells were cultured for an additional 24 hours in 96-well plates at a cell density of 10,000 cells per well in the presence of the different nanoparticles at the following concentrations: 10, 50 100 and 250 µg/ml.

iv. Morphological characterization and cellular viabilitiy

Morphology was assessed using a light microscope under a 40X objective lens, in order to determine morphological changes on treated cells. To determine cellular viability, the culture medium was replaced with PBS (Sigma:D8537) with 2 mg/ml MTT (Sigma: M2128). The cells were incubated at 37ºC, 5 % CO_2 during 35 minutes. After incubation the solution was removed, taking care of not damaging the precipitated tetrazolium crystals. The crystals were dissolved in 100 µl of dimetil sulfoxide (DMSO) (Sigma: D8418) with vigorous mixing for 60 seconds to ensure a homogeneous dissolution. Mitochondrial activity was determined by absorbance in a spectrophotometer. This test is based on the capacity of healthy mitochondria to reduce tetrazolium [3-(4,5-Dimetyl-2-Tiazolyl)-2,5-diphenyl-2H-Tetrazolium Bromide] to formazan, an insoluble form [1-(4,5-Dimethyl-2 Tiazolyl) 3,5, -Difenyl formazan], giving a purple coloring.

v. Statistical analysis

All experiments were performed with 4 replicates of each concentrations used in each NP tested. Results are shown as mean ± SD. Statistical significance was assessed with the t-Student test; p values <0.05 were considered statistically significant.

vi. Ethical considerations

The primary cultures were obtained from Balb/C mice, following the regulations for the management of laboratory animals, as well as the guidelines of the ethics committee of the University of Guadalajara.

Results

i. Particle size

The average particle sizes measured by DLS were as follows: $NPSiO_2$ 53 nm, NPAu@Si 51 nm and NPAg 47 nm.

figure 1 shows the average particle size obtained, versus to the intensity % in which they were located. A low polydispersity was observed (30 and 80 nm in diameter for the nanoparticles).

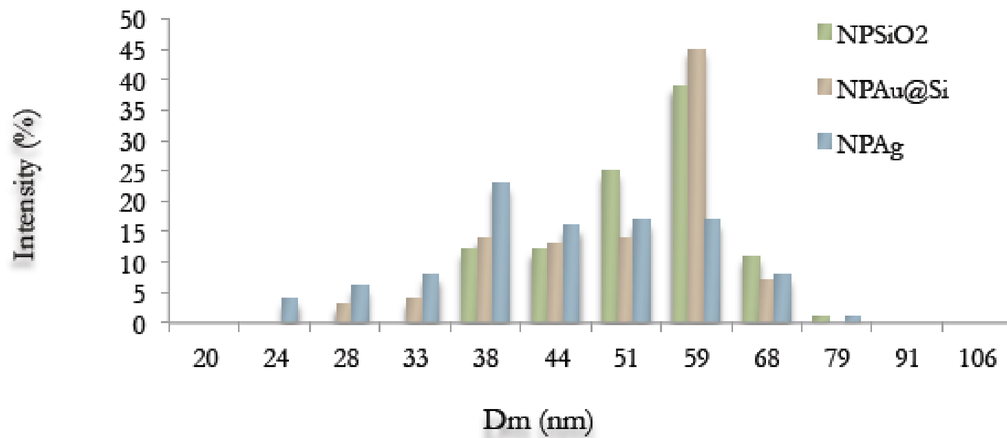

Figure 1. Intensity percentage for each particle size, the average for each nanoparticle is $NPSiO_2$: 53 nm, NPAu@Si: 51 nm and NPAg: 47 nm

ii. Morphological characterization and cellular viability:

After treating the cell cultures with NP, a microscopic inspection was performed in order to observe any morphological changes of the cells under study.

In figure 2, we show a photographic panel. In the control group cells with well-defined cell membranes were observed. In the groups treated with $NPSiO_2$, cells with a well-defined cell membrane were observed at all concentrations; however a possible accumulation of the nanomaterial inside the cell was observed, at concentrations of 100 and 250 µg/ml. Also, cell clumps were observed, being more apparent at 100 µg/ml, this behavior was noticed when

the cells detected a decrease of nutrients in the environment. The presence of detritus was observed at the maximum concentrations used. The groups treated with the NPAu@Si also presented an intact cell membrane. However, it was not possible to determine the presence of accumulated NP inside the cells as observed in the $NPSiO_2$ treatment. Cell debris is less apparent, compared to cells that were exposed to $NPSiO_2$.

NPAg treated cells presented structural changes in their cell membrane, and an accumulation of the material was observed. At the concentration of 10 μg/ml, agglomeration of NP was observed, being more apparent in the concentration of 50 μg/ml and 100 μg/ml. However it is not possible to

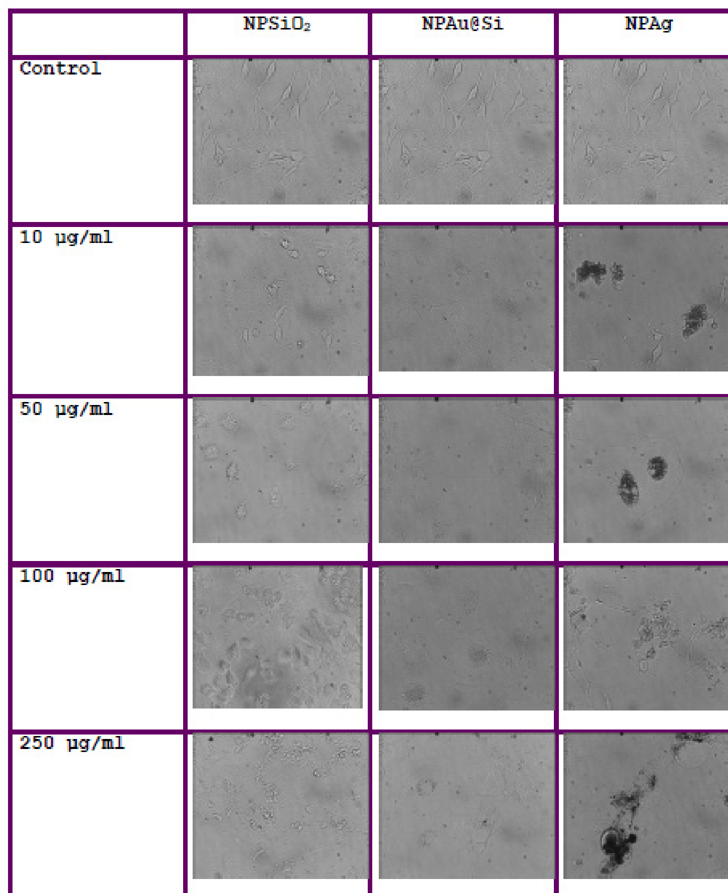

Figure 2. Photographs showing the different morphological changes of brain cells treated with different concentrations of $NPSiO_2$, NPAu@Si y NPAg.

determine if the NP are located inside or outside the cells. Detritus was observed at 10 µg/ml being more evident at higher concentrations.
After exposure to different concentrations of NP, the percentage of cytotoxicity was determined with the MTT method, in table 1 % viability are presented. A considerably decrease in cell viability was observed in the three types of nanomaterials, even at low concentrations, this effect was dose-dependant. Cells remained slightly more stable on when treated with NPAu@Si; on the other hand, a lower viability was registered in cells treated with NPAg. However, it is noteworthy that treatment with $NPSiO_2$, cell death was not evident, as there were no observable products of cell death. One possible hypothesis to account for this fact is that the glial cells were still active, collecting cell debris from surrounding dead neurons. Also, it is interesting to note that in the NPAu@Si treatment, lower levels of cell death were noticed, compared to the other two treatment ($NPSiO_2$ and NPAg).

Table 1. Viability percentages of primary cultures after treatment with $NPSiO_2$, NPAu@Si y NPAg.

	$NPSiO_2$	NPAu@Si	NPAg
10 µg/ml	66	63	55
50 µg/ml	58	62	52
100 µg/ml	49	56	25
250 µg/ml	33	47	22

In figure 3, the graph displays the % of viability for each different concentration of NP. Compared to the control group, a significantly lower cell viability was obtained. Indicating that all three types of NP are toxic to brain cells when exposed *in vitro.*

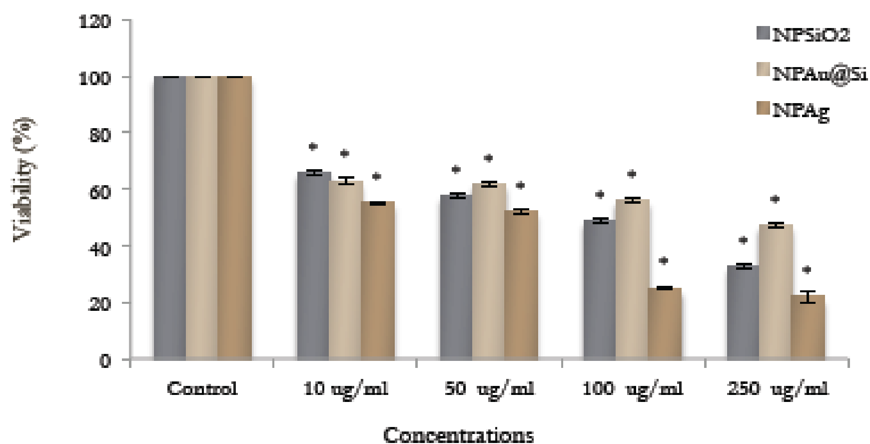

Figure 3. Graph showing the percentages of viability for brain cells in culture at different concentrations of $NPSiO_2$, NPAu@Si, NPAg. The results represent mean ± SD of 4 replicas and is expresed en % versus control, *$p<0.05$.

Discussion and conclusions

The use of metallic nanomaterials in the medical sciences has been increasing due to the characteristics and properties that these materials present at nanometer scale[18,23]. However, although some are considered inert or biocompatible in certain cell types and tissues, it should be considered whether these materials are capable of crossing the BBB and their toxicity to the brain cells should be determined[4, 6, 24, 25].

It is important to note that the three materials used in this work have been previously used as antimicrobial, anti-inflammatory agents or simply as drug vectors in other cell lines without affecting viability[6, 26-33].

The MTT assay, is widely utilized as first tool to determine cellular viability, followed by other more specific techniques to determine cellular damage[34]. Diverse authors have used techniques as: EthD-I[23], LDH[35], Tunel Assay[36], Annexin V-FITC/PI[37] to study cellular viability various cell lines, nevertheless, only a few works regarding brain cell viability are available[38].

The results shown in this work, exhibit a high cytoxicity of the NP tested over brain cells. Further studies are needed to fully determine viability changes in these cells.Previous studies have employed transmission electron microscopy to assess cell morphology, especially in vacuoles[38]. The particle size of the materials is of vital importance so that they can be considered useful for biomedicine (<100 nm)[6,15,39]. The NP used in this study ranged between 50-55 nm, however, they could still be considered voluminous, since there are some reports of NP that have not caused cell damage with diameters smaller than 20 nm[6]. The different synthesis methods employed showed similar particle sizes. Therefore, we cannot conclude whether the toxicity is due to the particle size or the material itself. Due to the fact that the three types of metallic materials caused cytotoxicity to the brain cells after exposure for 24 hs, observing cellular morphological and physiological alterations. Further research aimed at reducing the toxicity is warrented. Strategies such as: reducing particle size, using different types of materials or applying a coating with inert agents that may go unnoticed by the immune system; could improve their viability as drug carriers. As an example, PEG has been used to cover certain nanoparticles, this treatment could be tested in our culture model[6,40].

Although the results presented in this work show that the NP at the conditions employed are not innocuous to brain cells, the use of these NP should not be abandoned. Changing variables as already mentioned could minimize the detrimental effects observed. In addition, it would be of interest to know and determine details of the apoptotic pathway triggered in these cells; this data could be extrapolated and applied as a treatment in cell lines responsible for neoplasias such as gliomas.

The search for non-toxic materials that can be used in the area of neurosciences should be continued. SNC-compatible NP have enormous potential applications for the specific transport of drugs, neurotransmitters and hormones, which can not cross the BBB on their own.

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Referencias

1.Estevez A, Pritchard S, Harper K, Aston J, Lynch A, Lucky J et al. Neuroprotective mechanisms of cerium oxide nanoparticles in a mouse hippocampal brain slice model of ischemia. Free Radical Biol Med 2011; 51(6):1155-63.
2.Walters R, Kraig R, Medintz I, Delehanty J, Stewart M, Susumu K, et al. Nanoparticle Targeting to Neurons in a rat hippocampal slice culture model. ASN Neuro 2012; 4(6):AN20120042.
3.Dayem A, Kim B, Gurunathan S, Choi H, Yang G, Saha S, et al. Biologically synthesized silver nanoparticles induce neuronal differentiation of SH-SY5Y cells via modulation of reactive oxygen species, phosphatases, and kinase signaling pathways. Biotechnol J 2014; 9(7):934-43.
4.Yang J, Lee C, Ko H, Suh J, Yoon H, Lee K, et al. Multifunctional Magneto-Polymeric Nanohybrids for Targeted Detection and Synergistic Therapeutic Effects on Breast Cancer. Angewandte Chemie 2007;119(46):8992-5.
5.Khung Y, Barritt G, Voelcker N. Using continuous porous silicon gradients to study the influence of surface topography on the behaviour of neuroblastoma cells. Experimental Cell Research 2008; 314(4):789-800.
6.Park J, Gu L, von Maltzahn G, Ruoslahti E, Bhatia S, Sailor M. Biodegradable luminescent porous silicon nanoparticles for in vivo applications. Nature Materials 2009; 8(4):331-6.
7.Sailor M. Porous silicon in practice. Weinheim: Wiley-VCH; 2012.
8.Qin Z, Joo J, Gu L, Sailor M. Size Control of Porous Silicon Nanoparticles by Electrochemical Perforation Etching. Particle & Particle Systems Characterization 2013; 31(2):252-6.
9.Canham L. Handbook of Porous Silicon. EMIS; 1997.
10.Hong C, Lee J, Zheng H, Hong S, Lee C. Porous silicon nanoparticles for cancer photothermotherapy. Nanoscale Res Let 2011; 6(1):321.
11.Dos Santos C, Sekel M, Ingle A, Gupta I, Galdiero S, Galdiero M, et al. Silver nanoparticles: therapeutical uses, toxicity, and safety issues. J Pharmaceut Scien 2014;103(7): 1931-44.
12.Watanabe Y, Sakai T, Commun. Labs. Rev Electron 1971;19, 899.
13.Dan M, Tseng M, Wu Unrine, Grulke, Yokel. Brain microvascular endothelial cell association and distribution of a 5 nm ceria engineered nanomaterial. Internat J Nanomedicine 2012;4023.
14.Kim C, Kim T, Choi I, Soh M, Kim D, Kim Y, et al. Ceria Nanoparticles that can Protect against Ischemic Stroke. Angewandte Chemie 2012;124(44):11201-5.

15.Liu Q, Zhang J, Sun W, Reuben Q, Weiliang X, Hongchen G. Delivering hydrophilic and hydrophobic chemotherapeutics simultaneously by mesoporous silica nanoparticles to inhibit cancer cells. Internat J Nanomedicine 2012; 2(7): 999-1013.
16.Chen L, Miao Y, Chen L, Jin P, Zha Y, Chai Y, et al. The role of elevated autophagy on the synaptic plasticity impairment caused by CdSe/ZnS quantum dots. Biomaterials 2013; 34(38):10172-81.
17.Shen H, You J, Zhang G, Ziemys A, Li Q, Bai L, et al. Cancer therapy: cooperative, nanoparticle-enabled thermal therapy of breast cancer (Adv. Healthcare Mater. 1/2012). Advanced Healthcare Materials 2012; 1(1):128.
18.Xu F, Piett C, Farkas S, Qazzaz M, Syed N. Silver nanoparticles (AgNPs) cause degeneration of cytoskeleton and disrupt synaptic machinery of cultured cortical neurons. Molecular Brain 2013; 6(1):29.
19.Khanbabaie R, Jahanshahi M. Revolutionary Impact of Nanodrug Delivery on Neuroscience. Current Neuropharmacol 2012; 10(4):370-92.
20.Jodko-Piorecka K, Litwinienko G. First experimental evidence of dopamine interactions with negatively charged model biomembranes. ACS Chemical Neuroscience 2013; 4(7):1114-22.
21.Kefalopoulou Z, Politis M, Piccini P, Mencacci N, Bhatia K, Jahanshahi M, et al. Long-term clinical outcome of fetal cell transplantation for parkinson disease. JAMA Neurol 2014; 71(1):83.
22.Upadhyay R. Drug delivery systems, CNS protection, and the blood brain barrier. BioMed Res Internat 2014.
23.Xiao Y, Gao X, Taratula O, Treado S, Urbas A, Holbrook R, et al. Anti-HER2 IgY antibody-functionalized single-walled carbon nanotubes for detection and selective destruction of breast cancer cells. BMC Cancer 2009; 9(1):351.
24.Shade C. Liposomes as advanced delivery systems for nutraceuticals. Integrative Medicine 2016; 15(1): 33-6.
25.Jose J, Charyulu R. Prolonged drug delivery system of an antifungal drug by association with polyamidoamine dendrimers. Internat J Pharma Invest 2016; 6(2):123-7.
26.Rigo D, Ferroni L, Tocco I, Roman M, Munivrana I, Gardin C, et al. Active Silver nanoparticles for wound healing. Internat J Mol Science 2013; 1(14):4817-40.
27.Vazquez-García F, Tanomaru-Filho M, Chávez Andrade G, Bosso-Martelo R, Basso-Bernardi M, Guerreriro-Tanomaru J, Effect of silver nanoparticles on phsysicochemical and antibacterial properties of calcium silicate cements. Brazilian Dental Journal 2016; 27(5):508-14.
28.Jang S, Park J, Cha H, Jung S, Lee J, Jung S, et al. Silver nanoparticles moify VEGF signaling pathway and mucus hypersecretion in allergic airway inflammation. Internat J Nanomed 2012; 7:1329-43.
29.Boyles M, Kristl T, Andosch A, Zimmermann M, Tran N, Casals E, et al. Chitosan functionalization of gold nanoparticles encourages particle uptake and induces cytotoxicity and pro-inflammatory conditions in phagocytic cells, as well as enhancing particle interactions with serum components. J Nanobiotechnol 2015;18(13):84.
30.Wang L, Zhao W, Tan W. Bioconjugated silica nanoparticles:Development and applications. Nano Research. 2008; 1:99-115.
31.Chen X, Wong R, Khalidov I, Wang Y, Lee J, Wang Y, Jin M. Inflamed Leucocyte-mimetic nanoparticles for molecular imaging of inflammation. Biomaterials 2011; 32 (30):7651-61.
32.Liu Q, Zhang J, Sun W, Reuben Q, Weialiang X, Gu X. Delivering hydrophilic and hydrophobic chemotherapeutics simultaneously by magnetic mesoporous silica nanoparticles to inhibit cancer cells. Internat J Nanomed 2012; 7:999-1013.

33.Park H, Kim K, Jang S, Park J, Cha H, Lee J, et al. Attenuation of allergic airway inflammation and hyperresponsiveness in a murine model of asthma by silver nanoparticles. Internat J Nanomed 2010; 9(5): 505-15.
34.Bahadar H, Maqbool F, Niaz K, Abdollahi M. Toxicity of nanoparticles and on overview of current experimental models. Iranian Biomedical J 2016; 20(1):1-11.
35.Xie Y, Woolbright B, Kos M, McGill M, Dorko K, Kumer S, et al. Lack direct cytotoxicity of extracellular ATP against hepacyte: Role in the mechanism of acetaminophen hepatotoxicity. Clin Translational Res 2015; 1(2):100-6.
36.Darzynkiewicz Z, Galkowski D, Zhao H. Analysis of apoptosis by cytometry using TUNEL assay. Methods 2008; 44(3): 250-4.
37.Sun L, Li Y, Liu X, Jin M, Zhang L, Du Z, et al. Cytotoxicity and mitochondrial damage caused by silica nanoparticles. Toxicol 2011: 1619-29.
38.Choi J, Zheng Q, Katz H, Guilarte T. Silica-based nanoparticle uptake and cellular response by primary microglia. Environmental Health Perspectives 2010; 118:5.
39.Tran L, Wilson L. Nanomedicine: making controllable magnetic drug delivery possible for the treatment of breast cancer. Breast Cancer Res 2011; 13: 303.
40.Kim C, Kim T, Choi I, Soh M, Kim D, Kim Y et al. Ceria Nanoparticles that can protect against ischemic stroke, therapeutic. Angewandte Chemie Internat 2012; 51:11039-43.

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