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Original Article

In Vitro Toxicity of the Naked and Serum-treated Nanoparticles on Cardiomyocytes

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Background and Aims: Although metal and metal oxide nanoparticles are used in different medical applications, they may have considerable toxicity on various cells, such as myocytes. Therefore, this study aimed to evaluate the toxicity of the naked and serum-treated silver nanoparticles (Ag NPs) and magnesium oxide nanoparticles (MgO NPs) on the cardiomyocytes.

Materials and Methods: Cardiomyocytes were separately exposed to different concentrations of the naked and serum-treated nanoparticles for 24 hours at 37ºC. Then, MTT assay, cell metabolism assay and LDH assay were performed.

Results: Naked Ag NPs and MgO NPs had more toxicity than serum-treated nanoparticles. The highest cardiomyocyte toxicity was observed for naked Ag NPs, whereas the minimum toxicity was seen for the serum-treated MgO NPs.

Conclusions: Coating of nanoparticles with serum components leads to decrease in toxicity for cardiomyocytes, and MgO NPs have less toxicity on the myocytes than Ag NPs.

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Introduction

The use of nanoparticles in heart diseases is regarded as a new interesting field that offers diverse opportunities including treatment of defective heart valves, diagnosis and treatment of arterial plaque, understanding of cardiac pathophysiology at sub-cellular levels, and imaging of affected myocardial cells, amaong others [1,2]. Targeted therapy by nanoparticles has been applied for modulation of macrophages [3] and blood vessels [4] after myocardial infarction. Notably, these nanoparticles cross the endothelium by enhanced permeation and retention. The feasibility of this procedure has already been demonstrated [5, 6]. On the other hand, annexin-labeled nanoparticles and apoptosis-sensing nanoparticles have been utilized for detection and imaging of damaged cardiac cells [7]. Nanoparticles show a great efficacy in the field of cardiology as in nanopipette use for inserting drugs in certain portions of cardiac tissue [8], blocking low-density lipoproteins as well as cholesterol molecules by the use of nanolipoblockers [9] and application of polymer nanoparticles to dissolve the inflamed plaque [10].

In the last decade, metal and metal oxide nanoparticles have been widely studied for medical applications including implants, tissue engineering, drug delivery, antimicrobial effects, etc [11, 12]. These nanoparticles may be applied for diagnosis and treatment of heart diseases as well. Although these nanoparticles have different advantages, their toxicity must be taken into consideration. Nanoparticles can penetrate into the body via dermal, mucosal, intravenous, oral, inhalational and intra-peritoneal routes, which may then diffuse to various organs such as heart, liver, spleen, kidney and brain [13]. According to previous studies, decreased mitochondrial function and increased LDH leakage were observed after exposure of myocytes to nanoparticles [14]. As a matter of fact, they interact with different compounds in cells including membranes, proteins and DNA [15]. Although some studies have been conducted concerning toxicity of metal and metal oxide nanoparticles [16, 17], little information is published about their adverse effects on heart cells [18-21]. Jawad et al. demonstrated that TiO_2 nanoparticles at concentration of 10 µg/mL revealed no significant toxicity on myocytes over 24 hours, though at concentration of 100 µg/mL, the nanoparticles led the heart contraction to decrease [22]. Du et al. (2013) worked on cardiovascular toxicity of silica nanoparticles at different sizes and dosages in Wistar rats, and revealed that these nanoparticles could pass the alveolar-capillary barrier. Moreover, nanoparticle uptake and its related toxicity were dependent on the size and dosage of particles [23]. In another study, gold nanoparticles were reported to induce cardiac tissue damage, depending on both their size and time [24]. It may be postulated that the effect of naked nanoparticles used in laboratory conditions or cell cultures can be different from the same nanoparticles after entering the circulation and being covered by various plasma molecules, the latter being

inevitable after in vivo exposure. Modification of such physicochemical characteristics of nanoparticles as surface charge, size, shape, chemical reactivity, wettability, etc. seems quite possible after their coating by other molecules. Therefore, the aim of this study was to evaluate the toxicity of naked and serum-treated silver nanoparticles (Ag NPs) and magnesium oxide nanoparticles (MgO NPs) on cardiomyocytes of Balb/c mice.

Materials and methods

Ag NPs and MgO NPs were provided from the Lolitech Company, Germany. RPMI 1640, collagenase, hyaluronidase and 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) were purchased from the Sigma-Aldrich Chemical Co (St Louis, USA). Hanks' balanced salt solution (HBSS), and Alamar blue were obtained from the Invitrogen, USA. LDH kit was obtained from the Pars Azmoon Company, Iran, and Balb/c mice were sourced from the Pasteur Institute of Iran.

Preparation and characterization of the naked and serum-treated nanoparticles

Firstly, serial concentrations of each nanoparticle (31, 62, 125, 250 and 500 µg/mL) were separately prepared in RPMI 1640 medium, shaken for 5 minutes, and stored at 5ºC. In the next step, 1 mL of whole blood was obtained from each of 10 male Balb/c mice, and its serum was separated. In order to prepare serum-treated nanoparticles, one mL of each nanoparticle at concentration of 1000 µg/mL was added to one mL of serum. Then, they were incubated for 1 hour at 37ºC, followed by centrifugation at 5000 rpm and discard of supernatant.The nanoparticle pellets were re-suspended in RPMI 1640. Similar to naked nanoparticles, serial concentrations (31,62,125,250 and 500 µg/mL) of serum-treated nanoparticles were prepared in RPMI 1640. The size and structure of all nanoparticles were studied by scanning electron microscopy (SEM) (model S-2400, Hitachi, Japan) and Fourier transform infrared (FTIR) spectroscopy.

Isolation of Balb/c mouse cardiomyocytes

Three male Balb/c mice, weighing 18-20 g, were used. Their cardiomyocytes were isolated according to a reference [25]. Briefly, both left and right ventricles were removed, chopped, and incubated with an enzyme mixture (trypsine, collagenase and hyaluronidase) at 37ºC for 15 minutes. Isolated cells were centrifuged at 3000 rpm for 3 minutes. Ultimately, cardiomyocytes were washed and re-suspended in RPMI 1640 medium. The final concentration was 1000 cells/mL, which was used for all experiments**.**

MTT assay

At first, 100 µL of cardiomyocyte suspensions were separately added to serial concentrations of naked and serum-treated nanoparticles, incubated at 37ºC for 24 hours, followed by three washes with HBSS. Then 100 µL of RPMI 1640 and 25 µL of 5 mg/mL MTT were added to cells (1000 cardiomyocyte/well) in 96-well micro plate, and incubated at 37ºC for 4 hours. In the final step, 100 µL of isopropanol (70% v/v) was added before optical density (OD) of wells was read by a micro plate reader (Novin Gostar, Iran) at 490

nm. Finally, all ODs were normalized to control, *i.e*., the OD of each well was divided to OD of the negative control, which was the cells not treated with any nanoparticles.

Cell metabolic activity assay

Firstly, 100 µL of cardiomyocyte suspensions were separately added to the serial concentrations of naked and serum-treated nanoparticles, incubated at 37ºC for 24 hours, and then were washed three times with HBSS. In the next step, 100 µL of RPMI 1640 and 25 µL of the Alamar blue reagent were added to each well, and incubated at 37 ºC for 4 hours. The cell metabolism status was measured by reading OD at 590 nm using microplate reader (Novin Gostar, Iran), which were then normalized to control.

LDH assay

The quantity of released LDH enzyme was measured by LDH assay kit, according to the kit manufacturer instructions. At first, 100 µL of cardiomyocytes were separately added to serial concentrations of naked and serum-treated nanoparticles. After incubation at 37ºC for 24 hours, the cell media were centrifuged at 3000 rpm for 15 minutes, and their supernatant was used for LDH assay. Briefly, one mL of reagent 1 (lactate) and one mL of reagent 2 (NADH) were mixed, and then 10 µL of the supernatant was added to this mixture. The average OD of each sample was read by a micro plate reader at 340 nm after 5 minutes. In the negative control group, the cells were not treated with any nanoparticles. In the final step, all data were normalized to control, *i.e*., the OD of each well was divided to OD of the negative control.

Statistical analysis

All the tests were repeated five times, and the results were demonstrated as mean ± standard deviation (SD). Then, the student's t-test was applied to evaluate the significant differences utilizing SPSS software (V.16.0 for Windows; SPSS Inc., USA), and $P<0.05$ was considered as indicative of statistically significant difference.

Results

Characterization of the naked and serum-treated nanoparticles

Fig. 1a and Fig. 1b demonstrate the SEM images of the naked Ag NPs and MgO NPs, respectively. As is shown, all naked nanoparticles had high agglomeration in RPMI 1640. However, in the case of treated Ag NPs (Fig. 1c) and MgO NPs (Fig. 1d), no agglomeration was observed. Obviously, serum-treated nanoparticles display the same size and structure as of their naked counterparts. These images demonstrate round shapes of all coated nanoparticles with no agglomeration. According to FTIR spectrum, all serum-treated nanoparticles had the same surface composition, *i.e.*, they had amide band I (1650 cm^{-1}), amide band II (1550 cm^{-1}) and NH stretching vibrations – amide A and B (3170-3300 cm^{-1}). These spectra were not obsrved in the naked nanoparticles.

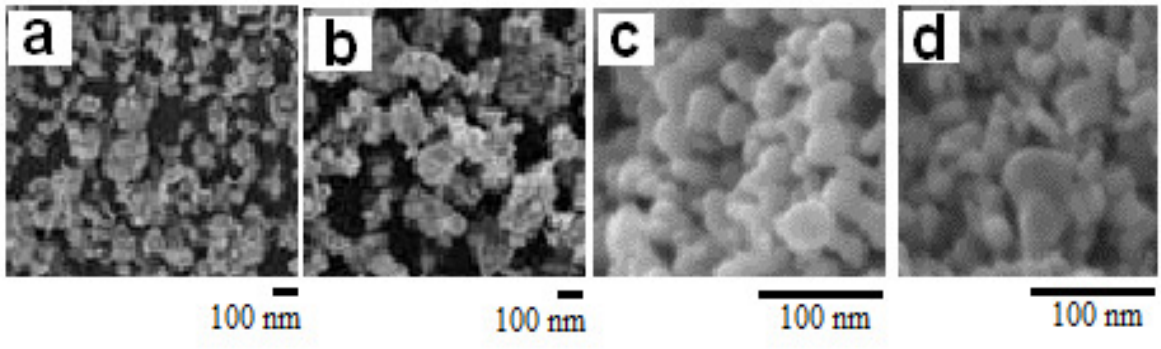

Fig. 1. SEM images of naked Ag NPs (a), MgO NPs (b), serum-treated Ag NPs (c) and serum-treated MgO NPs (d).

Cardiomyocyte toxicity results

The hematoxylin & eosin-stained cardiomyocytes are demonstrated in Fig. 2 (a) and Fig. 2 (b) after incubation with the naked Ag NPs and MgO NPs, respectively. As is indicated in Fig. 2 (a) and Fig. 2 (b), no frank difference in histopathology is detectable between the naked Ag NPs and MgO NPs, except nuclear enlargement in the former group. In order to compare toxicities of the naked and serum-treated nanoparticles, three assays were utilized including MTT assay, cell metabolic activity assay and LDH assay. The toxicity of the naked and serum-treated Ag NPs and MgO NPs are shown in Fig. 3 and 4, respectively. Each figure contains MTT (a), cell metabolic activity (b) and LDH (c) results. All nanoparticles affected cardiomyocyte, and led to a decrease in cell viability and cell metabolism, and increased release of LDH enzymes. As is depicted in figures, naked form of each nanoparticle led to significantly less cell viability and cell metabolism, and higher LDH enzyme release in comparison with serum-treated forms ($P<0.05$). As is seen in these figures, cardiomyocyte toxicities of all naked and coated nanoparticles were dose-dependent. In other words, higher concentrations of nanoparticles led to lower cell viability and lower cell metabolism, as well as higher LDH release. The highest toxicity was observed with naked Ag NPs, whereas the minimum toxicity was attributed to serum-treated MgO NPs.

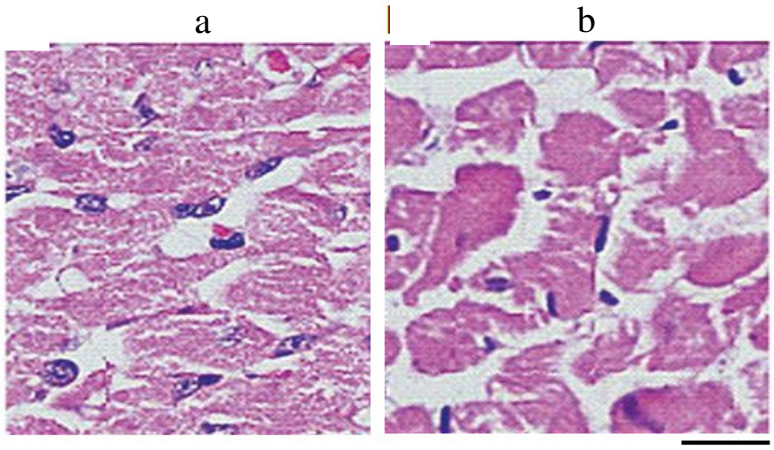

Fig. 2. Stained cardiomyocytes after incubation with naked Ag NPs (a) and MgO NPs (b). The scale bar is 100 µm. H&E staining, ×400.

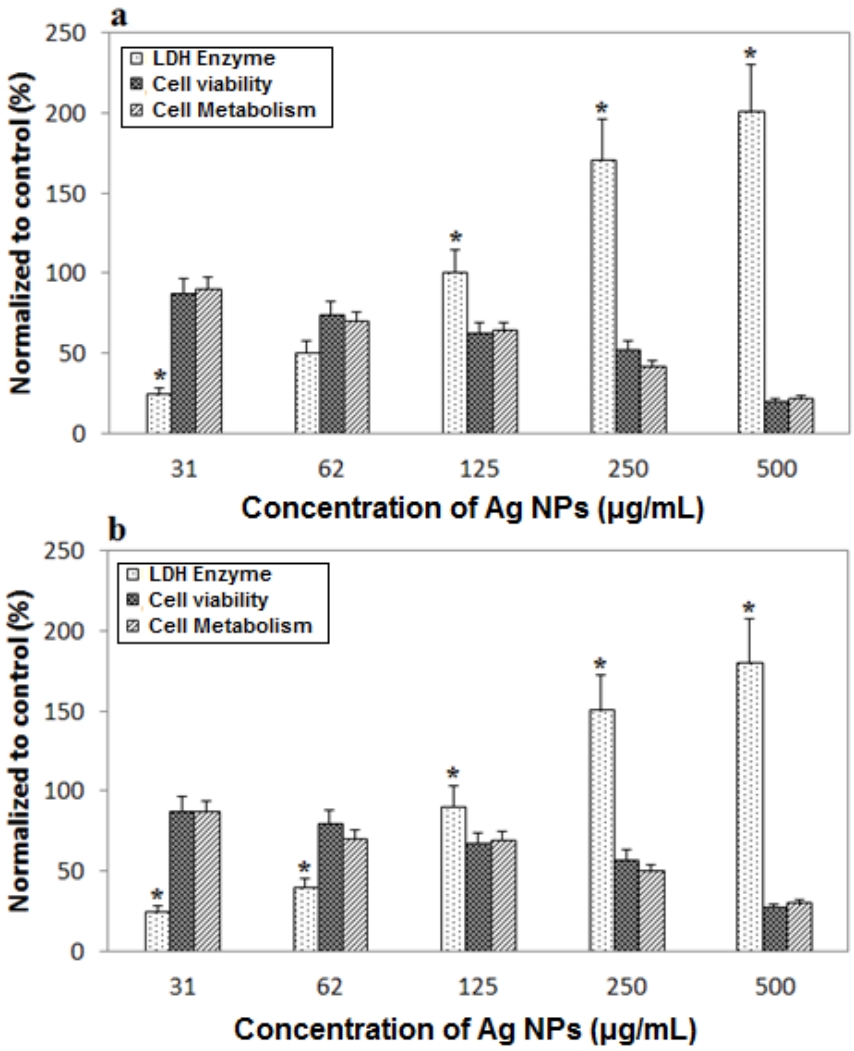

Fig. 3. The effects of Ag NPs on cardiomyocytes. At first, cardiomyocytes were separately incubated with serial concentrations of naked (a) and serum treated (b) Ag NPs for 24 hours at 37 ºC. Then, cell viability, LDH release and cell metabolism were measured. All data are shown as mean ± SD with n=5 (independent t-tests). *P<0.05 compared with cell viability and cell metabolism level, at the same concentration.

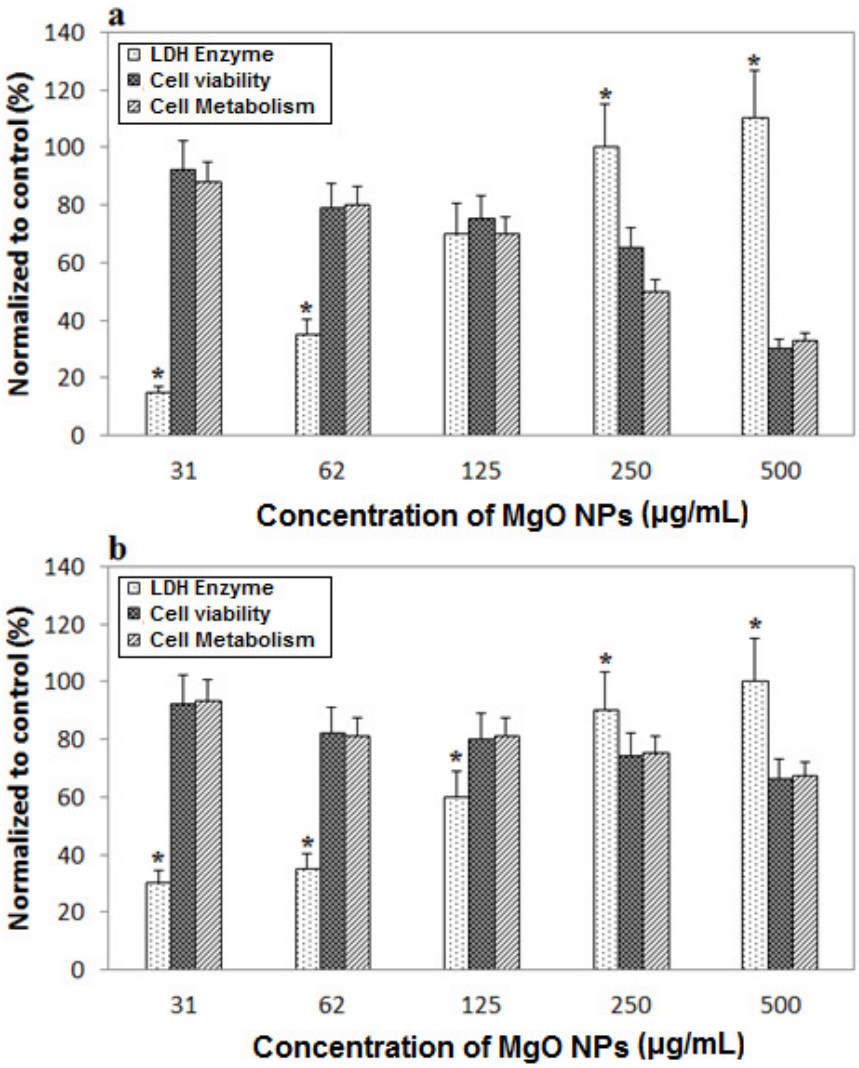

Fig. 4. The effects of MgO NPs on cardiomyocyte. Cardiomyocytes were separately incubated with serial concentrations of naked (a) and serum treated (b) MgO NPs for 24 hours at 37 ºC. Then, cell viability, LDH and cell metabolism were measured. All data are shown as mean ± SD with n=5 (independent t-test). *P<0.05 compared with cell viability and cell metabolism level, at the same concentration.

Discussion

Metal and metal oxide nanoparticles are good candidates for a wide range of medical applications due to their superb chemical and physical properties [11, 12]. In cardiology, nanoparticles can be utilized for imaging of affected myocardial cells, treatment of heart diseases and study of molecular functions [1, 2]. In spite of some advantages, they impart remarkable toxicity which depends on their size, shape and surface [16, 17]. Those variables may obviously be affected by serum molecules when they gain access to the circulation. Therefore, the purpose of this study was to evaluate the toxicity of naked and serum-treated Ag NPs and MgO NPs on the cardiomyocytes.

This study revealed that naked form of nanoparticles led to less cell viability, lower cellular metabolism and higher LDH enzyme release than serum-treated forms. The higher toxicity of naked nanoparticles than serum-treated nanoparticles has been reported in a previous study [26]. This pattern occurs due to protein adsorption. The layer of proteins on the surface of nanoparticles inhibits direct interaction between nanoparticles and such cell components as membranes, enzymes and DNA. On the other hand, naked nanoparticles aggregate in RPMI 1640, because of electrostatic forces. Authors hypothesize that the formation of micron-sized metal and metal oxide particles inside as well as outside of cells is responsible for higher cell damage.

The toxicity of naked Ag NPs was significantly more than MgO NPs ($P<0.05$). Interestingly, this order was also observed for the serum-treated nanoparticles. The authors assume that the reason for higher toxicity of Ag NPs is easier cellular uptake and increased generation of reactive oxygen species (ROS) which lead to more damage to cell components including enzymes. Other mechanisms, such as high ion release and oxidative property may be influential as well, which must be evaluated in future studies. Findings of the present study are consistent with those of other studies [16, 17]. Some reports have demonstrated that specific nanoparticles lead to DNA damage, caspase activation, condensation of chromatin, formation of micronuclei and lipid peroxidation [15, 27]. Moreover, a direct relationship was detected between nanoparticle uptake (and subsequent ROS generation) and LDH release, whereas an inverse relationship was observed between nanoparticle uptake and cell viability/metabolism level [28]. Nanoparticles can also affect myocardial cells by injuring epithelial tissues [29], oxidative stress response and inflammation [30, 31]. Some researchers have also indicated that oxidative stress as a response to nanoparticles can be regarded as an important mechanism of toxicity [32].

Mann et al. proposed that oxidative stress, inflammation and autonomic dysregulation can be possible mechanisms of toxic effect of nanoparticles on the cardiac cells. On the other hand, they declared that nanoparticles can change vascular endothelial cell integrity leading to disruption of heart rate, electrical activity and increased susceptibility to ischemia/reperfusion injury [33]. No data have been gleaned concerning the effect of serum-treated nanoparticles on the cardiac tissue, though some works have reported effects of naked nanoparticles on the heart cells, which are discussed as follows. Abdelhalim et al. demonstrated that the exposure to Au NPs produces cardiac damages depending on the size and duration of exposure. Au NPs the size of 10 and 20 nm were more toxic 50 nm-particles, and administration of nanoparticles for 7 days was more injurious than 3 days [24]. Interestingly, as reported in other studies, polyethylene glycol (PEGylated) Au NPs can affect cardiovascular function [34]. Jawad et al. demonstrated that TiO_2 NPs at concentration of 10 µg/mL for 24-hour had no effect on rat myocytes, though concentration of 100 µg/mL resulted in a

reduction of contraction amplitude [22]. Moreover, TiO_2 NPs reduced fibroblast proliferation and cell viability at concentration of 5–150 µg/mL after 4 days of exposure. In another study, Mallik et al. reported great uptake of protein-TiO_2 NPs by the cardiac cells, which caused higher oxidative stress and decreased mitochondrial membrane potential. Moreover, protein modification of TiO_2 NPs led to more cardiac toxicity [35]. Meanwhile, other metal oxide nanoparticles such as silica nanoparticles were found to be toxic to the myocardial tissue. According to Du et al., silica nanoparticles could pass through the alveolar-capillary barrier and reach the heart, with their uptake being dependent on the nanoparticle dosage and size [23]. This nanoparticle increases interleukin-1 beta, interleukin-6, tumor necrosis factor-alpha, intercellular adhesion molecule-l and vascular cell adhesion molecule-l. Stampfl et al. suggested Langendorff heart as a model for nanoparticle investigation, which enables observation and analysis of heart parameters over 24 hours. The findings of that study revealed that TiO_2 and SiO_2 increase heart rate and arrhythmia [25]. On the other hand, some nanoparticles were reported to be cardioprotective. Niu et al. demonstrated that cerium oxide nanoparticles protect the heart against progression of cardiac dysfunction by their antioxidant properties [36]. In order to reach a comprehensive understanding of the interactions between NPs and tissues, other tests are recommended to be done in the future, such as tissue antioxidant enzymes, lipid peroxidation, cytokine production and ultra-structural changes.

Conclusions

The findings of the present study showed that naked nanoparticle had higher toxicity than serum-treated nanoparticles on cardiomyocytes. Overall, Ag NPs revealed more toxicity compared to MgO NPs. This study may provide more understanding about the actual circumstances regarding toxicity of these nanoparticles, which should be considered at in vivo conditions in future studies.

Conflict of Interest

The authors declare no competing financial interest.

Acknowledgments

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