RESEARCH ARTICLE

The Investigation of the Toxicity of Palladium Nanoparticles on Human Lymphocyte

Maryam Zivari fard [1], Majid Sharifi [2,3], Arian Shojaei [1], Seyed Mahdi Rezayat [1], Seyyedeh Elaheh Mousavi [1,*], Mojtaba Falahati [2,*]

[1] Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran.

[2] Department of Nanotechnology, Faculty of Advanced Sciences and Technology, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran.

[3] Department of Animal Science, Faculty of Agriculture, University of Tabriz, Tabriz, Iran.

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ABSTRACT

Palladium nanoparticles (Pd-NPs) have various applications in industries, such as: Biomedicine, Sewage treatment, Electronics, and Catalytic processes. Different studies on NP have shown that they have negative impacts on different cell categories in vitro. Despite extensive considerations, the molecular mechanism of Pd-NPs toxicity has remained elusive thus far. Therefore, in this paper, we investigated the toxicity of Pd-NPs by evaluating their effects on human lymphocyte. Blood lymphocyte cells were initially isolated by ficoll solution and were exposed to Pd-NPs and Pd (II) ions. Then, we examined oxidative stress, cell cycle and apoptosis employing flow cytometry. We found the NPs administration suppressed cell growth,which in turn resulted in cell apoptosis. Also, cell cycle stopped at sub G1 phase, resulting in DNA damage as well as profound ROS increase. Our results showed that Pd-NPs treatment for 24 hours led to apoptosis, oxidative stress, as well as cell cycle blockage. It is notable that Pd (II) ions induced more severe toxicity. Our findings provide valuable insights on Pd-NPs toxicity.

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INTRODUCTION

Nanomaterials referred to particles which are smaller than 100 nm and considered as one of the strategic materials of the twentieth century [1]. One of the most important members of these materials is Pd-NPs that is used in various industries such as ornaments, dental alloys, and catalytic converters in automotive exhaust [2-4]. Therefore, the possible health impact of Pd-NPs when they enter the body is of great interest. The catalytic converters release Pd particles as well as very small amount of Pd-NPs in the human respiratory range [5]. After using the elements of Pd group as catalytic converters, the amount of Pd, especially Pd-NPs, will increase significantly around the roads [4]. There is not enough information about the role of Pd-NPs in allergic sensitivity [6, 7]. Also, it has been shown that Pd-NPs affect the secretion of INF-γ and Th1 cytokine in type IV immune reactions [3]. Petraca, Clemente, found that exposure to Pd-NPs increases the mitogen activation rate with DNA diploid content, which shows that G0 phase has increased in human peripheral blood mononuclear cells. This feature of Pd-NPs is very important, because preventing the progression of the cell cycle is a crucial factor in curing the human cancers [8]. Studies on several cell lines including human skin malignant melanoma cells and human ovarian A2780 cancer cells show that the induction of apoptosis and cessation of cell cycle happens after exposure to Pd-NPs [8-10] .

ROS performance in biological species has many

* Corresponding Author Email: *Mojtaba.falahati@alumni.ut.ac.*
semousavi@sina.tums.ac.ir

contradictions. It helps the cellular messaging and system defence of the body, also it plays an essential role in induction of apoptosis in order to prevent disease. On the other hand, damaging the important cellular macromolecules results to cancer and heart disease. Also, studies have shown that ROS plays a very important role in the immune system as well as the transmission paths of signalling. Nevertheless, its excessive increase leads to various damages to the cell and even to the body [11-13] . Therefore, due to the lack of a study on induction of apoptosis of Pd-NPs on human lymphocyte, this paper investigates the possible effects of Pd-NPs and Pd (II) ions on induction of cell apoptosis. It also studies the NPs-associated effects on oxidative stress and cell cycle arrest in human lymphocytes.

MATERIAL AND METHODS

Materials

Pd (II) ion and Pd-NPs were purchased from US Nano, USA, as a powder with a particle size of 15 nm, with a purity of 99.95%, and with spherical morphology. The metals composition RPMI1640 (biosera), Fetal Bovine Serum (Gibco BRL), NaCl (merck), NaOH (merck), penicillin/ streptomycin solution 100X, PBS tablet, MTT (3-(4, 5-dimethylthiazol-2-thiazol-2-tl)-2, 5-diphenyl tetrazolium bromide) (Sigma), dimethyl sulfoxide (DMSO), a AnnexinV, Dichlorodihydrofluorescein diacetate (DCFH-DA), phytohemagglutinin (Gibco), Trypan Blue in 0/9% NaCl solution 0/4% (wt/vol) (Gibco), propidium iodide (sigma Aldrich, Milan, Italy), RNase (DNase-free, Sigma Aldrich, Milan, Italy).

Isolation and stimulation of peripheral blood lymphocytes

We have isolated the human peripheral blood lymphocyte from three healthy donors by density gradient centrifugation and using Ficoll-Hypaque according to the standard manufacturer procedure. Cultured cells exposed to different concentrations of Pd-NPs or Pd (II) complexes (0, 0.01, 0.1, 1, 10, 50, 100, 200 µg/ml) in RPMI 1640 medium containing 10% fetal calf serum (complete culture medium), 1% penicillin and streptomycin, containing 2% phytohemagglutinin (sigma Aldrich, Milan, Italy), and incubated at 37°C under 5% CO2 95% air atmosphere.

Cell viability assays

The cytotoxicity is studied by MTT assay, 1×10^4 cells per well in 200 µL culture medium (three replicates), seeded in 96-well culture plate, incubated at 37°C, 5% CO2, and exposed to different concentration of Pd-NPs (0, 0.01, 0.1, 1, 10, 50, 100, 200 µg/ml) or Pd (II) ions (0, 0.01, 0.1, 1, 10, 50, 100, 200 µg/ml). The control group received only the culture medium plus solvent after 24 hours of incubation. At the end of exposure time, we had a washing step to remove the incubation medium containing Pd-NP or Pd (II) ion. Then, we added 20µL of the MTT solution (5mg/ml) to each well and then incubated it with MTT for 4 hours. The coloured formazan crystals formed by MTT in living cells dissolved in 200 µl of DMSO. After 10-15 minutes, the optical density (O.D.) values of the solutions are measured at 540 nm as the stimulation wavelength by using a spectrophotometer plate reader (Applied Biosystems, Life Technologies, Monza, Italy). Also negative controls (i.e., complete culture comprising Pd-NP or Pd (II) ions) at all of the concentrations that we used have been tested, and no interposition with the colorimetric assay observed. We considered the cells without treatment as negative controls (100% viability).

Detection and measurement of ROS

Intracellular reactive oxygen species (ROS) are measured by using Dichlorodihydrofluorescein diacetate (DCFH-DA), which is a non-fluorescent and cell permeant substance, which will be transformed to dichlorofluorescein (DCFH) by endogenous esterases. DCFH is a non-permanent non-fluorescent substance which will be oxidized by cellular ROS to fluorescent dichlorofluorescein (DCF). 1×10^6 cells per well in 1000 µL culture medium seeded in 24-well culture plate (three replicates). After 24 hours, the cell is washed with phosphate-buffered saline (PBS) and incubated with 10 µM DCFH-DA, for 30 minutes, at 37°C. The fluorescence emitted by the oxidized form of DCFH-DA is measured by using a FACSCalibur flow cytometer.

Cell cycle assay

For analysis of DNA content in each phase of the cell cycle, 1×10^6 cell per well in 1000 µL culture medium was seeded in 24-well culture plate (three replicates). After treatment with Pd-NPs or Pd (II) ions cells were washed, for 24 hours with PBS, and fixed in ethanol 70% ice- cold. The cell then stained with 50µg/mL propidium iodide in PBS buffer containing 200µg/ML RNase (DNase-free,

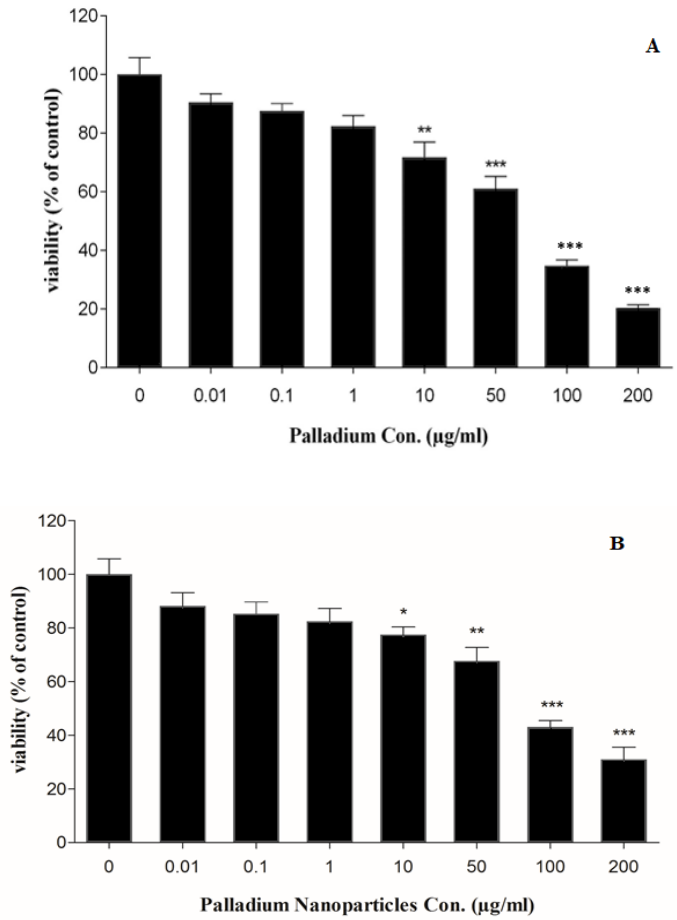

Fig. 1. Evaluation of *in vitro* cytotoxicity by MTT assay. A) Different concentrations of Pd (II) ions (0, 0.0, 0.1, 1, 10, 50, 100, 200 μgmL−1). B) Different concentrations of Pd-NP (0, 0.0, 0.1, 1, 10, 50, 100, 200 μgmL−1).

Sigma Aldrich, Milan, Italy) and analysed with FACSCalibur flow cytometer equipped with Cell Quest Software (Becton Dickinson, Mountain View, CA).

Apoptosis assay

Apoptosis in lymphocyte measured by using the Annexin V-propidium iodide. 1×10^6 cells per well in 1000 µL culture medium were seeded in 24-well culture plate with three replicates. After treatment with Pd-NPs (95 µg/ml) and Pd (II) ions (65 µg/ml) complexes the cell washed for 24 hours with PBS. After 15 minutes incubation in dark with 5 µL Annexin V-FITC and 5µL propidium iodide, the cells suspended and then diluted with 200 µL binding buffer and then the cells filtered and analysed by FACSCalibur flow cytometer.

Statistical analysis

The data is presented as mean values with standard deviations. Statistical analysis was performed with one-way ANOVA followed by least square means and also the statistical differences were considered significant at level of $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

RESULT AND DISCUSSION

The lymphocyte is known as inactive cell, which is used *in vitro* experiments to stimulate cells and start a cell cycle of phytohemagglutinin (PHA). After 24 hours exposure to Pd (II) and Pd-NPs, we observed a decrease in cell survival by MTT assay (Fig.1). The results showed that by increasing the concentration of Pd (II) ion and Pd-NPs in the culture medium, their toxicity increases

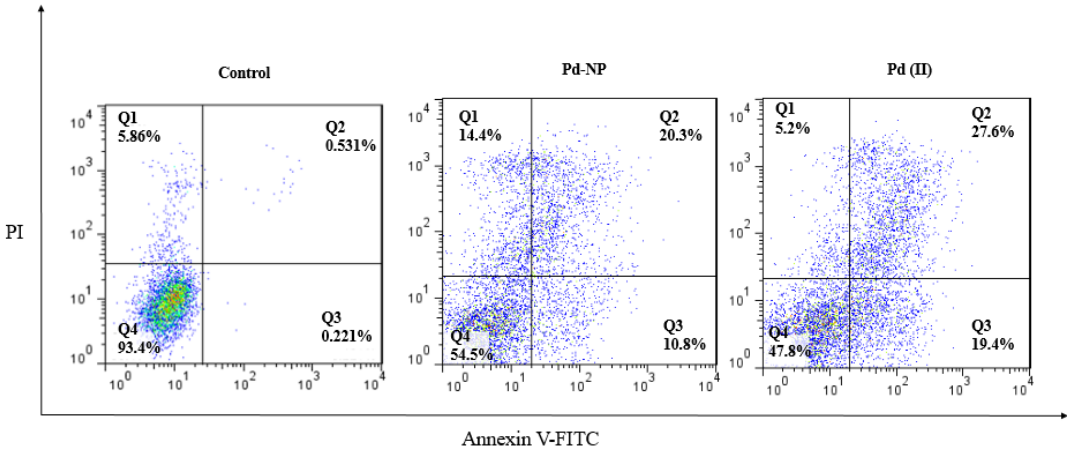

Fig. 2. Flow cytometry can analyse the AnnexinV-PI stained cells. Only PI positive population indicates necrotic cells whereas only AnnexinV positives are early apoptotic cells. Cells in the late apoptosis get both colours. Induction of apoptosis in human lymphocytes by NP-NP and Pd (II). Human lymphocytes were activated by treatment with PHA.

significantly, whereas ionic Pd was the most toxic (Fig. 1). Nevertheless, effect of Pd (II) ion and Pd-NPs cytotoxicity on the lymphocyte cells started at 10 µg/mL, with 21–25% (P<0.01) and 18–21% (P<0.05) decreases in cell survival, respectively (Fig. 1). The IC50 values for lymphocyte cells exposed to ionic Pd were lower than 70 µg/mL, which is indicating a strong cytotoxicity. While, the IC50 values for Pd-NPs were in the range 90–100 µg/mL for lymphocyte cell. Overall, despite the higher toxicity of Pd (II) ion compared to Pd-NPs, the pattern of cells vitality and their reduction in concentrations of 0 to 200 µg/mL was similar for both of them.

The effect of Pd-NPs and Pd (II) ions on cell cycle development was investigated for 24 hours (Fig. 4). When cells faced with DNA damage, they accumulate in one of the phases of cell cycles such as gap1 (G1), DNA synthesis (S), or in gap2/mitosis (G2/M). Both Pd (II) ions and Pd-NPs significantly increased the population of lymphocyte cells in the subG1 phase (24.32% and 19.38%, respectively) compared to the control phase. While the highest accumulation of the cells in the S and G2 phases were related to the Pd-NPs and Pd (II) ions, respectively. In addition, based on the increment of cellular population in the subG1 phase the results of the cell cycle indicate that Pd (II) ion is approximately 1.25 times more effective in stopping the cell cycle than Pd-NPs. The results of this study showed that the exposure to Pd (II) ions and Pd-NPs, reduced the cells vitality and maintains at G0 status or prolong/stop at subG1 stage. Studying the effect of toxicity in the G1 stage of the cell cycle is very important for both therapeutic and biological activities [14]. For example, the most healthy cells and even the cancer cells decide on the development of cell growth, such as increasing cell size and proliferation, at the G1 stage [15].

Apoptosis plays an important role in the biological activity of cells, which can be characterized by several morphological and biochemical changes in cells. As shown in Fig. 2, both Pd (II) ions and Pd-NPs significantly (P<0.001) caused cell death in 24 hours incubation period. The results of the Annexin V/PI assay showed that Pd-NPs significantly increase the apoptotic cell induction in Q_2 (early apoptotic cells) from 0.531% to 20.3% and Q_3 (late apoptotic cells) from 0.221% to 10.8% compared to the control group, while Pd (II) ions increased cell death in Q_2 (27.6%) and Q_3 (19.4%), respectively. Similarly, it was found that Pd (II) ions increased the 1.8 and 1.35-fold induction of apoptosis in regions Q_3 and Q_2 relative to Pd-NPs, respectively. If no corrections are made to reduce the toxicity of Pd (II) ions and Pd-NPs, the cell may go to the pathway of apoptosis. Increasing the level of apoptosis in this experiment confirms this hypothesis. As depicted in Fig. 3, the treatment of Pd (II) ions and Pd-NPs increased the intracellular levels of ROS to 196 and 173 units compared to the control group. On the other hand, the ROS results indicate that its increase is in line with apoptosis fluctuations. Therefore, it is possible to increase the induction of apoptosis with ROS. In agreement with our results, Reza et al. revealed that

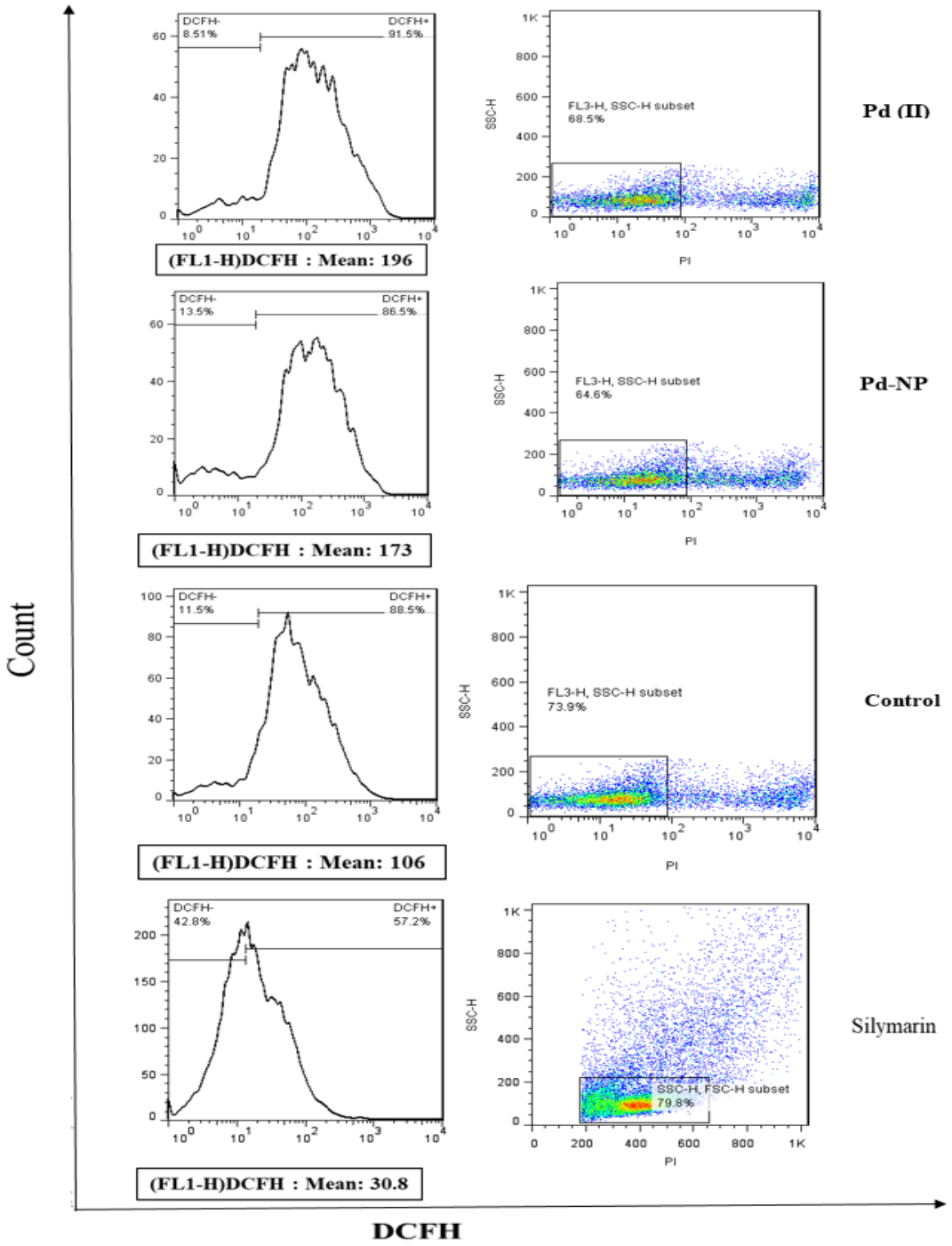

Fig. 3. ROS generation in lymphocytes exposed to Pd-NP and Pd (II) complexes. Cells treated with silymarin were considered as positive controls (as antioxidants).

using Pd (II) ion or Pd-NPs not only increases the cytotoxicity of cells through the enhancement of ROS, but also halts the activity of cells in the subG1 stage. Another study in 2020 confirms the effects of apoptosis induction and cellular toxicity of Pd-NPs in the ovarian cell [16]. Furthermore, similar to our results, they showed that Pd (II) ions are more toxic than the Pd-NPs [16]. In agreement with our

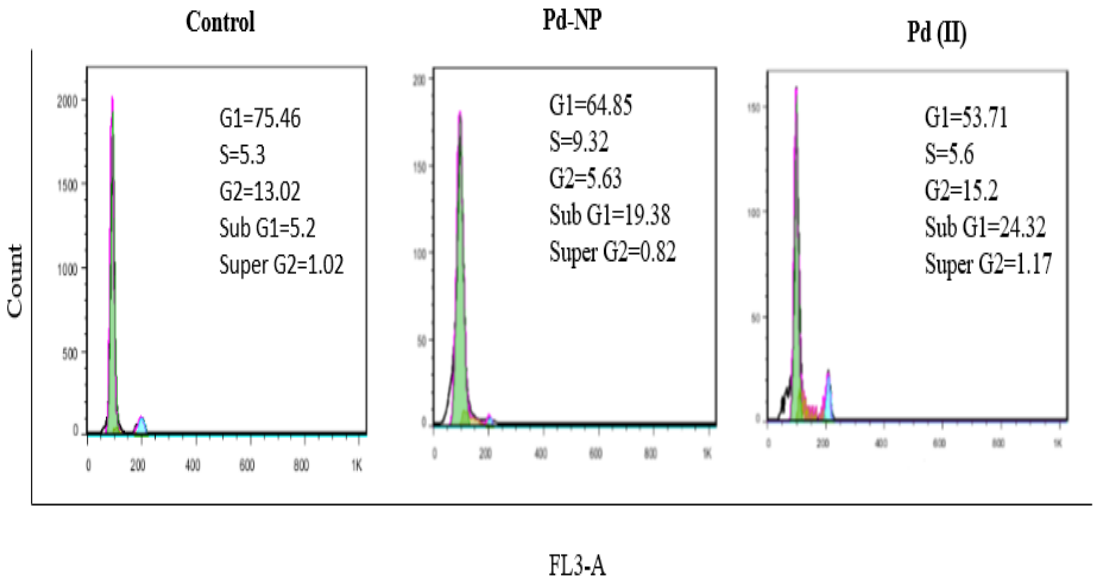

Fig. 4. Cells were treated with 95µg/ml of Pd-NP and 65µg/ml Pd (II) complexes for 24 hours and the ratio of sub-G1 was measured by flow cytometry analysis after PI staining.

results, by studying the human peripheral blood mononuclear model showed that the use of Pd (II) ion or Pd-NPs not only increases the cytotoxicity of cells through the enhancement of ROS, but also halts the activity of cells in the subG1 stage. In agreement with our results Gurunathan et al. showed that Pd-NPs increase ROS levels as well as induced apoptosis on the A549 cell line [17]. Also, similar to our results, it has been showed that Pd (II) ions are more toxic than the Pd-NPs [8]. Since part of the toxicity of Pd-NPs depends on the release of Pd (II) ions from their NPs, the Pd (II) ions toxicity is expected to be as high as that of NPs. Therefore, metal ions may accumulate in mitochondria and damage their function, which could induce mitochondrial apoptosis. However, these possible mechanisms of action should confirm through a deeper research [18, 19]. Likewise, Iavicoli et all by using Pd-NPs based on a dose-dependent manner were able to inhibit A549 and lung epithelial cells activity in a relatively long period (48 to 120 hours) at the subG1 phase [20]. In this work, the higher inhibition of Rat-1fibroblasts compared to A549 cells indicates the variability of cellular susceptibility to the cytotoxicity of Pd-NPs [20]. At the same time, it was shown that Pd-NPs not only inhibit the proliferation of melanoma A375 cells in a dose-dependent manner, but also they induce apoptosis by increasing ROS, damaging DNA, stopping the cell cycle in the G0/G1 phase, and accumulating of cytotoxicity of Pd-NPs in phases S and G2/M [9]. Whereas, contrary to provided reports,Chhat, Morphy-Marion showed that Pd-NPs do not have a negative effect on human 3D10 eosinophils and primary eosinophil cells survival which plays an important role in pulmonary diseases and allergies [21]. Nonetheless, Pd-NPs significantly increased the adhesion of eosinophil cells to endothelial cells. Recently, in the cellular models it has been shown that Pd-NPs and Pd (II) ions, based on the increase ROS and induction of apoptosis, led to the death of Human ovarian cancer cells (SKOV3 cells) and the stopping of cell cycles in the G1 phase due to DNA damage [18]. Also, Gutunathan, Qasim showed that Pd (II) ions, are increasing the rate of SKOV3 cells accumulation in the subG1 phase in comparison with Pd-NPs [18]. In general, the results indicate that Pd-NPs and its ions can be effective in reducing cell viability. Therefore, usage of these compounds is effective in biomedical activity,especially in controlling cancerous cells.

CONCLUSION

Since the use of Pd (II) ions and Pd-NPs in catalytic processes, electronics, and nanomedicine has attracted a lot of attention, their toxicity is considered to be very vital in biological processes. However, unlike other metallic NPs, there are

fewer reports of cytotoxic Pd-NPs. Therefore, this study aimed to investigate the potential cytotoxicity of Pd (II) ions and Pd-NPs based on cell proliferation, apoptosis, ROS and cell cycle stopping in lymphocyte cells. The results showed that Pd-NPs and Pd (II) ions inhibit the growth of lymphocyte cells in a dose-dependent manner, while the intensity of inhibition was higher by Pd (II) ions. Furthermore, Pd (II) ion or Pd-NPs not only increase the cytotoxicity of cells through the enhancement of ROS, but also halt the activity of cells in the subG1 stage, whereas Pd (II) ions provide a higher level of apoptosis and ROS.

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CONFLICTS OF INTEREST

The authors have none to declare.

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