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Effect of Polystyrene Nanoplastics on Ovarian Granulosa Cells

Zhenghao Wang, Chuanyao Huang and Yongmei Chen *

College of Chemical Engineering, Sichuan University of Science & Engineering, Zigong Sichuan, 643000, China

*** Corresponding author:** Yongmei Chen

Abstract: Existing studies have shown that microplastics can cause male reproductive damage, but the effect of nanoplastics on female reproductive ability is rarely studied. The effect of PS-NPs (polystyrene nanoplastics) on the morphology of human granulosa-like tumor cells (KGN) was observed and photographed by optical microscope. CCK8 and LDH kits were used to detect the viability and damage of KGN cells exposed to 0, 25, 50, 75, 100, 125 and 150 μg/mL of PS-NPs, and the IC50 value of half inhibition of KGN cells by PS-NPs is 130.4 μg/mL. The ROS accumulation in KGN cells after exposure to 200 μg/mL of PS-NPs is significantly increase. The results showed that with the increase of the concentration of 50 nm PS-NPs, the morphology of KGN cells was significantly changed, the cell viability was significantly decreased, and the cell damage rate was significantly increased. These results provide evidence for the toxicity of PS-NPs in female reproduction.

Keywords: Female Reproduction; Nanoplastics; Cell Viability; Oxidative Stress.

1. Introduction

Plastics are widely used, and plastic waste degrades into small plastic particles under the influence of physical and chemical interactions in the environment. The particle size in the range of 100nm-5μm is microplastics (MPs) and the particle size less than 100nm is nanoplastics (NPs) [1]. Due to their nano-size, NPs may be more widely distributed than MPs particles, because of the larger specific surface area, and can pass through biological barriers to enter blood, cells and tissues and organs, which may be more harmful to organisms [2]. And it is increasingly becoming a new focus of research on plastics in the environmental field [3]. NPs enter the human body through the digestive tract and respiratory tract, and cause different degrees of toxic damage [4]. Among them, polystyrene NPs (PS-NPs) accounted for the highest proportion of the environment. Previous studies have shown that PS-NPs can cause male reproductive toxicity. Gao et al. compared the effects of MPs/NPs with different particle sizes on spermatogenic function and morphological changes of spermatogenic cells in the mouse testis, and the results showed that MPs/NPs exposure caused reproductive toxicity, including spermatogenesis disorders and sperm physiological changes, oxidative stress and inflammation. At the same time, the morphological structure of seminiferous tubules, Sertoli cells and Leyden cells also changed significantly [5]. In addition, studies have shown that due to the large specific surface area of NPs, it adsorbs other pollutants, such as phthalate lipids, which further aggravates the toxicity to the reproductive system [6]. Ovarian health directly affects female reproduction [8]. Some studies have shown that oxidative stress can cause damage to ovarian granulosa cells [9]. Some studies have shown that PS-NPs can cause oxidative stress in animals and cells [10]. These results suggest that PS-NPs may affect female reproductive health through oxidative stress and apoptosis. In this study, we used 50 nm PS-NPs to expose ovarian granulosa cells and explore the effect of PS-NPs on KGN cells, so as to provide evidence for female reproductive toxicity caused by micro/nano-plastics.

2. Materials and Methods

2.1. Materials and Chemicals

50nm polystyrene microplastics were purchased from Wuxi Rege Biotechnology Co. Ovarian granulosa cells KGN were purchased from Wuhan Pricella, DMEM/F12 medium and Fetal bovine serum (FBS) were purchased from Beijing Dongge Boye Biotechnology Co. LTD, CCK8 kit and LDH kit were purchased from Beiren Chemical Science and Technology (Beijing) Co., LTD. Reactive oxygen species detection kit, Shanghai Biyun Tian Biotechnology Co., LTD.

2.2. Instruments and Equipment

Scanning electron microscopy (SEM, Thermo Scientist-Apreo 2, USA), critical point desiccator (Tousimis Autosamdri-815, Series), Fourier transform infrared spectroscopy (Thermo Fisher Scientific, Nicolet Nexus, USA), cell incubator (Thermo Fisher, USA), 1300 SERIES A2 biosafety cabinets (Thermo Fisher, USA), High speed cryogenic centrifuge (Xiangyi cence H1750R, China), fluorescence inverted microscope (Leica DMi8, Germany), ultrasonic cleaning instrument (WM3200-DE, China) ultra-pure water instrument (Milli-Q direct8, Germany).

2.3. Scanning Electron Microscope

50 nm PS-NPs were diluted to 1.5 g/mL with ultra-pure water, fully sonicated for 15 min to disperse them, fixed on a silicon wafer, and then dehydrated in a gradient of 30%, 50%, 70%, 100%, and 100% ethanol for 10 min each. After dehydration, the sample was removed from the 100% ethanol. The samples were placed in a critical point desiccator and dried for 1h. After drying, the samples were removed and fixed to the sample table with conductive glue, sprayed with gold and tested.

2.4. Fourier Infrared Spectroscopy

The functional groups and chemical bonds of PS-NPs were measured by Fourier transform infrared spectroscopy.

2.5. Cell Culture

4mL DMEM/F12+14%FBS was added into a 25 cm^2 cell culture bottle, and 1 mL KGN cells were added and cultured in a 37℃, 5% CO2 cell incubator. When the cells reached 80%-90% confluence, the old medium was discarded, the cells were washed with PBS twice, and 2 mL trypsin was added to the cells, and digested for 3 min. The cells were transferred to a 15 mL centrifuge tube and centrifuged at 1500 rpm/min for 3.5min. The supernatant was discarded, medium was added to resuspend the cells, and the cells were subcultured at a ratio of 1:3, two to three times a week.

2.6. Cell Viability Assay

CCK8 kit were used to detect cell viability and cell damage. KGN cells were seeded at 1×10^4 cells per well in 96-well plates and cultured overnight. KGN cells were exposed to PS-NPs (0, 50, 100, 200, 400 μg/mL), and the medium without cells was set as a blank control. After 24 h, 10 μL of CCK-8 solution was added to each well, incubated for 3 h, and the absorbance at 450 nm was measured using a multifunctional fluorescent microplate reader.

2.7. Cell Damage Assay

KGN cells (1×10^4 cells per well) were seeded in 96-well plates, cultured to confluence, and exposed to PS-NPs for 24 h. A high control well without PS-NPs, a low control well without PS-NPs, a high control blank without PS-nps, and a background blank well without PS-NPs were set up. After 24 h of MPs/NPs exposure, 10 μL Lysis Buffer was added to the high control well and incubated for 30 min. 100 μL Working Solution was added to each well and incubated at room temperature in the dark for 15 min. Finally, 50 μL terminating reaction solution was added and the absorbance at 490 nm was measured by microplate reader. Normalized LDH release values were obtained.

$$\text{Cell damage rate}(\%) = \frac{A-C}{B-C}\times 100\%$$

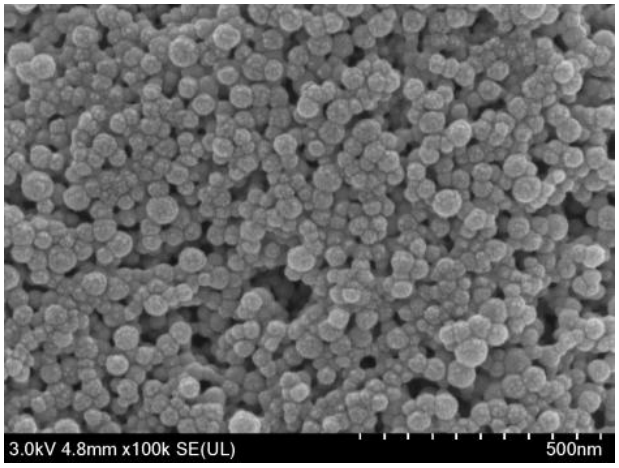
: A is the sample absorbance (sample hole minus sample blank hole); B is the absorbance of high control (high control well minus high control blank well); C is the absorbance of the low control (the background blank was subtracted from the low control well).

2.8. Intracellular ROS Levels

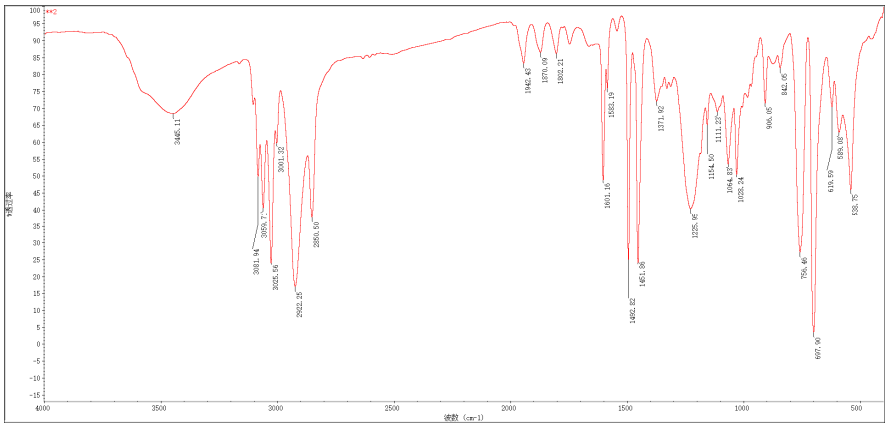
KGN cells (1×10^4 cells per well) were seeded in black 96-well plates. After 24h of exposure, 10 μmol/L DCFH-DA probe was added to the cells, and the cells were incubated at 37℃ in the dark for 20min. The cells were washed with serum-free cell medium 3 times, and observed under a fluorescence microscope and photographed. Fluorescence images were analyzed by imageJ for fluorescence intensity, and three fields were selected for each group.

3. Results

3.1. Characterization of Nanoplastics



(a)



(b)

Fig 1. Characterization of PS-NPs:(a)Morphological characterizations of PS-NPs. Scanning electron microscope (SEM) photomicrographs;(b) Fourier infrared spectra of PS-NPs

The 50 nm PS-NPs were stored at 4℃ in the dark. The surface morphology was observed by scanning electron

microscopy (SEM). Fig.1(a)shows the SEM results of 50nm PS-NPs. PS-NPs is spherical particles with relatively uniform particle size, and was well dispersed in water without obvious agglomeration. The surface functional groups/chemical bonds of the sample were determined by Fourier transform infrared spectroscopy (FTIR). The FTIR results are shown in Fig.1(b). The stretching vibration peak of PS-NPs group at the wavelength of 3025.56 cm^{-1}-3059.71 cm^{-1} is related to the C-H bond on the phenyl group. The characteristic absorption peaks of PS-NPs at 1601.16 cm^{-1}-1583.19 cm^{-1} and 1492.82 cm^{-1}-1451.86 cm^{-1} were related to benzene ring, The absorption peak of PS-NPs at 1028.24 cm^{-1} to 906.05 cm^{-1} was related to the C-H bond bending in the benzene ring, The characteristic absorption peak of PS-NPs at 756.45 cm^{-1}-697.90 cm^{-1} was related to the phenyl substitution, The absorption peaks detected at other positions were caused by the contraction of C-C/C-H bond or the bending vibration of $C-H_2$, and no other functional group absorption peaks were detected, indicating that the surface of PS-NPs in the experiment was not modified and the composition was polystyrene.

3.2. PS-NP Caused Morphological Changes in the Cells

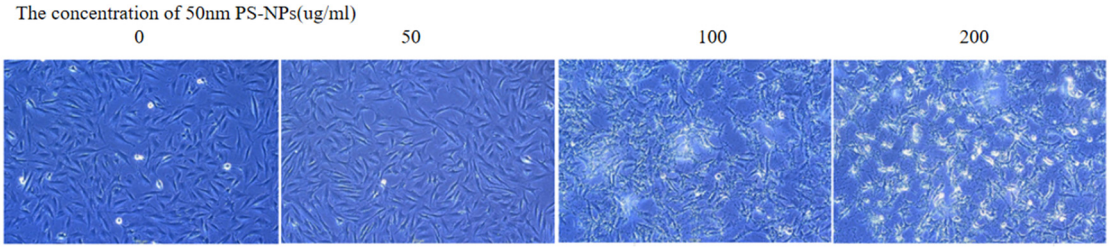
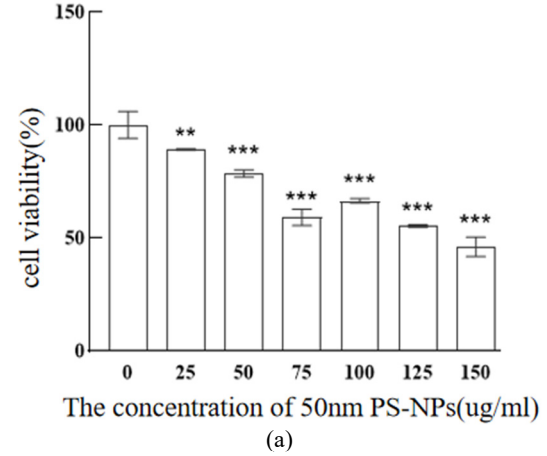


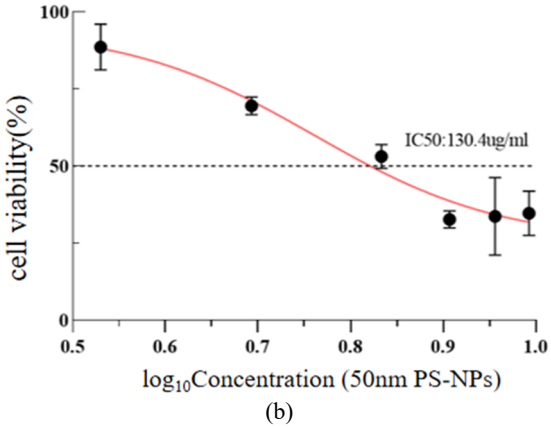
Fig 2. Morphology of KGN cells

The morphology of KGN cells exposed to 50 nm PS-NPs at concentrations of 0, 50, 100, 200 μg/mL for 24 hours was observed. The cells in the control group grew dense and fibrous, and the morphology was consistent. With the increase of concentration, the cell morphology gradually lost its fibrous shape, the intercellular space increased, and more floating cells appeared (Fig.2).

3.3. PS-NPs Inhibited Cell Viability



(a)



(b)

Fig 3. Effects of PS-NPs exposure on(a)Cell viability and(b)IC50

KGN cells were exposed to 50 nm PS-NPs at the concentrations of 0, 25, 50, 75, 100, 125, and 150 μg/mL, respectively. As shown in the Fig.3(a), The cell viability was significantly decreased at 50 μg/mL, and the later concentrations were significantly lower than that of the control group (**P < 0.01, ***P < 0.001).

As shown in Fig.3(b), the IC50 of KGN cells after exposure to 50nm PS-NPs, that is, the concentration of 50 nm PS-NPs that inhibited 50% of KGN cells was 130.4 μg/mL.

3.4. PS-NPs Caused Cell Damage

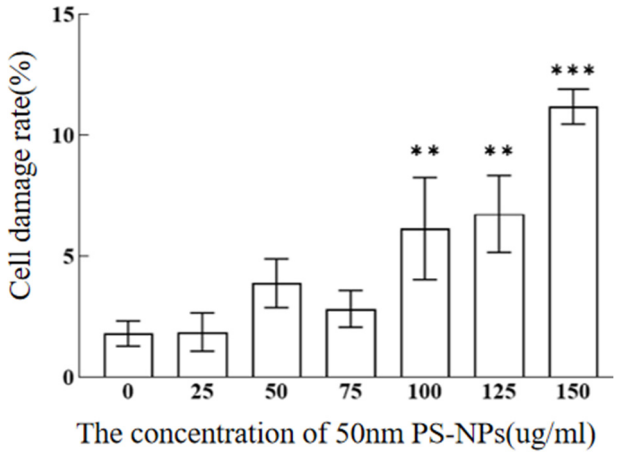


Fig 4. cell damage rate after PS-NPs exposure

When KGN cells were exposed to 50nmPS-NPs at concentrations of 0, 25, 50, 75, 100, 125 and 50 μg/mL, the percentage of cell damage at the concentration of 100 μg/mL was significantly higher than that of the control group, indicating significant cell damage (**P < 0.01, ***P < 0.001).

3.5. Reactive Oxygen Species Detection

To explore whether 50 nm PS-NPs caused oxidative stress in granulosa cells, ROS levels in KGN cells exposed to different concentrations of 50 nm PS-NPs were measured. As shown in Fig.5(a)The intensity of ROS fluorescence was significantly enhanced in KGN cells after PS-NPs exposure. In addition, after PS-NPs exposure. Fig.5(b) shows the fluorescence intensity of intracellular ROS in DCFH-DA dye-

bound KGN cells after PS-NPs exposure. When the concentration of PS-NPs reached 200μg/mL, the fluorescence intensity increased significantly. (***P < 0.001).

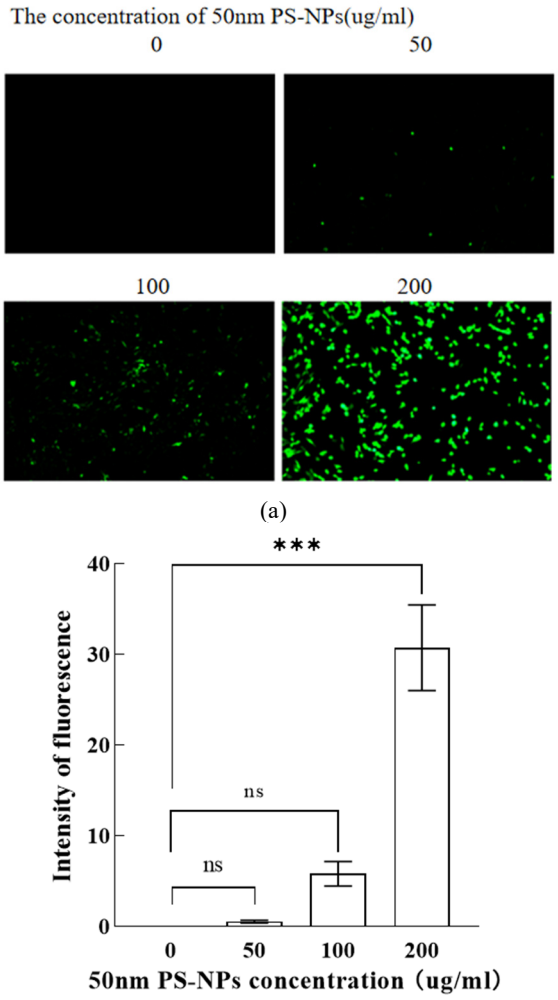


Fig 5. (a)Effects of PS-NPs exposure on reactive oxygen species (ROS)(b)Fluorescence intensities were randomly taken from three fields

4. Discussion

Due to the wide distribution and persistence of MPs in the environment, it is urgent to fully understand the potential hazards of MPs, including smaller NPs, to human health and their mechanisms [12]. Previous studies have shown that MPs/NPs are abundant in soil [13], fresh water, and ocean [14], and that MPs/NPs enter the human body through inhalation, ingestion, and skin contact [15]. Increasing evidence has shown that microplastics are ubiquitous in human tissues, including lung, blood, feces, kidney, liver and breast milk [16]. It has been reported that loss of intercellular contacts, increased cytoplasmic processes, including pseudopodia, cilia, microprocesses and focal adhesion, and significant differences in surface cell morphology were observed after exposure of human lung A549 cells to 1 μm and 10 μm PS-MPs. Cell proliferation was inhibited in the 1 μm and 10 μm groups [17].In addition, the situation of LDH release reflects the degree of cell damage, and our data showed that the degree of KGN cell damage gradually increased with the increasing concentration of 50 nm PS-NPs, which began to increase significantly at 100 μg/mL. The exposure of Balb/c male mice to 5-5.9 μm PS-MPs for 6 weeks resulted in a significant decrease in sperm count and motility, a significant increase in sperm malformation rate, a significant decrease in LDH level, and a significant increase in ROS and MDA levels, these results suggest that PS-MPs may cause male reproductive toxicity [18]. However, the toxic effects of NPs on the female reproductive system remain to be evaluated. In this study, we demonstrated that PS-NPs inhibited KGN cell proliferation. Specifically, KGN cell viability gradually decreased with increasing concentration of 50 nm PS-NPs, and half inhibition was reached at 130.4 μg/mL. These results provide new clues about the reproductive toxicity of PS-NPs in females and provide a reference for the toxicity of NPs on female reproduction

5. Summary

Nano-plastics are widely present in the environment and have great harm. When taken into the human body, they can cause human reproductive damage. In this study, KGN cells were exposed to 50 nm PS-NPs. The results showed that the cell viability gradually decreased with the increase of PS-NPs concentration, and the degree of cell damage gradually increased. The IC50 showed that 50 nm PS-NPs inhibited half of KGN cells at 130.4 μg/mL, and the ROS level of KGN cells was detected. These results indicated that with the increase of 50 nm PS-NPs concentration, the oxidative stress level of KGN cells increased. This suggests that 50nm PS-NPs can cause changes in cell morphology and affect cell proliferation, in addition to causing ROS accumulation in cells, which may provide a basis for PS-NPs to damage female reproduction.

Acknowledgments

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