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Cytotoxic and Apoptotic Effects of Polyethylene Nanoparticles on Normal Monkey Kidney Vero E6 Cell Line

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ABSTRACT

Background: Nanoplastics (NPs) become one of the most threatening substances for living organisms in the aquatic and terrestrial environments. Due to their small size and high surface area, few studies have revealed their toxic impact on mainly marine organisms. However, there is scarcity on NPs impact on mammalian cells.

Methods: We used 200-900 nm polyethylene nanoparticles (PE-NPs) to evaluate their cytotoxic and genotoxic effects on mammalian Vero E6 cell line using MTT and Comet assays.

Result: Characterization of the selected PE-NPs confirmed their nano spherical shape, size and their low-density type. Serial dilution of PE-NPs decreases cell viability with 50% inhibition concentration (IC_{50}) 91 µg/mL (at 24 h sampling time), 26 µg/mL (48 h) and 22 µg/mL (72h) and an average IC_{50}= 46 µg/mL. Treatment of Vero E6 cells with the average IC_{50} significantly affects the genomic integrity by decrease of % DNA in head, increase of % DNA in tail, tail length (µm) and tail moment, in a time-dependent manner. Therefore, PE-NPs of the low-density type, that is widely used in different products including coatings and films, have cytotoxic and genotoxic effect on mammalian cells. NPs deserves more attention and further studies are needed to focus on their molecular mechanisms *in vitro*.

Key words: Comet assay, Cytotoxicity, Genotoxicity, MTT, Nanoplastic, Polyethylene.

INTRODUCTION

Plastics represent the main litter found in marine and terrestrial environments worldwide. They accumulate in different environments due to their excessive use, especially in the pandemic COVID period and the improper waste management schemes (Gong and Xie, 2020), that could have impacts on human, animals (Rouabah *et al*., 2022) and plants. The main problem of plastics' accumulation is their fragmentation to small sized particles and fragments with higher surface area through physical, chemical, or biological routes (Singh and Sharma, 2008; Song *et al*., 2017; Syranidou *et al*., 2019). Small particles of plastic pollution, that are degraded from larger particles, termed microplastics (MPs, <5 mm) or nanoplastics (NPs, <1000 nm) according to their size. These small particles are especially concerning because of their potential to translocate in the bodies of organisms as well as their ability to act as vectors to other contaminants (Al Malki *et al*., 2021; Hussien *et al*., 2021).

Recently, it was documented the ecotoxicological effects of MPs and NPs on marine biota and plants (Yong *et al*., 2020). However, the nanoscale plastics might have more impacts on environmental fate, human health and biota than microplastics due to their transport properties, interactions with natural colloids and light, bioavailability and diffusion time (Gigault *et al*., 2021). MPs and NPs could accumulate in human body from drinking water (Koelmans *et al*., 2019), food sources (Smith *et al*., 2018) and by inhalation (Prata, 2018). They have been detected in human stool (Schwabl *et al*., 2019), blood (Leslie *et al*., 2022) and placenta (Ragusa *et al*., 2021), but their impact on human health remains unclear (Wright and Kelly, 2017). A recent report of World Health Organization's (WHO) related to "Microplastic in drinking water" indicates that there is not yet proof of harm, however it recommends for more research studies to be carried out on this issue (WHO, 2019).

Polyethylene (PE) is one of the most commonly microplastic present in the marine environment (Andrady, 2017) and has a stronger affinity than any others to adsorb persistent organic pollutants (Rochman *et al*., 2013). Due to its thermal stability, versatile nature and effectiveness, low density PE (LDPE) is used in a variety of applications. LDPEs are used in a wide range of products, including packaging, film, wire and cable insulation, coating and molding (Kyaw *et al*., 2012; Maraschin, 2001). Therefore, the present study aims to investigate the cytotoxic and

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apoptotic effects of polyethylene nanoparticles (PE-NPs) on mammalian cells represented by the normal monkey kidney Vero E6 cell line using MTT and Comet assays.

MATERIALS AND METHODS

The experiment was conducted at the High attitudes research centre and the central lab of Taif University, Taif, Saudi Arabia from the period January 2021 to March 2022.

Polyethylene nanoparticles

The polyethylene nanoparticles (PE-NPs) used in this study were >90% spherical particles that were purchased from Cospheric LLC (Santa Barbara, CA, USA) in the form of odorless and white powder in the nanoscale range from 200 -900 nm. They are hydrophobic nanoparticles with density: 0.95 g/cc. Polyethylene nanoparticles were characterized using X-ray diffraction (XRD), fluorescence microscopy, scanning electron microscopy (SEM) and FTIR to ensure their polymer type, shape and nano particle size.

Characterization of PE-NPs

Different techniques were done to characterize and ensure the polymer shape, structure and size of the present nano polymer. Scanning electron microscope (SEM, JEOL instrument) was used to determine the surface shape of PENPs. X-ray diffraction (XRD, at 30 kV, 100 mA) was used to determine the morphological properties of the polymer. The spectrum of XRD was recorded by CuKα radiation with a wavelength of 1.5406 Å in the 2θ (from range 20-80). Fourier Transform Infrared Spectroscopy (FTIR, Agilent technologies) was carried out at 450 to 4000 cm^{-1}. Powder of PENPs was loaded on glass slides and stained with a few drops of 1 mg/ml Nile red dye in acetone. Slides were kept in the dark box for 20 mins before fluorescence microscope examination (Carl Zeiss Microscopy GmbH, Jena, Germany) using epifluorescence mode (× 40 objective lens) with Alexa Fluor 660 filter for red fluorescence color (with excitation at bandpass (BP) 600±50 nm; emission BP 685±50 nm) (Sturm *et al*., 2021).

Toxicity assessment

Cell lines and culture conditions

Normal African green monkey kidney cell line, Vero E6, (ATCC ® CRL-1586™) was cultured into 75 cm^2 flasks in DMEM-high glucose media (Sigma-Aldrich, Taufkirchen, Germany) supplemented with 4500 mg/L glucose, 2mM L-glutamine, 1mM sodium pyruvate, 10% fetal bovine serum (FBS) and antibiotics (penicillin 100 IU/mL, streptomycin 100µg/mL). The cell culture was kept under standard culture conditions (37°C, 95% humidified air and 5% CO_2).

Cytotoxicity MTT assay and IC_{50} determination

Vero E6 cells were cultured in 96-well culture plates in DMEM-high glucose (Sigma) supplied with 10% FBS at a concentration of 1×10^5 cells/mL. After 24 h, the culture medium was discarded and then the cells were treated with serial concentrations of PE-NPs (40, 60, 80, 100, 120, 140, 160, 180, 200 µg/mL) in Dimethylsulfoxide (DMSO). After 24, 48 and 72 h of incubation, the culture media were discarded and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 50 µL of 0.5 mg/mL PBS) was added per each well. The cells were incubated for 4 h (37°C, 95% humidified air and 5% CO_2) then 50 µL of DMSO was added per each well. Afterwards, the plates were shaken for 10 min and absorption was measured using an ELISA microplate reader at wavelength 570 nm (Mosmann, 1983; Vajpeyee *et al*., 2022). DMSO was used as a negative control and each concentration was assayed in triplicate. The following equation was utilized to calculate cell viability (percentage):

$$\text{Cell viability (\%)} = \frac{\text{Mean OD of treated cells}}{\text{Mean OD of control (untreated cells)}} \times 100$$

Data were plotted by using the Microsoft Excel program. The 50% inhibition concentration (IC_{50}) for PE-NPs at different sampling times were determined using the IC50 Calculator of AAT Bioquest ® 2019. Average IC_{50}, of different sampling time, were used for cell line treatment for further evaluation.

DNA fragmentation (comet) assay

The alkaline Comet assay was carried to assess the effect of PE-NPs on the genomic DNA integrity in normal Vero E6 cells according to the Tice *et al*. (2000). A mixture of 10 µL cell suspension (untreated or PE-NPs treated, separately) and 75 µL of 0.5% low melting point agarose was spread on a half frosted slide pre-coated with normal melting agarose (1%). After gel solidification, the slides were kept in pre-chilled lysis buffer (2.5M NaCl, 100 mM EDTA, 10 mM Tris base, 20 mM NaOH, (PH=10.0) with freshly added 1% Triton X-100 and 10% DMSO) at 4°C for 2 weeks. Then slides were incubated in freshly prepared electrophoresis buffer (300mM NaOH and 1mM EDTA, pH 13) for 20 minute and then electrophoresed in the same buffer for 30 minute at 300mA and 25 V (0.90 V/cm). Later, the slides were kept in the neutralization Buffer (0.4 mM Tris base, PH=7.5) for 5 mins. Finally, slides were immersed in 100% cold ethanol for fixation, dried and stored in a dark box at room temperature until photographed. The cells were stained with ethidium bromide (20 µg/ml) for 20 min before examination and imaging using an epi-fluorescent microscope at (400 x). For each sampling time (24, 48 and 72 h) 150 randomly selected cells (50 cells on each of three replicate slides) were scored and analyzed using COMETSCORE software. To quantify the DNA damage, the percentage of DNA in comet tail and head and tail moment (TM) were evaluated.

Statistical analysis

One-way ANOVA was conducted to differentiate between different groups within the same parameter, followed by Tukey's multiple comparisons test using GraphPad software (GraphPad® 2017, San Diego, CA, USA). **** indicates P≤0.0001, *** indicates P≤0.001,** indicates P≤0.01, * indicates P≤0.05 and ns (non-significant) means P>0.05.

RESULTS AND DISCUSSION

Nanoplastics (NPs) characterization

Firstly, polyethylene nanoparticles (PE-NPs) were characterized by different techniques prior to their toxicity assessment. Fig 1(a) shows the fluorescence photo micrograph of PENPs stained with Nile Red dye in acetone as a solvent, it represents a rapid-screening approach to detect and quantify microplastics/nanoplastics from different samples (Maes *et al*., 2017). Fig 1(b) shows the X-ray diffraction (XRD) patterns of pure PENPs, in which two sharp peaks are seen at 2θ = 21.43° (110 reflection) and 23.82° (200 reflection), while there is a broad peak at 2θ = 14.6°. Those patterns referred to the triclinic unit cell of the low-density polyethylene (LDPE) (Boz and Wagener, 2006; Esmaeili *et al*., 2013). In addition, FTIR was used to identify the type of PENPs because it represents a fingerprinting technique extensively used to identify particles by their unique spectra (Löder *et al*., 2015). FTIR spectra of pure PE-NPs show two split peaks at 2916 and 2849 cm^{-1} that are assigned to the asymmetric and symmetric stretching vibration bands of -CH_2- of LDPE (Fig 1c). While the deformation vibration and in-plane rocking vibration bands of -CH_2- were shown at 1465 and 721 cm^{-1}, respectively (Dogan *et al*., 2018). Therefore, XRD and FTIR confirm the LDPE type of the present used nanoplastic. SEM images show varied sizes of spherical shape PENPs, but at high magnification (× 3000) nanoscale particles are found as shown in Fig 2.

Cytotoxicity of nanoplastics (NPs) in the vero E6 cell line

MTT assay was done to evaluate the cytotoxic effects of the PE-NPs on the Vero E6 cell line. According to the results in Fig 3 (a-d), nanoparticles of PENPs exhibited cytotoxic effects against Vero cells in a time and dose-dependent manner. Cell viability decreased significantly at the concentrations of 40, 60, 80, 100, 120, 140, 160, 180, 200 µg/mL when treated with nanoplastics (PE-NPs) at different sampling times but cells' damage was highly recorded after 72 h. The 50% inhibition concentration (IC_{50}) for PE-NPs at different sampling times were determined to verify their cytotoxicity in the Vero E6 cell line. After 24, 48 and 72 h exposure, the IC_{50} value of PE-NPs on Vero cells occurs at 91, 26 and 22 µg/mL, respectively, with an average IC_{50}= 46 µg/mL. These findings are consistent with an earlier study by González-Fernández *et al*. (2021), who recorded high cytotoxic effect of nanoplastics (polystyrene (PS) type) on a brain-derived cell line (SaB-1) from gilthead seabream (*Sparus aurata*) using MTT assay. However, they have determined LC_{50} dosage (12 µg/mL) lower than the present study. In addition, Guimarães *et al*. (2021) have reported nanoplastics (PS type) cytotoxicity in *Ctenopharyngodon Idella* juveniles using erythrocyte morphometry assay. They have recorded that erythrocyte treated with PS nanoplastics have higher micronuclei frequency and nuclear abnormalities such as binucleated cells, nuclear constriction (symmetrical and asymmetric), blebbed, notched, kidney-shaped and nuclear vacuole in comparison to untreated

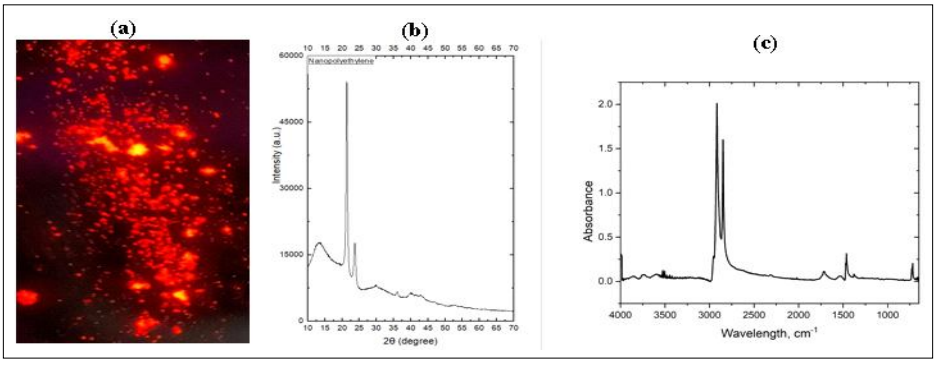

Fig 1: Fluorescence photomicrograph of polyethylene nanoparticles (PE-NPs) stained with Nile Red in acetone (× 400 magnification) (a), XRD patterns (b) and FTIR spectra of PE-NPs (c).

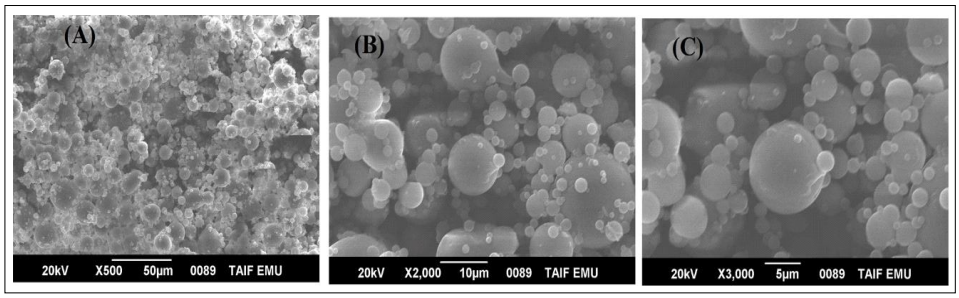

Fig 2: Scanning electron microscope photos showing the spherical shape of PE-NPs at different magnifications × 500 (A), × 2000 (B) and × 3000 (C).

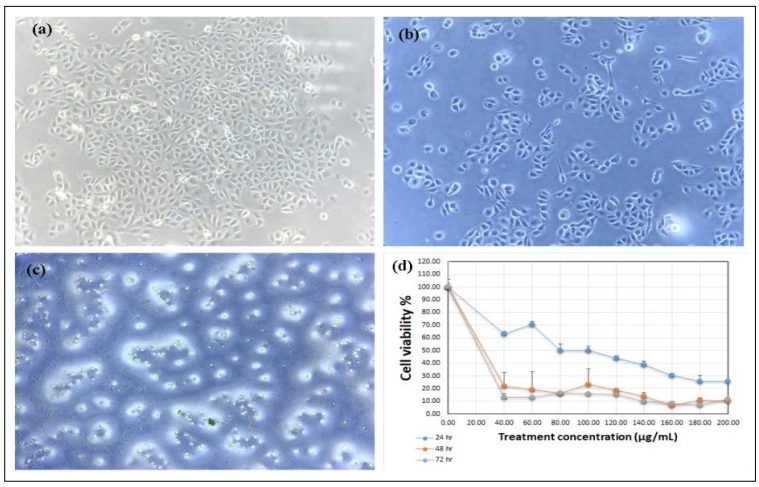

Fig 3: Photomicrograph of untreated Vero E6 cells (a), treated with PE-NPs at 24 h (b) and 72 h sampling times (c) that show high cell damage. Percentage of cell viability of Vero E6 cells according to serial dilutions treatment of PE-NPs (d) at different sampling time 24 h, 48 h and 72 h.

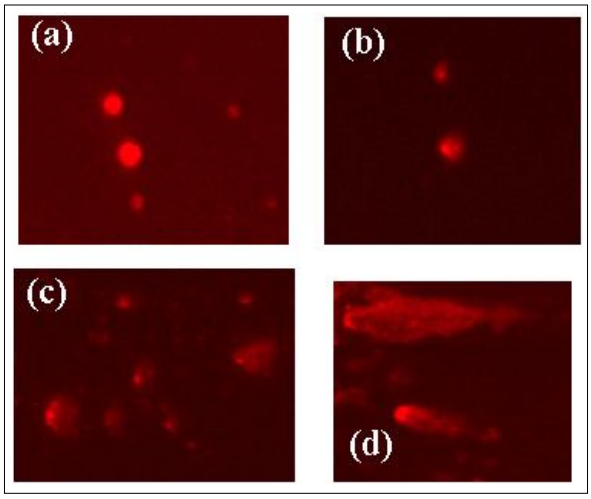

Fig 4: Examples for the observed comet nuclei in the Vero E6 cells showing intact undamaged DNA (a) and damaged DNA with various degrees at 24 h (b), 48 h (c) and 72 h (d) sampling times.

cells. Recently, it was known that NPs are more toxic than MPS on cells and that was attributed to the difference in their size, because MPs are not able to cross cellular membranes as NPs (Banerjee and Shelver, 2021). Once NPs penetrate the cellular lipid membrane, they might change membrane structure by softening membrane and reduce its molecular diffusion that can severely affect cellular functions and lead to cytotoxicity (Bochicchio *et al*., 2017). It was suggested that NPs cell internalization was done through clathrin-mediated endocytosis, phagocytosis or macropinocytosis, depending on the plastic particle size, cell type and surface functionalization (Teleanu *et al*., 2019).

Genotoxicity of polyethylene nanoparticles (PE-NPs)

Initially, data have shown that Vero E6 cells treated with an average IC_{50}= 46 µg/mL of PE-NPs induced DNA damage, as observed in the comet assay (single cell gel electrophoresis assay). Fig 4 shows examples for the DNA damage in the Vero E6 cells treated with PE-NPs that is increased in time-dependent manner. It was clear that PE-NPs treatment decreases % head DNA, increases % tail DNA, tail length and tail moment as shown in Fig 5 (a-d). It was recorded that increased concentration (Guimarães *et al*., 2021) and sampling time (Choi *et al*., 2019) of nanoplastics exposure were the major factors to the extension of DNA damages. These results are in compliance with recent data that have been associated with increase in the DNA damage by time (Choi *et al*., 2019). They have reported that PS (MPs/NPs) toxicity on marine copepod *Tigriopus japonicus* increases according to smaller PS size (50 nm vs. 10 µm) and by time (24 vs. 48 h). They thought that DNA damage by PS could be related to the oxidative stress based on high nitric oxide, lipid peroxidation and hydrogen peroxide levels. In addition, Guimarães *et al*. (2021) have determined the genotoxic and mutagenic effect of PS, even at low concentration (0.04 ng/L), on *Ctenopharyngodon idella* juveniles by using comet assay. They have reported an increase in % tail DNA, tail length and olive tail moment due to PS treatments characterizing a concentration-dependent (0.04 ng/L, 34 ng/L and 34 µg/L) effect.

Based on the present outcomes, the observed time-response effect of PE-NPs causes cell and DNA damage. In our peer review, it is the first time to report the effect of the widely used LDPE nanoparticles on mammalian cells. Especially, the present nanoplastic type was incorporated in various products that can be easily transferred to human tissues as others. The main problem is the accumulation of genetic material damage and/or non-sufficient DNA repair due to long-term exposure of nanoplastics. Guimarães *et al*. (2021) have reported different nuclear abnormalities due to nanoplastic (PS) treatment to cells and they have suggested that these abnormalities could be biomarkers to chromosomal instability, gene amplification and cytokinesis

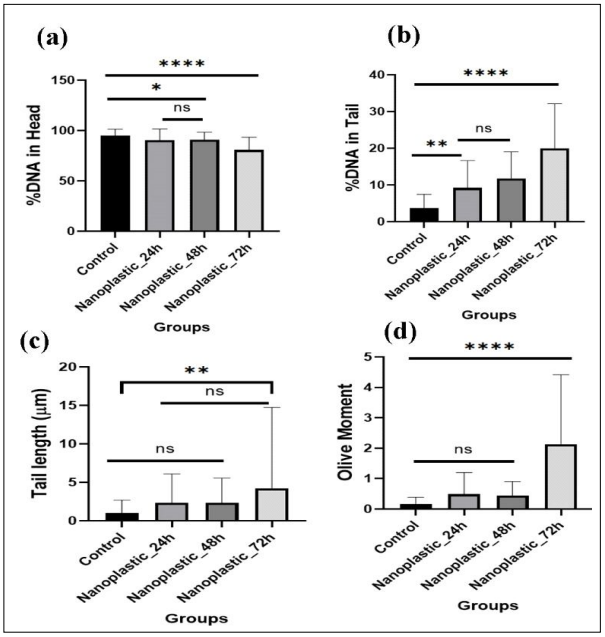

Fig 5: DNA damage represented as percentage of DNA in head (**a**), percentage of DNA in tail (**b**), tail length (im) (c) and tail moment (**d**) in different groups. Results are expressed as mean ± SE and different groups were compared using one-way ANOVA followed by Tukey's test. **** indicates P≤0.0001, ***, indicates P≤0.001, ** indicates P≤0.01, * indicates P≤0.1 and (ns) refers to non-significant differences between groups at P≤0.05.

arrest due to aneuploidy. These changes could lead to abnormal cellular responses, harmful events, incapability to block abnormal cell cycles or apoptosis of altered cells that could end to carcinogenesis (Araújo *et al*., 2019; Singh *et al*., 2009). However, it is still not clear the mechanisms underlying the genotoxic effects of MPs/NPs. MPs/NPs are chemically inert substances that have an ability to induce reactive oxygen species (ROS) production, causing oxidative stress (Cole and Galloway, 2015; Imhof *et al*., 2017; Jeong *et al*., 2016) that ends with chain breaks of the DNA molecule (Avio *et al*., 2015). In addition to their toxicity, NPs could act as vectors of other toxic compounds, such as synthetic stabilisers, phthalates, bisphenol A, polychlorinated biphenyls, flame retardants and pigments, due to their hydrophobic nature (Hamlin *et al*., 2015; Lithner *et al*., 2011).

CONCLUSION

There is a rapid increase in the total amount of MPs/NPs particles everywhere in our environment due to the continuous consumption of plastic products. Those particles found their way to our body, but we don't know much about their impact. Therefore, in the present work, we studied the cytotoxic and genotoxic effect of polyethylene nanoparticles at different sampling times on mammalian Vero E6 cells. PE-NPs are highly toxic to inhibit cell growth at low concentration especially after 72 h from treatment. In addition, there is a statistically significant increase in DNA damage that is represented by comet assay compared to untreated cells. More in-depth studies are needed to determine NPs molecular pathway in damaging DNA.

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Author contributions

All the authors participated in the study's design, practical work, writing manuscript and approved the final manuscript.

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Data availability statement

All data generated or analyzed during this study are included in this published article.

Conflicts of interest

The authors declare no conflict of interest.

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