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Low doses of nanodiamonds and silica nanoparticles have beneficial hormetic effects in normal human skin fibroblasts in culture

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HIGHLIGHTS

- ND and SiO_2-NP can induce biphasic dose response in human facial skin fibroblasts (FSF1) in culture.
- ND and SiO_2-NP enhance wound healing ability *in vitro* and slow down aging.
- ND and SiO_2-NP activate Nrf2- and FOXO3A-mediated cellular stress responses.
- ND and SiO_2-NP at low doses are potential hormetins.

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ABSTRACT

Nanodiamonds (ND) and silica nanoparticles (SiO_2-NP) have been much investigated for their toxicity at high doses, little is known about their biological activity at low concentrations. Here we report the biphasic dose response of ND and SiO_2-NP in modulating normal human facial skin fibroblasts (FSF1) in culture. ND and SiO_2-NP at low concentration (up to 0.5 μg/ml) had beneficial effects on FSF1 in terms of increasing their proliferation and metabolic activity. Exposure of FSF1 cells to low levels of NP enhanced their wound healing ability *in vitro* and slowed down aging during serial passaging as measured by maintenance of youthful morphology, reduction in the rate of loss of telomeres, and the over all proliferative characteristics. Furthermore, NP treatment induced the activation of Nrf2- and FOXO3A-mediated cellular stress responses, including an increased expression of heme oxygenease (HO-1), sirtuin (SIRT1), and DNA methyltransferase II (DNMT2). These results imply that ND and SiO_2-NP at low doses are potential hormetins, which exert mild stress-induced beneficial hormetic effects through improved survival, longevity, maintenance, repair and function of human cells.

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1. Introduction

Nanoparticles (NP), owing to their unique physico-chemical properties, have gained tremendous attention for their numerous biological and biomedical applications (Hoet et al., 2004). The attention is mainly focused on nanodiamonds (ND) and silica nanoparticles (SiO_2-NP), which are generally considered as biocompatible, and have been employed as drug delivery systems (Chen et al., 2013b), as MRI contrast enhacers (Manus et al., 2010; Moraru et al., 2014) and as biosensors for intracellular pathogens (Armstead and Li, 2011; Clemens et al., 2012). However, their widespread use and increased human exposure has raised concerns about their potentially toxic effects at relatively high doses. For example, SiO_2-NP were found to cause cardiovascular system impairment (Du et al., 2013), liver fibrosis (van der Zande et al., 2014), and granuloma formation in the liver and spleen of rats (Ivanov et al., 2012). In *in vitro* system, using HUVEC cell line model, NP were found to decrease cell viability and to induce the generation of reactive oxygen species (Duan et al., 2013). SiO_2-NP-mediated NF-κB activation, upregulation of inflammatory factors in human endothelial cells (Corbalan et al., 2011) and micronulei formation in mouse fibroblasts was also shown (Park et al., 2011). The toxicity of ND was also demonstrated in HeLa cells in terms of an increase in oxidative stress, induction of DNA fragmentation, as well as pro-senescent and hypermethylating effects (Mytych et al.,

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2014). In human peripheral lymphocytes, ND-mediated reduction in chromatin stability, increased DNA single strand breaks and oxidative damage were observed (Dworak et al., 2014). Recently, Karpukhin et al. have reported toxic effects exerted by ND at high concentrations, and protective response to bacterial agents and anti-inflammatory effects at low concentrations in mouse neutrophils (Karpukhin et al., 2011). Similar biphasic effects were observed for silver NP on HepG2 cells, with cytotoxic effects at high concentrations (de Lima et al., 2012), but accelerated cell proliferation through p38 MAPK activation at low doses (Jiao et al., 2014).

Such a biphasic dose–response to an agent, characterized by a low dose stimulation or beneficial effect and a high dose inhibitory or toxic effect is termed as hormesis (Calabrese et al., 2007; Mattson, 2009). The hormetic effects are due to the defensive and adaptive responses of cells to stress, typically involving several kinases, deacetylases and transcription factors, such as HSF, Nrf2 and NF-κB, followed by synthesis of cytoprotective and restorative proteins (Mattson, 2009). Some of the main hormetic agents, termed hormetins, studied in relation to their beneficial effects include heat, ethanol, food restriction, exercise, irradiation, heavy metals, hypergravity, antibiotics, pro-oxidants and dietary components such as polyphenols and flavonoids (Rattan, 2012; Rattan and Le Bourg, 2014). Some well documented effects of hormetins in human cells in culture are slowing down aging, extension of replicative lifespan, faster wound healing, increased differentiation, increased angiogenesis, and improved tolerance to other stresses (Rattan et al., 2009). There are some reports on the hormetic effects of silver NP at non-cytotoxic doses on human hepatoma cells (Jiao et al., 2014). However, there are no published reports so far about the effects of low doses of ND and SiO_2-NP on normal healthy human cells. In this study, we evaluate the potential hormetic effects of low concentrations of ND and SiO_2-NP, using normal human facial skin fibroblasts undergoing serial passaging in culture.

2. Materials and methods

2.1. Nanoparticles

Nanodiamonds (ND) (<10 nm particle size) and silica nanoparticles (SiO_2-NP) (12 nm primary particle size) were purchased from Sigma (Poland) (#636428, CAS Number 7782-40-3 and #718483, CAS Number 112945-52-5, respectively). ND were previously characterized by atomic force microscopy (AFM), Fourier transform infrared spectroscopy (FTIR) and Zetasizer Nano ZS (Dworak et al., 2014; Mytych et al., 2014; Mytych et al., 2015a). SiO_2-NP were also characterized by AFM, as described earlier (Mytych et al., 2015b).

2.2. Cell culture

Normal diploid human facial skin fibroblast cell strain, designated FSF1 (kindly provided by LVMH Research, St. Jean de Braye, France), was established from a skin biopsy of an eyelid reduction of a healthy 40-year-old woman, as described previously (Demirovic et al., 2014; Jorgensen et al., 2014; Demirovic et al., 2015). Cells were cultured in an incubator at 37 °C in a humidified atmosphere in the presence of 5% CO_2 using Dulbecco's Modified Eagle's Medium (DMEM; Lonza, USA) supplemented with 10% fetal bovine serum (Lonza, USA), and antibiotic and antimycotic mix solution (100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B; Lonza, USA). For sub-culturing and serial passaging, cells were trypsinized and divided into new culture flasks at 1:2 or 1:4 ratio. Accordingly, 1 or 2 passages (P) were added to the age of the cultures, respectively. FSF1 cells have been previously used extensively for studies on cellular aging, senescence, stress responses and replicative lifespan *in vitro* (Demirovic et al., 2014; Jorgensen et al., 2014; Demirovic et al., 2015).

2.3. Nanoparticle dose selection – MTT assay

Effects of ND and SiO_2-NP on the survival of FSF1 were determined by using the so-called MTT assay, measuring the mitochondrial activity, as described earlier (Mytych et al., 2014). Briefly, P14 cells were seeded into 96-well plates at a density of 3×10^3 cells/well and allowed to settle for 24 h before the medium was replaced with fresh medium containing a wide range of concentrations of NP (from 0.1 to 100 μg/ml), followed by further incubation for 48 h. Afterwards, MTT assay was performed and absorbance was read at 595 nm and at 655 nm (measurment and reference wavelength, respectively), using Biorad 550 microplate reader. The results were presented as percent MTT activity where the readings for the untreated control cells were considered as 100%. Based on the results from MTT assays, we chose 0.5 μg/ml concentration for both types of nanoparticles for further experiments.

FSF1 were cultured without (control conditions) or with (treated conditions) continuous presence of either 0.5 μg/ml ND or 0.5 μg/ml SiO_2-NP, for 20 passages (from P15 to P34). Experiments were performed on younger (P19/20) and older (P32/34) cells.

2.4. Cell viability, morphology and lysosomal staining

After trypsynization, cells were mixed with one volume of 0.4% trypan blue (Life Technologies, USA) and the percentage of live cells was estimated using Countess Automated Cell Counter (Life Technologies, USA). Cellular morphology was monitored with a Zeiss Axiovert 25 inverted microscope and a computer image analysis system Zeiss AxioVision v. 4.6.3.0. For intracellular lysosomal detection, cells were seeded into 6-well plates at a density of 1×10^4 cells/well (for micrographs) or into 96-well plates at a density of 3×10^3 cells/well (spectroscopy analysis). After 24 h, the medium was replaced by fresh medium containing 0.001% Neutral Red and incubated for another 3 h in 37 °C. The cells were fixed in 0.5% formaldehyde in 1% $CaCl_2$ solution for 20 min at RT and washed extensively with water to remove the residual dye. Micrographs were taken in the same way as for morphology or the dye was extracted from the cells with 1% acetic acid in 50% ethanol solution and the absorbance was read at 540 nm, using Biorad 550 microplate reader. The results were presented as percent Neutral Red Uptake where the readings for the untreated control cells were considered as 100%. Mean Autophagic Arbitary Units were calculated according to the formula created by Martins et al. (Martins et al., 2013).

2.5. One-step growth curve

Short-term growth experiments were performed as described earlier (Demirovic et al., 2014; Jorgensen et al., 2014; Demirovic et al., 2015). Briefly, P19 cells were seeded onto 24-well plates at a density of 1×10^4 cells/well. Cell numbers were counted on days 1, 3, 6 and 8, and at the end of the experiment the cells were stained with Giemsa for permanent record and microphotography.

2.6. Wound healing assay

The extent of cell migration was determined by employing a so-called wound healing assay as described earlier (Demirovic and Rattan, 2011). Briefly, 2×10^5 P20 cells were seeded in each well of a 6-well plate and after 24 h the scratch was done. The medium

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