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Magnetic ferroferric oxide nanoparticles induce vascular endothelial cell dysfunction and inflammation by disturbing autophagy

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HIGHLIGHTS

- B-Fe_3O_4NPs did not induce cell apoptosis or necrosis in HUVECs within 24 h.
- B-Fe_3O_4NPs induced HUVEC dysfunction and inflammation.
- B-Fe_3O_4NPs induced enhanced autophagic activity and blockade of autophagy flux.
- Suppression of autophagy dysfunction attenuated B-Fe_3O_4NP-induced HUVEC dysfunction.

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ABSTRACT

Despite the considerable use of magnetic ferroferric oxide nanoparticles (Fe_3O_4NPs) worldwide, their safety is still an important topic of debate. In the present study, we detected the toxicity and biological behavior of bare-Fe_3O_4NPs (B-Fe_3O_4NPs) on human umbilical vascular endothelial cells (HUVECs). Our results showed that B-Fe_3O_4NPs did not induce cell death within 24 h even at concentrations up to 400 μg/ml. The level of nitric oxide (NO) and the activity of endothelial NO synthase (eNOS) were decreased after exposure to B-Fe_3O_4NPs, whereas the levels of proinflammatory cytokines were elevated. Importantly, B-Fe_3O_4NPs increased the accumulation of autophagosomes and LC3-II in HUVECs through both autophagy induction and the blockade of autophagy flux. The levels of Beclin 1 and VPS34, but not phosphorylated mTOR, were increased in the B-Fe_3O_4NP-treated HUVECs. Suppression of autophagy induction or stimulation of autophagy flux, at least partially, attenuated the B-Fe_3O_4NP-induced HUVEC dysfunction. Additionally, enhanced autophagic activity might be linked to the B-Fe_3O_4NP-induced production of proinflammatory cytokines. Taken together, these results demonstrated that B-Fe_3O_4NPs disturb the process of autophagy in HUVECs, and eventually lead to endothelial dysfunction and inflammation.

1. Introduction

Today, engineered nanoparticles (NPs) and nanotechnology are widely used in the field of healthcare and life sciences, with numerous medical and biotechnological applications [1,2]. NPs created in the diameter range between 1 and 100 nm, display unique physicochemical characteristics due in part to their smaller size, large surface-to-volume ratio, and increased reactivity [3]. Because of the diverse potential of NPs, it is estimated that the occupational and public exposure to NPs will dramatically increase in the future. Understanding the potential adverse effects of NPs has become a priority both for health protection and for regulating the safe development of nanotechnologies [4].

A growing number of epidemiological studies clearly demonstrate that exposure to NPs is linked to multiple adverse cardiovascular effects in both healthy individuals and in individuals with preexisting cardiovascular disease [5]. It is well accepted that NPs can enter the vascular system intentionally by injection in the form of nanodiagnostics or nanomedicines [5]. In addition,

Abbreviations: AO, acridine orange; Alexa 488-Ac-LDL, Alexa Fluor 488-acetylated low-density lipoprotein; Baf, bafilomycin A1; B-Fe_3O_4NPs, bare ferroferric oxide nanoparticles; CRP, C-reactive protein; eNOS, endothelial NO synthase; FBS, fetal bovine serum; FGF-2, fibroblast growth factor 2; HUVECs, human umbilical VECs; HAVECs, human aortic VECs; IL, interleukin; LDH, lactate dehydrogenase; 3-MA, 3-methyladenine; NPs, nanoparticles; NO, nitric oxide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick endlabeling; TEM, transmission electron microscopy; TNF, tumor necrosis factor; VECs, vascular endothelial cells.

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NPs rapidly translocate to the systemic circulation and reach different organs even after inhalation [6,7]. Vascular endothelial cells (VECs), which form the inner cellular lining of the entire cardiovascular system, have direct contact with these NPs, making NP-endothelial interactions potentially pathogenically relevant [8]. Therefore, research on the adverse effects of NPs on VECs is urgently needed.

Magnetic NPs are among novel materials that have received extensive attention due to their high biocompatibility and biodegradability [9,10]. Ferroferric oxide (Fe_3O_4)-containing NPs, which represent one example of this type of material, have been developed in recent years for use as contrasting agents in magnetic resonance imaging (MRI), carriers in drug delivery systems and heating elements for hyperthermia [11–13]. It is necessary to further identify any potential toxicity associated with the use of Fe_3O_4-containing NPs. Currently, studying the biological behavior of Fe_3O_4-containing NPs in VECs is important for evaluating the safety of these NPs. It has been demonstrated that at a concentration of 100 μg/ml, bare Fe_3O_4NPs (B-Fe_3O_4NPs) with a primary particle diameter of 43 nm induce apoptosis in ECV304 cells (a human umbilical VEC (HUVEC) line) after treatment for 24 h [14]. Su et al. reported that Fe_3O_4@APS@AA-co-CA, a type of modified Fe_3O_4NP, has no measurable effects on the viability of cultured HUVECs at low concentrations (1–200 μg/ml). However, at a concentration of 400 μg/ml, these Fe_3O_4NPs decrease cell viability by inhibiting cell proliferation [15]. Studies on the toxic effects of Fe_3O_4NPs have yielded disparate results, depending on the NP size, surface modification, cell type, cell medium composition, protein-NP interaction, etc. In addition, the biological behavior and the precise mechanisms of the toxicology of Fe_3O_4NPs on VECs are not well understood. In the present study, we investigated the direct effects of B-Fe_3O_4NPs with a diameter of 10–15 nm on HUVECs, and focused particularly on the role of autophagy in the VEC dysfunction caused by B-Fe_3O_4NPs.

2. Materials and methods

2.1. Cell culture

HUVECs were obtained in our laboratory as previously described [16]. Cells were cultured on gelatin-coated plastic dishes in MCDB131 medium (Sigma–Aldrich Co., St Louis, MO, USA) supplemented with 20% fetal bovine serum (FBS, HyClone Laboratory, Logan, UT) and 70 ng/ml fibroblast growth factor 2 (FGF-2, GIBCO, Carlsbad, CA, USA) in a humidified incubator at 37 °C with 5% CO_2; they were not used for the experiments beyond passage 8. HUVECs used in our experiments were obtained from the umbilical cords of 3 healthy donors. Because the umbilical cord is medical waste, it is not restricted by ethical considerations and laws.

2.2. Exposure to B-Fe_3O_4NPs

The size distribution, morphology, purity, stability and crystal structure of B-Fe_3O_4NPs were characterized in our previous study [17]. B-Fe_3O_4NPs were sterilized at 121 °C for 30 min and were then suspended in MCDB131 plus 20% FBS. Stock solutions were sonicated for 5 min before they were diluted with complete MCDB131 culture medium to various concentrations (1, 10, 100, 200 and 400 μg/ml).

2.3. Measurement of cell density

After treatment, the HUVECs in each group were resuspended in MCDB131 culture medium following digestion. A drop of the cell suspension from each sample was placed on a hemacytometer (QiuJing, Shanghai, China) and the number of cells was counted under a phase-contrast microscope (Nikon, Japan). The cell density (cells/ml) = average number of cells/mm^2 area × 10^4/ml.

2.4. Cell viability analysis

Cell viability was measured with the WST-8 assay using the cell counting Kit-8 (Sigma–Aldrich Co., St. Louis, MO, USA) according to the manufacturer's protocols. Briefly, HUVECs were seeded in 96-well plates (Corning Costar, The Netherlands) and incubated overnight to allow for cell attachment. After the cells were exposed to various concentrations of B-Fe_3O_4NPs for 20 h, 10 μl of WST-8 solution was added to each well, and the plates were incubated for 4 h. After exposure, the medium was collected and the aggregated B-Fe_3O_4NPs were removed by centrifugation at 1500 rpm for 10 min. Then, the supernatants were transferred into a new 96-well plate and the absorbance was measured at 450 nm using an Epoch™ spectrophotometer (BioTek Instruments, Inc., Vermont, USA). Cell viability (%) was expressed as = (optical density (OD) of control or B-Fe_3O_4NP treated groups/OD of the control group) × 100%. The viability of the control group was considered to be 100%. To investigate whether B-Fe_3O_4NPs affect the WST-8 assay, an interference control (400 μg/ml B-Fe_3O_4NPs without cells) was performed.

2.5. Lactate dehydrogenase (LDH) assay

Cell necrosis was detected using an LDH kit (Nanjing Jiancheng Co., Nanjing, People's Republic of China) according to the manufacturer's protocol. Briefly, after treatment with various concentrations of B-Fe_3O_4NPs for 24 h, cell culture medium was collected, and the aggregated B-Fe_3O_4NPs were removed by centrifugation at 1500 rpm for 10 min. Then, the supernatants were transferred into a new 96-well plate, and LDH reaction solution was added to each well. After further incubation for 30 min, absorbance was measured at 340 nm using an Epoch™ spectrophotometer. LDH activity was expressed relative to the basal LDH release in untreated control cells. To investigate whether B-Fe_3O_4NPs affect the LDH assay, an interference control (400 μg/ml B-Fe_3O_4NPs without cells) was also performed.

2.6. Analysis of apoptotic cells

Apoptotic cells were assessed using terminal deoxynucleotidyl transferase-mediated dUTP nick endlabeling (TUNEL, Promega, Madison, WI, USA) according to the manufacturer's protocol. Cells were observed under a fluorescence microscope (Leica, Wetzlar, Hessen, Germany) fitted with a filter cube I3 (Bandpass filter 450–490 and Longpass filter 515). The apoptosis rate was quantified based on the TUNEL-positive rate.

2.7. Alexa Fluor 488-acetylated low-density lipoprotein (Alexa 488-Ac-LDL) uptake assay

VECs are functionally defined by their capacity to take up Ac-LDL from the plasma [18]. To detect whether B-Fe_3O_4NPs induced VEC dysfunction, the cells were exposed to various concentrations of B-Fe_3O_4NPs for 24 h; then 10 μg/ml Alexa 488-Ac-LDL (Invitrogen, Carlsbad, CA, USA) was added and the plate was incubated for an additional 4 h. The media was removed and cells were washed twice with basal MCDB131 medium. Cells were observed under a fluorescence microscope (Leica, Wetzlar, Hessen, Germany) fitted with a filter cube I3 (Bandpass filter 450–490 and Longpass filter 515).