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Original Research Article

Comparative study of the effects of different magnetic nanomaterials on vascular endothelial cells

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ABSTRACT: Objective To study the effects of three kinds of magnetic nanomaterials on reactive oxygen species, cell junction of vascular endothelial cells in vitro and their relationship. **Methods** Primary human umbilical vein endothelial cells (HUVECs) were randomly divided into control and magnetic nanoparticles exposed groups. Dynamic light scattering (DLS) was used to characterize the particle size and charge of nanomaterials. CCK 8 method was used to measure cell viability. Flow cytometry was used to observe the level of ROS in cells by DCFH-DA fluorescent probe labeling. Using iron staining and transmission electron microscopy (TEM) to observe the uptake uptake of magnetic nanomaterials by endothelial cells. The cells were fluorescently labeled with VE-cadherin antibody, and the cell junctions were observed under a laser confocal microscope. The expression of VE cadherin was analyzed by western blot. **Results** Magnetic nanomaterials could increase ROS in endothelial cells, decrease VE-cadherin levels and the gap bebetween cells increased. When treated with the antioxidant N-acetylcysteine. the level of reactive oxygen decreased, while the cell junction tends to be intact. The effects of magnetic materials on the activity of endothelial cells, ROS level, VE-cadherin expression were different due to components, surface modification, and size. **Conclusions** Different magnetic nanoparticles have different effects on the ROS and cell junction of endothelial cells; the exposure dose used in experiments can affect the integrity of endothelial cell junction.

KEYWORDS: Magnetic nanomaterial; Endothelial cells; Reactive oxygen species; Cell junction

0 Introduction

Magnetic nanoparticles are being increasingly used in biomedical research, and the development of magnetic resonance imaging contrast agents, cell tracing techniques, tumor magnetothermal treatment methods, targeted drug delivery systems, and biomolecule or cell capture and separation techniques based on magnetic nanoparticles have shown significant promise for applications[1–3], which to a large extent will allow magnetic nanoparticles to enter the circulating blood and come into contact with the vascular

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endothelium. The vascular endothelium is composed of tightly connected vascular endothelial cells. Endothelial cells are involved in the immune response of the body and have functions in anticoagulation, regulation of extracellular matrix components and regulation of blood flow, and these regulatory roles are inextricably linked to the integrity of vascular endothelial junctions[4]. Adhesion junctions between endothelial cells are mediated by vascular endothelial calmodulin (VE-cadherin), which plays a role in connecting adjacent cells and determining intracellular signaling such as cell location, proliferation and apoptosis, and is one of the key factors affecting endothelial cell integrity. If the cell adhesion junctions are reduced, the gap between adjacent endothelial cells will increase, leading to vascular endothelial leakage, causing increased vascular permeability and subsequently the corresponding pathological changes[5–6]. A number of studies have shown that nanoparticles are readily taken up by a variety of tumor cells and affect intracellular reactive oxygen species (ROS) levels[7–9], but whether nanoparticles are taken up by vascular endothelial cells and affect intercellular connections needs to be systematically investigated. In this paper, we focus on the effects of three different magnetic nanoparticles on intracellular ROS and intercellular junctions in vascular endothelial cells, and explore and establish the correlation between them, with the aim of providing basic research data for the safe in vivo application of magnetic nanoparticles for the sustainable development of magnetic nanoparticles in the biomedical field.

1 Materials and methods

1.1 Dynamic light scattering measurement

Stock solutions of three magnetic materials including dextran-modified iron oxide nanoparticles (dextran-Fe_2O_3, Dex -Fe_2O_3), dimercaptosuccinic acid-modified iron oxide nanoparticles (meso-2, 3-dimercaptosuccinic acid-Fe_2O_3, DMSA Fe_2O_3) and $MnFe_2O_4$ were dispersed in 1 mL of double-distilled water and serum-containing medium using a Zetasizer Nano ZS90 (Malvern Instruments) analyzer to determine the hydrodynamic diameter and zeta potential of the particles. All measurements were performed at room temperature.

1.2 Cell culture

Human umbilical vein endothelial cells (HUVECs) were selected as the vascular endothelial cell model. The cells were cultured in endothelial cell medium (ECM) containing 5% fetal bovine serum, 1% endothelial growth factor, and 1% penicillin/streptomycin at 37°C and 5% CO_2.

Before cell culture, the culture flasks/plates were pre-coated with 1 μg/cm^2 of poly-L-lysine (PLL) (all of the above were purchased from ScienCell Research Laboratories). Cells were passaged at a ratio of 1 : 3 every 2 d or so. Cells of generation 1 ~ 6 were selected for the experiment.

1.3 Cell activity analysis

HUVECs cell activity was assayed with a cell counting kit (CCK-8, Dojindo Molecular Technologies, Inc). HUVECs cells were inoculated into 96-well cell culture plates at a density of 1.5 × 10^4 cells/well, and the medium was discarded after walling, washed twice with PBS, and magnetic nanoparticles were added. In the reduction assay, the cells were pretreated with 3 mmol/L N-Acetyl-Lcysteine (NAC, Sigma-Aldrich) for 1 h, and then 100 μL of magnetic nanoparticles were added. After incubation for 24 h, the medium was discarded and washed twice with PBS. 100 μL of medium and 10 μL of CCK8 reagent were added to each well, and 90 μL was aspirated into the enzyme plate after 2 h. The absorbance at 450 nm was measured. Each group was set up in 3 parallel, the group containing only culture medium and CCK8 was set up as blank control, and the cell activity of HUVECs not treated with magnetic nanoparticles was set up as 100%.

1.4 Measurement of intracellular reactive oxygen species

HUVECs cells were inoculated in 24-well cell culture plates at a density of 1 × 10^5 cells/well.

After the cells were treated with different magnetic nanomaterials, PBS containing 10 μmol/L DCFH-DA probe was added to each well and incubated for 30 min, after which the cells were collected by digestion. Positive controls were spiked with 5 μL hydrogen peroxide (H_2O_2) (concentrations of 30% ~32%) and the fluorescence intensity was detected by flow cytometry.

1.5 Prussian blue iron staining

HUVECs cells were inoculated in 30 mm cell culture dishes (4×10^5 cells/dish) and incubated against the wall. The cells were co-incubated with magnetic nanomaterials for 24 h and then washed with PBS and fixed with 4% paraformaldehyde solution for 30 min. Equal volumes of 2% aqueous potassium ferricyanide and 2% hydrochloric acid were added and kept at room temperature for 10 min before washing with double-distilled water and staining with eosin dye for 15 s.

1.6 Transmission electron microscopy observation

HUVECs cells were inoculated at a density of 1.5×10^6 cells/dish and incubated against the wall in 100 mm culture dishes. After coincubation with magnetic nanoparticles for 24 h, the cells were washed twice with PBS, gently scraped off from the culture dish and centrifuged, fixed with 2.5% glutaraldehyde (Fluka), dehydrated, embedded, sectioned, and placed on a copper grid for observation through a transmission electron microscope (TEM) manufactured by JEOL Ltd. Ltd. and observed by transmission electron microscope (TEM).

1.7 Laser confocal observation

HUVECs cells were inoculated at a density of 1×10^5 cells/well in a 24-well cell culture plate for wall culture. Cells were treated with different materials and then fixed with 4% paraformaldehyde and 0.3% Triton X-100 (Farco Chemical Supplies) permeabilized and closed. The cells were incubated with VE-cadherin rabbit antibody and Alexa 488-labeled goat anti-rabbit antibody (Cell Signaling Technology), respectively, at room temperature for 1 h. After washing with PBS, the slices were sealed with a blocker containing DAPI (Nakasugi Golden Bridge). The slices were observed under a laser confocal microscope and analyzed by FluoView software (Fluo View FV1000, Olympus).

1.8 Western blot analysis of cell-linked protein VE-cadherin expression

HUVECs cells were inoculated at a density of 6×10^5 cells/well in a 6-well plate and cultured against the wall. After 1 h of magnetic nanoparticle treatment, cells were lysed on ice, supernatants were collected, and protein concentrations were determined using a BCA protein assay kit (Thermo Fisher Scientific). The proteins were denatured by cooking in a boiling water bath for 5 min and analyzed by Western blot. Add primary antibody, VE-cadherin rabbit antibody (1:1000 dilution, Cell Signaling Technology), incubate overnight at 4°C, wash the membrane and incubate for secondary antibody development.

1.9 Statistical analysis

Statistical significance was analyzed by t-test using SPSS software (SPSS 17.0). Experimental data were expressed as mean plus or minus standard deviation (mean±SD). P-values less than 0.05 were considered statistically significant differences.

Table 1 Particle sizes and Zeta potentials of three kinds of magnetic nanoparticles in different dispersion systems

Category	Dispersion system	Particle size/nm	Polydispersity coefficient	Zeta potential/mV
Dex-Fe_2O_3	Water	80.1±2.5	0.29±0.04	-25.60±0.91
	ECM	50.4±1.2	0.26±0.02	-6.51±0.03
DMSA-Fe_2O_3	Water	299.4±2.8	0.22±0.04	-29.37±0.90
	ECM	273.3±1.5	0.50±0.09	-8.54±0.66
$MnFe_2O_4$	Water	105.6±3.5	0.41±0.03	-24.40±0.73
	ECM	108.1±1.2	0.35±0.05	-5.39±0.81

2 Results and Discussion

2.1 Magnetic nanoparticle characterization

To confirm the stability of the three magnetic nanoparticles in water and medium, the particle size and zeta potential were examined using dynamic light scattering (DLS). The results are shown in **Table 1**. The particle sizes of the three particles in the two dispersion systems are basically the same; combined with the PDI index analysis, the three magnetic nanoparticles have good dispersion in the medium. In addition, the Zeta potential of magnetic nanoparticles in both dispersion systems was negative, and the potential was close to the Zeta potential in the medium (-6.76 mV ± 0.49 mV) due to the easy adsorption of protein on the surface of nanoparticles in the medium.

2.2 Effect of magnetic nanoparticles on endothelial cell activity

The effect of different magnetic nanoparticles on the cellular activity of HUVECs was examined using the CCK 8 method, and the results are shown in Figure 1. In the experimental concentration range, Dex-Fe_2O_3 showed lower vascular endothelial cytotoxicity compared with the control, and the cytotoxicity of the other two magnetic nanoparticles showed concentration-dependent effects. Comparing Dex-Fe_2O_3 and DMSA-Fe_2O_3, the same Fe_2O_3 component exhibited greater differences in cytotoxicity due to different surface modifications. In addition, $MnFe_2O_4$ resulted in enhanced cytotoxicity due to the doping of Mn[10]. According to the International Standardization Organization (ISO) criteria for the evaluation of cytotoxicity of biomaterials[11], cellular activity greater than 80% is considered as primary toxicity. In this study, an exposure concentration less than primary toxicity was selected for each magnetic nanoparticle for subsequent cellular experiments (marked by red dashed boxes).

2.3 Uptake of magnetic nanoparticles by vascular endothelial cells

The endocytosis of DMSAFe_2O_3 by HUVECs was further verified by iron staining using DMSA-Fe_2O_3 as an example (**Figure 2A**). Compared with the control group, blue particles appeared in the cytoplasm of the DMSAFe_2O_3-treated group, and the number of blue particles increased with increasing concentration,

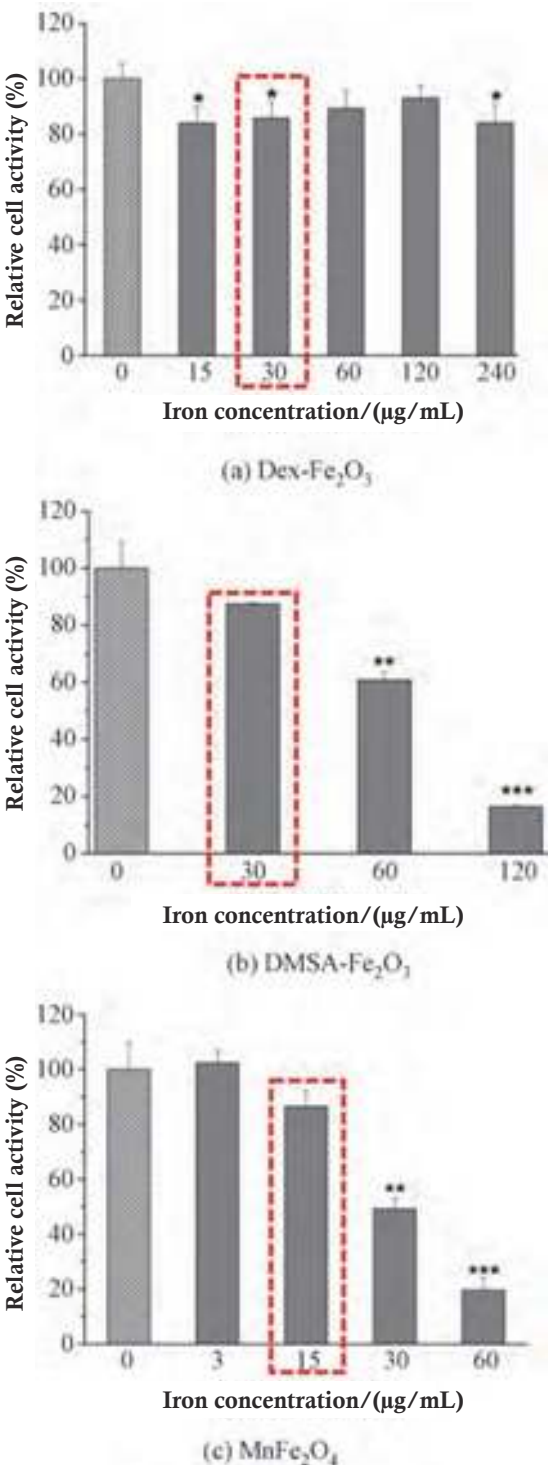
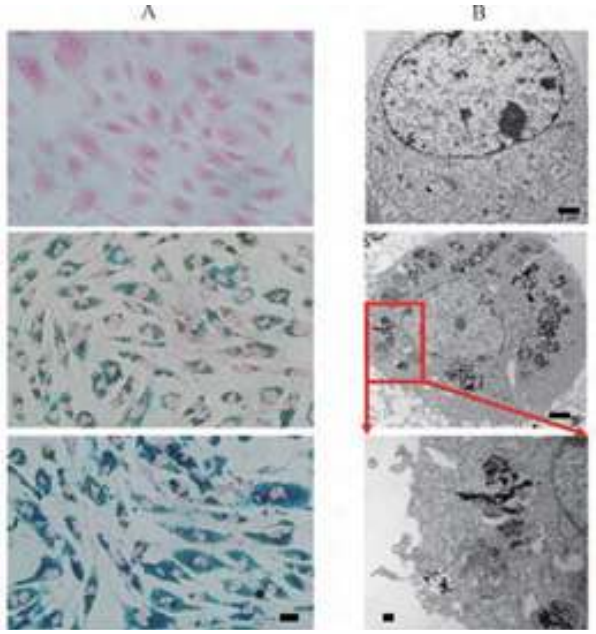


Figure 1 Effect of different magnetic nanomaterials on the viability of HUVECs cells

indicating that the magnetic nanoparticles could be endocytosed by HUVECs, and the endocytosis was positively correlated with the exposed dose. The localization of magnetic nanoparticles in the cells was further observed by transmission electron microscopy (TEM). The results were shown in Figure 2B, where DMSA Fe_2O_3 could be taken up by endothelial cells and was mainly present in the intracellular lysosomes after uptake.



A: Prussian blue iron staining results, from top to bottom, control, 30 μg Fe/mL DMSA-Fe_2O_3-treated group, 60 μg Fe/mL DMSA-Fe_2O_3-treated group, Bar = 20 μm; B: electron microscopy results, from top to bottom, control, 30 μg Fe/mL DMSA-Fe_2O_3-treated group The magnification of the red box is shown. The scale bar of the full figure is 1 μm, and the scale bar of the magnified figure is 200 nm

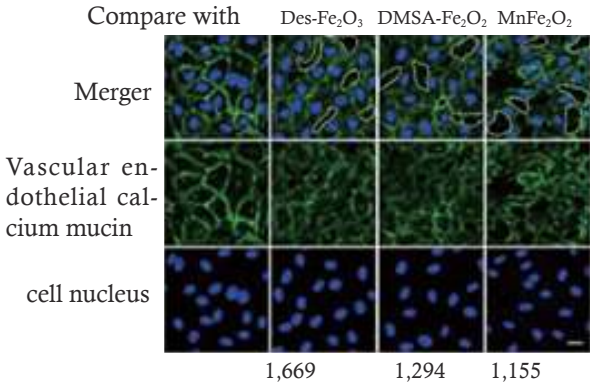
Figure 2 Uptake results of DMSA-Fe_2O_3 by HUVECs

2.4 Effect of magnetic nanomaterials on vascular endothelial cell junctions

VE-Cadherin plays a key role in angiogenesis and maintenance of vascular stability[12]. By fluorescently labeling VE Cadherin and using confocal laser scanning microscopes (CLSM), the state of cell junctions could be observed, as shown in Figure 3(a). Western blot results further demonstrated that the VE-cadherin expression level of HUVECs in the Dex Fe_2O_3 and DMSA Fe_2O_3 nanoparticle treatment groups was reduced [**Figure 3 (b)**]. The results suggest that magnetic nanoparticles affect endothelial cell junctions even at very low exposure concentrations of cytotoxicity.

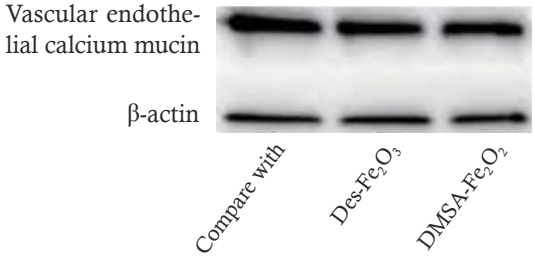
2.5 Effect of magnetic nanomaterials on ROS levels in vascular endothelial cells

The upregulation of reactive oxygen species (ROS) levels after cellular engulfment of nanoparticles is the most important mechanism of nanomaterial toxicity[13–15], but is ROS associated with cell attachment? The results of flow analysis (see **Figure 4**, Figure H_2O_2 as positive control) showed that all three magnetic nanomaterials induced different increases in ROS levels in endothelial cells after 1 h of exposure and showed concentration and time dependence.



Laser confocal observation of the effect of magnetic nanoparticles on cell junctions with fluorescently labeled nuclei (blue) and VE-Cadherin (green), white dashed circles for cell gaps, and Dex-Fe_2O_3, DMSA-Fe_2O_3, and $MnFe_2O_4$ concentrations of 30 ug Fe/mL, 30 ug Fe/mL, and 15 ug Fe/mL, respectively.

(a) results of CLSM



Western results of magnetic nanomaterials on the expression level of cellular connexin VE-cadherin,-Dex-Fe_2O_3 and DMSA-Fe_2O_3 concentrations were 30 ug Fe/mL

(b) results of Westem

Figure 3 Effect of different magnetic nanomaterials on the conjunction of endothelial cells

To further verify the correlation between the altered intercellular junctions induced by magnetic nanomaterials and changes in intracellular

ROS levels, cells were treated with the antioxidant N-acetylcysteine (NAC), a commonly used sulfhydryl-containing antioxidant that interferes with free radical production and scavenges generated free radicals to regulate cellular metabolic activities and protect the body from reactive oxygen species-induced oxidative stress damage[16]. The results of laser confocal observation showed that the cell boundaries in the group without NAC were blurred and the intercellular gaps were increased; after treatment with NAC, the endothelial cell gaps were correspondingly reduced, the cell junctions were more intact, and the boundary staining became clear (**Figure 5**), indicating that the ROS generation induced by magnetic nanoparticles was closely related to the reduction of intercellular junctions. The above results suggest that the excess ROS induced by magnetic nanoparticles in HUVECs may lead to the reduction of intercellular adhesion junctions and eventually induce cell gap formation and increase the permeability of the endothelial layer.

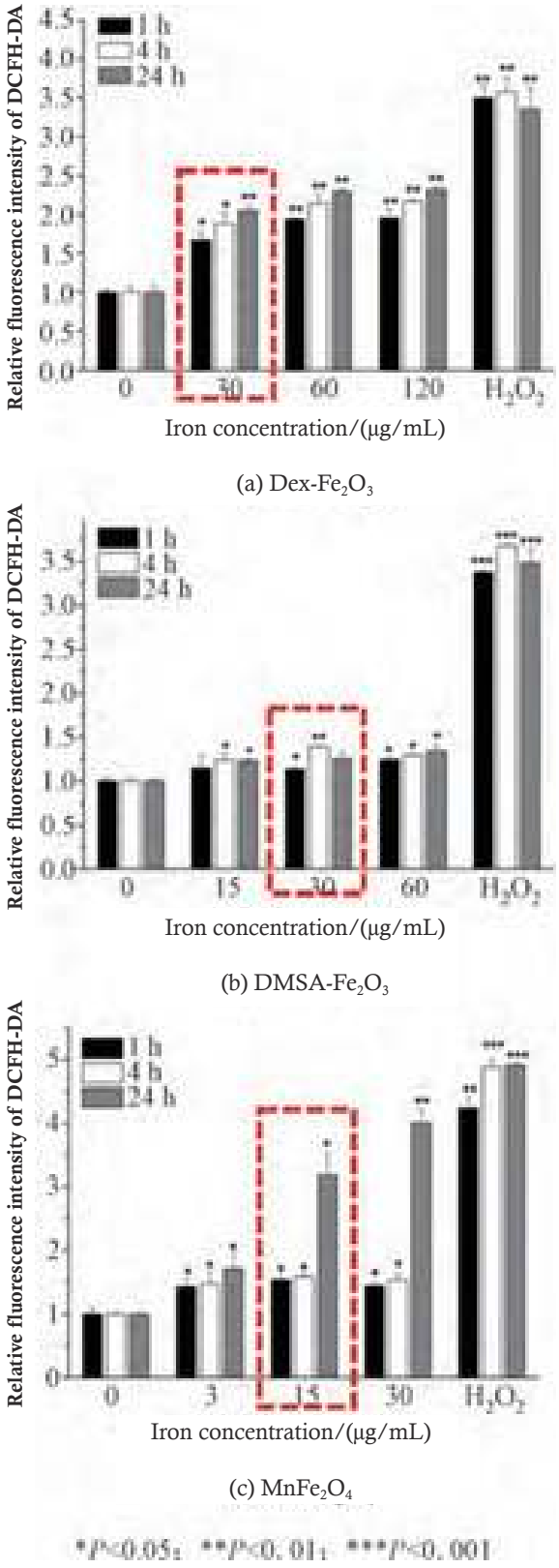


Figure 4 ROS levels of magnetic nanomaterials on vascular endothelial cells

3 Conclusion

Magnetic nanoparticles induced upregulation of ROS in vascular endothelial cells at primary cytotoxic exposure concentrations, causing a decrease in VE-cadherin, leading to an increase in cell gaps and increased endothelial layer permeability; treatment with the antioxidant N-acetylcysteine significantly attenuated reactive oxygen species upregulation-mediated weakening of endothelial cell junctions, indicating that the integrity of vascular endothelial cell junctions was induced by magnetic nanomaterials regulation of endothelial cell ROS levels.

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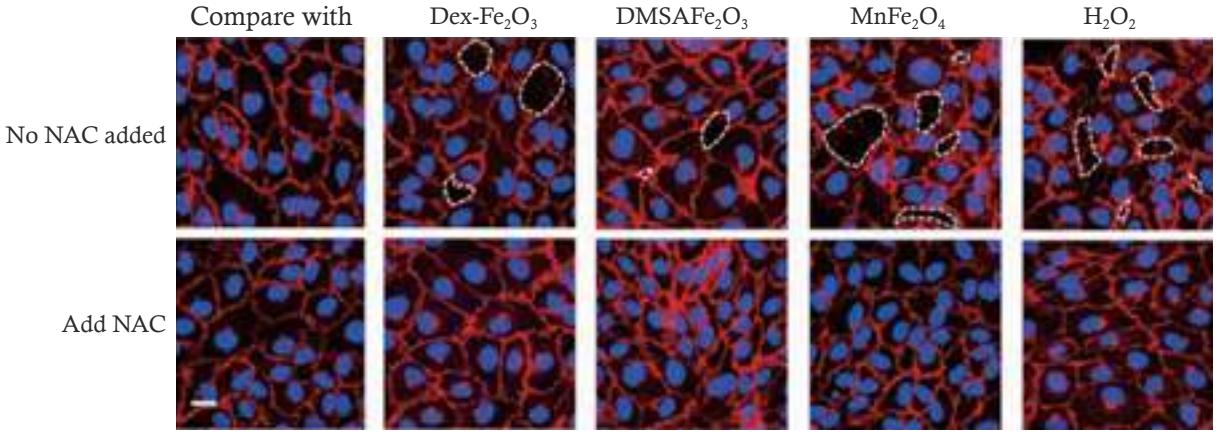

Fluorescently labeled nuclei (blue) and VE-cadherin (red) were incubated for 1 h. Cells treated with 7.5 mg/mL H_2O_2 were set as positive controls; Dex-Fe_2O_3, DMSAFe_2O_3, and $MnFe_2O_4$ concentrations were 30

Figure 5 Effect of magnetic nanomaterials on endothelial cell junction after NAC treatment

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