Toxicology: Open Access
ISSN: 2476-2067

Zhang et al., Toxicol Open Access 2017, 3:3
DOI: 10.4172/2476-2067.1000130

Review Article Open Access

Research on Nanotoxicity of an Iron Oxide Nanoparticles and Potential Application

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Received date: July 17, 2017; **Accepted date:** August 28, 2017; **Published date:** September 01, 2017

Abstract

The iron oxide nanoparticles (FeNPs) are widely used in biomedicine for good biocompatibility. To promote its safe application, any potential nanotoxicity should be thoroughly and carefully investigated. This paper systematically summarizes our lab's research on the nanotoxicity of iron oxide nanoparticles coated with dimercaptosuccinic acid (DMSA), including the effects of FeNPs on viability, apoptosis, cycle, and oxidative stress at cell level. *In vitro* studies revealed that the FeNPs showed obvious apoptosis of human acute monocyte cells (THP-1) and human hepatoma cells (HepG2) at the highest concentration. FeNPs resulted in common and cell type-specific nanotoxicities of the FeNPs to both human and mouse cells at the gene, disturbed cell's iron and osmosis homeostasis by the internalization of FeNPs through releasing iron ion in cells, resulted in cytotoxicity of DMSA as coating molecules of FeNPs and the inhibitor of DNA binding/differentiation (Id) related nanotoxicity of FeNPs at gene level. The studies of our lab shed many new insights into the nanotoxicity of the nanoparticle. Furthermore, the toxicity may play more value if it is guided and applied reasonably, such as iron supplement, anti-oxidation, and immunotherapy.

Keywords: Iron oxide nanoparticles; Nanotoxicity; Gene expression profile

Introduction

The iron oxide nanoparticles (FeNPs) are widely applicated in biomedicine, such as magnetic resonance imaging (MRI) contrast agents, drug vectors and hyperthermia [1-3]. FeNPs are well-known unique magnetic properties and good biocompatibility relative to other popular metal nanomaterials [4]. Therefore, FeNPs have been the most intensively studied to commercialize in recent years. However, its potential nanotoxicity has been overlooked by academia and not masked by its bright application [5,6]. To promote its safe application, especially as a clinical agent, any potential nanotoxicity should be thoroughly and carefully evaluated. Some important nanotoxicities of FeNPs have been therefore discovered, such as inductions of cell inflammation [7], mitochondrial injury [8-10], detriment to cell viability [9,11], reactive oxygen species (ROS) [8,9,12,13], apoptosis [9,14,15], oxidative stress [15-17], cell motility impairment [12], autophagy [8,9], and DNA damage [16,17].

As most of these applications are based on the delivery of large numbers of nanoparticles onto or into the cells of interest [18], more and more studies have paid attention to the resulted effects on cells and their degradation. Cells can promptly and moderately regulate its gene expression profile in response to any changes of intra- or extracellular environments. Therefore, detection of gene expression profile is helpful to explore the potential nanotoxicity of nanomaterials [13,19,20]. There are two advanced gene expression profiling techniques, including DNA microarray (genechip) [20,21], and RNA-seq [22]. Finding all genes differently expressed at the cell or tissue levels by a nanomaterial can provide valuable clues to investigate any potential toxicity and analyze the relevant molecular mechanism [13,19,20,23]. Therefore, more and more studies started to evaluate the nanotoxicity of various nanomaterials at the molecular level [13,20,22]. Many previous unknown nanotoxicities of a nanomaterial were thus exposed. For example, Kedziorek et al. studied the gene expression profile of neural stem cell line C 17.2 with DNA microarray and revealed early cellular responses to intracellular magnetic superparamagnetic iron oxide nanoparticles (SPIONs) [24]. The analysis of the gene expression profile found that various nanoparticles produced intracellular ROS and resulted apoptotic cell death, such as silica nanoparticle [19], silver nanoparticles [20], and magnetic nanoparticles [25].

Hamed Arami et al. reported that the effects of size, various additional molecular parameters on the surface of the iron oxide nanoparticles, molecular structure of the coating molecules, administered dose on their degradation rates, and transformation to plasma ferritin still require to be studied systematically in more accurate ways through developed characterization tools with higher mass sensitivities [26]. Our lab continuously and systematically studied the nanotoxicity of iron oxide nanoparticles coated with dimercaptosuccinic acid (DMSA), which possessed a mean particle size of 11 nm through TEM observation. This paper gives a summarily review of our studies on the iron oxide nanoparticle in recent years.

Characteristics and Internalization of FeNPs

FeNPs showed monodispersed in water solution and had an average size of 11.0 ± 1.25 nm by TEM observation Figure 1. FeNPs were negatively charged and superparamagnetic properties. IR spectral analysis demonstrated that DMSA successfully coated the nanoparticles [27]. The results of Prussian blue staining displayed that FeNPs could be internalized into mouse mononuclear macrophages (RAW264.7), mouse hepatoma cells (Hepa1-6), human acute monocyte cells (THP-1), human hepatoma cells (HepG2), human

cervical cancer (HeLa), and human normal liver cells (HL-7702). Thereamong, RAW264.7 was labeled more effectively than other cells at any concentration of the nanoparticles [28]. FeNPs were proved to locate in the cytoplasmic inclusions [29].

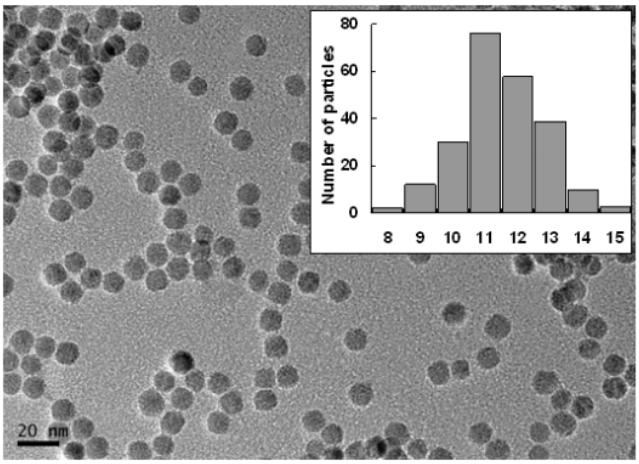

Figure 1: Dispersibility and size of FeNPs through a transmission electron microscope (TEM) observation [29].

Evaluation Biocompatibility of FeNPs at Cell Level

Evaluation biocompatibility of FeNPs at cell level mainly includes effects on viability, apoptosis, cycle, and oxidative stress, which are indispensable assessment of nanotoxicity. We investigated six different mammalian cell lines (RAW264.7, THP-1, Hepa1-6, HepG2, HL-7702, and HeLa) at six different concentrations (0,20,30,40,50 and 100 μg/mL) of FeNPs for 48 h. It is found that the viability of all the cells were not significantly repressed except HepG2 exposed to 100 μg/mL FeNPs. The detection of oxidative stress displayed that the levels of total superoxide dismutase and xanthine oxidase showed no significant changes, while the levels of malonyldialdehyde activity significantly increased. The nanoparticles did not produce any significant influence in cell cycles at any of the doses, but caused obvious apoptosis of THP-1 and HepG2 cells at the highest concentration Figure 2. These results reveal that 30 μg/mL FeNPs used in human studies with an intravascular nanoparticle imaging agent (Combidex) efficiently labeled all the cells studied, but did not produce any significant effects on their viability, oxidative stress, cycle, and apoptosis, indicating better biocompatibility and more useful clinical applications [11].

Effects of FeNPs on Global Gene Expression Profile of Five Different Mammalian Cells

To investigate the nanotoxicity of FeNPs, we firstly detected gene expression profile of five different mammalian cells treated with different dose for different time, including two mouse cell lines, the RAW264.7 and Hepa1-6 cells, and three human cell lines, the THP-1, HepG2, and HL7702 cells. Thereamong, RAW264.7 and THP-1 are blood cell and belong to monocyte-macrophage system, while Hepa1-6, HepG2, and HL7702 are liver-derived hepatoma cells. These five cell lines are suitable for evaluating the nanotoxicity of FeNPs because the blood and liver cells are most intensively enriched the nanoparticles *in vivo* due to the intravenous administration and passive targeting. After data analysis, all differentially expressed genes of five cells under each treatment were thus identified. Thereamong, few genes were differentially expressed in HL7702 because of normal liver cells. We analyzed effects of FeNPs on global gene expression of RAW264.7 cells treated with two different doses (50 and 100 μg/mL) for 4 h, 24 h, and 48 h. The results demonstrated that FeNPs display cytotoxicity in this type of macrophage at high doses [27]. Then, by annotating the functions of the differentially expressed genes of four cancer cells, those common and cell type-specific nanotoxicities of the FeNPs to both human and mouse cells at the gene, biological process and pathway levels affected by the FeNPs were characterized, developing new insights into the nanotoxicity of the FeNPs to these cell lines [30,31].

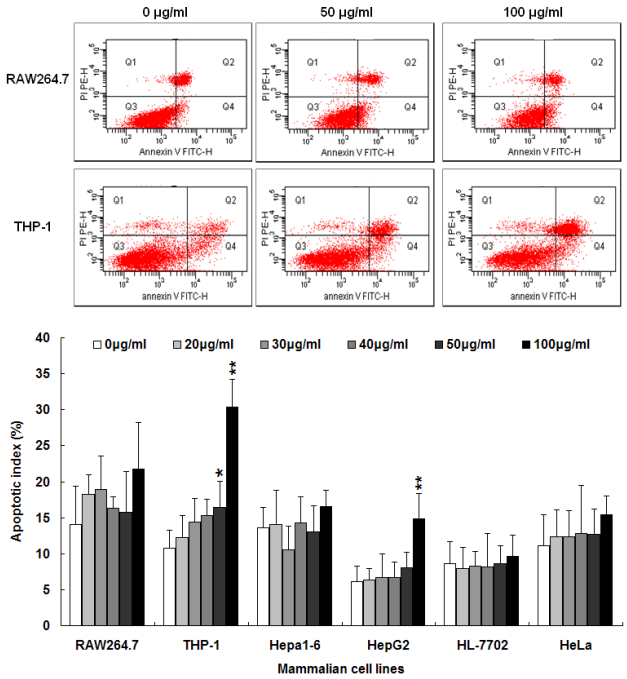

Figure 2: Cell apoptosis [11]. Copyright© 2010, Springer Science +Business Media BV.

Effects of FeNPs on Apoptosis Response Genes

Our previous study has revealed that high concentration of FeNPs could increase cell apoptosis and decrease cell viability of some specific mammalian cell types [32]. Apoptosis is an important biological process, which is closely related to evaluate toxicity of nanoparticles. We then investigated the global gene expression profiles of human THP-1 monocytes. It is found that 35 significant up-regulation genes at the concentrations of 100 μg/ml enriched three GO terms related to cell apoptosis, including apoptosis, death and programmed cell death by GO analysis [29]. However, it is only reported a small number of genes correlated with cell apoptosis were determined to be expressed differently *via* RT-PCR detection in some previous studies restricted by low throughput [33]. A few previous studies have already been performed to understand the mechanism of cell apoptosis induced by nanoparticles. Here, our studies demonstrate that the cell apoptosis was triggered by FeNPs *via* the caspase-10 mediated apoptosis pathway Figure 3. A large number of gene of cytokine were differentially

expressed *via* NLR signaling pathway and TLR signaling pathway, such as IL-1, PIK3R1, NFKBIA, and TNFSF10. Consequently, the excessive activation of cytokines induced extrinsic apoptosis pathway [29].

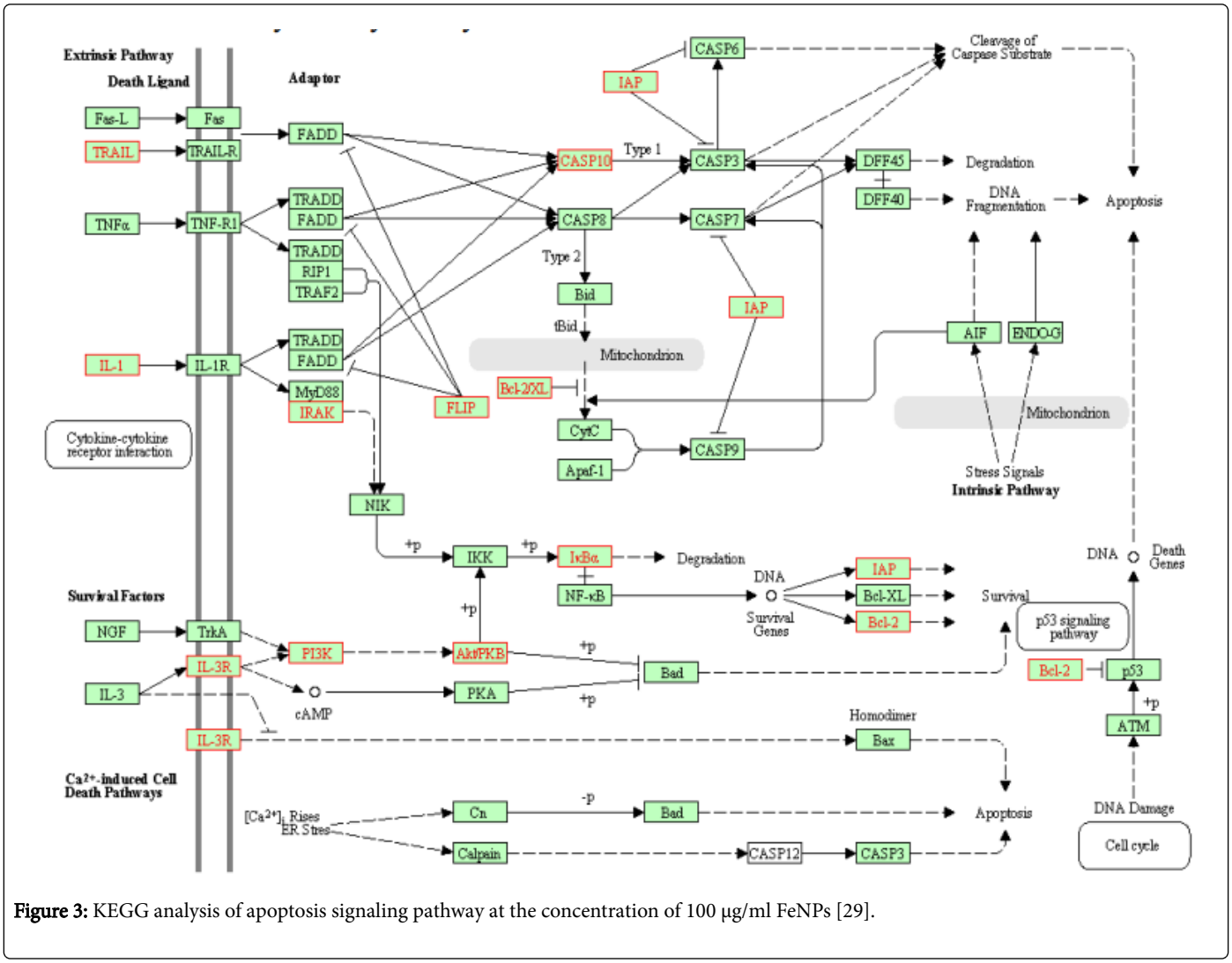

Figure 3: KEGG analysis of apoptosis signaling pathway at the concentration of 100 μg/ml FeNPs [29].

Effects of FeNPs on the Transcription of Genes Related to Iron and Osmosis Homeostasis

By checking effects of FeNPs on gene expression profile of RAW264.7 cells, we found that several important genes were responsible for intracellular iron homeostasis, which indicate that the iron homeostasis must be disturbed by the internalization of FeNPs through releasing iron ion in cells. Further experiments proved that the transcriptions of *Trf*, *Tfrc*, *Lcn2*, and *Hfe*, relevant to iron metabolism, and *Slc5a3*, *Slc6a12*, related to osmosis were significantly changed by the FeNPs treatment [34]. The subsequent detection of cellular iron content demonstrated that the internalized FeNPs were degraded in lysosomes for its acid environment and thus released iron ions in cells, which damaged the iron and osmosis homeostasis of cells and thus lead to cell's complementary responses, including repressing the expressions of *Trf*, *Tfrc*, and *Hfe* to prevent the transfer of extracellular iron ions into cells, inducing the expression of *Lcn2* to promote the transfer of intracellular iron ions out of cells, and down regulating the expressions of *Slc5a3* to prevent the transfer of extracellular myo-inositol (very important organic osmolyte) into cells Figure 4 [34]. The results provided valuable mechanistic insights into various FeNPs-induced toxicities [35].

Iron is essential to virtually all living organisms and involves in multiple metabolic functions, including oxygen transport in hemoglobin [36]. Iron deficiency anemia is prevalent. Furthermore, it is reported that 90% of patients older than 65 years with iron deficiency anemia suffer from a gastrointestinal cancer [35]. Iron oxide nanoparticles are attracting attention to be developed as iron supplement. For example, feraheme is approved by FDA for the treatment of iron deficiency anemia in adult patients with chronic kidney disease (CKD) [37]. FeNPs may be improved as another iron supplement in future.

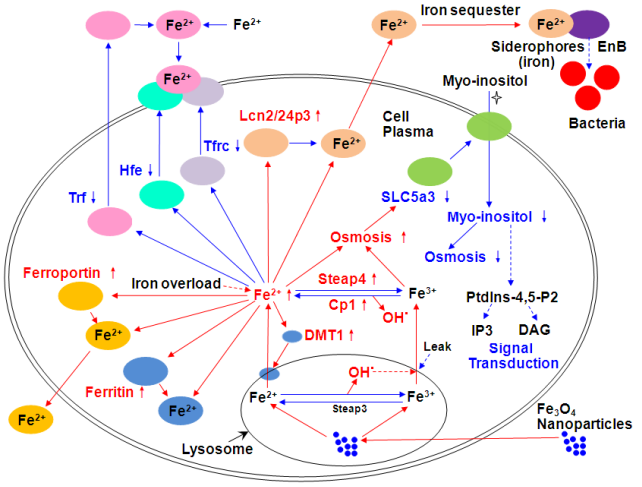

Figure 4: Schematic of effects of FeNPs on transcription of genes related to iron and osmosis homeostasis. Upward and downward arrows mean up- and down-regulation of transcription after nanoparticle treatment, respectively [34].

Effects of DMSA Coating on the Expression of Genes Coding Cysteine-Rich Proteins

We found that about one fourth of the differentially expressed genes (DEGs) coded cysteine-rich proteins (CRPs) in all four mammalian cells under each treatment through analysis of DEGs [38]. DMSA contains abundant thiol groups. The results indicate that FeNPs greatly affected the expressions of CRP-coding genes. Furthermore, about 26% of CRP-coding DEGs were enzyme genes in all cells. Our further experiments revealed that the effect mainly resulted from DMSA carried into cells by the nanoparticles [38]. FeNPs were internalized into cells by endocytosis and positioned in lysosomes [39]. The lysosomal thiol reductase (GILT) catalyzes disulfide bond reduction under acidic conditions of lysosome [40]. The produced abundant thiol groups can directly bind with both cysteines and metal ions in cysteine-rich proteins, which are nearly transcription factors. Therefore, a large number of target genes were regulated differently Figure 5. The bare FeNPs have hydrophobic surfaces and a propensity to agglomerate [41]. It has been proved that the uncoated Fe_3O_4 nanoparticles are toxic to cells [42]. Thus, FeNPs are often produced with various coating molecules, such as 2,3-dimercaptosuccinic acid (DMSA) [43]. It was reported that DMSA coating could improve stability, internalization, biodistribution, and biocompatibility of FeNPs *in vivo* and *in vitro* [44]. DMSA is used as orally administered metal chelating agent receiving the approval of Food and Drug Administration of USA [45]. DMSA alone showed low toxicity in various biological systems due to its extracellular distribution [46]. Our study thus firstly reported the cytotoxicity of DMSA as coating molecules of FeNPs at the gene transcription level [38].

It is well known that free radicals cause the oxidation of biomolecules, such as protein, lipid, and DNA, which results cell injury and death [47]. Free radicals is deleterious to mammalian cells and involves in the pathogenesis of many chronic diseases [48]. FeNPs with rich thiol groups may be available antioxidants to remove excess free radicals by redox reactions.

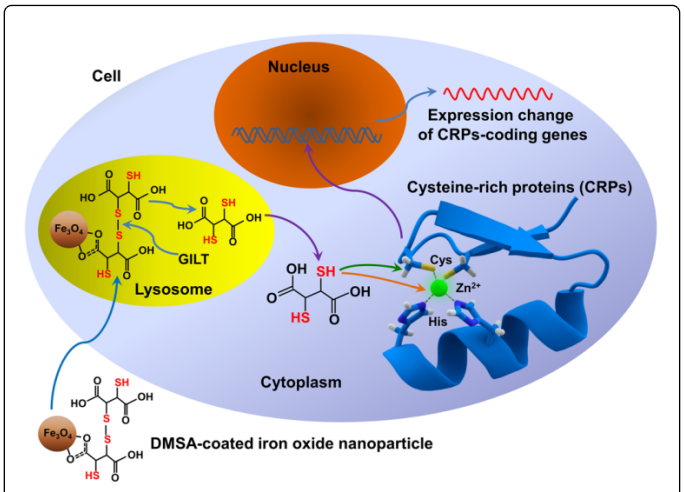

Figure 5: Schematic of effect of FeNPs on expression of CRP-coding genes and the potential molecular. mechanisms [38]. Copyright© 2015, American Chemical Society.

Effects of FeNPs on the Expression of Id Genes

In our DNA microarray studies, it is worth to note that the transcription of the Id3 gene was significantly down-regulated in five cell lines (RAW264.7, Hepa1-6, THP-1, HepG2, and HL7702) treated with FeNPs at two doses. Id genes of the inhibitor of DNA binding/differentiation code for a class of well-known helix-loop-helix (HLH) transcription factors, which are related to cell growth, proliferation, differentiation, lineage commitment, tumor cell aggressiveness and metastatic behavior [49,50]. We then did more investigations on the effect of FeNPs upon the Id genes. We detected the expression of Id genes in six cell lines (the above cell lines plus HeLa) at the same conditions through quantitative PCR. Under each treatment, the Id1, Id2, and Id3 gene was significantly down-regulated in both cell lines and the liver tissues of mice, while Id4 gene was obviously up-regulated. These results reveal that the nanoparticle exerts a significant effect on the *in vitro* and *in vivo* expression of Id genes [51]. FeNPs may regulate the Id genes *via* hypoxia, iron ions or redox and hydroxyl radicals Figure 6. To our knowledge, it is the first report of this cellular effect of FeNPs. Our study thus provides new insights into the Id-related nanotoxicity of FeNPs and the close relationship between the regulation of Id genes and iron [51].

Since Id3 gene was significantly down-regulated in six cell lines, which can be identified as a nanotoxicity biomarker of the FeNPs. It is reported that overexpressed Id3 inhibited cell proliferation and induced apoptosis and may be a potential target for tumor suppression [52]. Here, downregulated Id3 may activate cell proliferation, such as T cell, macrophages and may be a potential drug for immunodeficiency therapy.

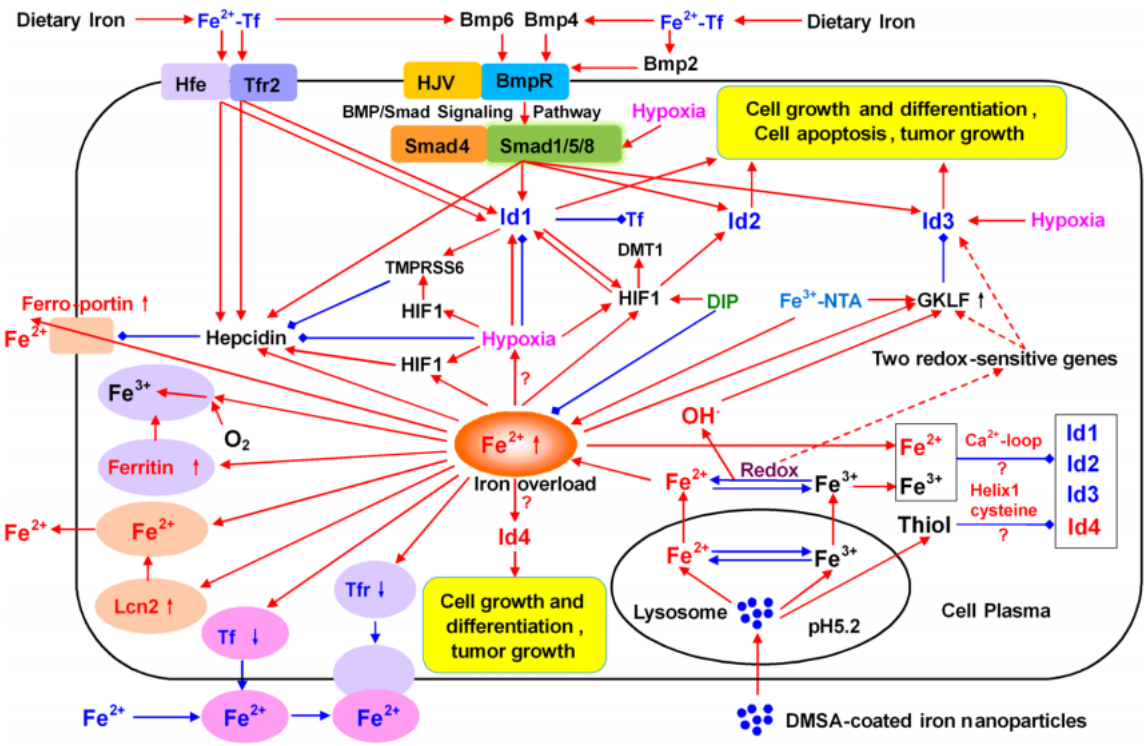

Figure 6: Potential mechanism of the Id genes regulated differently [51]. Copyright© 2015, American Chemical Society.

Effects of FeNPs on the Immune System

Our previous study revealed that intravenously-injected FeNPs were passive targeted to the liver and spleen [51], which are known classically part of the important immune system termed mononuclear phagocytic system (MPS) [53,54]. The MPS system includes macrophages located in different organs and monocytes circulating in the blood [55], clearing pathogens or foreign bodies such as viruses [56]. The previous data demonstrated that FeNPs induced response to virus and hepatitis C pathway in the THP-1 cells, TLR signaling pathway and JAK-STAT signaling pathway in the RAW264.7 cells, indicating that the nanoparticles may influence in immune responses of MPS like virus [30,31]. Immune system plays the most critical role in immunotherapy [57]. We are systematically investigating the effect of FeNPs on immune cells and explore the application of FeNPs in immunotherapy.

In summary, we investigate systematically the nanotoxicity of FeNPs at cell level and gene level Figure 7. The results show that FeNPs did not produce any significant influence in cell level at any of the doses, except that obvious apoptosis of THP-1 and HepG2 cells at the highest concentration. At gene level, we analyzed gene expression profile in depth. It is found that many genes were differently expressed, which were involved in cell growth, cell apoptosis, iron homeostasis, cysteine-rich proteins, and immune response. The data analysis is helpful for extracting the useful biological information, finding new biological phenomena, postulating the possible underlying molecular mechanism and conceiving new hypothesis. More importantly, it can provide key clues and instructions for designing and performing new experiments for verify the conceived hypothesis, which shed many new insights into the nanotoxicity of the nanoparticle. The iron oxide nanoparticles show great application potential in biomedical field. The research on their nanotoxicity is most significant. At the same time, the toxicity may play more value if it is guided and applied reasonably.

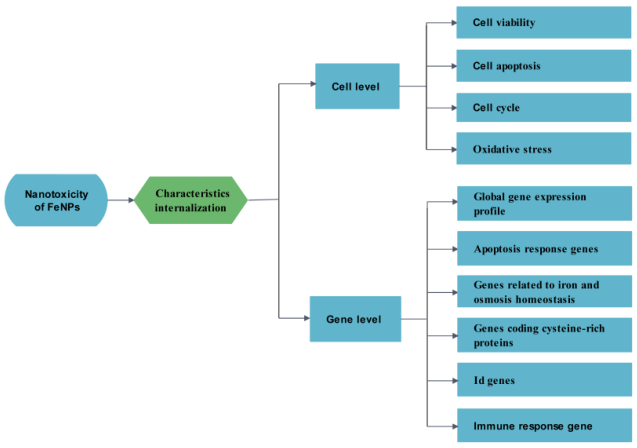

Figure 7: The nanotoxicity evaluation of FeNPs at cell level and gene level.

Acknowledgements

This work was supported by the National Key Research and Development Program of China (2017YFA0205502) and the National

Natural Science Foundation of China (61571119). We greatly thank LI-COR Biosciences (Shanghai) for providing a free Pearl Imager machine for using in our lab in animal research.

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