

CD44⁺ fraction of A549 cells. MgNPs-Fe₃O₄ induced the expression of heme oxygenase-1 at a concentration of 1 µg/mL, and in a dose-dependent manner. Despite these effects, MgNPs-Fe₃O₄ had minimal effect on cell viability and elicited only a small increase in the number of cells undergoing apoptosis. Together, these data suggest that MgNPs-Fe₃O₄ exert little or no cytotoxicity until a high exposure level (100 µg/mL) is reached. This dissociation between elevated indices of cell damage and a small effect on cell viability warrants further study.

Keywords: magnetic nanoparticles; cytotoxicity; genotoxicity; A549; CD44

1. Introduction

Nanotechnology—the manipulation and production of matter sized between 1 and 100 nm—has grown markedly with the promise of substantial benefits and applicability to such diverse areas as clothing, electronics, engineering, and healthcare [1]. The principal goal of nanotechnology is to develop new materials in the nanometer scale, including nanoparticles, defined as particulate materials with at least one dimension of less than 100 nm. The design and development of nanomaterials have been of fundamental importance to the industry, given their novelty and the benefits conferred by their physicochemical properties.

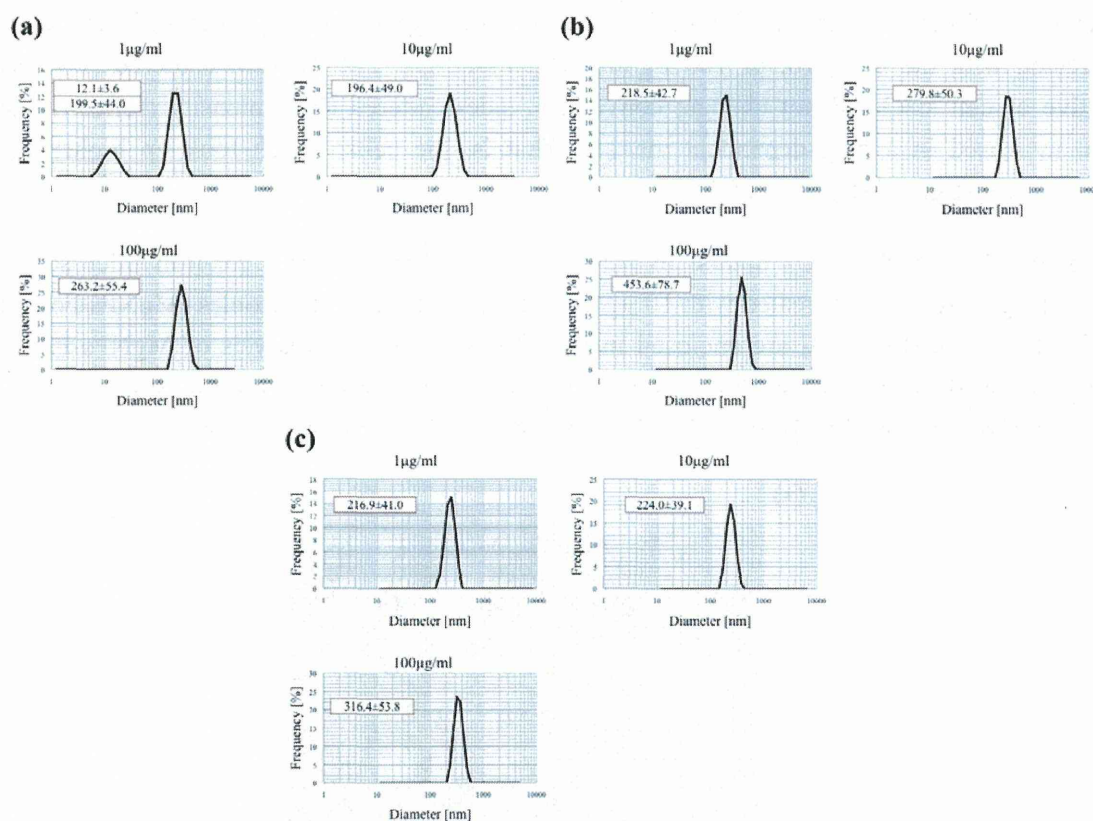
Magnetic nanoparticles (MgNPs) are a subclass of nanomaterials. Among MgNPs, Fe₃O₄-containing MgNPs (MgNP-Fe₃O₄; magnetite) are the only MgNPs approved for clinical use. Magnetite has a cubic inverse spinel structure with oxygen forming a face-centered cubic (FCC) closed packing; the interstitial tetrahedral and octahedral sites are occupied by Fe cations [2]. Due to their unique physical, chemical, and mechanical features, MgNPs-Fe₃O₄ have been used as magnetic resonance imaging contrast agents, targeted drug delivery systems, and hyperthermic agents when placed in an external magnetic field [3,4]. Modified/unmodified MgNP-Fe₃O₄ has been reported to improve the efficiency of anticancer drugs and reverse multidrug resistance [5,6]. However, these same properties of MgNPs can induce cytotoxicity and genotoxicity [7]. Studies have shown that MgNPs-Fe₃O₄ are less toxic than MgNPs containing SiO₂, TiO₂, CuO, and TiO₂ [7–9]. However, results regarding the potential of MgNPs-Fe₃O₄ to induce cytotoxicity, genotoxicity, and oxidative stress, have been inconsistent [7–11], necessitating further study to identify any potential toxicity associated with their use. Therefore, the objective of this study was to investigate the cytotoxicity and genotoxicity of MgNPs-Fe₃O₄. Experiments were designed to examine the effect of MgNPs-Fe₃O₄ on indices of oxidative stress, and resultant cellular and nuclear damage in A549, human alveolar epithelial-like type-II cells. We also assessed the effect of MgNPs-Fe₃O₄ on the expression of CD44, a transmembrane glycoprotein involved in inflammation, cell migration, signaling, and tumor metastasis [12,13].

2. Results and Discussion

Studies regarding the toxicological impact of MgNPs-Fe₃O₄ have yielded disparate results, depending on the cell type, surface modification, cell medium composition, protein-MgNP interaction,

and oxidation state of iron [7,14]. We evaluated the cytotoxic effects of MgNPs-Fe₃O₄ in A549 cells. We report that MgNPs-Fe₃O₄ caused LDH leakage only at a concentration of 100 µg/mL; increased ROS production and 8-OH-dG content, and decreased glutathione (GSH) levels were found with 10 µg/mL MgNPs-Fe₃O₄. Despite these responses, MgNPs-Fe₃O₄ caused only a small decrease and increase in cell viability and apoptosis, respectively.

Figure 1. Measurement of MgNPs-Fe₃O₄ size by dynamic light scattering. MgNPs-Fe₃O₄ were suspended at a concentration of 1, 10 or 100 µg/mL in (a) Ham's F-12 Medium with 10% fetal bovine serum (FBS); (b) Ham's F-12 Medium alone; (c) Phosphate-buffered saline (PBS).



2.1. Characterization of MgNPs-Fe₃O₄ Suspension in Various Conditioned Medium

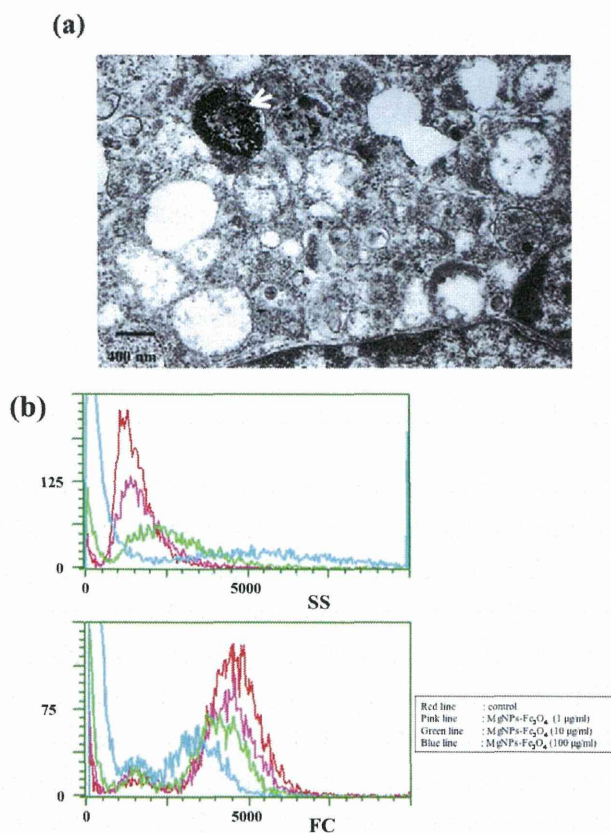
One of the most important significant factors for analysis of the toxicity of nanoparticles is size. As well as the sizes of the primary nanoparticles, the hydrodynamic sizes of secondary nanoparticles in dispersion are important as their sizes have a dramatic effect on cell response to exposure. The high ionic nature of the solution and the electrostatic/van der Waals interaction between protein and nanoparticles results in the formation of secondary particles. The mean hydrodynamic diameter of MgNPs-Fe₃O₄ in Ham's F-12 medium, without FBS or supplements, increased in a dose-dependent manner (Figure 1b). In Ham's F-12 medium with 10% FBS and supplements or PBS, the mean hydrodynamic diameter was comparable between MgNP-Fe₃O₄ suspensions of 1 and 10 µg/mL (Figure 1a,c); mean hydrodynamic diameter of MgNPs-Fe₃O₄ was greater at 100 µg/mL in both media.

Together, these data suggest that MgNPs-Fe₃O₄ agglomerate at a high concentration. The presence of FBS appeared to enhance the stability of MgNPs-Fe₃O₄ in suspension. These data are consistent with a previous report showing that MgNPs show increased stability against aggregation in the RPMI-1640 with an increasing amount of FBS [15]. Therefore, the influence by the sedimentation rate of the secondary nanoparticles (NPs) and ratios of protein to NPs could be taken into consideration in the *in vitro* toxicity of NPs. These results shows the hydrodynamic sizes of secondary nanoparticles in Ham's F-12 medium with 10% FBS used in this study.

2.2. MgNPs-Fe₃O₄ Uptake

A representative micrograph shows that after 24 h, MgNPs-Fe₃O₄ aggregate within intracellular vesicles in A549 cells (Figure 2a). Figure 2b shows the flow cytometric light scatter histograms of the cells treated with the 0, 1, 10, or 100 µg/mL MgNPs-Fe₃O₄. The forward-scattered (FS) intensity (reflective of cell size) did not change; conversely, side-scattered (SS) intensity (reflective cellular uptake) increased in a dose-dependent manner. That is, the cells, which took up higher doses of MgNPs showed higher intensities of SS.

Figure 2. MgNPs-Fe₃O₄ uptake in A549 cells; (a) Transmission electron microscopy imaging of A549 cells treated with 10 µg/mL Fe₃O₄ magnetic nanoparticles (MgNPs-Fe₃O₄) for 24 h. MgNPs-Fe₃O₄ are enclosed in vesicles (arrow); (b) Analysis of MgNPs-Fe₃O₄ uptake by flow cytometric light scatter. A549 cells were treated with 0 (control), 1, 10 or 100 µg/mL MgNPs-Fe₃O₄ for 24 h. SS: side-scattered; FS: forward-scattered.



2.3. Effect of MgNPs-Fe₃O₄ on Cell Viability, Cell Membrane Damage, and Apoptosis

Treatment with MgNPs-Fe₃O₄ for 24 h did not affect cell viability as assessed by the Alamar Blue assay. However, treatment with 100 µg/mL MgNPs-Fe₃O₄ for 72 h caused a significant reduction in cell viability (Figure 3). Significant LDH leakage was detected following treatment with 100 µg/mL MgNPs-Fe₃O₄; lower concentrations had no effect (Figure 4). As shown in Figure 5a, treatment with 100 µg/mL MgNPs-Fe₃O₄ for 24 h caused a small but significant increase in the percentage Annexin V-staining cells; however, these values were greatly below that caused by H₂O₂ (Figure 5b).

Figure 3. Effect of Fe₃O₄ magnetic nanoparticles (MgNPs-Fe₃O₄) on viability of A549 cells. A549 cells were treated with 0 (control), 1, 10 or 100 µg/mL MgNPs-Fe₃O₄ for 24 or 72 h. Cell viability was assessed using the Alamar Blue assay. Data are presented as the mean ± SD of 3 independent experiments. * $p < 0.05$ vs. control.

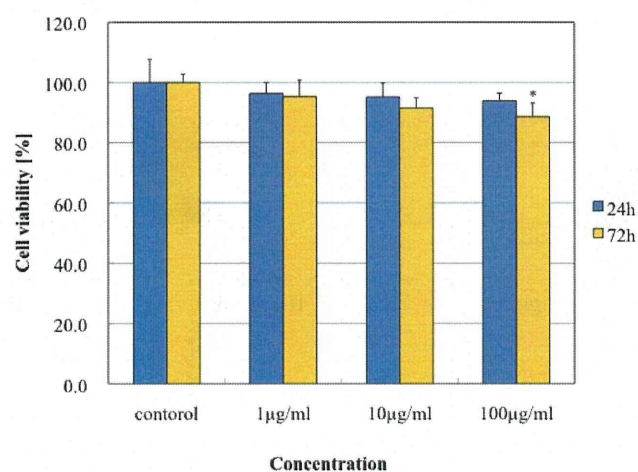


Figure 4. Effect of MgNPs-Fe₃O₄ on lactate dehydrogenase (LDH) release by A549 cells. A549 cells were treated with 0 (control), 1, 10 or 100 µg/mL MgNPs-Fe₃O₄ for 24 h. LDH release was assessed by formazan absorbance (LDH Cytotoxicity Assay Kit). Data are presented as the mean ± SD of 3 independent experiments. ** $p < 0.01$ vs. control.

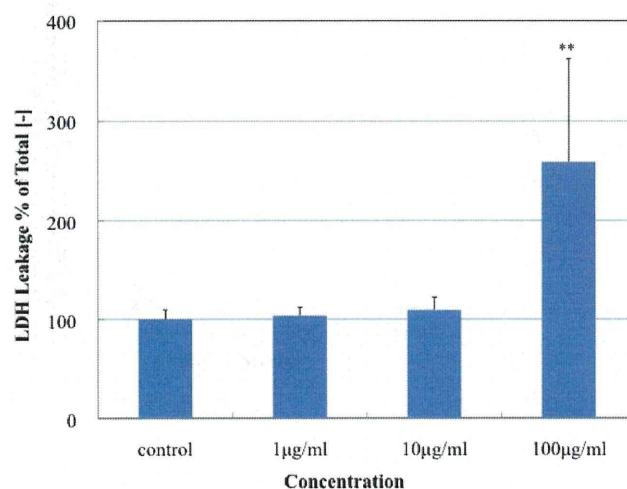
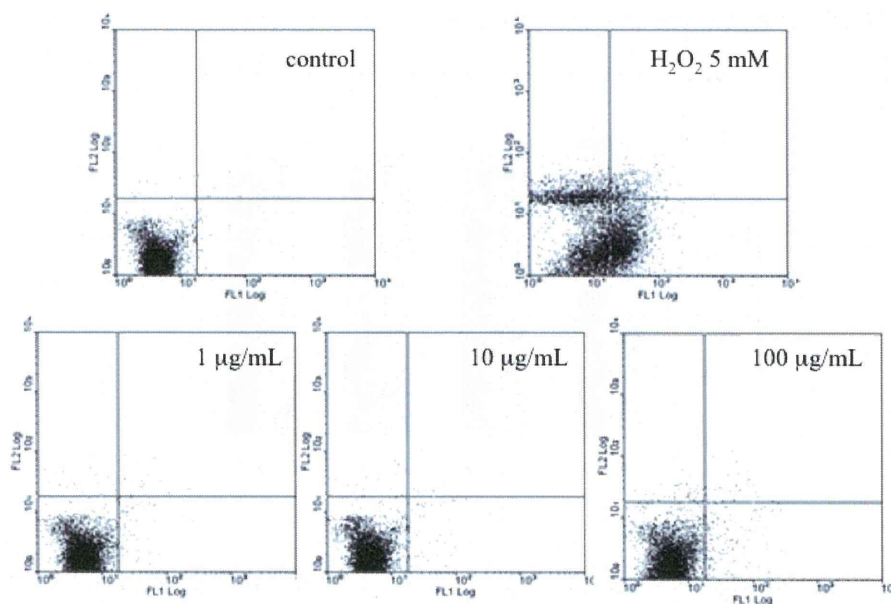
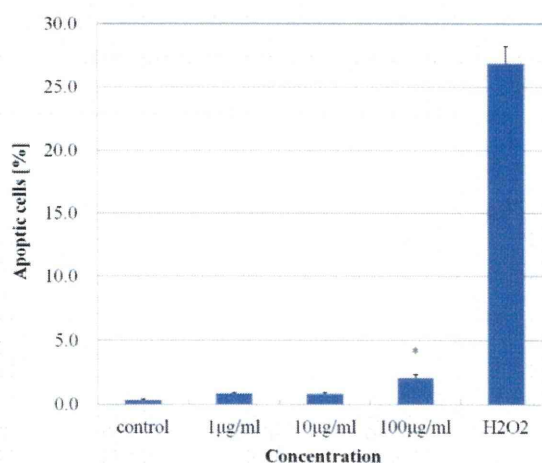


Figure 5. Effect of Fe₃O₄ magnetic nanoparticles (MgNPs-Fe₃O₄) on apoptosis in A549 cells. A549 cells were treated with 0 (control), 1, 10 or 100 µg/mL MgNPs-Fe₃O₄ for 24 h; cells were treated with 5 mM H₂O₂ for 24 h as a positive control. Apoptosis of A549 cells treated with MgNPs-Fe₃O₄ or H₂O₂ was determined by flow cytometry based on propidium iodide/Annexin V staining patterns; (a) Representative flow cytometry of one set of triplicate experiments; (b) Percentages of apoptotic cells from flow cytometry analysis. Apoptotic cells include early apoptotic cells (AnnexinV+/PI-) and late apoptotic or necrotic cells (AnnexinV+/PI+). Data are presented as the mean ± SD of three independent experiments. * *p* < 0.05 vs. control.

(a)



(b)



2.4. Effect of MgNPs-Fe₃O₄ on ROS Production, Intracellular Glutathione, and 8-OH-dG Levels in DNA

As shown in Figure 6, MgNPs-Fe₃O₄ caused a dose-dependent increase in ROS production with concentrations of 10 and 100 µg/mL. Figure 7 demonstrates that MgNPs-Fe₃O₄ caused a dose-dependent decrease in the GSH level; GSH was reduced by 65% with 100 µg/mL MgNPs-Fe₃O₄. The 8-OH-dG levels were increased approximately 8- and 14-fold above control with 10 and 100 µg/mL MgNPs-Fe₃O₄, respectively (Figure 8). ROS production by MgNPs-Fe₃O₄ is well known to be involved in the cytotoxic response in various cell types. Fe₃O₄, a mixture of FeO and Fe₂O₃, is unstable and can readily undergo oxidation to yield γ -Fe₂O₃ + Fe²⁺ [7,9,16]. The free Fe²⁺ ions can react with hydrogen peroxide and oxygen produced by the mitochondria to produce highly reactive hydroxyl radicals and Fe³⁺ ions [17] that can damage DNA, proteins, polysaccharides, and lipids *in vivo*. Similar to our findings, previous studies have shown that Fe₃O₄ elicited an increase in oxidative DNA lesions in A549 cells with minimal effect on cell viability [7–9]. Of the non-enzymatic antioxidants, GSH represents the major intracellular redox buffer in all cell types. Abundant in all cell compartments, it constitutes the first line of the cellular defense mechanism against oxidative injury [16]. Previous studies demonstrated that ROS generation following GSH depletion caused mitochondrial damage and up-regulation of pro-apoptosis mediators [2,7,18]. We found MgNPs also significantly reduced the GSH level. However, our data suggest that the shift in balance toward pro-oxidant mechanisms exerts little impact on cell viability.

Figure 6. Effect of MgNPs-Fe₃O₄ on production of reactive oxygen species (ROS) by A549 cells. A549 cells were treated with doses 0 (control), 1, 10 or 100 µg/mL MgNPs-Fe₃O₄ for 24 h. ROS production was determined using the CM-H₂DCFDA assay. Data are presented as the mean ± SD of three independent experiments. ** $p < 0.01$ vs. control.

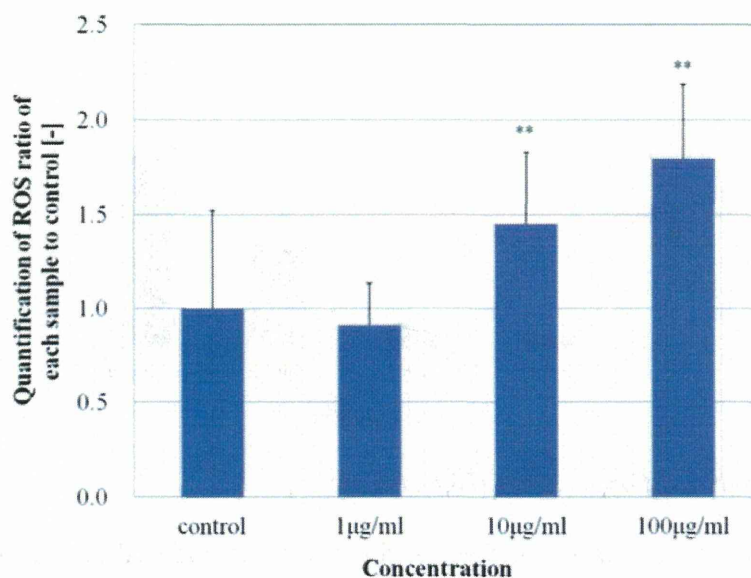


Figure 7. Effect of MgNPs-Fe₃O₄ on intracellular glutathione (GSH) levels in A549 cells. A549 cells were treated with 0 (control), 1, 10 or 100 µg/mL MgNPs-Fe₃O₄ for 24 h. GSH levels were assessed by luciferin bioluminescence (GSH-Glo Glutathione Assay Kit). Data are presented as the mean ± SD of three independent experiments. Significantly different from the untreated control at ** $p < 0.01$ vs. control.

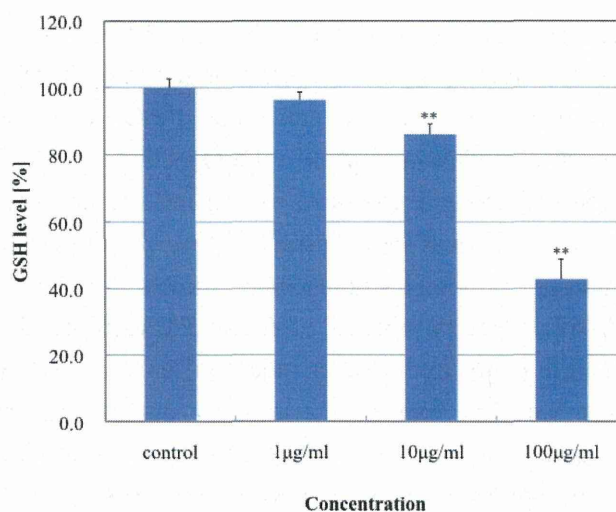
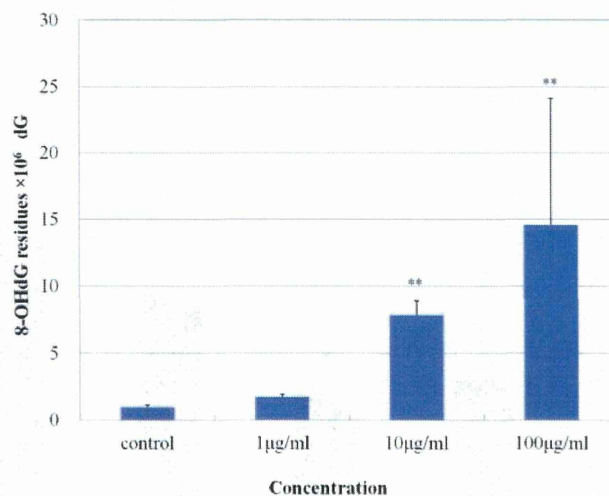


Figure 8. Effect of MgNPs-Fe₃O₄ on 8-hydroxy-deoxyguanosine (8-OH-dG) levels in A549 cells. A549 cells were treated with 0 (control), 1, 10 or 100 µg/mL MgNPs-Fe₃O₄ for 72 h. DNA was extracted by the sodium iodide method; 8-OH-dG levels were determined using HPLC-ECD. Data are presented as the mean ± SD of three independent experiments. * Significantly different from the untreated control at ** $p < 0.01$ vs. control.

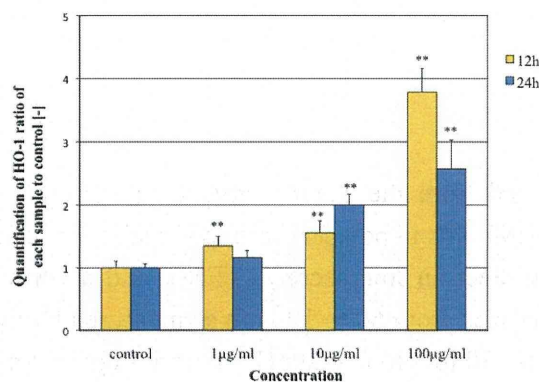


2.5. Expression of the Heme Oxygenase-1 (HO-1) Gene

As shown in Figure 9, the transcript level of the *HO-1* was induced in a dose-dependent manner after 12 and 24 h of MgNPs-Fe₃O₄ exposure, however its transcription level at 100 mg/mL exposure after 24 h was reduced compared to after 12 h. Oxidative stress is caused by an imbalance in the level

of ROS and a biological system's ability to detoxify the reactive intermediates [16]. Cells possess both enzymatic and non-enzymatic mechanisms to counterbalance the cytotoxicity and genotoxicity caused by ROS [16]. In the lungs, the major enzymatic antioxidants are superoxide dismutases (SODs), catalase, and glutathione peroxidase (GSH-Px); others include those examined in this study, HO-1, thioredoxin (TR), and glutaredoxin (GLRX). HO-1 is involved in playing a major role in degradation of heme to biliverdin, but has recognized potent anti-inflammatory and anti-apoptotic effects [17,19]. HO-1 is induced mainly at the transcriptional level by oxidative stress, pro-inflammatory mediators, and some growth factors [18]. HO-1 mRNA expression is known to mediate antioxidant and cytoprotective effects and has been considered useful as a marker for particle-induced oxidative stress. Park *et al.* [20] showed that treatment of a human bronchial epithelial cell line with TiO₂-MgNPs for four hours caused dose-dependent increases in mRNA expression of HOG-1, glutathione-*S*-transferase, and catalase; mRNA expression level of HO-1 had returned to baseline by 24 h [20]. Napierska *et al.* [21] also showed a marked induction of HO-1 mRNA in the endothelial cell at six hours after treatment of SiO₂-NPs, but reduction of HO-1 mRNA at 24 h. Our results appear to be same as these two studies.

Figure 9. Effect of MgNPs-Fe₃O₄ on mRNA expression of the *HO-1* gene in A549 cells. The expression level of the *HO-1* was normalized according to the expression level of β -actin. Data are presented as the mean \pm SD of three independent experiments. ** $p < 0.01$ vs. control.

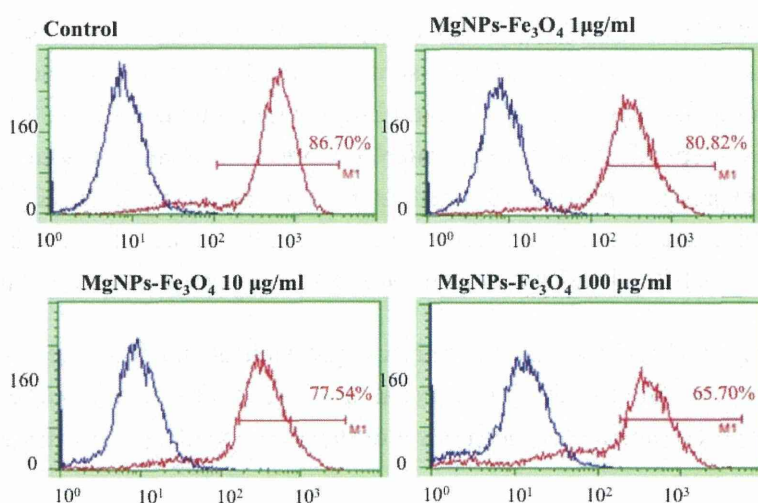


2.6. Effect of MgNPs-Fe₃O₄ on CD44⁺ Cell Fraction in A549 Cells

MgNPs-Fe₃O₄ caused a dose-dependent reduction in the CD44⁺ subpopulation (Figure 10). CD44 is a cell surface glycoprotein that mediates cellular adhesion to the extracellular matrix and is involved in multiple processes, including inflammation, cell migration, signaling, and tumor metastasis [13,22]. CD44 is up-regulated in the damaged epithelium of asthma patients, and is believed to be involved in tissue repair by localizing chemokines and growth factors to the disrupted epithelium [23]. CD44 is also a marker of certain cancer stem cells [24], in which it functions to defend cancer cells against oxidative stress by increasing GSH synthesis [25]. CD44 has also been reported to be involved in the protective effect of hyaluronate on constitutive DNA damage by ROS in A549 cells [26]. Consistent with the previously noted reduction in GSH and increase in 8-OH-dG levels, we found that MgNPs-Fe₃O₄ markedly decreased the CD44⁺ cell fraction of A549 cells. Thus, these results highlight another mechanism by which MgNPs-Fe₃O₄ impair redox control and damage DNA in A549 cells. Our

results also offer the possibility that CD44 may be a marker MgNP-Fe₃O₄-induced cytotoxicity; however, further study is warranted.

Figure 10. Effect of Fe₃O₄ magnetic nanoparticles (MgNPs-Fe₃O₄) on CD44⁺ cell fraction in A549 cells. A549 cells were treated with 0 (control), 1, 10 or 100 µg/mL MgNPs-Fe₃O₄ for 24 h. Cells were labeled with mouse anti-human CD44 monoclonal antibody; level of CD44⁺ cells was determined by flow cytometry.



3. Experimental Section

3.1. MgNPs-Fe₃O₄

MgNPs-Fe₃O₄ were obtained from the Toda Kogyo Corporation (Otake, Hiroshima, Japan). As specified by the manufacturer, MgNPs in powder were spherical, with an average particle size of 10 nm, as measured by transmission electron microscopy (TEM), and a surface area of 100–120 m²/g. In suspension, the particle size ranged from 60 to 100 nm as measured by dynamic light scattering (DLS) and zeta potential ranged from –30 to –40 mV at pH 10. Bare MgNPs-Fe₃O₄ were used in this study.

3.2. Preparation of MgNPs-Fe₃O₄ in Culture Medium

MgNPs-Fe₃O₄ were sterilized by ultraviolet (UV) irradiation and suspended in phosphate-buffered saline (PBS), Ham's F-12 alone, and Ham's F-12 medium containing 10% fetal bovine serum (FBS) and 100 U/mL penicillin-streptomycin to yield a concentration of 1, 10 or 100 µg/mL. Suspensions were sonicated at 30 W for 10 min using an Ultrasonic HomogenizerVP-050 (TAITAEC, Koshigaya, Saitama, Japan).

3.3. Cell Line

A549 human lung epithelial cells were purchased from American Tissue Type Culture Collection (Manassas, VA, USA). Cells were incubated in Ham's F-12 Medium with 10% fetal bovine serum

(FBS) and 100 U/mL penicillin–streptomycin in 5% CO₂ at 37 °C. Cells were maintained at a density of 60%–70% confluence in 100 mm dishes, and used in log-phase of growth.

3.4. Characterization of MgNP-Fe₃O₄ Suspensions in Cell Culture Medium

The average hydrodynamic size and size distribution of MgNPs-Fe₃O₄ in cell culture media and their intracellular localization were determined by DLS using a Fiber-Optics Particle Analyzer FPAR-1000 (Otsuka Electronics, Hirakata, Osaka, Japan). MgNPs-Fe₃O₄ were suspended in Ham's F-12 Medium with or without 10% FBS and supplements, or in phosphate-buffered saline (PBS).

3.5. Cellular Uptake of MgNPs-Fe₃O₄ in A549 Cells

The cellular uptake of MgNPs-Fe₃O₄ in A549 cells was analyzed as follows.

3.5.1. Transmission Electron Microscopy (TEM)

A549 cells were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) at 4 °C for 4 h. Samples were post-fixed with 2% osmium tetroxide at 4 °C for 2 h, dehydrated, and embedded in epoxy resin. Ultrathin sections (80 nm) were then stained with uranyl acetate and lead citrate, and observed by TEM.

3.5.2. Flow Cytometry Assay

A549 cells were treated with 0, 1, 10 or 100 µg/mL MgNPs-Fe₃O₄ for 24 h, and then trypsinized and suspended in medium. Cellular uptake of MgNPs-Fe₃O₄ was analyzed using flow cytometry (Millipore, Billerica, MA, USA), in which the intensities of forward-scattered (FS) and side-scattered (SS) light are proportional to cell size and intracellular density of MgNPs-Fe₃O₄, respectively. A total of 30,000 cells were measured per sample.

3.6. AlamarBlue Assay

Cell viability was determined using the alamarBlue assay (Alamar Biosciences, Sacramento, California, USA) according to the manufacturer's instructions. Briefly, cells (1.0×10^4 cells/well) were incubated with MgNPs-Fe₃O₄ (0, 1, 10 or 100 µg/mL) for 72 h at 37 °C. AlamarBlue (10%) was added to each well and incubated for 200 min. Metabolically active cells reduced the dye to a fluorescent form, which was measured using a plate reader (excitation/emission: 570 nm/600 nm; Viento XS, DS Pharma Biomedical, Suita, Osaka, Japan). Cell viability was determined by linear interpolation of the emission from cells treated with 0.1% saponin (0% viability) and untreated cells (100% viability).

3.7. Lactate Dehydrogenase (LDH) Release Assay

LDH release assay to assess membrane integrity was performed using LDH-cytotoxicity assay kit (BioVision, CA, USA) according to the manufacturer's instructions. Cells cultured in 24-well plates (1.5×10^4 cells/well) were treated with 0, 1, 10 or 100 µg/mL MgNPs-Fe₃O₄ for 24 h at 37 °C. Plates were then centrifuged at 250× g for 5 min. The supernatant of each well was transferred to a fresh, flat