Dose-, treatment- and time-dependent toxicity of superparamagnetic iron oxide nanoparticles on primary rat hepatocytes

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Aim: As a first study in literature, to investigate concentration-dependent (0–400 µg/ml) and exposure-dependent (single dosing vs cumulative dosing) effects of superparamagnetic iron oxide nanoparticles (d = 10 nm) on primary rat hepatocytes in a time-dependent manner. **Materials & methods:** Sandwich-cultured hepatocyte model was used to evaluate viability, hepatocyte specific functions and reactive oxygen species level. **Results:** In terms of all parameters, generally statistically more significant effects were observed in a concentration- and time-dependent manner. In terms of hepatocyte-specific functions, cumulative dosing caused significantly ($p < 0.05$) more deleterious effects at 48th hour. **Conclusion:** A combination of various biomarkers should be employed for the evaluation of the effect of superparamagnetic iron oxide nanoparticles on liver, and each biomarker should be analyzed in a time- and exposure-dependent manner.

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Superparamagnetic iron oxide nanoparticles (SPION), which possess unique physicochemical features such as ultrafine sizes and a large surface area to mass ratio [1,2], are currently the only clinically approved metal oxide nanoparticles and the most commonly used superparamagnetic nanoparticles [2,3]. SPION have a vast variety of biomedical applications [1,4,5] including magnetic resonance imaging [6–8], targeted delivery of drugs [5,9] or genes [10,11], tissue engineering [12,13], hyperthermia of cancer [14,15], magnetic transfections [16,17], among others. This is despite the ban on some commercial SPION due to toxicity; ferumoxytol (Feraheme), ferumoxides, ferucarbotran, ferumoxtran-10, ferristene and ferumoxsil are SPION based magnetic resonance imaging contrast agents at current clinical trials [6–8]. In parallel to their increasing use in clinical applications and the vast variety of their biomedical applications, safety concerns about SPION have increased and to date, there is neither adequate information regarding their safety nor standardized method to establish such parameters [18–20]. Investigations on the influences of physicochemical properties of SPION on their interactions with tissues, uptake by lymphatic or blood components and correlations with toxicity or safety risks provide no consensus to date [21,22].

Recent report [20] indicates that iron oxide magnetic nanoparticles accumulate primarily in the liver and spleen on the first day postinjection. Thus, investigation of hepatotoxicity profile of SPION is critical to their successful translation into the clinic. To date, there are a few studies that reported controversial results related to SPION-induced liver toxicity, such as *in vivo* studies focusing on major organs including liver [23–26] and *in vitro* studies using hepatic cell lines, L02 [23] and HepG2 [2].

Use of animal models is a costly and sometimes inefficient method of generating toxicity data. In contrast, the use of *in vitro* models for toxicity testing has several advantages: it provides a significant reduction in the use of

animals, which is expensive due to maintenance and care costs; much smaller quantities of chemicals are required for testing; it can potentially shorten the time for assessing toxicity or adverse effects; it provides a much higher throughput allowing the rapid evaluation of multiple chemicals at various concentrations and their metabolites; it allows to study chemical metabolism, evaluate the mechanisms of toxicity, measure enzyme kinetics and examine dose–response relationships [27–29]. Thus, *in vitro* toxicity tests for screening prior to animal studies are urgently required. While immortalized liver derived cell lines (Fa2N-4, HepG2, Hep3B, PLC/PRF5, Huh7, HBG and HepaRG) are commonly used for SPION toxicity testing, they present serious limitations: they do not possess phenotypic characteristics of the liver tissue; they do not illustrate consistent expression profile of genes involved in phase 1 and phase 2 metabolism between passages, and as a result, data can be difficult to interpret across laboratories and passages; they show much lower expression of liver-specific functions on average compared with primary hepatocytes; they represent a phenotype from a single donor, which reduces their predictive value for the human population [29]. Although the use of 3D systems with dynamic flow for primary cell and/or stem cell cultures appears to be promising for toxicological studies, further research is needed to establish whether they may provide increased benefit over traditional culture systems [29]. In this context, the sandwich-cultured hepatocyte model has proven to be a valuable *in vitro* system that maintains specific hepatic cytomorphology and function relevant to drug metabolism, disposition and toxicity, and closely resembles the *in vivo* setting for short-term and medium-term studies [29–32]. To the best of our knowledge, to date, there have been no reports in the literature that have assessed the SPION-induced toxicity on primary rat hepatocytes in a concentration-dependent manner; SPION concentration is more critical than any other factor, such as surface modification or size [33]. In addition, to the best of our knowledge, to date, there have been no reports in the literature that have assessed the SPION-induced toxicity on primary rat hepatocytes in terms of their exposure contrasting a single dose exposure with accumulated exposure. Given the fact that in many real-life cases and biomedical applications, nanoparticles accumulate and affect their target tissues over a long period of time [2,34–36]; we posit that this is an important aspect that has been overlooked.

In this study, our goal was to establish a method that can be used toward identifying toxicity, adverse effects and mechanism of action of SPION on the liver and its parenchymal cells, hepatocytes. Specifically, we have investigated the concentration-dependent (0–400 µg/ml) and exposure-dependent (single dosing vs cumulative dosing) effects of SPION (d = 10 nm) on primary rat hepatocytes in a time-dependent manner, which to the best of our knowledge has not been reported previously. To this end, we have used a sandwich-cultured hepatocyte model and evaluated the effects of SPION on the viability and hepatocyte-specific functions (the secretion of albumin and urea) with respect to time. In addition, to investigate the role of reactive oxygen species (ROS) in SPION-induced hepatotoxicity, we also studied the time-dependent ROS production upon treatment of primary rat hepatocytes with SPION in a concentration-dependent manner.

Materials & methods

Chemicals

Superparamagnetic magnetite (Fe₃O₄) nanoparticles (10 nm transmission electron microscopy [TEM]; magnetization: >45 emu/g, at 4500 Oe; 5 mg/ml in H₂O) were purchased from Sigma-Aldrich (MO, USA) and used as received. There is no reported toxicity in terms of all toxicity categories (acute toxicity; skin corrosion/irritation; serious eye damage/eye irritation; respiratory or skin sensitization; germ cell mutagenicity; carcinogenicity [except from expression from the International Agency for Research on Cancer]; reproductive toxicity; specific target organ toxicity – single exposure; specific target organ toxicity – repeated exposure; aspiration hazard) on the ‘safety data sheet’ of the product [37]. We note that while these are not medical grade SPION that are used in the clinic, we used them here to develop an effective *in vitro* system and identify markers to detect toxicity induced by SPION. Further studies with medical grade SPION need to be conducted in the future to confirm the findings of the current study. PrestoBlue cell viability reagent was purchased from Thermo Fisher Scientific (MA, USA); RedoxSensor™ Red CC-1 was purchased from Thermo Fisher Scientific; Williams’ Medium E and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich; L-glutamine was purchased from Thermo Fisher Scientific. Penicillin/streptomycin was purchased from Invitrogen (CA, USA); albumin ELISA kit and urea colorimetric assay were purchased from Abcam (MA, USA) and Stanbio Laboratory (TX, USA), respectively.

Rat hepatocyte isolation & culture

Hepatocytes were isolated from adult female Lewis rats (Charles River Laboratories, MA, USA) weighing 180–200 g, as described previously [38,39]. Routinely, 200–300 million cells with 90–95% viability (determined by trypan blue exclusion) were isolated, and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, CA, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich), 0.5 U/ml insulin, 7 ng/ml glucagon, 20 ng/ml EGF, 7.5 µg/ml hydrocortisone, 200 U/ml penicillin, 200 µg/ml streptomycin and 50 µg/ml gentamycin. Rat hepatocytes were seeded into 12-well tissue culture plates and covered with a collagen gel (bottom coating: 1 ml/well [nine parts phosphate buffered saline [PBS]/one part collagen]; top gel: 200 µl/well [nine parts collagen/one part 10 × DMEM]), as described previously [30].

Cell viability assay

Cells at a density of 100,000/cm² were plated into 12-well tissue culture plates (Corning, NY, USA) and stabilized for 24 h before application of the top gel. After 48 h, following the top-gel application, viability of the cultured cells was determined (540 nm excitation; 590 nm emissions) (Figure 1) by PrestoBlue cell viability reagent (Thermo Fisher Scientific) that quickly, sensitively and quantitatively evaluates the mitochondrial conversion of resazurin to resorufin by accepting electrons from NADPH, FADH, FMNH, NADH and cytochromes [40]. PrestoBlue reagent (10×) was added to each well by diluting (1×) in the culture medium. PrestoBlue assay allows practical analyses (contains ready to use aqueous solution and fewer protocol steps) and also allows time course experiments in the same plate (as explained in the legend of the Figure 1); therefore, we preferred this assay.

Experimental & control groups

Immediately, following the viability measurement, three different SPION concentration groups (100, 200 and 400 µg/ml) were introduced to nine wells (three wells for each concentration) of the 12-well tissue culture plates in one-shot style (100, 200 and 400 µg/ml concentrations were applied once at 0 h) or cumulative style (100/3, 200/3 and 400/3 µg/ml were applied in 4-h intervals, i.e., 0, 4 and 8 h). In each 12-well tissue culture plate (one plate for one-shot style and the other for cumulative style), three wells were for the control group with no SPION treatment. Viability of the SPION-treated and control wells was measured at 24 and 48 h after the start of SPION treatment (Figure 1). Five different experiments were performed in triplicate in five different weeks; in each experiment, primary hepatocytes used for one-shot treatment group and cumulative treatment group were isolated from the same female Lewis rat. From the PrestoBlue assay results, LD₅₀ (the lethal dose that causes death in 50% of the test samples) values were calculated.

Live/dead assay

To visualize the dose-dependent effects of SPION on cell viability of primary rat hepatocytes, a fluorescent live/dead assay was performed at the seventh day after the start of treatment with iron oxide nanoparticles using a live/dead viability/cytotoxicity kit (L-3224; Invitrogen, NY, USA). Briefly, primary hepatocytes were washed with PBS and incubated at 37°C for 30 min with assay reagent (4 µM EthD-1 and 2 µM Calcein in PBS). The cells were washed three-times with PBS and viewed with an Axiovert 40 CFL (Zeiss, Germany) and X-Cite 120Q (Lumin Dynamics, Ontario, Canada).

Albumin ELISA & urea assay

Media was collected from all the wells at the start of the SPION treatment (prior to PrestoBlue viability assay), and 24 and 48 h after the start of treatment with SPION (Figure 1), and stored at -80°C until analyses for albumin and urea secretion, as described previously [41].

Albumin analysis

To measure the amount of albumin production, an in-house developed inverse competitive ELISA method was used. In each well of 96-well high-binding plates, 100 µl of 50 µg/ml albumin (MP Biomedicals LLC, CA, USA) was plated and the plates were sealed and kept at 4°C overnight for the albumin to bind. The plates were washed four-times using PBS-Tween and 50 µl of both samples and standards (100–0 µg/ml) were introduced as duplicates in separate wells. Albumin antibody (~2 µg/ml) was introduced immediately to provide competition for the antibodies between the bound albumin on the plate and albumin in samples, and then the plates were sealed again and incubated at 37°C for 90–120 min. The plates were washed using PBS-Tween, and by introducing to

Figure 1. Schematic representation of the experimental protocol excluding reactive oxygen species generation. All steps between T = 0 h and T = 240 h were made with the same two 12-well plates (one for one-shot group, one for cumulative group) for each of the five different experiments. SPION: Superparamagnetic iron oxide nanoparticle.

wells at regular intervals the plates were developed with an o-phenylenediamine dihydrochloride (OPD) solution containing H_2O_2 , then by introducing to wells at the same intervals the reaction was stopped with 8N H_2SO_4 . Using dual readings at 490 and 650 nm with a Benchmark Plus microplate reader (Biorad Inc., CA, USA), the absorbance at each well was analyzed. At last, standard curves were constructed for each plate, and sample concentrations were calculated accordingly.

Urea analysis

To measure the amount of urea production, a colorimetric urea detection assay (Stanbio Inc., TX, USA) was used. Following the manufacturer's instructions, in clear 96-well assay plates, 10 μ l of both samples and standards (200–0 μ g/ml) were introduced along with 150 μ l of blood/urea/nitrogen (BUN) reagent mixture. Then the plates were sealed tightly and incubated at 60°C for 90 min. After the plates were cooled on ice for 20 min, absorbances of the samples were read at 520 and 650 nm with the Benchmark Plus microplate reader (Biorad Inc.). At last, standard curves were constructed for each plate and sample concentrations were calculated accordingly.

ROS generation

Primary rat hepatocytes at a density of 100,000/cm² were plated into six-well tissue culture plates (Corning). Cell ROS experiments were started one day after cell seeding. Red CC-1 was dissolved in DMSO to make a stock solution at the concentration of 5 mM. Nanoparticle solutions were prepared at three different concentrations of 100, 200 and 400 μ g/ml. Cultured rat hepatocytes were washed by Williams' Medium E supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin (WME for short, similarly hereinafter). Then cells were labeled with Red CC-1 stain by incubating the cells with 5 μ M Red CC-1 at 37°C for 15 min. After being washed by WME, 1 ml of WME (for the control group) and 1 ml of nanoparticle solutions of 100, 200 and 400 μ g/ml were added to the wells, respectively. Then the plate was placed on microscope for the time-lapse experiment.

Time-lapse experiments were assessed by Zeiss Axiovert 200 M inverted fluorescence microscope with a heating unit and temperature control. The temperature was kept at 37°C. Five locations per well were chosen for image recording. Bright field and fluorescence images were recorded every 30 min at the same locations automatically. The image capture lasted for 18 h. The area integral intensity of the red fluorescence (indication of ROS levels) was calculated for each location and each time point using Image J software. All time points for each location are normalized relative to their own T = 0 reference frame and the results are expressed as relative ROS levels. Videos were made by the image sequences of each location. Five different experiments were performed in triplicate in five different weeks for all groups.

Statistical analysis

To determine whether there were any statistically significant differences between the means of the groups, one-way analysis of variance with Tukey honest significant difference test was used. To determine whether there were any statistically significant differences between the treatment styles (one-shot vs cumulative) for each concentration, unpaired one-tailed student *t*-test was used. Data were expressed as the mean $\pm$ standard deviation from five independent experiments with three replicates. For all the statistical analyses, as the threshold for significance, *p* < 0.05 was used.

Results

Cell viability

The cytotoxicity of SPION was assessed by PrestoBlue assay (Thermo Fisher Scientific) (Figure 2). From the PrestoBlue assay results, for 48th hour after the start of treatment (the range of concentrations from 100 to 400 μ g/ml with 24-h exposures is reflective for the LD₅₀ data at 48th hour after the start of treatment as seen in Figure 2), LD₅₀ values were calculated as 328.51 $\pm$ 18.25 and 319.79 $\pm$ 25.73 for one-shot and cumulative treatment styles, respectively.

Live/dead assay

To visualize the dose-dependent effects of SPION on cell viability of primary rat hepatocytes, live/dead assay was performed with DAPI (blue) counterstain at the seventh day after the start of treatment with iron oxide nanoparticles (no treatment for control groups); and live (green) and dead (red) cells were observed (Figure 3).

Albumin ELISA & urea assay

The effect of dose-dependent SPION treatment on the liver-specific function of primary rat hepatocytes was assessed by analyzing albumin (Figure 4A & B) and urea (Figure 4C & D) synthesis.

Figure 2. Cell viability assay of the primary rat hepatocytes for 24th and 48th hours after the start of treatment (A: One-shot; B: Cumulative) with SPION in three different concentrations (100 µg/ml, 200 µg/ml and 400 µg/ml). Y axis values of the bars: (A) 1st Day: 100 µg/ml: 92.49 ± 3.46; 200 µg/ml: 86.69 ± 4.85; 400 µg/ml: 78.39 ± 6.84; 2nd Day: 100 µg/ml: 96.28 ± 3.90; 200 µg/ml: 82.69 ± 5.42; 400 µg/ml: 35.75 ± 2.47. (B) 1st Day: 100 µg/ml: 91.55 ± 3.88; 200 µg/ml: 85.94 ± 4.38; 400 µg/ml: 77.03 ± 8.88; 2nd Day: 100 µg/ml: 92.46 ± 3.79; 200 µg/ml: 81.05 ± 4.26; 400 µg/ml: 34.38 ± 3.83. The values are the mean (percent of control group) ± standard deviation of five different samples (the methodology used to convert spectrophotometric values to percentages (percent of control group) has been described in the Supplementary File). Significant difference with respect to control is denoted as *p value < 0.05 and **p value < 0.01.

ROS generation

Accumulating evidence strongly suggests that metal oxide nanoparticles induce toxicity that is primarily mediated by increased ROS production [2,25,42,43]. Therefore, ROS generation in primary rat hepatocytes upon treatment with SPION in three different concentrations was investigated (Figure 5). Here we focused on total ROS generation as a major factor of SPION-induced hepatotoxicity, rather than one or more of the elements of total ROS generation using the Red-CC1 assay kit and time lapse experiments that allow quantitative assessment of ROS generation and accumulation over time.

Discussion

In the current study, considering time factor, we investigated dose- and treatment-dependent toxicity of SPION having a vast variety of biomedical applications.

Dose-dependent toxicity

According to the cell viability, as expected, when the concentration of the SPION increased, the viability of the primary rat hepatocytes decreased. Both in one-shot and cumulative treatment groups, statistically significant effects on the viability of the primary rat hepatocytes were observed upon treatment with SPION with 200 µg/ml ($p < 0.05$) and 400 µg/ml ($p < 0.01$) at the 24th hour after the start of treatment. In addition, both in one-shot and cumulative treatment groups, compared with the 24th hour, more dramatic effects were observed at 48th hour (Figure 2). There was no statistically significant difference ($p > 0.05$) between LD₅₀ values of one-shot and cumulative treatment groups.

When the representative live/dead images obtained at the seventh day after the start of treatment (Figure 3) are compared with quantitative viability results obtained at the 24th and 48th hours after the start of treatment (Figure 2), it can be inferred that damaging effect of SPION on primary rat hepatocytes is progressive. While at 48th hour, nearly 65% of the primary rat hepatocytes treated with 400 µg/ml of SPION died both in one-shot and cumulative treatment groups (Figure 2); at the seventh day, nearly all of the hepatocytes treated with 400 µg/ml of SPION died both in one-shot and cumulative treatment groups (Figure 3). The mechanisms of the staining of the primary rat hepatocytes by Ethd-1, Calcein AM and DAPI can be used to interpret the images in Figure 3, thus

Figure 3. Live/dead assay at the 7th day after the start of treatment with SPION. Upper panel: One-shot; Lower panel: Cumulative. **(A):** No treatment; **(B):** 100 µg/ml of SPION; **(C):** 200 µg/ml of SPION; **(D):** 400 µg/ml of SPION. Green: Live cells; Red: Dead cells; Blue: DNA/nuclei of the cells; Image scale bar: 20 µm. SPION: Superparamagnetic iron oxide nanoparticle.

Figure 4. The effect of SPION on the liver-specific function of primary rat hepatocytes. Albumin (A & B) and urea (C & D) assays to quantify the specific function of the primary rat hepatocytes for 24th and 48th hours after the start of treatment (A & C: one-shot; B & D: cumulative) with superparamagnetic iron oxide nanoparticles in three different concentrations (100, 200 and 400 µg/ml). Y-axis values of the bars: (A) First day: 100 µg/ml: 78.20 ± 7.29 ; 200 µg/ml: 59.65 ± 8.50 ; 400 µg/ml: 32.39 ± 4.78 ; Second day: 100 µg/ml: 83.20 ± 7.59 ; 200 µg/ml: 46.42 ± 8.39 ; 400 µg/ml: 15.19 ± 4.92 ; (B) First day: 100 µg/ml: 71.79 ± 5.81 ; 200 µg/ml: 53.80 ± 4.79 ; 400 µg/ml: 37.01 ± 3.31 ; Second day: 100 µg/ml: 73.77 ± 4.81 ; 200 µg/ml: 31.34 ± 2.17 ; 400 µg/ml: 7.66 ± 0.60 ; (C) First day: 100 µg/ml: 121.56 ± 9.21 ; 200 µg/ml: 115.72 ± 3.56 ; 400 µg/ml: 118.81 ± 6.66 ; Second day: 100 µg/ml: 103.10 ± 5.85 ; 200 µg/ml: 85.91 ± 7.59 ; 400 µg/ml: 68.75 ± 7.42 ; (D) First day: 100 µg/ml: 114.20 ± 4.55 ; 200 µg/ml: 125.82 ± 5.71 ; 400 µg/ml: 125.56 ± 7.33 ; Second day: 100 µg/ml: 88.05 ± 3.78 ; 200 µg/ml: 98.78 ± 6.52 ; 400 µg/ml: 57.46 ± 3.48 . The values are the mean (percent of control group) $\pm$ standard deviation of five different samples (the methodology used to convert spectrophotometric values to percentages [percent of control group] has been described in the Supplementary File). Significant difference with respect to control is denoted as **p < 0.01. SPION: Superparamagnetic iron oxide nanoparticle.

can be used to clarify the mechanism of toxicity induced by SPION. The polyanionic dye calcein is well retained within live cells and gives out green fluorescence upon the enzymatic conversion of the virtually nonfluorescent cell-permeant calcein AM to the intensely fluorescent calcein by intracellular esterases (only live cells have active esterases). EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of a bright red fluorescence upon binding to nucleic acids in dead cells (EthD-1 cannot penetrate the intact plasma membrane of live cells). DAPI, a popular nuclear and chromosome counterstain, emits blue fluorescence upon binding to AT regions of DNA; due to its cell impermeant feature, higher concentrations are needed to enter live cells [44].

Figure 5. The effect of SPION on ROS generation in primary rat hepatocytes. The state of oxidative stress in primary rat hepatocytes upon treatment with SPION in three different concentrations (100, 200 and 400 $\mu\text{g/ml}$) for a duration of 18 h (A); and for specific time points within the 18-h time-lapse experiment as a percent of control (B). Y-axis values of the bars: 30th minute: 100 $\mu\text{g/ml}$: 114.44 $\pm$ 1.38; 200 $\mu\text{g/ml}$: 114.97 $\pm$ 1.52; 400 $\mu\text{g/ml}$: 118.02 $\pm$ 3.53; 150th minute: 100 $\mu\text{g/ml}$: 148.16 $\pm$ 8.53; 200 $\mu\text{g/ml}$: 156.04 $\pm$ 12.83; 400 $\mu\text{g/ml}$: 191.67 $\pm$ 15.38; 210th minute: 100 $\mu\text{g/ml}$: 145.64 $\pm$ 7.65; 200 $\mu\text{g/ml}$: 156.64 $\pm$ 12.31; 400 $\mu\text{g/ml}$: 204.65 $\pm$ 16.41; 480th minute: 100 $\mu\text{g/ml}$: 155.52 $\pm$ 3.57; 200 $\mu\text{g/ml}$: 184.29 $\pm$ 6.81; 400 $\mu\text{g/ml}$: 346.60 $\pm$ 10.89; 1050th minute: 100 $\mu\text{g/ml}$: 153.10 $\pm$ 2.14; 200 $\mu\text{g/ml}$: 226.58 $\pm$ 9.39; 400 $\mu\text{g/ml}$: 245.40 $\pm$ 7.90. The values are the mean $\pm$ standard deviation (SD) (A), and the mean (percent of control group) $\pm$ SD (B) of five different samples. Statistically significant difference with respect to control for a duration of 18 h (A), and for specific time points within the 18-h time-lapse experiment (B) is denoted as ** $p < 0.01$; statistically significant differentiation time points (150th, 210th and 480th minutes) between two different concentrations in treatment groups is denoted as * $p < 0.05$ (e.g., the statistically significant differentiation [$p < 0.05$] between 100 $\mu\text{g/ml}$ of SPION-treated group and 400 $\mu\text{g/ml}$ of SPION-treated group started at 150th minute) (B).

ROS: Reactive oxygen species; SPION: Superparamagnetic iron oxide nanoparticle.

While, for both one-shot and cumulative treatment groups, the obtained dose-dependent images are consistent with the mentioned mechanisms in Ethd-1 and calcein AM channels, they are not consistent with the mentioned mechanisms in DAPI channel. Due to live cell semi-impermeant feature of DAPI, the images belonging to primary rat hepatocytes treated with 400 $\mu\text{g/ml}$ of SPION should have the most intense DAPI signal and the images belonging to control groups should have the faintest DAPI signal. Then this situation deserves further explanation; we hypothesize that the situation results from the binding mechanism of DAPI to DNA. DAPI binds to minor groove of DNA with a preference for AT-rich sequences [45,46] as a consequence of three factors: the intrinsically narrower minor groove in AT regions than in GC regions of DNA, leading to a snug fit of the flat aromatic drug rings between the walls of the groove; the more negative electrostatic potential within the minor groove in AT regions, attributable in part to the absence of electropositive- NH_2 groups along the floor of the groove; and the steric advantage of the absence of those same guanine- NH_2 groups, thus permitting DAPI molecule to sink deeper into the groove [47]. Thus, DAPI staining pattern provides a mixture of information on both DNA structure and DNA sequence [48]. Therefore, we hypothesize that increase in SPION concentration resulted in DNA damage due to mutation(s) leading to a decrease in the proportion of AT-rich sequences in DNA, and thus, leading to deteriorations in the above-mentioned three factors that are crucial for binding of DAPI to DNA. And further studies are needed to clarify this issue. On the other hand, the other DNA binding dye used in live/dead assay, Ethd-1, is not a sequence-selective dye and it can strongly bind to dsDNA (one molecule of EthD-1 binds per four base pairs in dsDNA), ssDNA, RNA, oligonucleotides and triplex nucleic acid structures in dead cells [44]. Therefore, DNA damage did not prevent the binding of Ethd-1 to DNA, and as expected, Ethd-1 could give stronger signal with the increase in concentration of treated SPION.

Albumin and urea are considered to be the two of the most reliable markers for assessing the metabolic competency of the liver [49]. The current study also revealed the loss of hepatocyte-specific functions, albumin and urea synthesis, upon treatment of SPION in a dose-dependent manner (Figure 4). Albumin is synthesized primarily in the liver and it is the most abundant protein in plasma (at physiological concentrations of 40–50 g/l), constituting 50% of the total plasma protein content and accounting for 70% of plasma's oncotic pressure. Albumin's main function is to maintain colloidal osmotic pressure; it also neutralizes endogenous or exogenous toxins and transports metabolic products, regulatory mediators, nutrients and proteins [50,51]. Urea is the chief metabolic product of nitrogen metabolism in mammals, and the urea cycle is the metabolic pathway responsible for ammonia detoxification in which ammonia (formed in the breakdown of amino acids) is converted to urea, a less toxic and more water-soluble product which is easily excreted into urine [49,52]. Therefore, the above-mentioned results (Figure 4) illustrate that in parallel with increase in concentration of applied SPION, the metabolic pathways containing albumin and ammonia detoxification will be more seriously impaired.

Consistently with the accumulating evidence in the literature [2,25,42,43], we demonstrated that SPION exposure resulted in ROS production in primary rat hepatocytes in a dose-dependent manner (Figure 5). Highly significant differences ($p < 0.01$) were found between control and test groups (control vs 100 $\mu\text{g/ml}$ of SPION-treated group; control vs 200 $\mu\text{g/ml}$ of SPION-treated group; and control vs 400 $\mu\text{g/ml}$ of SPION-treated group) and also found among treatment groups (100 $\mu\text{g/ml}$ of SPION-treated group vs 200 $\mu\text{g/ml}$ of SPION-treated group; 100 $\mu\text{g/ml}$ of SPION-treated group vs 400 $\mu\text{g/ml}$ of SPION-treated group; and 200 $\mu\text{g/ml}$ of SPION-treated group vs 400 $\mu\text{g/ml}$ of SPION-treated group) (Figure 5A). However, when the data belonging to specific time points within 18 h were analyzed, the situation was not as simple as the analyses of the data belonging to whole 18-h experiment. Interestingly, all concentrations of SPION-induced highly significant ($p < 0.01$) ROS production at 30th minute compared with control group. However, there was no statistically significant difference ($p > 0.05$) among the treatment groups at the 30th minute (Figure 5B). The further analyses illustrated that statistically significant differentiation ($p < 0.05$) among treatment groups took place at certain time points within 18-h time-lapse experiment (Figure 5B). An interesting relationship between 200 $\mu\text{g/ml}$ of SPION-treated group and 400 $\mu\text{g/ml}$ of SPION-treated group was observed in terms of dose-dependent ROS response. While there were highly significant differentiations ($p < 0.01$) between 200 $\mu\text{g/ml}$ of SPION-treated group and 400 $\mu\text{g/ml}$ of SPION-treated group within 18 h (e.g., 480th minute as clearly seen in Figure 5B), the differentiation largely decreased toward the end of the experiment. From the 960th minute (237.63 ± 10.67 and $262.65 \pm 8.77\%$ of control upon treatment with SPION with 200 and 400 $\mu\text{g/ml}$, respectively) to the end of the time-lapse experiment at the 1050th minute, there was no statistically significant ($p > 0.05$) differentiation between the two treatment groups in terms of ROS production (the last statistically significant [$p < 0.05$] difference between the two treatment groups was at the 930th minute; 241.42 ± 10.83 and $269.49 \pm 9.02\%$ of control upon treatment with

SPION with 200 and 400 µg/ml, respectively) (Figure 5A & B). And, the mentioned ROS response behaviors of 200 µg/ml of SPION-treated group and 400 µg/ml of SPION-treated group are not consistent with the viability results of these groups. To determine whether there is a correlation between total ROS response and the viability of the primary rat hepatocytes, we calculated the relative total ROS response for each treatment group throughout the time-lapse experiment of 18 h. The ratio of the total ROS response of control: 100 µg/ml of SPION-treated group: 200 µg/ml of SPION-treated group: 400 µg/ml of SPION-treated group was 1:1.50:2.03:2.59. Thus, total ROS response throughout the time-lapse experiment of 18 h is not consistent with the viability results given in Figures 2 & 3.

Upon internalization of SPION via various intracellular uptake mechanisms including passive diffusion, receptor-mediated endocytosis, clathrin-mediated endocytosis, caveolin-mediated internalization and other clathrin and caveolin-independent endocytosis, the SPION are degraded into iron ions in the lysosomes [2,53,54]. This intracellular unbound 'free iron' can potentially cross the nuclear or mitochondrial membrane and the latter case is a major source of ROS production induced by SPION within a cell [2,55,56]. Superoxide ($O_2^{\bullet-}$), as a byproduct of oxidative phosphorylation, is produced from one-electron reduction of oxygen by the mitochondrial electron transport chain, and is converted to hydrogen peroxide (H_2O_2) by superoxide dismutases [55,56]. Unbound 'free iron' from SPION induces a wide range of ROS by serving as catalyst through the Fenton reaction (Equations 1 & 2, in which H_2O_2 is converted into highly reactive hydroxyl or superoxide radicals catalyzed by ferrous [Fe^{2+}]/ferric [Fe^{3+}] ions released from SPION) and the Haber–Weiss reaction (Equation 4, which is the net reaction of Fenton reaction [Equation 1] and the reaction 3 (in which superoxide [$O_2^{\bullet-}$] reduces the ferric ion [Fe^{3+}] released by the Fenton reaction) as shown below [2,34,55–59].

Ferric ion (Fe^{3+}) might also be reduced at varying reaction rates by a range of cellular reductants, such as glutathione, l-cysteine, NAD(P)H and FADH₂ (Equation 5) [58,60]. Fenton and Haber–Weiss reactions yield hydroxyl radical, which is by far the most ROS and is generally indicative of an overtly toxic event; $OH^{\bullet}$ reacts at a diffusion-controlled rate with biomolecules to form a series of additional free radicals (the thiyl radical [$RS^{\bullet}$] in Equation 6; a carbon-centred radical ($(R)_3C^{\bullet}$) in Equation 7; and the peroxy radical ($(R)_3COO^{\bullet}$) in Equation 8) that can lead to oxidative stress, mitochondrial damage, lipid peroxidation, protein modification, DNA damage, cytotoxicity, genotoxicity and cancer [25,57,58,61].

Above-mentioned detailed mechanisms of ROS production induced by SPION clearly illustrate the major role of ROS in observed viability results. However, these mechanisms also illustrate that ROS production induced by SPION is dependent on two reactants, hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet-}$). The total ROS response ratio of control: 100 $\mu\text{g/ml}$ of SPION-treated group: 200 $\mu\text{g/ml}$ of SPION-treated group: 400 $\mu\text{g/ml}$ of SPION-treated group was 1:1.50:2.03:2.59. That is, in primary rat hepatocytes, up to a concentration of 200 $\mu\text{g/ml}$, every 100 $\mu\text{g/ml}$ increment in concentration resulted in approximately 50% increment in total ROS production compared with control group (from control to 100 $\mu\text{g/ml}$, the increment was 50% of control; from 100 to 200 $\mu\text{g/ml}$, the increment was 53% of control). On the other hand, 200- $\mu\text{g/ml}$ increment in concentration from 200 to 400 $\mu\text{g/ml}$ resulted in nearly the same increment in total ROS production (the increment was 56% of control). This situation illustrates that at around a specific concentration between 200 and 400 $\mu\text{g/ml}$, the increase in ROS production stopped (most probably at ~ 300 $\mu\text{g/ml}$; the increment in $\sim 50\%$ of control is supposed to be due to the 100 $\mu\text{g/ml}$ increment in concentration) due to the depletion of the reactants, H_2O_2 and $O_2^{\bullet-}$, produced by mitochondria and due to no further production of the reactants, H_2O_2 and $O_2^{\bullet-}$, by disrupted mitochondria caused by the treatment of SPION [62,63]. This finding is consistent with previous studies reporting that Fenton reaction follows pseudo-first-order kinetics [64,65].

On the other hand, at the 48th hour after the start of treatment, there was a highly significant difference ($p < 0.01$) between viability values belonging to 200 $\mu\text{g/ml}$ of SPION-treated group and 400 $\mu\text{g/ml}$ of SPION-treated group in both one-shot and cumulative treatment groups. Consistently with a previous study (using a murine fibroblast cell line [L-929]) suggesting that toxicity in cells exposed to SPION might be attributed to another mechanism due to nonexact correlation between ROS production and cell viability [33], the discrepancies between ROS production and viability results (in terms of concentrations of 200 and 400 $\mu\text{g/ml}$) illustrate that ROS production is not the sole reason behind the viability parameter. The toxicity due to protein–SPION interaction was also reported; for example, upon interaction with SPION, an irreversible conformational change in the secondary structure of proteins (e.g., transferrin) was observed [66]. Downstream protein–protein interactions, cellular signaling and also DNA transcription may be affected by SPION-induced protein conformational changes [2,67]. Thus, in addition to ROS, protein adsorption on SPION may also contribute to the underlying mechanisms associated with hepatotoxicity induced by SPION.

Beyond all, as emphasized in previous studies using other metal nanoparticles, for example, gold nanoparticles [68] and titanium dioxide nanoparticles [43], the time-dependent ROS production data from the current study illustrates that ROS can be used as an early and potent diagnostic marker for SPION-induced toxicity as well as other nanotoxicological inquiries in the liver. According to the data revealed by the current study, logarithmic increase in ROS level occurred in the time period between 300th and 480th minute after the start of treatment with 400 $\mu\text{g/ml}$ of SPION; in the time period between 420th and 720th minute after the start of treatment with 200 $\mu\text{g/ml}$ of SPION. Thus, rather than the value of the ROS production, the time periods, at which the logarithmic phases form, are good candidates for the sensitive and early detection of SPION-induced hepatotoxicity. That is, we hypothesize that the analyses of the correlation between ROS and cytotoxicity should be similar with the analyses of the amplification plots in real-time PCR technique (the technique focuses on Ct or Cp values [the number of cycles required to reach threshold] that are inversely proportional to the initial amounts of target molecules [diluted sample crosses the threshold line later than more concentrated sample] rather than final fluorescence signals that are not always proportional to the initial amounts of target molecules) [69–71].

Treatment-dependent toxicity

Many studies investigating nanoparticle-induced toxicity use excessively high exposures, which bear little resemblance to true exposure levels; except for some exceptions (e.g., SPION usage to monitor cell engraftment and graft survival after hepatocyte transplantation, which means single dose exposure), the real situation is low exposure to nanoparticles that accumulate in cells over time, which represents a higher cumulative dose [2,34,35]. The most promising application of magnetic particles is targeted delivery of anticancer drugs for cancer therapy (to improve stability, biodistribution and pharmacokinetics of the anticancer drugs, and to decrease adverse effects and toxicity thanks to the selective accumulation of the drug in tumor sites) [1,5,72], which contains repeated exposure to anticancer drugs; for example, topotecan (a US FDA-approved potent anticancer drug that is water-soluble

derivative of camptothecin) was applied to the patients in 1.0 mg/m^2 daily for 5 days, on Days 1–5 and 22–26 concomitantly with radiotherapy in the context of Phase I study [36]. In the case of doxorubicin, the most commonly used dose schedule when used as a single agent is $60\text{--}75 \text{ mg/m}^2$ as a single intravenous injection administered at 21-day intervals; when used in combination with other chemotherapy drugs, the most commonly used dosage is $40\text{--}60 \text{ mg/m}^2$ given as a single intravenous injection every 21–28 days [73]. The average blood volume of an adult is 5 l [74]; detailed study reported overall mean body surface area of 3613 adult male and female cancer patients receiving chemotherapy as approximately 1.8 m^2 [75]. Reported loading capacity of the nanodrug delivery formulation (total entrapped drug weight/total nanoparticle weight) based on SPION is very low; surface functionalized SPION-based nanoformulations containing less than 10% by weight of drug illustrated successful antitumor effects [76]. Based on the mentioned values (average body surface area: 1.8 m^2 ; average blood volume: 5 l; and an optimistic 10% drug loading capacity), if a topotecan-loaded SPION-based nanoformulation is translated to clinic, this means that approximately $18 \text{ }\mu\text{g/ml}$ of SPION accumulates after 5-day application every 3 weeks ($1 \text{ mg/m}^2/\text{day}$ of topotecan concomitantly with radiotherapy); thus, after 18 weeks, accumulated SPION concentration exceeds $100 \text{ }\mu\text{g/ml}$ that is the safe concentration in the current study in terms of most of the viability and hepatocyte-specific functions. However, if a doxorubicin-loaded SPION-based nanoformulation is translated to clinic, this means that over $100 \text{ }\mu\text{g/ml}$ of SPION accumulates only after first injection; over $200 \text{ }\mu\text{g/ml}$ of SPION accumulates only after second injection ($60 \text{ mg/m}^2/\text{day}$ of doxorubicin). These calculations illustrate that the SPION concentrations used in the current study are realistic according to clinical applications of anticancer drugs; and targeted delivery of anticancer drugs using nanoparticles (e.g., topotecan- or doxorubicin-loaded SPION) represents cumulative accumulation of nanoparticles in cells over time. Thus, in the current study, we also investigated the treatment-dependent toxicity of SPION on primary rat hepatocytes by employing cumulative treatment with SPION, in addition to single dose exposure in one-shot treatment. In terms of the viability of the primary rat hepatocytes and the calculated LD_{50} values from the viability results, statistically insignificant ($p > 0.05$) differences were observed between the one-shot treatment group and the cumulative treatment group at 24th and 48th hours after the start of treatment. On the other hand, in terms of hepatocyte-specific functions (albumin and urea synthesis), while there were statistically insignificant ($p > 0.05$) differences between the one-shot treatment group and the cumulative treatment group at the 24th hour after the start of treatment, there were statistically significant ($p < 0.05$) differences between these groups at the 48th hour after the start of treatment. We hypothesize that the different results in different treatment groups were caused by different agglomeration of SPION due to the treatment styles. While in one-shot treatment group, three different concentrations (100 , 200 and $400 \text{ }\mu\text{g/ml}$) were applied once at 0 h, in cumulative treatment group, a third of each concentration was applied with 4-h intervals (e.g., $100/3 \text{ }\mu\text{g/ml}$ at 0 h, $100/3 \text{ }\mu\text{g/ml}$ at 4 h and $100/3 \text{ }\mu\text{g/ml}$ at 8 h) and after third application (eighth hour), accumulated dose reached the single applied dose in one-shot treatment group. More concentrated SPION (compared with the cumulative treatment group, primary rat hepatocytes were treated with threefold more concentrated SPION at $T = 0$ in one-shot treatment group) may result in formation of larger sized agglomerates. Nanoparticle size affects cytotoxicity upon internalization, smaller nanoparticles appear to be more toxic than larger ones, since small nanoparticles possess a high surface area relative to their total mass, which increases the chance to interact with surrounding biomolecules (e.g., biomolecules involved in pathways of albumin and urea synthesis) and, as a consequence, to trigger adverse responses [77]. This may explain the general more dramatic effect of SPION on primary rat hepatocytes in terms of the viability and the hepatocyte-specific functions at the 24th and 48th hours after the start of treatment, and significantly ($p < 0.05$) more dramatic effect on the hepatocyte-specific functions (except for urea synthesis upon treatment with $200 \text{ }\mu\text{g/ml}$ of SPION) at the 48th hour after the start of treatment in the cumulative treatment group. The treatment-dependent toxicity results revealed by the current study illustrate that treatment style is an important factor for evaluation of possible toxicities induced by SPION. And this situation deserves further research investigating the treatment-dependent toxicity of SPION by better mimicking real cumulative exposure. Since, cumulative treatment style used in the current study is still far from the real situation characterized by low exposure to nanoparticles that accumulate in cells over time, changing the growth medium every 24 h forms an obstacle to mimic the real cumulative treatment style. To this end, microfluidic liver-on-a-chip models might be a better platform to mimic real liver structure and real cumulative exposure.

The majority of the liver consists of parenchymal cells, mostly hepatocytes, which can form 80% of the liver volume, while the nonparenchymal cells (Kupffer cells, sinusoidal endothelial cells and stellate cells) represent 40% of the total number of liver cells but only 6.5% of the organ volume [78,79]. The major specialized tissue-resident

macrophages are Kupffer cells in liver, and they are the most active phagocytes in uptaking SPION from the bloodstream [80]. In addition, it was proposed that iron-induced hepatotoxicity occurs at early times after treatment and is largely dependent on the activity of Kupffer cells through promotion of free-radical reactions associated with the respiratory burst in iron-overloaded rats [81]; thus, Kupffer cells should be involved for comprehensive evaluation of possible liver toxicity induced by SPION. Recently, the liver-on-a-chip platform mimicking real liver structure (containing primary cryopreserved human hepatocytes and three cell lines of human origin EA.hy926, LX-2 and U937 as substitutes for endothelial, stellate and Kupffer cells, respectively) and allowing continuous flow of growth media (thus allows to mimic the real cumulative treatment style much better compared with the static conditions used in the current study) was reported by our group [82]. Further investigations of SPION-induced liver toxicity continue in our laboratory using the reported liver-on-a-chip platforms.

Conclusion

The criteria to define toxicity of nanoparticles need to be clearly defined. In terms of the measure of biocompatibility, most studies (e.g., studies on clinically approved MRI contrast agents such as Feridex and Resovist) largely focus on the extent of cytotoxicity observed [2,83]. In emerging studies, SPION-induced aberrant cellular responses (e.g., DNA damage, oxidative stress, mitochondrial membrane dysfunction and changes in gene expression) have been reported in the absence of cytotoxicity [2]. In the current study, both in one-shot treatment group and cumulative treatment group, SPION induced more dramatic effect on albumin production compared with the viability in primary rat hepatocytes. While there were statistically insignificant ($p > 0.05$) differences between the two treatment groups in terms of viability and LD₅₀ values, statistically significant ($p < 0.05$) differentiation between the two treatment groups appeared at the 48th hour after the start of treatment in terms of hepatocyte-specific functions (for both albumin and urea synthesis). The discrepancies among the results of the assays of viability, live/dead, hepatocyte-specific function (albumin and urea synthesis) and ROS production illustrate that consideration of a sole marker (e.g., cell viability) is a weak biomarker for the evaluation of the effect of SPION on liver. Rather, a combination of various biomarkers should be employed for this purpose. And, importance of time factor and treatment style revealed by the current study illustrate that each biomarker should be analyzed by taking into account the two parameters. It should be pointed out that Fe₃O₄ nanoparticles (10 nm [TEM]; magnetization: >45 emu/g, at 4500Oe) were used in the current study; thus, the results and evaluations revealed

Summary points

Objective

- To investigate the concentration-dependent (0–400 µg/ml) and exposure-dependent (single dosing vs cumulative dosing) effects of superparamagnetic iron oxide nanoparticles (SPIONs) on primary rat hepatocytes in a time-dependent manner, which to the best of our knowledge has not been reported previously.

Methods

- Sandwich-cultured hepatocyte model was used to evaluate the effects of SPION on the viability and hepatocyte-specific functions (the secretion of albumin and urea) with respect to time.
- 18-h time-lapse experiment was carried out to investigate the role of reactive oxygen species in SPION-induced hepatotoxicity in a concentration-dependent manner.

Results & discussion

- Both in single dosing and cumulative dosing groups, statistically significant effects on the viability of the primary rat hepatocytes were observed upon treatment with SPION with 200 and 400 µg/ml at the 24th and 48th hour after the start of treatment.
- Live/dead assay carried out at the seventh day after the start of treatment illustrated that damaging effect of SPION on the viability of the primary rat hepatocytes was progressive with time.
- The results also revealed the loss of hepatocyte-specific functions, albumin and urea synthesis, upon treatment with SPION in a dose- and time-dependent manner; significantly more deleterious effects on the functions were observed at the 48th hour after the start of treatment in the cumulative dosing group.
- All concentrations of SPION induced highly significant production of reactive oxygen species at the 30th minute; and then, statistically significant differentiation among concentration groups took place at certain time points within the 18-h time-lapse experiment.

Conclusion

- Thus, the current study reveals that a combination of various biomarkers should be employed for the evaluation of the effect of SPION on liver, and each biomarker should be analyzed in a time- and exposure-dependent manner.

by the current study reflect effects of these nanoparticles. The variations in physicochemical properties (size, shape, magnetic and surface properties) can change the behavior of the SPION in biological environment [84,85].

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/nnm-2017-0387

Financial & competing interests disclosure

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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