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Comprehensive cytotoxicity studies of superparamagnetic iron oxide nanoparticles

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ABSTRACT

Recently lots of efforts have been taken to develop superparamagnetic iron oxide nanoparticles (SPIONs) for biomedical applications. So it is utmost necessary to have in depth knowledge of the toxicity occurred by this material. This article is designed in such way that it covers all the associated toxicity issues of SPIONs. It mainly emphasis on toxicity occurred at different levels including cellular alterations in the form of damage to nucleic acids due to oxidative stress and altered cellular response. In addition focus is been devoted for in vitro and in vivo toxicity of SPIONs, so that a better therapeutics can be designed. At the end the time dependent nature of toxicity and its ultimate faith inside the body is being discussed.

1. Introduction

Superparamagnetic iron oxide nanoparticles (SPIONs) have been found promising candidate in nanobiotechnology for wide range of applications such as magnetic separation, drug delivery, magnetic resonance imaging (MRI) and magnetic hyperthermia (MH) [1–4]. Most importantly the site-specific drug and diagnostics agent delivery by using SPIONs is the most exciting applications in cancer theranostics [5,6]. The wide ranges of potential bio-applications of SPIONs are influenced by its physical, chemical, and magnetic properties along with its shape and size. The toxicity of SPIONs towards normal cells are hindering its successful implication as therapeutic agent. High degree of nonspecific binding to cell components and biological fluids by SPIONs as well as colloidal instability of SPIONs during their delivery into biological media are the main cause of the toxicity [7]. The response of these particles to living system both in terms of acute and chronic toxicity is main concern in terms of clinical activity [8]. Moreover the degradation and it's accumulation inside the body of this nanoparticles following administration is very important point of study. Currently the most trusted and easiest approach to study the In vitro cytotoxicity studies of nanoparticle is by using different cell lines varying their incubation times and evaluating by colorimetric assays [9,10]. This approach has gained lots of publicity. However, the main drawbacks of these studies include a wide range of nanoparticle concentrations and exposure time [11,12].

In addition, various researchers used different cell lines with varying culturing conditions which made things more difficult, as direct comparisons between the available studies and their own results are not validated. It is to be note that while working on SPIONs, the reported toxicity taken into consideration includes, inflammation, diminished mitochondrial activity, the cellular stress mediated generation of reactive oxygen species (ROS) and chromosome condensation [13–18].

This article is designed in such way that it covers all the associated toxicity issues of SPIONs. SPIONs are manufactured in higher quantities in order to meet the demands for rapidly growing field of nanomedicine for biomedical applications. But exposure to human body and ecosystem needs to address. This review mainly aims to collect the toxicological in vitro and in vivo data along with major adverse effects of SPIONs [19]

2. Why toxicity study of SPIONs?

SPIONs are the most preferred candidate in biomedical applications for diagnostics and therapeutics. Many in vivo toxicity appliances of SPIONs are needed in most of biomedical applications. Hence it is important to study the overall toxicity associated with them. SPIONs are

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very small in size, comparable with the biomolecules. Such a small size can cause sequestration of these moieties into various body systems and can interfere with their normal functioning. They might cross blood-brain barrier and damage neural functions, also can cross nuclear membrane and cause mutations. The bare SPIONs have very low solubility which can lead to agglomeration which can obstruct blood vessels [11].

SPION are coated with a suitable biocompatible material for increase in stability, water dispersibility and biocompatibility.

3. In vitro toxicity studies of SPIONs

In order to confirm the toxicity, different assays are available. Each assay is based on some different principle, for more accurate results it is recommended to carry multiple assay for same samples. Some of the widely used assay are lactate dehydrogenases assay (LDH), Sulphorhodamine B (SRB) assay, protein assay, neutral red, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

3.1. In vitro assays for cytotoxicity studies of SPIONs

MTT assay is a widely accepted, non-radioactive, colorimetric based assay [20,21]. MTT is derivative of a tetrazolium salt, which is converted into purple formazan insoluble complex by enzyme within the mitochondrial dehydrogenases [22]. Recent reports suggest that that reduction of MTT can also be facilitated by NADH or NADPH within the cells and also outside of mitochondria [22]. Therefore further modification of the initial protocol by Mossmann was proposed [23,24] in order to increase the repeatability and the sensitivity of the assay. Only active mitochondria contain these enzymes; therefore, the reaction only occurs in living cells [25].

The neutral red uptake assay is based on the ability of viable cells to incorporate and bind the supra vital dye neutral red. This assay is widely used cytotoxicity assay used for biomedical and environmental applications. The principle behind this is the weak cationic dye penetrates cell membranes by the mechanism of nonionic passive diffusion and concentrates in the lysosomes.

The dye binds to lysosomal matrix by electrostatic interaction, which is then extracted from the viable cells by using an acidified ethanol solution, and the absorbance of the solubilized dye is quantified using a spectrophotometer [26].

Another important assay commonly used is, LDH leakage assay which is based on the measurement of lactate dehydrogenase activity in the extracellular medium. The silent features like reliability, speed, and simple evaluation are the major strengths of this assay [27].

The most widely used assay for viability study is the trypan blue. The assay is simple method of determining cellular viability [28]. In this the cells are sedimented onto slides and fixed in a mixture of trypan blue and paraformaldehyde. The nonviable cells a stain with dark blue color, whereas viable cells exclude the dye [29]. The major concern with trypan blue assay is its difficulty to interpret because of staining artefacts.

A number of techniques for detecting DNA damage (e.g. micronuclei, mutations, structural chromosomal aberrations) have been used to identify substances with genotoxic activity. The comet assay, also known as single-cell gel electrophoresis (SCGE), is so named because damaged cells form a comet-shaped pattern after electrophoresis. It is a sensitive method to measure genotoxicity and cytotoxicity of chemical and physical agents. The comet assay has also been used to analyse the capacity of cellular DNA repair [30].

Continues metabolic process produces reactive oxygen species (ROS) such as superoxide and hydrogen peroxide. ROS generation is normally counterbalanced by the action of antioxidant enzymes and other redox molecules. However, higher levels of ROS can lead to cellular injury and may damage biomolecules such as DNA, lipids and proteins [31]. This excess reactive oxygen species should be eliminated from the cell. The cellular antioxidant enzymes and other redox molecules take care of excessive ROS and counterbalance ROS generated in the cell [32].

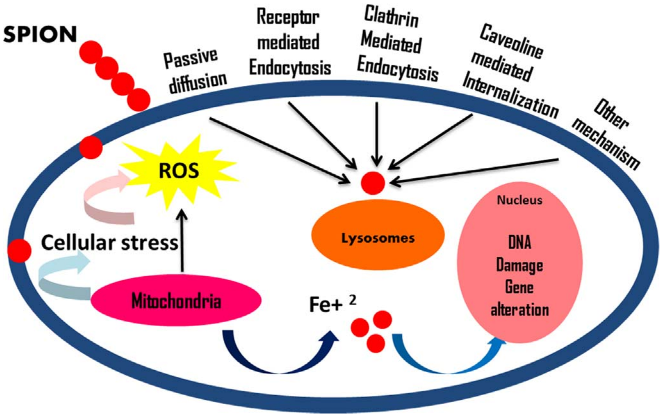

Fig. 1. Schematic representation of possible mechanism of SPIONs interaction and SPIONs-induced toxicity at cellular level.

3.2. Mechanism associated with in vitro toxicity of SPIONs

The most beautiful features of SPIONs is they can be easily attracted and manipulated by using external magnetic field and in addition the superparamagnetic properties, enables them to work as magnetic switches. In addition the least toxic effect shown on human body has attracted researcher to explore this system for maximum biomedical applications [33,34].

Fig. 1 represents the possible mechanism of SPIONs interaction with cell and toxicity at cellular level. The figure suggests that SPION can interact with cell by different mechanisms. The prominent one are, a) passive diffusion b) Receptor mediated endocytosis c) clatharin mediated endocytosis d) and caveoline mediated endocytosis. After entering inside the cell SPION are degraded by enzymes present in lysosomes and breaks the assembly to form ions. This Fe + 2 ions generates reactive oxygen species (ROS) by altering mitochondrial and other organelle functions and induction of cell signalling pathways which leads to activation of inflammatory tells [35,36]. Possible mechanism of SPIONs interaction and SPIONs-induced toxicity at cellular level is shown in Fig. 1.

3.2.1. SPION associated plasma membrane toxicity

The SPION also shows toxicity by damaging the plasma membrane and proteins. In addition to induction of cell signalling pathways, SPION can stimulates the redox reactions and up regulate plasma membrane proteins which results in the generation of cellular stress and ultimately cell death [37,38].

It is observed that the toxicity assay based upon mitochondrial functionality (e.g., MTT and XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide)), which are based upon reductase enzyme may show large errors [39]. The reason behind this is the redox active surface of SPIONs could widely impact electron flow and change the mitochondrial functionality [40–42]. The study done by Jeng and Swanson [16] showed that SPIONs had a major effect upon mitochondrial function and maximum concentration tested was ([Fe] $\approx$ 2.5 mM) at this concentration there was statistically significant change in the mitochondrial function. In another study done by Au et al. [40] similar results were observed and the authors have concluded that SPION alters mitochondrial function as well as decreased cell viability.

The study lead by the Stroh et al. [14] confirmed that citrate-coated SPIONs results in a substantial increase in protein oxidation and oxidative stress [14]. The study also concluded that iron was the source to

Table 1
A brief account of in vitro toxicity of SPIONs (bare as well as coated) on different cell types using different cytotoxic assays Adapted from Ref. [58].

Organ	[a]Cell type	[b]Coating material on SPIONs	Assay used	Concentration of SPIONs	Exposure time (h)	Observation	Refs.
CNS	astrocytes (human Nerve cells)	–	MTS and LDH	10 μg/mL	6	significantly ($p < 0.01$) increased MTS production revealed alteration in mitochondrial function	[40]
	Schwann cell	Dextran	dyes (PI)	up to 4 mg/mL	48	No change in cell viability	[59]
	Glioma	tetramethyla mmonium11-aminoundecanoate		0.1–100 μg/mL	24	concentration dependent toxicity	[60]
	GL261 (mouse brain)	Dextran	MTT	1–200 μg/mL	24	Higher toxicity was exhibited as compared to bare one	[61]
Liver	BRL 3A (rat)		MTT	0–250 μg/mL	24 h	concentration dependent and 50% decrease in viability at 250 μg/mL	[12]
	BRL 3A (rat)		LDH	0–250 μg/mL	24	toxic effect at 250 μg/mL was reported	[15]
	HepG2 (human)	Baavi-b USPIO	MTT			no indication of cytotoxicity	[62]
	HepG2 (human)	amino-surface	MTT	0.03 μg/mL to 3 mg/mL	5 days	LD50 of Gal-ASPIO-278 = 1500 μg/mL	[63]
	HepG2 (human)	amine-surface	Cytochrome C	0.03–3000 μg/mL	4 h to 5 days	The toxicity is associated with the zeta potential of NPs	[63]
	SMMC-7721 (human hepatocellular)	Chitosan	MTT	0–123.52 μg/mL		Bare MNPs showed decreased cell viability as compared to coated one	[64]
Pancreas	human islet	Dextran	dyes (PI)	280 μg/mL		viability of labelled islets were similar to the control islets	[65]
Kidney	Cos-7 (monkey)		MTT	0.2–23.05 Mm		no toxicity detected	[66]
Skin	dermal fibroblasts (human)	PEG, insulin	MTT	0–1 mg/mL	24 h	25–50% decrease in viability for bare particles (250 μg/mL); 99% viability for PEG-coated (1 mg/mL)	[67,68]
	HEK	Dextran	MTT, alamar blue	0–26 μg/cm^2	24 h	Size depended toxicity has been seen. 20 nm particles had shown a decrease in cell viability, while the 15 and 50 nm particles were not cytotoxic.	[69]
	Murine epidermal cells (JB6 P$^+$)	Dextran	MTT, alamar blue	0–26 μg/cm^2	24 h	activation of AP-1, 5% reduction in cell viability at the highest dose evaluated (26 μg/cm^2)	[69]
	dermal fibroblasts (human)	sodium oleate	MTT	0–1000 μg/mL	24 h	bare SPIONs shown disrupted cytoskeleton Lactoferrin or ceruloplasmin coated SPIONs attached to the cell membrane	[42]
	hTERT-BJ1 (human)	dextran and albumin- derivatized	dyes (BrdU)	0.05 mg/mL	24–72 h	Albumin-coated particles shown more cell viability as compared to bare and dextran coated	[70]
	L929 (mouse)	PVA	dyes (crystal violet)	800 mM	72 h	confirmed the presence of gas vesicles inside Cells	[71,72]
	L929 (mouse)	PVA	MTT	0.2 mM	24 h	morphology and size dependent toxicity	[73]
	L929 (mouse)	PEGF and PVA	MTT	0.4–1.6 M	24–72 h	morphology and size dependent toxicity	[71,74]
	L929 (mouse)	PEGF	dyes (NR)	800 mM	24–72 h	concentration@800 mM did not change the cell shapes notably and cells appeared not to be damaged	[74]
	L929 (mouse)	PAA	MTT		48 h	No observable toxicity was found	[55]
	L929 (mouse)	Chitosan	MTT		48 h	No observable toxicity was found	[20]
	L929 (mouse)	Chitosan/Glutar-aldehyde	MTT		24 h	No observable toxicity was found	[75]
	L929 (mouse)	Oleic acid/betain HCl	MTT		24 h	No observable toxicity was found	[76]
	3T3 (mouse)		MTT	0–30 ppm	72 h	no significant difference in the toxicity	[77]
	HS68 (human foreskin)	ethylene glycol	MTT	1 mg/mL	24 h	no significant difference in the viability of Cells	[78]
	Melanoma (human)	PVA and vinyl alcohol/vinyl amine copolymer	MTT	12, 61, and 123 μg/mL	2 and 24 h	polymer alone (was more toxic than polymer-coated SPIONs;	[79]
	SK-MEL-37 (human melanoma)	DMSA, citric acid or lauric acid	MTT	up to 840 μg/mL		cell viability decreased in a dose-dependent manner	[80]
	HaCaT		MTT	0.01–100 mg/mL	24 h	cell viability decreased in a dose-dependent manner	[81]

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Table 1 (*continued*)

Organ	[a]Cell type	[b]Coating material on SPIONs	Assay used	Concentration of SPIONs	Exposure time (h)	Observation	Refs.
Blood	J774 (murine)	Tween 80	MTT	25–500 μg/mL	1–6 h	Enhanced ROS generation, leading to cell injury and death; concentration- and time- dependent damage	[82]
	macrophages(human)	dextran	MTS and dyes (BrdU)	100 μg/mL	7 days	20% of macrophages were viable after 7 days	[83]
	Mouse		WST-1			higher degree of necrosis due to rod shaped	[84]
	macrophage cells (RAW264.7)		LDH			$Fe_2O_3$was in correlation with both the higher degree of membrane damage and ROS Production	[84]
	human monocyte macrophage	dextran	MTT and NBT	1 and 10 mg/mL	up to 14 days	only mildly toxic at the highest applied dosage (i.e., particle concentration of 10 mg/mL)	[85]
	K562 (human leukemia)	Tetraheptyl- ammonium	MTT	2.5 μg/mL	72 h	inhibition rate = 46% for the cell system incubated with Fe_3O_4-PLA	[86]
	K562 and K562/A02 (human leukemia)	ADM conjugated	MTT	20 μg/mL to 5 mg/mL	48 h	cell proliferation significantly ($P < 0.001$)	[87]
	T lymphocyte cell line (rat)	scAbCD3	Tetrazolium	0.15 μg	48 h	no detectable toxicity	[88]
Muscles	A10 (rat)	polylactide	Redox	10–50 μg/mL	72 h	no detectable toxicity	[89]
Lung	A549 (human)		dyes (TB) and ROS	up to 80 μg/mL	18 h	no or low toxicity	[90]
	A549 (human)		Comet	up to 80 μg/mL	4 h	oxidative DNA lesions in cultured A549 cells after exposure to 40 μg/mL and 80 μg/mL SPIONs	[90]
	A549 (human)	silica	MTT	4 mg/mL		IC50 = 4 mg/mL	[91]
	H441 (human)	PEI	MTT	90 μg/mL	24–48 h	Toxicity of tested complexes was acceptable (cell viability > 80%)	[92]
	LLC (mouse)	poly(TMSM A-*r*- PEGMA)	MTT	1–100 μg/10^5 cells	12 h	no indication of toxicity	[93]
Mesenchyma	MSC (human)	PLL	MTT		1–43 days	long-term viability, and apoptotic indices were unaffected	[94]
	MSC (human)		comet	50–250 μg/mL	24–72 H	Did not affect the apoptosis	[95]
	rMSC (rat) and MSC (human)	PDMA	Tetrazolium	15 μg γ-Fe_2O_3/mL	24 h	The viability with coating was more as compare to bare	[96]
	rMSC (rat)	HEDP	MTS	25, 50, and 100 μg iron/mL	48 h	Concentration dependent toxicity	[97]
Heart	BAECs		redox	90 μg/mL	24 h	cell viability was not adversely affected by internalized SPIONs;	[98]
Breast	B16/DNS and B16/phOx (mouse breast)	DNS hapten covalently attached to CLIO	ATP	100 mM	48 h	DNS-CLIO was nontoxic to B16/DNS (DNS receptor positive) and B16/phOx (control receptor positive) cells	[99]
	MCF-7 (human breast)	dextrane and phosphatidyl choline/ cholesterol	MTT	100 μg/mL	1–3 days	presence of SPIONs in culture medium led to alterations in mitochondria ultrastructural organization and decrease of oxygen uptake by mitochondria in sensitive and anticancer drugs resistant cells	[100]
	H184B5F5/M10, SKBR3 (normal breast), MB157, and T47D (human breast cancer)		MTS	0.1, 1, 10, and 100 μM	72 h	no observable change in cell viability	[101]
	B16F10 (mouse breast)	CMC	XTT	1–5 mM	24 h	after 48 h, cell viability was reduced (81%) at concentrations > 1 mM	[102]
Prostate glands	PC3 (human prostate)	TCL-SPIONs	MTT	0.1 mg/mL	48 h	cytotoxicity was comparable to free Dox	[103]
Cervix	HeLa (human cervical)	PLL	MTT		1–43 days	long-term viability, growth rate, and apoptotic indices of the labelled cells were unaffected by the endosomal incorporation of SPIONs	[94]
	HeLa (human cervical)	dextran, amino-dextran, heparin, and dimer-captosuccinic acid	MTT	0.05–0.5 mg/mL	24 h	viability of cell culture was not significantly Affected	[104]
	KB (human carcinoma)	PAMAM and G3	XTT	0–80 mg/mL	4 days	dendrimer-stabilized SPIONs did not display cytotoxicity to KB cells in the predetermined concentration range	[64]

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Table 1 (*continued*)

Organ	[a]Cell type	[b]Coating material on SPIONs	Assay used	Concentration of SPIONs	Exposure time (h)	Observation	Refs.
Cancer	MSTO-211H (human)		MTT	0–30 ppm	72 h	Concentration depended toxicity	[77]
	HMMs (human)	dextran	MTT	0–10 mg/mL	24, 48, 72 h	not toxic at particle concentration of 1 mg/mL and mildly toxic at particle concentration of 10 mg/mL after 72 h	[85]

[a] Abbreviations of cell types: hTERT-BJ1, Infinity Telomerase Immortalized primary human fibroblasts; L929, mouse fibroblast cell; rodent 3T3, Swiss mouse fibroblast cells; HS68, human foreskin fibroblast cells; HEK, normal human epidermal keratinocytes; A549, human lung adenocarcinoma epithelial cells; H441, human lung adenocarcinoma epithelial cells; BRL 3A, rat liver cells; HepG2, human liver hepatocellular cells; MSCs, mesenchymal stem cells; rMSCs: rat mesenchymal stem cells; Cos-7, obtained by immortalizing a CV-1 cell line derived from kidney cells of the African green monkey; OCTY, mouse kidney cells; J774, murine macrophage cells; Schwann, principal glia of the peripheral nervous system; BAECs, bovine aortic endothelial cells; HeLa, cervical cancer cells; LLC, mouse Lewis lung carcinoma; GL261, mouse brain tumor cells; K562, human immortalized myelogenousleukemia cells; k562/A02, human leukemiacells; B16, mouse melanoma cells; B16/DNS, mouse melanoma cells with DNS receptor positive; B16/phOx, mouse melanoma cells with control receptor positive; SMMC-7721, human hepatocellular carcinoma cells; PC3, human prostate cancer cells; A2780, human ovarian cancer cells; MCF-7, human breast cancer cells; SK-MEL-37, human melanoma cells; KB, human epithelial carcinoma cells; H184B5F5/M10, normal breast epithelial cells; B16F10, SKBR3, MB157, and T47D, three types of breast cancer cells; MSTO-211H, human lung mesothelioma cells; HMMs, human malignant mesothelioma cells; HaCaT, human keratinocyte cells; A10, rat aortic smooth muscle cells.

[b] Abbreviation of coatings: Baavi-bUSPIO, (Avidin-coated baculoviral vectors-biotinylated ultra-small superparamagnetic iron oxide nanoparticles); PEG, poly(ethylene glycol); PEGF, poly(ethylene glycol-*co*-fumarate); PLL, poly(L-lysine); PVA, poly(vinyl alcohol); PEI, polyethyleneimine; ADM, adriamycin; TCL-SPIONs, thermally cross-linked SPIONs; DMSA, meso-2,3-dimercaptosuccinic acid; HEDP, 1-hydroxyethylidene-1.1-bisphosphonic acid; PAA, poly(acrylic acid); MPEG, methoxypoly(ethylene glycol)-oligo(aspartic acid)); WSC, water-soluble chitosan; LA, linoleic acid; PAMAM, dendrimer-stabilized (carboxyl-functionalized poly(amidoamine); G3, dendrimers of generation 3; CMC, carboxymethylCurdlan; CLIO, cross-linked iron oxide; PDMA, poly(N,Ndimethylacrylamide); scAbCD3, nonviral gene delivery agent bearing CD3 single chain antibody. c Abbreviation of toxicity methods: MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide); MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); XTT, (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide); BrdU, bromodeoxyuridine; LDH, lactate dehydrogenase; ATP, adenosine triphosphate; NBT, Nitrobluetetrazolium; WST, water-soluble tetrazolium; PI, propidium iodide.

generate the reactive oxygen species (ROS). This was supported by a dramatic reduction in these levels of ROS via co-administration of an iron chelator.

Van den Bos et al. [43] also reported a study in which he used dextran coated SPIONs in dose-dependent manner. It was observed that there was increase in lipid peroxidation with simultaneous increase in dose [43]. The key factor for generation of ROS was ferritin which was reported in rat synaptosomes and which lead to neurodegeneration in vivo [44].

It is also observed that surface coating has particular effect at the same time the length of a coating can play a significant role and it is seen that it bear a negative correlation with toxicity [17]. At the same time longer tails coated SPION may undergo degradation into shorter tails within the intracellular environment and cause toxicity.

The SPIONs being in nanometre size can easily enter into the nuclear membrane and may cause damage to DNA and which may results in generation of ROS. In addition the released ROS further causes damage to nucleic acid and at high concentration may lead to breaking of hydrogen bonding in DNA structure.

Damage or injury to cytoskeletal structure is very important area of research. The toxicity created by SPION needs to confirm, as these filaments are essential element in maintaining cellular and structural morphology. The study suggests that high doses of SPION lead to interference with the actin cytoskeleton resulting in decreased cell proliferation [45]. The study done by Soenen et al. clearly shown that SPION encapsulated in liposomes also called magntoliposomes shown direct effect on actin cytoskeleton architecture and which leads to formation of focal adhesion complexes and cell has shown decreased proliferation ability. The study also reveals that the effect was reversal and took 7 days to return to normal [45]. Disruption of a cytoskeleton protein, tubulin, and dynamic cortical meshwork of F-actin are some other reported effects of SPION [46–48]. Resovit is commercially available MRI agent formulated with carboxy dextran coated SPION. When pancreatic islet cells labelled with Resovist, there was increasing expression in insulin levels [49]. In another study of Resovist on mesenchymal stem cells showed amplified cellular growth and cell cycle progression. This was accompanied by alterations in the expression of cell cycle regulatory proteins [50].

Primary human fibroblasts (hTERT-BJ1) cell line shown increase in cell proliferation in response to transferrin-coated SPIONs [46].

Recent invitro studies have shown the effect of SPION on macrophages. The study revealed that there was change in cellular behaviours with cytokine expression. In addition there was increased expression of IL-1, 4, and 10, TNF- α and inhibition of tumor necrosis factor-α (TNF-α) which suggest the potential effect on immuno modulatory capabilities [51–53].

Our group has also studied rigorously on in vitro cytotoxicity associated with different ferrite and other MNPs such as Fe_3O_4, $CoFe_2O_4$, Ni-$ZnFe_2O_4$, $ZnFe_2O_4$ nanoparticles with different coating materials using MTT and trypan blue assays on different cell lines, both cancerous and normal cell lines [54–57].

Table 1: A brief account of in vitro toxicity of SPIONs (bare as well as coated) on different cell types using different cytotoxic assays is discussed in detail.

4. In vivo toxicity studies of SPIONs

4.1. Mechanism associated with in vivo toxicity of SPIONs

The SPIONs are aggregated in a particular tissue by using a magnet for maximum effects for therapy or diagnosis application, which can leads to high concentrations in that area [105]. Now this may lead to high levels of free Fe ions in the exposed tissue which may lead to cellular damage which can lead to or have a significant impact on future generations if the fidelity of the genome in germ cells is not maintained [106–108]. It also to be note that iron has been associated with cancer different researchers has explained various mechanisms for these effects [109,110].

The physical and chemical characteristics of SPIONs are considered as crucial factors to determine pharmacokinetics, toxicity and bio distribution of magnetic nanoparticles [57]. Till date very few studies are available on humans which can discuss the detail property of SPION. One such study is done on Ferumoxtran-10, which is a dextran-coated USPIO (ultra-small SPIONs). It has seen that this NPs have shown to induce the transient effects including urticaria, diarrhoea and nausea [111,112]. The same system when it was exposed as commercial contrast agent in living system, adverse events from USPIO were reversible and diminish with the time [113].

Chertok et al. [114] checked the possibility of SPIONs as a drug delivery vehicle for magnetic targeting of brain tumors. Animals were intravenously injected with nanoparticles (12 mg Fe/kg), no observable toxicity was found. Pradhan et al. [115] found no significant changes in haematological and biochemical parameters and suggested that the high dose had raised the Serum glutamic pyruvic transaminase (SGPT) levels suggesting the hepatic toxicity while the detail histopathological images suggested that there was no morphological changes was noted.

The study done by Lübbe et al. [116] developed a stable nanomedicine of magnetic nature and to which different molecules of drugs, cytokines and other molecules are chemically attached and directed inside the cells through magnetic field. Various concentrations of the magnetic fluid were tested in rats and immunosuppressed nude mice. As a result, the Ferro-fluid did not cause major laboratory abnormalities. Hu et al. [117] coupled PEG-coated Fe_3O_4 nanocrystals with a cancer-targeting antibody, rch 24 mAb as a MRI contrasting agent. After completion of successful invitro cell line study the assembly was used for in vivo experiments for identification of human colon carcinoma After the experiment the nude mice recover anaesthesia and lived normally for weeks, which demonstrates that the bioconjugates have no acute fatal toxicity.

4.2. Genotoxicity

It has been seen that the any type of cellular stress has shown to have expression of different signalling factor. Similarly, the SPIONs exposure uplifts the expression of genes which are involved in cell signalling and shows the impact on signalling transduction pathways. The, uplifted genes includes; tyrosine kinases, integrin subunits members of the protein kinase C family, Ras-related protein, extracellular matrix proteins (ECM proteins) and matrix metalloproteinases [46]. It is also reported that in vivo administration of dietary iron in rats had increased number of DNA breaks [118]. Polyaspartic acid-coated magnetite NPs in vivo study demonstrated a time and dose-dependent increase in micronucleus frequency [16].

Fig. 1 explain the possible mechanism of ROS after exposure of SPIONs following internalization via a number of possible mechanisms is shown in Fig. 1, [119,120].

4.3. Immunotoxicity

Immunotoxicity is the study of toxicity effect of NPs on immune cells [47]. Till date very limited data is available which can suggest the interaction between immune system and SPION [121]. The study done by Shen et al. [122] shown that administration of iron oxide nanoparticles, in a dose-dependent manner significantly weakened inflammatory reactions and delayed the expression of interferon-γ, interleukin-6 and tumor necrosis factor-α at the inflammatory site [123].

4.4. Cellular stress

Cellular stress due to SPION is important factor for expression stress molecules. Gao et al. [124] reported that SPIONs lowers p53 expression. He also studies the effects of SPIONs on cell cycle regulatory proteins [124]. Spindle cell sarcoma and pleomorphic sarcoma in rats was reported after I/M exposure of iron-dextran complex [125]. Expression of hepcidin was observed in iron-overload in vivo [126–128].

5. Fate of SPIONs

In the literature, most of work was carried out to study the toxic effects of SPIONs but a very less data was available on the final destination of SPIONs after exposure in vitro or after administration in vivo. It is a prime importance to study the clearance or use of SPIONs after exposure to body for a particular therapy application such as in drug delivery, MRI and hyperthermia.

5.1. Fate of SPIONs in vitro

In vitro studies suggested that SPIONs are avidly taken up by fibroblasts, macrophages and tumor cells. The surface property of the SPION has greater impression on the uptake inside the cell. For example, the system of carboxydextran-coated SPIONs of size ranging less than dextran-coated SPIONs had shown the higher percentage internalization inside the macrophage cell, but this uptake is not associated with cell activation as no interleukine-1 release is observed [129]. Muller et al. [130] hypothesized that the cell toxicity was only conferred after internalization into the cells [130]. Furthermore, Muller et al. confirmed particle internalization into the granulocytes by labeling the particles with luminal, a chemiluminescent dye, which nicely correlate with intracellular iron uptake [85].

5.2. Fate of SPIONs in vivo

SPION once administered, the fate inside the body is dependent on various parameters which include size, shape, and most important coating done on the surface of the particle. One study has reported that initially the SPION once administered, enters into liver and spleen [131,132]. The system developed of oleic acid/pluronic-coated SPIONs had shown that more than half of the drug were accumulated inside the liver of rats [133,134]. Similarly one study has reported that following internalization of dextran coated SPIONs, the particles are accumulated in lysosomes. The iron oxide is broken into iron ions via change in pH and ultimately gets incorporated into haemoglobin. The dextranase further helps to break the dextran coating and facilitate the degradation [129]. The important question here arise that this degree of degradation is highly dependent upon the protein corona present on the surface of SPION.

6. Conclusions

This review discusses the properties of SPIONs that may contribute to their toxicity as well as some methods of assessing this toxicity in vitro. The importance of in vitro toxicity testing has increased in recent times, mainly due to its desirable qualities over in vivo testing. Specifically, in vitro tests are easier to manipulate, more cost effective and easier to interpret.

Toxicity of SPIONs is proved to be concentration dependent and it also depends on exposure time. No observable toxicity is seen at lower levels of SPIONs as these particles can be cleared from body. While in the case of high dose exposure, the particles may trigger cellular stress and altered response. Hence some more studies in this direction are needed. In addition it is noted that the functionalization of SPION with biological moiety has shown least toxic effects, but it is critical to design functionalized SPIONs which are able to meet sufficient internalization property and are appropriately magnetizable, and also meet the demands of a particular application without compromising on cellular toxicity. The criteria to define toxicity of SPIONs needs to be redefined, particularly as studies on SPIONs have begun to highlight aberrant cellular responses including DNA damage, oxidative stress, mitochondrial membrane dysfunction and changes in gene expression all in the absence of cytotoxicity. Hence terms such as biocompatibility need to be revaluated when commenting on the safety of these SPIONs. This will ensure the safer use of SPIONs in nanomedicine and will help to establish novel targeted therapies with improved design that are able to deliver their beneficial promises to the medical field.

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Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2017.12.002.

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