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Potential toxicity of engineered nanoparticles in mammalian germ cells and developing embryos: treatment strategies and anticipated applications of nanoparticles in gene delivery

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BACKGROUND: Engineered nanoparticles (ENPs) offer technological advantages for a variety of industrial and consumer products as well as show promise for biomedical applications. Recent progress in the field of nanotechnology has led to increased exposure to nanoparticles by humans. To date, little is known about the adverse effects of these ENPs on reproductive health, although interest in nanotechnology area is growing. A few biocompatible ENPs have a high loading capacity for exogenous substances, including drugs, DNA or proteins, and can selectively deliver molecular cargo into cells; however, they represent a potential tool for gene delivery into gametes and embryos.

OBJECTIVE AND RATIONALE: Understanding the reprotoxicological aspects of these ENPs is of the utmost importance to reliably estimate its potential impact on human health. In addition, a search for protective agents to combat ENP-mediated reproductive toxicity is warranted. Therefore, in this review we summarize the toxic effects of a few ENPs (metal and metal oxides, carbon-based nanoparticles, quantum dots and chitosan) in mammalian germ cells and developing embryos, and propose some treatment strategies that could mitigate nanoparticle-mediated toxicity. In addition, we outline the anticipated applications of ENPs in transgenic animal production in order to generate models for investigations into the mechanisms for human disease.

SEARCH METHODS: A literature search was performed using the National Center for Biotechnology Information PubMed database up until March 2016 and relevant keywords were used to obtain information regarding mammalian germ cell-specific toxicity and embryotoxicity of ENPs, possible treatment strategies, as well as the anticipated applications of nanoparticles in gene delivery in germ cells and embryos. Only English language publications were included.

OUTCOMES: Here, we demonstrate the toxicological effects of ENPs in mammalian germ cells and developing embryos by considering both *in vitro* and *in vivo* experimental models based on the existing literature. The biodistribution and cellular uptake of ENPs and the observed toxicities are mostly dependent on ENP size and surface-coating agents (surface functional groups/surface charge). ENPs have been shown to induce toxicity via oxidative stress, inflammation and DNA damage in both human and mouse germ cells. Use of antioxidant, anti-inflammatory drugs and selective metal chelators would be beneficial against nanoparticle-induced toxicity.

WIDER IMPLICATIONS: Our review provides the reproductive scientists a mechanistic insight into the reprotoxicological aspects of ENPs to reliably estimate its potential impact on human health and help to select/design protective agents to combat ENP-mediated toxicity. Furthermore, research regarding the detailed mechanism(s) of ENP toxicity in mammalian germ cells and developing embryos as well as the search for protective agents to combat ENP-mediated reproductive toxicity is warranted. Furthermore, we anticipate that investigations into the possibility of applying nanovectors to gene delivery in germ cells and early embryos will open new horizons in reproductive biology.

Key words: nanoparticles / germ cell toxicity / embryotoxicity / reactive oxygen species / antioxidants / gene transfer

Introduction

Engineered nanoparticles (ENPs) have been used in various fields in recent decades, including biomedicine, diagnosis, biological imaging, drug and gene delivery, catalyst, fuel additives, electronics, agriculture, cosmetics, pharmaceuticals, etc. (Modestov and Lev, 1998; Jordan *et al.*, 1999; Ravi Kumar, 2000a; Mitra *et al.*, 2001; Mills *et al.*, 2003; Penn *et al.*, 2003; Kaida *et al.*, 2004; Wang *et al.*, 2004; Shelley, 2005; Liu, 2006; Nel *et al.*, 2006; Zhang *et al.*, 2007; Mccarthy and Weissleder, 2008; Park *et al.*, 2008; Slowing *et al.*, 2008; Na *et al.*, 2009; Robichaud *et al.*, 2009; FAO/WHO, 2010; Mahmoudi *et al.*, 2010, 2011; Cassee *et al.*, 2011; Hajipour *et al.*, 2012; Krol *et al.*, 2012; Weir *et al.*, 2012; Giri *et al.*, 2013; Sack *et al.*, 2014). ENPs are typically defined as particles that are designed and produced to have a dimension or size that is <100 nanometers (Oberdörster *et al.*, 2005a,b; ASTM, 2006; SCENIHR, 2007). Such nanosized particles possess nanostructure-dependent characteristics, such as electrical, mechanical, magnetic, chemical, optical and biological (Lee *et al.*, 2016; Mozumder *et al.*, 2016; Namin *et al.*, 2016; Rodzinski *et al.*, 2016; Yue *et al.*, 2016) that differ from the bulk properties of the constituent chemicals and compounds. Therefore, ENPs provide a better performance over traditional products and are being used to develop innovative goods. These unique and interesting properties of nanoparticles are mainly attributed to the large surface area and increased particle number (Roduner, 2006). Since the commercialization of products using ENPs has expanded, the possible health risk of ENPs is to be expected to be addressed over the coming decade (Pietroiusti *et al.*, 2013).

Nanotoxicology is defined as a new branch of toxicology that deals with the adverse health effects of nanoparticles (Donaldson *et al.*, 2004; Oberdörster *et al.*, 2005b; Buzea *et al.*, 2007). Nanoparticles can readily enter into cells and are able to cross biological membranes owing to their extremely small size (Holsapple *et al.*, 2005; Pietroiusti *et al.*, 2013). They can easily gain access to the circulatory system via inhalation, ingestion, oral routes and sometimes via skin penetration (Hoet *et al.*, 2004; Oberdörster *et al.*, 2005a,b; Ryman-Rasmussen *et al.*, 2006; Hagens *et al.*, 2007; Braydich-Stolle *et al.*, 2010a; Yildirimer *et al.*, 2011; Schäfer *et al.*, 2013; Yah, 2013; Gaillet and Rouanet, 2015; Shakeel *et al.*, 2015). Nanoparticle injections and implants are other routes of exposure (Yah, 2013). Once they enter the blood stream, they can translocate to body organs and tissues. Nanoparticles show toxic effects in human tissues and cell cultures by increasing oxidative stress and inflammatory cytokine production (Oberdörster *et al.*, 2005a; Choi *et al.*, 2015; Senapati *et al.*, 2015; Tarantini *et al.*, 2015). They can also be taken up by the mitochondria and the nucleus, causing mitochondrial damage, DNA mutation and even cell death (Li *et al.*, 2003; Savic *et al.*, 2003; Geiser *et al.*, 2005; Porter *et al.*, 2007; Foldbjerg *et al.*, 2011; Xu *et al.*, 2014; Yoisungnern *et al.*, 2015; Zhang *et al.*, 2015b).

Recently, reproductive toxicity due to nanoparticle exposure has become a major component of risk assessment. In mammals, the gametes and developing embryos are highly vulnerable and situated in a protected place in the body. Since gamete quality plays an important role in gametogenesis, the influence of nanoparticles on a single gamete may cause notable developmental differences (Gandolfi and Brevini, 2010). Nanoparticle-mediated gamete injury may either affect reproductive function or have pathological influences on offspring (Campagnolo *et al.*, 2012). Ema *et al.* (2016) demonstrated that embryonic or fetal alterations, such as morphological and functional alterations, growth restriction and death, can be caused from direct

effects on the embryo/fetus (when the nanoparticles reach the fetal tissues), indirect effects (occurring as a consequence of maternal stress and/or toxicity) or a combination of the two. Several studies have shown that nanoparticles can effectively cross the biological barriers protecting reproductive tissues. It has been reported that nanoparticles can cross the blood–testis barrier (BTB) and accumulate inside the testis (Kim et al., 2006a,b; Balasubramanian et al., 2010). Nanoparticles can also be translocated to the ovaries and accumulate within ovarian cells (Austin et al., 2012; Gao et al., 2012; Zhao et al., 2013; Tassinari et al., 2014). The transplacental crossing ability of several nanoparticles, e.g. gold (Semmler-Behnke et al., 2007), titanium dioxide (Takeda et al., 2009; Yamashita et al., 2011), quantum dots (Chu et al., 2010), fullerene (Sumner et al., 2010), silicon (Refuerzo et al., 2011), iron oxide (Di Bona et al., 2014), carbon nanotubes (CNTs; Qi et al., 2014), silver (Austin et al., 2012; Wang et al., 2013) and silica (Yamashita et al., 2011), has also been reported in experimental animals. Hougaard et al. (2015) demonstrated that fullerene, CNTs, gold, silver and silica nanoparticles (SiO_2NPs) could cross the placenta in pregnant animals, which were exposed to nanoparticles either via oral or intravenous routes. Therefore, understanding the adverse effects of these widely available and widely used nanoparticles on reproductive health is imperative.

A few ENPs show high loading capacity for exogenous DNA or proteins, and can selectively deliver molecular cargo into target cells (Kneuer et al., 2000; Sandhu et al., 2002; Csogor et al., 2003; Thomas et al., 2003; Kakizawa et al., 2004; Tsai et al., 2004; Liu et al., 2005; Singh et al., 2005; Wisher et al., 2006; Guillot-Nieckowski et al., 2007; Sokolova and Epple, 2007; Tripathi et al., 2012; Park et al., 2014; Du et al., 2015). Due to its spontaneous internalization into the target cells, nanoparticle-mediated gene delivery is associated with a number of benefits as compared to conventional viral- and electro-transfers. Nanovectors combine the key advantages of viral vectors, such as high specificity and non-invasive delivery, together with the main advantage of electroporation, such as excluding the possibility of viral incorporation into the host genome. Therefore, ENPs represent a potential tool for gene delivery into gametes and embryos.

ENPs include several metal and metal oxide nanoparticles (Ag, Au, Al, TiO_2, SiO_2, Fe_3O_4, CeO_2, MoO_3, ZnO are among the most common), carbon-based nanoparticles (CNTs, carbon black, fullerene, graphene oxide), quantum dots and polymer nanoparticles (such as chitosan), which are being used in a large number of commercial products, biomedicine, diagnosis, biological imaging, and drug and gene delivery. As a consequence, humans are frequently exposed to nanoparticles via occupational or consumer exposure (breathing or skin contact), ingestion or sometimes via direct injection. Therefore, in this review, we mainly focus on the toxic effects of the above-mentioned ENPs in mammalian germ cells and developing embryos by considering both *in vivo* and *in vitro* models based on the available literature. Finally, we propose some of the treatment strategies that could mitigate nanoparticle-mediated toxicity and outline the anticipated applications of ENPs in transgenic animal production.

Methods

A computerized literature search was performed using the National Center for Biotechnology Information PubMed database until March 2016. We searched the database using the following keywords: nanoparticles [Ag, Au, Al, TiO_2, SiO_2, Fe_3O_4, CeO_2, MoO_3, ZnO, CNTs, carbon black, fullerene, graphene oxide, Quantum dots (QDs) and chitosan], testes, sperm, ovary, follicles, oocytes, embryo, placenta, toxicity, antioxidants and gene delivery. Only English language publications were included. We only focused on mammalian germ cell-specific toxicity and the embryotoxicity of ENPs, and we propose possible therapeutic strategies as well as their anticipated applications for gene delivery into germ cells and embryos.

Results

Application of nanoparticles in different areas, probable route of exposure in humans and their associated health hazards

Table I summarizes the potential applications of nanoparticles in different fields. Table II depicts the probable routes of exposure for nanoparticles in humans and their associated health hazards.

Toxicological effects of metal and metal oxide-based nanoparticles on germ cells and embryos

The *in vivo* and *in vitro* effects of metal and metal oxide-based nanoparticles on male gametes/testes, female gametes/ovary and embryo development are shown in Table III.

Silver nanoparticles

Silver nanoparticles (AgNPs) exhibit antimicrobial properties and are widely used in cosmetics, paints, pharmaceutical products, food and textile industries, construction materials and electronic products (Chen and Schluesener, 2008; Jain et al., 2009; Ahamed et al., 2010; Kalishwaralal et al., 2010; Radetic, 2013; Banach et al., 2014; Zarei et al., 2014; Smulders et al., 2015).

Effects on male and female germ cells. Extensive research has been conducted on the effects of AgNPs on male germ and testicular cells both *in vivo* and *in vitro*. Kim et al. (2008) showed that 60 nm AgNPs could cross the BTB and are distributed inside the testis after repeated oral exposure at a dose of 30–1000 mg/kg body weight in rats for 4 weeks. In their study, AgNPs showed a dose-dependent accumulation in testis. Similarly, Park et al. (2010) studied the testicular accumulation of 22, 42, 71 and 323 nm AgNPs after repeated oral administration with a 1 mg/kg body weight dose for 2 weeks in mice and showed that only 22 and 42 nm AgNPs significantly accumulated in the testes compared with controls. These studies indicate that very small-sized AgNPs can readily cross the BTB even after administration of low doses.

Thakur et al. (2014) checked the chronic effects of orally administered AgNPs on rat spermatogenesis and testicular morphology. They showed that oral administration of 5–20 nm AgNPs to rats (20 µg/kg body weight) for 90 consecutive days causes degenerative changes in seminiferous tubules and increases proinflammatory responses (Thakur et al., 2014). Han et al. (2016) also demonstrated that single intraperitoneal injection of 20 nm (0.5, 1 mg/kg) AgNPs in male ICR mice caused histopathological changes in testis and increased proinflammatory responses after 15 days. In addition,

Table I Applications of nanoparticles in different areas.

Nanoparticles	Abbreviation/ chemical formula	Applications
Silver	Ag	Antimicrobials, cosmetics, paints, pharmaceutical products, food and textile industries, electronics, construction materials (Chen and Schluesener, 2008; Jain *et al.*, 2009; Ahamed *et al.*, 2010; Kalishwaralal *et al.*, 2010; Radetic, 2013; Banach *et al.*, 2014; Zarei *et al.*, 2014; Smulders *et al.*, 2015)
Gold	Au	Catalyst, electronics, cancer diagnosis and therapy, biological sensors, drug delivery (Jeong *et al.*, 2014; Jafri *et al.* 2015; Liu *et al.*, 2015)
Aluminum	Al	Electronics, antimicrobials, prevent scratch in plastic lenses, new tissue-biopsy tools, military devices (Braydich-Stolle *et al.*, 2010a,b; Ema *et al.* 2010)
Titanium dioxide	TiO_2	Rubber, inks, paints, plastics, paper, cosmetics, toothpaste, food colorants (Ema *et al.*, 2010; Shakeel *et al.*, 2015)
Iron oxide	Fe_3O_4	Diagnosis, drug delivery, cellular therapy, magnetofection, magnetic imaging (Makhluf *et al.*, 2006)
Molybdenum trioxide	MoO_3	Catalyst, gas sensor, display material (Kim *et al.*, 2010a,b)
Silica	SiO_2	Biomedical imaging, gene delivery, photodynamic therapy, catalytic supports (Roy *et al.*, 2005; Kim *et al.*, 2006a, b; Lin *et al.*, 2007; Lu *et al.*, 2007; Ohulchanskyy *et al.*, 2007)
Zinc oxide	ZnO	Gas sensors, solar cells, biosensors, ceramics, photo detectors, catalysts, UV filters, antivirus agent (Chen and Lia, 2003; Hu *et al.*, 2003; Li and Wu, 2003; Hernandez Battez *et al.*, 2008; Gerloff *et al.*, 2009; Jin *et al.*, 2009; John et al., 2010; Rasmussen *et al.*, 2010; Schilling *et al.*, 2010; He *et al.*, 2011)
Cerium dioxide	CeO_2	Catalyst, additive for diesel, cancer therapies (Park *et al.*, 2008; Cassee *et al.*, 2011; Giri *et al.*, 2013; Sack *et al.*, 2014)
Carbon nanotubes	CNT	Paper batteries, cables and wires, transistors and electrical circuits, biomedical imaging (Jerosz *et al.*, 2011; Cheng *et al.*, 2013; Islam *et al.*, 2015; Bhattacharya *et al.*, 2016)
Carbon black	CB	Pigment, automobile tires, radar-adsorbent materials, and in laser printer toner (Donaldson *et al.*, 2005; Ema *et al.*, 2010)
Fullerene	C_{60}	Lubricants, dietary supplements, electronics, cosmetics, fuel cells (Loutfy *et al.*, 2002)
Graphene oxide	GO	Biological imaging, drug delivery, cancer photothermal therapy, biosensing, tissue engineering (Sun *et al.*, 2008; Artiles *et al.*, 2011; Zhang *et al.*, 2011a,b; Sheng *et al.*, 2013)
Quantum dots	QDs	Biological imaging (Feugang *et al.*, 2012)
Chitosan	CSNP	Biotechnology, textile, cosmetics, drug delivery, agriculture, food preservatives (Li *et al.*, 1992)

AgNPs dose-dependently disrupted the function of Sertoli and Leydig cells in the testis, as evident from the downregulation of the Sertoli cell-specific genes necessary for supporting gametogenesis and Leydig cell-specific steroidogenesis-related genes necessary for male steroid hormone production. Zhang *et al.* (2015a) demonstrated that AgNPs caused size-dependent (10 and 20 nm) and dose-dependent (10–100 μg/ml) toxicity via oxidative stress and induced apoptosis/necrosis in mouse TM3 Leydig cells and mouse TM4 Sertoli cells when treated for 24 h. AgNPs also reduced the expression of tight junction genes in TM4 cells and steroidogenesis-related genes in TM3 cells.

Zhang *et al.* (2015a) showed that the growth of spermatogonial stem cells (SSCs) was inhibited when the AgNP-treated TM3 and TM4 cells were used as feeder cells for SSC culture. They proposed that altered physiological functions of Leydig and Sertoli cells due to AgNP treatment could negatively affect SSC proliferation and self-renewal mechanisms. The anti-proliferative effects of AgNPs on SSCs were also studied by Braydich-Stolle *et al.* (2010b): In their study, AgNPs size dependently (10–15, 25–30, 80 and 130 nm) and dose-dependently (5, 10, 25, 50 and 100 μg/ml) decreased the cell viability of SSCs after 24 h of treatment. They also showed that AgNPs decrease the proliferation of SSCs by disrupting the glial cell-derived neurotrophic factor/Fyn kinase signaling pathways.

AgNPs can induce apoptosis in all kinds of spermatogenic cells, such as spermatogonia, spermatocytes and spermatids (Thakur *et al.*, 2014; Han *et al.*, 2016). Han *et al.* (2016) investigated the effects of AgNPs on the development and maturation of germ cells. They showed that AgNP treatment dose-dependently decreased the expression level of premeiotic and meiotic markers markedly, compared to its effect on post-meiotic markers. These results support the notion that AgNPs deliver an immediate adverse effect on spermatogonia and spermatocytes, followed by a secondary effect on spermatids due to the death of the former two cell types. In another study, Castellini *et al.* (2014) investigated the long-term effects of AgNPs (45 nm) on the reproductive activity of rabbit buck, which were given a single intravenous injection (0.6 mg/kg body weight). AgNPs were observed inside the spermatids and ejaculated sperm after 21 days of AgNPs exposure. AgNPs affected the sperm concentration and increased reactive oxygen species (ROS) production in seminal plasma only after short-term treatment (7 and 21 days). AgNPs reduced the percentage of normal sperm, whereas acrosome-reacted sperm increased. These negative effects partly abated within 7 weeks, after which sperm traits progressively declined until 126 days. They proposed that the resistance to harmful substances is affected by the differentiation stage of male germ cells: developing male germ cells are more susceptible to exogenous substances during the leptotene-zygotene phase and are probably more resistant at other stages. On the other hand, the impaired reproductive function over the long term implies that some injuries occurred

Table II Route of exposure of nanoparticles and associated health hazards.

Major exposure routes	Mode of intake	Affected organs
Air: TiO_2, Ag,Au, Al, MoO_3, CeO_2, SiO_2, Fe_3O_4, ZnO, CNT, CB, C_{60}, QDs, CSNP	Breathing (Occupational and/or consumer exposure)	Respiratory tract (lung) Nervous system (brain) Circulatory system (transportation to other organs) Gastrointestinal tract Lymphatic system
Dermal: TiO_2, Ag, Au, MoO_3, SiO_2,Fe_3O_4, ZnO, CNT, C_{60}, GO, QDs, CSNP	Skin contact (Occupational and/or consumer exposure)	Neuronal system Lymphatic system
Food: Ag, TiO_2, C_{60} Toothpaste: TiO_2 Oral drug delivery: Au	Eating and drinking	Circulatory system (transportation to other organs) Gastrointestinal tract Lymphatic system
Injection: Au, Fe_3O_4	Intravenous, intraperitoneal, intradermal, intramuscular	Neuronal system Circulatory system (transportation to other organs) Lymphatic system

Affected organs	Associated health hazards
Respiratory tract (lung)	Inflammation, fibrosis, emphysema, tumor
Nervous system	Neurodegenerative disease, Parkinson's disease, Alzheimer's disease
Gastrointestinal tract	Crohn's disease, colon cancer
Lymphatic system	Podoconiosis, Kaposi's sarcoma
Circulatory system	Thrombosis, artheriosclerosis, cardiovascular disease
Other major organs (e.g. liver, kidney, spleen, testes, ovary, etc.)	Organ dysfunction and disease occur depending on translocation and accumulation of nanoparticles

during the epididymal transport and storage, as the testis showed almost normal spermatogenesis. Ordzhonikidze *et al.* (2009) showed that a single injection of 9 nm AgNPs at a dose of 1.6×10^{-3} g.ions/l in BALB/c male mice increased the frequency of abnormal sperm heads after 30 days. Gromadzka-Ostrowska *et al.* (2012) reported a size-dependent (20 and 200 nm), dose-dependent (5 and 10 mg/kg) and time-dependent (24 h, 7 and 28 days) decrease in epididymal sperm count in male rats after intravenous administration of AgNPs. They also showed that 20 nm AgNPs caused significant sperm DNA damage after 24 h, but decreased thereafter at 7 and 28 days. However, 200 nm AgNPs did not result in any DNA damage at all the measured time points. Therefore, these studies clearly showed that AgNPs can cause damage to testis and impair spermatogenesis at doses smaller than the AgNPs doses used in the clinic.

In another study, Miresmaeili *et al.* (2013) checked the adverse effects of AgNPs at very high doses. They showed that oral feeding of 70 nm AgNPs every 12 h for 48 consecutive days caused impairment of the acrosome reaction in sperm treated with 25 mg/kg body weight and reduction in spermatogenic cell number, such as spermatogonia (50 mg/kg body weight treated group), spermatocytes, spermatids and spermatozoa (50, 100 and 200 mg/kg body weight treated group) in male Wistar rats.

Adverse effects of AgNPs on neonatal and prepubertal testes development and spermatogenesis were also reported by several researches (Sleiman *et al.*, 2013; Mathias *et al.*, 2015; Zhang *et al.*, 2015b). Mathias *et al.* (2015) showed that oral administration of AgNPs (6 nm) starting from postnatal day 23 (PND23) to PND58 increases sperm abnormalities, and reduces acrosome and plasma membrane integrity and mitochondrial activity in male Wistar rats when treated with both 15 and 30 µg/kg/day and sacrificed at PND102. Similarly, Sleiman *et al.* (2013) showed that the total and daily sperm production of male Wistar rats were decreased at PND90 when they were treated with either 15 or 50 µg/kg/day AgNPs (60 nm) orally, starting from PND23 to PND53. However, the total and daily sperm production of male Wistar rats was decreased at PND53 when they were treated with 50 µg/kg/day. Zhang *et al.* (2015b) also assessed the sperm concentration at PND100 after the mice were treated with 1 and 5 mg/kg AgNPs (10–15 nm) five times from PND8 to PND21 by abdominal subcutaneous injection and showed that the sperm concentration was decreased significantly only in case of the 5 mg/kg AgNPs administered dose.

Yoisungnern *et al.* (2015) thoroughly investigated the adverse effects of AgNPs on mouse spermatozoa. In their study, mouse spermatozoa were treated with 0.1, 1, 10 or 50 µg/ml AgNPs (40 nm) for 3 h. AgNPs dose-dependently inhibited sperm viability and the acrosome reaction, increased sperm abnormalities and induced mitochondrial dysfunction due to oxidative stress. They showed that AgNPs could be internalized into spermatozoa. Furthermore, they checked the effects of AgNP-treated sperm on the IVF of oocytes and showed that AgNPs treatment dose-dependently decreased the fertilization rate.

On the other hand, Garcia *et al.* (2014) showed that intravenous injection (1 mg/kg, five injections were given every 3rd day) of AgNPs (10 nm) in CD-1 male mice did not affect sperm concentration and motility after 15, 60 or 120 days, although they observed significant alteration in Leydig cell function after 15 days, but this became insignificant 60 or 120 days post-treatment. Similarly, after 15, 60 and 120 days of treatment, the percentage of apoptotic germ cells was significantly increased, although the number at 120 days was

Table III Effects of nanoparticles on male and female germ cells and embryo development.

Nanoparticles	Effects on male germ cells	Effects on female germ cells	Embryo development
AgNPs	(i) Cross BTB; (ii) increases inflammatory responses; (iii) affects spermatogenesis mainly via apoptosis of spermatogonia, spermatocytes and spermatids; (iv) alters sertoli and Leydig cell functions; (v) affects SSCs proliferation/self-renewal mechanisms; (vi) induces oxidative stress, DNA and mitochondrial damage; (vii) impairs acrosome reaction and sperm fertilizing ability; (viii) can penetrate sperm *in vitro* (Kim *et al.*, 2008; Ordzhonikidze *et al.* 2009; Park *et al.*, 2010; Braydich-Stolle *et al.*, 2010a,b; Gromadzka-Ostrowska *et al.*, 2012; Miresmaeili *et al.*, 2013; Sleiman *et al.*, 2013; Castellini *et al.*, 2014; Thakur *et al.*, 2014; Mathias *et al.*, 2015; Yoisungnern *et al.*, 2015; Zhang *et al.*, 2015a,b; Han *et al.*, 2016)	(i) Can accumulate in ovary; (ii) increases inflammatory responses; (iii) reduces the number of primordial and growing follicles; (iv) induces apoptosis in granulosa cells and theca cells; (v) affects ovarian steroidogenesis; (vi) inhibits oocyte maturation *in vitro* (Austin *et al.*, 2012; Tiedemann *et al.*, 2014; Han *et al.*, 2016)	(i) Can accumulate in embryos; (ii) affects blastocyst-stage embryo development and induce apoptosis *in vitro*; (iii) reduces post-implantation success of embryos in host mice (Li *et al.*, 2010)
AuNPs	(i) Cross BTB; (ii) can aggregates on sperm membrane but cannot penetrate *in vitro*; (iii) reduces sperm fertilizing ability *in vitro* (Wiwanitkit *et al.*, 2009; Taylor *et al.*, 2014a,b; Lee *et al.*, 2015)	Do not show toxicity (Chen *et al.*, 2013a,b; Tiedemann *et al.*, 2014)	Does not show toxicity (Taylor *et al.*, 2014a,b)
TiO_2NP	(i) Cross BTB; (ii) affects spermatogenesis via oxidative stress, DNA damage and apoptosis in spermatozoa during spermiogenesis and sperm maturation and affect fertilizing ability; (iii) alters sertoli and Leydig cell functions; (iv) can penetrane inside head and plasma membrane of sperm *in vitro* (Gao *et al.*, 2013; Kyjovska *et al.*, 2013; Jia *et al.*, 2014; Pawar and Kaul, 2014; Hong *et al.*, 2015; Meena *et al.*, 2015; Smith *et al.*, 2015)	(i) Can accumulate in ovary; (ii) induce oxidative stress and apoptosis as well as affects ovarian sex hormone balance; (iii) also induces ovarian inflammation and necrosis; (iv) affects primary and secondary follicle development (follicular atresia) (Gao *et al.*, 2012; Zhao *et al.*, 2013; Tassinari *et al.*, 2014)	No reports
SiO_2NP	(i) Cross BTB; (ii) affects spermatogenesis via oxidative stress, DNA damage and mitochondrial damage in spermatozoa during sperm maturation in epididymis; (iii) induces apoptosis in germ cells via downregulation of miR-98 and its host gene Huwe1 expression and caspase-3 activation (Morishita *et al.*, 2012; Xu *et al.*, 2014; Hassankhani *et al.*, 2015; Xu *et al.*, 2015a,b)	No reports	No reports
CeO_2NP	(i) Cross BTB; (ii) induces DNA damage in *vitro*; (iii) can aggregates on sperm membrane but cannot penetrate *in vitro*; (iv) reduces sperm fertilizing ability *in vitro* (Geraets *et al.*, 2012; Zhao *et al.*, 2013)	(i) Induces DNA damage in follicular cells and oocytes *in vitro* by oxidative stress; (ii) decreases *in vitro* fertilization rate of oocytes (Courbiere *et al.*, 2013; Greco *et al.*, 2015; Preaubert *et al.*, 2015)	No reports
ZnONP	(i) Cross BTB; (ii) affects spermatogenesis mainly via apoptosis of spermatids; (iii) alters sertoli and Leydig cell functions; (iv) induces cytotoxicity on spermatozoa *in vitro* (Li *et al.*, 2012a,b; Barkhordari *et al.*, 2013; Talebi *et al.*, 2013; Moridian *et al.*, 2015)	No reports	Does not show toxicity (Hong *et al.*, 2014)
MoO_3NP, AlNP	Induces cytotoxicity and apoptosis in SSC line C18-4 (Braydich-Stolle *et al.*, 2005)	No reports	No reports
Fe_3O_4NP	Does not show any toxicity (Makhluf *et al.*, 2006)	No reports	No reports
CNT	(i) Cross BTB; (ii) affects spermatogenesis mainly via loss of spermatogonia; (iii) alter Sertoli and Leydig cell functions; (iv) induces oxidative stress (Bai *et al.*, 2010; Farombi *et al.*, 2014)	No reports	Affects embryo development in pregnant mice (Pietroiusti *et al.*, 2011)
CBNPs	Affects spermatogenesis mainly via disruption of steroidogenesis (Yoshida *et al.*, 2009, 2010; Kyjovska *et al.*, 2013)	No reports	No reports
Fullerene (C_{60})	No reports	No reports	Affects cell proliferation of embryonic midbrain cells in pregnant mice via oxidative stress (Tsuchiya *et al.*, 1996)

Continued

Table III *Continued*

Nanoparticles	Effects on male germ cells	Effects on female germ cells	Embryo development
Reduced graphene oxide (GO, rGO)	Shows nearly no or very low toxicity for male reproduction (Liang *et al.*, 2015)	rGO injection before pregnancy or during early gestation does not affect the reproductive health. rGO injection during late gestational causes abortions of all surviving females (Xu *et al.*, 2015a,b)	No reports
QDs	Does not show toxicity (Feugang *et al.*, 2012, 2015)	Affects oocytes maturation and *in vitro* fertilization rate (Hsieh *et al.*, 2009; Xu *et al.*, 2012)	(i) Affects blastocyst development after *in vitro* fertilization of oocytes; (ii) induces apoptosis in developing embryos *in vitro*; (iii) reduces post-implantation success of embryos in host mice and induces apoptosis (Chan and Shiao, 2008; Hsieh *et al.*, 2009)
CSNPs	No reports	No reports	(i) Affects blastocyst-stage embryo development and induces apoptosis in both TE and ICM cells *in vitro*; (ii) reduces post-implantation success of embryos in host mice (Park *et al.*, 2013)

BTB, blood-testis barrier; SSC, spermatogonial stem cell; TE, trophectoderm; ICM, inner cell mass.

reduced relative to 15 and 60 days, reflecting the capacity of the testes to recover initial damage due to clearance of deposited silver from testis. Several other *in vitro* studies also showed that AgNPs did not show any harmful effect on porcine and human spermatozoa (Moretti *et al.*, 2013; Tiedemann *et al.*, 2014). Moretti *et al.* (2013) treated human spermatozoa with AgNPs (65 nm) at a concentration range 30–500 µM for 1 h and did not find any harmful effects, which may be due to the low exposure time. Similarly, Tiedemann *et al.* (2014) reported that 11 nm AgNPs did not affect sperm viability when porcine sperm were treated with 10 µg/ml AgNPs for 2 h. Taken together, all these studies clearly showed that AgNP exposure has deleterious effects on Leydig and Sertoli cell functions, induces apoptosis in all kinds of spermatogenic cells, decreases sperm count, increases germ cell DNA damage, increases sperm abnormalities and decreases acrosome and plasma membrane integrity as well as mitochondrial activity via oxidative stress.

Only a few studies have been conducted on the effects of AgNPs on female germ cells. Austin *et al.* (2012) checked the AgNP distribution on gestation day (GD) 10 in pregnant CD-1 mice after the mice were injected intravenously at a dose of 35 or 66 µg AgNPs/mouse on GD7, 8 and 9. They showed that AgNPs accumulated in the ovaries, myometrium and endometrium in a dose-dependent manner. Han *et al.* (2016) investigated the effects of AgNPs on the ovaries and developing follicles in female mice after a single injection (0.5, 1 mg/kg) of AgNPs (20 nm). They showed that AgNPs increased the expression of proinflammatory cytokines, such as tumor necrosis factor alpha (Tnfa), interferon gamma (Ifng), interleukin 6 (Il6), interleukin 1 beta (Il1b) and monocyte chemoattractant protein 1 (Mcp1) genes in a dose-dependent manner after 15 days of treatment and induced apoptosis in granulosa and theca cells in a time-dependent manner (13, 16.7 and 18% after 2, 3 and 4 weeks compared to control, 11.2%) when administered at 1 mg/kg. AgNP treatment reduced the expression of genes, such as cytochrome P450 11a1 (Cyp11a1), cytochrome P450 19 (Cyp19), 3 beta hydroxysteroid dehydrogenase (3bhsd), 17 beta hydroxysteroid dehydrogenase III (17bhsd3) and cytochrome P450 17a1 (Cyp17a1) that are essential for ovarian steroidogenesis as well as forkhead box O3 (Foxo3a), Stella and Figla, essential for maintenance and survival of primordial follicles until ovulation in both a dose-dependent and time-dependent manner (2–4 weeks). AgNPs treatment (1 mg/kg) was shown to decrease the number of primordial and growing follicles (primary, secondary and Graafian follicles). They proposed that AgNPs-induced impaired ovarian steroidogenesis might be the cause for the downregulation of the genes responsible for follicular development and follicular apoptosis. In one *in vitro* study, Tiedemann *et al.* (2014) investigated the effects of 11 nm AgNPs (10 µg/ml) for 46 h on porcine oocytes. They showed that AgNPs could accumulate in the cumulus cell layer surrounding the oocyte and inhibited cumulus-oocyte maturation. All these studies show that AgNPs have severe toxicological effects on female reproductive functions.

Effects on embryo development. Li *et al.* (2010) checked the effects of AgNPs (13 nm) on pre-implantation embryo developments. In their study, blastocysts (obtained from ICR female mice) were treated with 25 or 50 µM AgNPs for 24 h. They demonstrated that AgNPs decreased the cell numbers in the inner cell mass (ICM) and trophectoderm (TE) as well as increased apoptosis rates in blastocysts in a

dose-dependent manner following 8 days of culture. However, Taylor *et al.* (2014b) demonstrated that AgNPs (21.5 nm) treatment at 50 µg/ml in mouse 2-cell embryos did not have any detrimental effects on development up to blastocyst stage, which might be due to larger size compared to that used in the study of Li *et al.* (2010). Yoisungnern *et al.* (2015) checked the effects of AgNP-treated sperm on the IVF of oocytes and subsequent embryo development after treating mouse spermatozoa with 0.1, 1, 10 or 50 µg/ml AgNPs (40 nm) for 3 h. They showed that AgNP treatment dose-dependently decreased the rate of blastocyst formation after the IVF of oocytes. AgNPs also decreased the number of both TE and ICM cells in a dose-dependent manner in blastocysts. Li *et al.* (2010) also checked the effects of AgNPs on post-implantation development (treated with 25 or 50 µM AgNPs for 24 h) after transfering blastocyst embryos into the uterine horns of pseudopregnant mice. AgNPs reduced the implantation success rate and post-implantation development in a dose-dependent manner into recipient mice.

The ability of AgNPs to cross the placenta has been reported by Austin *et al.* (2012). They showed that AgNPs (50 nm) could accumulate in the placenta, visceral yolk sacs and embryos in a dose-dependent manner on GD10 in pregnant CD-1 mice after the mice were injected intravenously at a dose of 35 or 66 µg AgNPs/mouse on GD7, 8 and 9. Han *et al.* (2016) showed that the number of fetuses in pregnant mice was reduced after mating with either AgNP-treated (1 mg/kg, single injection) female or male mice or both male and female mice treated with AgNPs. Therefore, the possible embryotoxic effects of AgNPs are also evident from the above discussion.

Gold nanoparticles

Gold nanoparticles (AuNPs) are among the most commonly used nanoparticles, which have extensive uses in catalysis, electronics, cancer diagnosis and therapy, biological sensors and drug delivery (Jeong *et al.*, 2014; Jafri *et al.*, 2015; Liu *et al.*, 2015).

Effects on male and female germ cells. Studies on the effects of AuNPs on male and female germ cells are limited. Lee *et al.* (2015) showed that AuNPs (15 nm) could cross the BTB and accumulate in the testis of mice after long-term (6 months) single exposure to AuNPs at a dose (i.v.) of 1 mg/kg body weight. Chen *et al.* (2013a,b) showed that AuNP (4.4, 22.5, 29.3 or 36.1 nm) exposure in mice (14 injections at a dose of 4 mg/kg every 2 days) did not induce a toxicological response in the male reproductive system. Taylor *et al.* (2014a) investigated the effects of AuNPs (10.8 nm) on bovine spermatozoa *in vitro*. The sperm was treated with 0.1, 1 or 10 µg/ml AuNPs for 2 h and used for IVF of oocytes. They showed that AuNPs only attached as aggregates to the sperm membrane, but could not penetrate the sperm. AuNPs decreased sperm motility and fertilization rates only at the highest used dose, whereas the sperm morphology and viability remained unaltered. However, conjugation with oligonucleotide partly inhibited the adverse effects of AuNPs. They proposed that AuNPs impaired sperm functions during fertilization, primarily by interacting with the sperm surface membrane. Wiwanitkit *et al.* (2009) showed that AuNPs (9 nm) could reduce sperm motility by up to 25% when human spermatozoa were treated with a very high concentration of AuNPs (44 ppm or 44 µg/ml) for 15 min. On the other hand, Moretti *et al.* (2013) demonstrated that 50 nm AuNPs did not show any harmful effects on spermatozoa that were treated with 30–500 µM AuNPs for 1 h. Similarly, Tiedemann *et al.* (2014) showed that 20 nm AuNPs did not affect sperm motility, membrane integrity and morphology using porcine spermatozoa that were treated with 10 µg/ml AuNPs for 2 h. Therefore, only very small AuNPs can cause sperm defects. Surface coating with oligonucleotides also partly inhibits the adverse effects of AuNPs in spermatozoa.

Tiedemann *et al.* (2014) studied the effects of different-sized AuNPs (6, 8 or 20 nm) on porcine oocytes treated either with 10 or 30 µg/ml AuNPs for 46 h. They showed that AuNPs could be taken up by the oocytes without any harmful effects on oocyte maturation. Chen *et al.* (2013a,b) demonstrated that AuNPs (4.4, 22.5, 29.3 or 36.1 nm) did not show any toxicological responses in the female reproductive system after 14 injections at a dose of 4 mg/kg every 2 days in the mice.

Effects on embryo development. In only one study, Taylor *et al.* (2014b) evaluated the toxic effects of 10.8 nm AuNPs on mouse 2-cell embryos that were treated with 50 µg/ml AuNPs and cultured to the blastocyst stage. AgNPs did not show any detrimental effects on the development of mouse embryos. Therefore, AuNPs do not appear to be harmful to developing mouse embryos.

Titanium dioxide nanoparticles

Titanium dioxide nanoparticles (TiO_2NPs) are extensively used as a pigment in rubber, inks, paints, plastics, paper, cosmetics, toothpaste and food colorants (Ema *et al.*, 2010; Shakeel *et al.*, 2015).

Effects on male and female germ cells. A number of studies have been conducted on the effects of TiO_2NPs on male germ and testicular cells both *in vivo* and *in vitro*. In one *in vivo* study, Gao *et al.* (2013) treated the CD-1 male mice by intragastric administration of TiO_2NP (5–6 nm) at a dose of 2.5, 5 or 10 mg/kg for 90 consecutive days and showed that TiO_2NPs can cross the BTB, accumulate in testicular cells in a dose-dependent manner and caused severe histological changes in the testis, including Sertoli cell apoptosis. Meena *et al.* (2015) also demonstrated a similar kind of dose-dependent accumulation of TiO_2NPs in the testis and abnormal testicular morphology with disrupted/disorganized seminiferous tubules of male Wistar rats, which were given intravenous injections (5, 25 or 50 mg/kg) of 21 nm TiO_2NPs for 30 days at weekly interval. In addition, TiO_2NPs caused inflammation in the testicular cells. Hong *et al.* (2015) also investigated the effect of TiO_2NPs (5–6 nm) on spermatogenesis suppression in ICR mice. In their study, ICR male mice were given intragastric administration of TiO_2NPs (2.5, 5 or 10 mg/kg) for 60 consecutive days. They showed that TiO_2NPs impaired testis and epididymis architecture, and presented severe histopathological changes. On the other hand, Smith *et al.* (2015) demonstrated that TiO_2NPs could accumulate in the scrotal adipose tissues, but none were detected in the testis of male ICR mice, which were given three injections (5 mg/kg) of TiO_2NPs (50 nm) intraperitoneally for three consecutive days and sacrificed 120 h post-injection. Histological analysis showed enlarged interstitial spaces in the testis. However, when the mice were injected with either 2.5 or 5 mg/kg TiO_2NPs and sacrificed 1–3 weeks later, both dosages showed similar testicular abnormalities. In another study, Jia *et al.* (2014) checked the effects of pubertal exposure to TiO_2NP (25 nm) in male Kunming mice,

which were treated orally either with a relatively low dose (10 mg/kg/day) or very high doses (50 or 250 mg/kg/day orally) starting from PND23 to PND70. At the lowest doses, TiO_2NP did not show any histological changes in the testis, whereas at the higher doses, TiO_2NP showed vacuoles in seminiferous tubules. TiO_2NP also decreased the layers of spermatogenic cells when applied at the highest dose. TiO_2NPs also reduced testosterone levels dose-dependently via the downregulation of steroidogenesis-related genes. The decrease in serum testosterone level due to TiO_2NP exposure has also been reported by Meena *et al.* (2015) and Gao *et al.* (2013).

Gao *et al.* (2013), Hong *et al.* (2015) and Meena *et al.* (2015) reported that TiO_2NPs decreased sperm numbers and sperm motility, and sperm abnormalities increased in a dose-dependent manner. Kyjovska *et al.* (2013) studied the effects of maternal airway exposure to TiO_2NPs on the daily sperm production in their offspring. They showed that the F1 male offspring, born from TiO_2 (20.6 nm) exposed mothers (exposed via whole body inhalation, 1 h/day from GD8 to GD18 with 42 mg/m^3 aerosolized TiO_2 powder) tended to display reduced sperm production (but this was not statistically significant). Meena *et al.* (2015) demonstrated that TiO_2NPs induce oxidative stress, which causes DNA damage and apoptosis in spermatozoa during spermiogenesis or sperm maturation, thereby affecting their fertilization potential. Therefore, Hong *et al.* (2015) hypothesized that the TiO_2NP-induced suppression of spermatogenesis might be closely related to alterations in testicular enzymatic activity (i.e. lactate dehydrogenase, sorbitol dehydrogenase, succinate dehydrogenase, glucose-6-phosphate dehydrogenase, ATPases, acid phosphatase, alkaline phosphatase and total nitric oxide synthase), oxidative stress and DNA damage. Smith *et al.* (2015) showed that TiO_2NP exposure in male ICR mice given three injections (2.5 or 5 mg/kg) of TiO_2NPs (50 nm) intraperitoneally for three consecutive days, decreased the sperm acrosome reaction and motility rates at 48 and 120 h dose-dependently after the last injection. The highest dose treatment decreased the mitochondrial membrane potential and hyperactivated motility, and increased apoptosis and ROS formation at 120 h. However, all these TiO_2NP-induced structural and functional sperm defects were transient, as they were not detected 10 days to 5 weeks post-injection. Therefore, they proposed that TiO_2NP exposure mainly affects epididymal sperm maturation and function via DNA damage and oxidative stress.

In one *in vitro* report from Pawar and Kaul (2014) showed the adverse effects of TiO_2NPs on mammalian spermatozoa where buffalo sperm was treated with 1, 10 or 100 µg/ml TiO_2NPs (30–90 nm) for 6 h. They found TiO_2NPs inside the head and plasma membrane of the spermatozoa. TiO_2NPs decreased sperm viability, membrane integrity, and increased DNA fragmentation in a dose-dependent manner. They demonstrated that TiO_2NPs increased DNA damage either via direct interaction within the sperm head or indirectly via ROS formation.

Tassinari *et al.* (2014) investigated the short-term effects of TiO_2NP (20–60 nm) exposure on female reproductive systems in Sprague Dawley female rats administered TiO_2NPs orally at a dose of 1 or 2 mg/kg for 5 consecutive days and sacrificed at day 6. They found a dose-dependent increase in the total Ti tissue levels in ovaries and apoptosis in ovarian cells. In the other two studies, the effects of long-term exposure to TiO_2NPs (5–6 nm) on ovarian dysfunction in ICR female mice by intragastric administration of TiO_2NPs at a dose of 2.5, 5 or 10 mg/kg (Gao *et al.*, 2012; Zhao *et al.*, 2013) for 90 consecutive days were investigated. Zhao *et al.* (2013) showed that TiO_2NPs could be translocated to the ovaries and accumulated within ovarian cells, decreased mouse body weight (34, 28 and 22 g for 2.5, 5 or 10 mg/kg treatment groups, respectively, compared to control, 38 g), and the relative weight of the ovary (0.8, 0.5 and 0.3 mg/g for 2.5, 5 or 10 mg/kg treatment groups, respectively, compared to control, 1.25 mg/g). Gao *et al.* (2012) demonstrated that TiO_2NPs could accumulate both in the cytoplasm and in the nucleus of ovarian cells and cause severe ultrastructural changes, such as mitochondrial swelling and cristae breakdown, irregular nuclear membrane and nuclear chromatin condensation when administered at a 10 mg/kg dose. At the same dose TiO_2NPs also induced apoptosis in the ovaries, caused atresia of primary and secondary follicles, and decreased fertility or pregnancy rate. Zhao *et al.* (2013) showed that TiO_2NPs caused follicular atresia, inflammatory cell infiltration and necrosis in all the treated groups (2.5, 5 or 10 mg/kg), although the ovarian injury was increased with increasing doses. They also showed that the pregnancy rate (81%, 72% and 58% for 2.5, 5 or 10 mg/kg treatment groups, respectively, compared to control) and the number of fetuses (10, 8 and 6 for 2.5, 5 or 10 mg/kg treatment groups, respectively, compared to control, 14) decreased in mice in a dose-dependent manner. Gao *et al.* (2012) reported that TiO_2NPs cause ovarian dysfunction, which is closely associated with increased ROS production (five and four times more superoxide and hydrogen peroxide formation, respectively, compared to control), peroxidative DNA damage (three times more compared to control), disturbances in the sex hormone (estradial, progesterone, LH and FSH) equilibrium and alterations in functional gene expression levels, such as increased Cyp17a1 (responsible for increased estradiol biosynthesis), aldo-keto reductase family 1, member C18 (Akr1c18, increased progesterone metabolism, thereby reducing progesterole level) and legumain (Lgmn, increased steroid metabolism) as well as upregulation of pro-apoptotic genes, such as Bcl2a1b and Bcl2-modifying factor (Bmf) in the ovaries.

Zhao *et al.* (2013) also reported that ovary injury and fertility reduction due to TiO_2NPs are closely associated with disturbances in the sex hormone equilibrium, and alterations in inflammation-related or follicular atresia-related cytokine expression [upregulation of insulin-like growth factor-binding protein (Igfbp2), epidermal growth factor (Egf), Tnfa, tissue plasminogen activator (Tpa), Il1b, Il6, Fas and Fas ligand (Fasl); downregulation of insulin-like growth factor 1 (Igf1), luteinizing hormone receptor (Lhr), inhibin alpha (Inha) and growth differentiation factor 9 (Gdf9) in the ovaries].

Effects on embryo development. Gao *et al.* (2012) and Zhao *et al.* (2013) reported that the number of offspring decreased in female mice after intragastric administration of TiO_2NPs (at a dose of 2.5, 5 or 10 mg/kg for 90 consecutive days) in a dose-dependent manner. All these studies clearly indicate that TiO_2NPs have reprotoxicological effects of on both males and females as well as fetotoxic effects.

Silica nanoparticles

SiO_2NPs have a wide variety of applications in biomedical imaging, gene delivery, photodynamic therapy, catalytic supports, etc. (Roy *et al.*, 2005; Kim *et al.*, 2006a,b; Lin *et al.*, 2007; Lu *et al.*, 2007; Ohulchanskyy *et al.*, 2007).

Effects on male and female germ cells. The effects of SiO_2NPs on male germ and testicular cells have been studied thoroughly in both *in vivo* and *in vitro* models. Xu *et al.* (2014) checked SiO_2NP (64.43 nm) internalization in the spermatogenic cells of ICR male mice that were given five injections (20 mg/kg) every 3 days and were sacrificed at 15, 35 and 60 days after the first dose. They demonstrated that on day 60, SiO_2NPs in spermatogenic cells mainly accumulated in the perinuclear region, but did not penetrate the nucleus after intraperitoneal injection in male mice. In another study, Morishita *et al.* (2012) checked the biodistribution of SiO_2NPs (70 and 300 nm) in the testis of BALB/c mice, which were given 0.8 mg SiO_2NPs intravenously on two consecutive days and analyzed 1 day after the last injection. They showed that SiO_2NPs (70 nm) could be found in Sertoli cells near the spermatozoa and in both the cytoplasm and the nucleus of spermatocytes, whereas 300 nm-sized SiO_2NP was not observed in the testis. Later, they (Morishita *et al.*, 2012) also treated the BALB/c mice with four injections of SiO_2NP (70 nm) at either 0.4 or 0.8 mg doses every other day and testes were collected 2 days or 4 weeks after the last injection. They demonstrated that SiO_2NP did not cause significant testicular injury. Leclerc *et al.* (2015) checked the testicular biodistribution of 70 nm gold-core SiO_2NPs after intramuscular injection at a dose of 1.6×10^{13} particles per mouse and showed that these nanoparticles neither caused histopathological changes nor were incorporated into the testes up to 45 days post-injection. Hassankhani *et al.* (2015) checked the effects of 10–15 nm-sized SiO_2NPs in male Wistar rats, which were treated at a dose of 333.33 mg/kg bw/day orally for 5 days and demonstrated that SiO_2NP caused testicular histopathological changes at very high doses. In one *in vitro* study, Xu *et al.* (2015a,b) investigated the effects of SiO_2NPs in TM4 Sertoli cells and GC-2 spermatocytes that were treated with 0.1–100 µg/ml SiO_2NPs (11.6 nm) for 24 h. SiO_2NPs did not have cytotoxic effects in TM4 cells but induced apoptosis in GC-2 cells only at the highest dose via mitochondrial dysfunction and caspase-3 activation. They also demonstrated that SiO_2NP exposure negatively regulated miR-98 and its host gene expression (Huwe1), which might cause enhanced caspase-3 expression and subsequent apoptosis in GC-2 cells.

Xu *et al.* (2014) demonstrated that SiO_2NPs caused damage to the mitochondrial cristae, decreased ATP levels and induced oxidative stress in testis, reduced epididymal sperm concentration and sperm motility, whereas sperm abnormalities increased by days 15 and 35 after five injections (20 mg/kg) of SiO_2NPs in ICR male mice. SiO_2NP also caused DNA damage in spermatozoa, but did not affect sperm acrosome integrity or fertility by days 15 and 35. However, all these adverse effects of SiO_2NP recovered by day 60. They proposed that SiO_2NP did not affect the spermatogonia and spermatocytes, but primarily affected the sperm maturation process in the epididymis by causing oxidative stress and mitochondrial damage, resulting in energy metabolism dysfunction. Wolterbeek *et al.* (2015) performed a two-generation reproductive toxicity study with SiO_2NP (200 nm) in Wistar rats. In their study, male and female rats were treated at doses of 100–1000 mg/kg bw/day orally for two generations. SiO_2NPs did not show any adverse effects on the reproductive performance of the rats, nor did it affect the growth and development of their offspring for two consecutive generations.

In another two *in vitro* reports, Barkalina *et al.* (2014, 2015) demonstrated that SiO_2NP (hydrodynamic diameter 147–322 nm) showed good biocompatibility (did not affect sperm viability, motility, acrosomal status and DNA fragmentation) with boar sperm that was treated with 10–30 µg SiO_2NPs per 10^7 sperm for 2 or 4 h. All these studies suggest that SiO_2NPs possess very little or no harmful effects on spermatogenesis.

Yamashita *et al.* (2011) checked pregnancy complications caused by different-sized SiO_2NPs (70, 300 and 1000 nm) in mice, which were injected with 0.8 mg of SiO_2NPs per mouse via tail vein intravenously on two consecutive days, at GD16 and GD17. The maternal body weight was significantly decreased at GD17 and GD18 after treatment with 70 nm SiO_2NPs. The 70 nm SiO_2NPs also decreased the uterine weight at GD18. However 300 and 1000 nm SiO_2NPs did not result in any changes. Surface modification of 70 nm SiO_2NPs with either carboxyl or amine groups could significantly prevent all these alterations.

Effects on embryo development. Yamashita *et al.* (2011) also checked the biodistribution of different-sized SiO_2NPs (70, 300 and 1000 nm) in the fetuses and placenta of pregnant mice injected with 0.8 mg of SiO_2NPs via the tail vein at GD16. They showed that only the 70 nm SiO_2NPs accumulated in the placenta and fetuses, whereas 300 and 1000 nm SiO_2NPs could not. Treatment with 70 nm SiO_2NPs also showed variable structural abnormalities in the placenta, such as failure of spiral artery canal formation and reduced blood flow in the fetal vascular sinuses. SiO_2NP treatment reduced the area of the spongiotrophoblast layer and the ratio of the spongiotrophoblast layer area to the total placental area compared to those observed in control mice. SiO_2NPs induced apoptosis in spongiotrophoblasts and decreased the surrounding lengths of the villi in the labyrinth layer compared to those of control mice. The plasma level of soluble fms-like tyrosine kinase-1 (sFlt-1) in SiO_2NPs-treated mice was significantly lower than in control mice. However, all these SiO_2NPs-induced structural and functional abnormalities in the placenta were abolished after coating with carboxyl or amine groups. Treatment with 70 nm SiO_2NPs decreased the fetal weight as well as increased the fetal resorption rates significantly. Seventy nanometer SiO_2NPs-treated mice also had smaller fetuses than untreated ontrol groups. However, 300 and 1000 nm SiO_2NPs did not show any changes. Surface modification of 70 nm SiO_2NPs with either carboxyl or amine groups could significantly prevent resorption and fetal growth restriction. Therefore, SiO_2NPs may be associated with pregnancy complications and fetal growth restriction, and the detrimental effects of SiO_2NPs can be lessened via suitably modifying their surfaces.

Ceria nanoparticles

Ceria nanoparticles (CeO_2NPs) are widely used as an additive for diesel due to their catalytic and oxidative properties as well as having applications in cancer therapies (Park *et al.*, 2008; Cassee *et al.*, 2011; Giri *et al.*, 2013; Sack *et al.*, 2014).

Effects on male and female germ cells. Geraets *et al.* (2012) first showed that CeO_2NPs (5–40 nm) could accumulate inside the testis and epididymis after a single and repeated inhalation exposure in male Wistar rats for 6 h per day for 28 days. Preaubert *et al.* (2015) showed the genotoxic effects of CeO_2NPs (7 nm) in mouse spermatozoa that were treated with 0.01 mg/l CeO_2NP for 1 h (% tail DNA was 3.64 times higher than control). Preaubert *et al.* (2015)

quantified the DNA damage in sperm by analyzing the percentage of DNA in the tail, which is total DNA that migrates from the nucleus into the comet tail during the electrophoresis. However, CeO_2NPs did not affect sperm viability and motility after treatment with a similar dose and time. Transmission electron microscopy (TEM) detected CeO_2NPs along the spermatozoa plasma membrane when the spermatozoa were treated with 0.01 mg/ml CeO_2NP for 2 h. However, TEM did not detect any CeO_2NPs in the cytoplasm of the spermatozoa even after treatment with 100 mg/l CeO_2NP for 2 h. Falchi *et al.* (2014) reported that CeO_2NP did not induce cytotoxicity in ram spermatozoa incubated with CeO_2NPs (22, 44 or 220 μg/ml) for up to 24 h at 4°C.

Courbiere *et al.* (2013) first assessed the cellular internalization and genotoxic effects of CeO_2NPs (3 nm) in mouse oocytes and follicular cells, which were treated with 2–100 mg/l CeO_2NPs for 2 h. TEM analysis showed accumulated CeO_2NPs in follicular cells when treated with the highest dose. In oocytes, CeO_2NPs accumulation was observed around the zona pellucida. However, CeO_2NPs cannot enter the oocyte cytoplasm even in the absence of the zona pellucida. CeO_2NPs induced DNA damage in both follicular cells and oocytes in a dose-dependent manner (mean olive tail moment, assessed in the single cell gel electrophoresis, or comet, assay, was 2–5 times higher than control). However, treatment with antioxidants significantly decreased the CeO_2NP-induced DNA damage in both follicular cells and oocytes. They also proposed that immature oocytes, with no or immature ZP and fewer follicular cells, could be more vulnerable to the DNA damage induced by CeO_2NPs. Greco *et al.* (2015) also demonstrated similar DNA-damaging effects for CeO_2NPs in mouse oocytes when treated with 100 mg/l CeO_2NPs for 2 h. However, Preaubert *et al.* (2015) reported that CeO_2NPs (7 nm) could induce DNA damage (mean olive tail moment was five times higher than control) in mouse oocytes even at a very low dose (0.01 mg/l) and low exposure time (1 h). Preaubert *et al.* (2015) also checked the effects of CeO_2NPs (7 nm) on IVF. In their study, they performed IVF (containing both cumulus-oocyte complexes and spermatozoa) with 0.01 mg/l CeO_2NPs for 5 h and found that the IVF rate significantly decreased after CeO_2NP treatment (55% compared to control, 68%). They also checked the cellular internalization of CeO_2NP during IVF and found CeO_2NP accumulation around the oocyte zona pellucida after treatment with 100 mg/l for 2 h. However, CeO_2NPs could not enter into the cytoplasm of oocytes and embryos. Finally, they proposed that CeO_2NPs decreased the IVF rate via oxidative stress, DNA damage and a mechanical effect, which disrupted gamete interactions. All these studies show that CeO_2NPs impart xenotoxic effects in germ cells and affect their fertilizing ability. However, effects of CeO_2NPs on embryo development have not been investigated yet.

Zinc oxide nanoparticles

Zinc oxide nanoparticles (ZnONPs) have been used in several products, such as gas sensors, solar cells, biosensors, ceramics, photo detectors, UV filters and antivirus agents as well as being used as catalysts (Chen and Lia, 2003; Hu *et al.*, 2003; Li and Wu, 2003; Hernandez Battez *et al.*, 2008; Gerloff *et al.*, 2009; Jin *et al.*, 2009; John *et al.*, 2010; Rasmussen *et al.*, 2010; Schilling *et al.*, 2010; He *et al.*, 2011).

Effects on male and female germ cells. The first report regarding the biodistribution of ZnONPs in the testis of mice was published by Li *et al.* (2012a,b). In their study, ICR male mice received a single dose (2.5 g/kg) of ZnONPs (50 nm) through intraperitoneal injection or oral administration and the tissue biodistribution of ZnONP was checked at 3 days post-dosing. They demonstrated that only intraperitoneally administered ZnONPs accumulated in the testis. Later, Talebi *et al.* (2013) investigated the effects of ZnONPs on spermatogenesis in mice. In their study, rats received ZnONPs (5, 50 or 300 mg/kg) orally every day for 35 consecutive days and were sacrificed on day 36. In the 5 mg/kg treated group, the percentage of vacuolized seminiferous tubules was increased. ZnONPs caused a reduction in the diameter of the seminiferous tubules as well as height of the seminiferous epithelium, dose-dependently, when applied at very high doses (50 and 300 mg/kg). Moridian *et al.* (2015) also checked the adverse effects of ZnONPs in male mice exposed to similar doses and time-periods as mentioned by Talebi *et al.* (2013). ZnONP significantly decreased the seminiferous tubular volume/diameter and testicular volume/weight as well as the number of Leydig cells and testosterone concentration in a dose-dependent manner from 50 to 300 mg/kg.

Talebi *et al.* (2013) predicted the possibility for Sertoli cell dysfunction and induction of apoptosis or autophagy in testicular germ cells after ZnONPs exposure. They also have demonstrated varying degrees of germ cell degenerative changes due to ZnONPs exposure, such as detached and vacuolized seminiferous tubules, loss of elongated spermatids and disorganization of germ cell layers, including sloughing of germ cells, in 50 and 300 mg/kg treated groups. In addition, multinucleated giant cells were detected in seminiferous tubules of the 300 mg/kg treated group. Finally, they showed that ZnONPs caused a reduction in epididymal sperm number, motility and increased sperm abnormalities in a dose-dependent manner from 50 and 300 mg/kg, whereas treatment with 5 mg/kg did not show any harmful effects on spermatogenesis and epididymal sperm parameters. Moridian *et al.* (2015) have proposed that impaired steroidogenesis due to the loss of Leydig cells could induce germ cell death, leading to decreased seminiferous tubular volume/diameter and testicular volume/weight.

There is only one *in vitro* report on the adverse effects of ZnONPs on mammalian (human) spermatozoa (Barkhordari *et al.*, 2013). Human semen samples were incubated with 50 nm ZnONPs (10–1000 μg/ml) and sperm viability was measured at different time intervals (45, 90 or 180 min). ZnONPs showed both dose and time-dependent cytotoxicity. The highest concentration (1000 μg/ml) led to the highest cell death (20.8%, 21.2% and 33.2% after 45, 90 and 180 min exposure, respectively). The lowest concentration (10 μg/ml) did not show any toxicity at up to 180 minutes exposure, whereas 15% cell death was observed when treated with 100 μg/ml for 180 minutes. These studies clearly demonstrate that ZnONPs have negative effects on spermatogenesis and induce sperm toxicity only when applied at very high doses. The toxicological effect of ZnONPs on female germ cells has not been investigated yet.

Effects on embryo development. In only one study, Hong *et al.*, (2014) checked the toxic effects of ZnONPs on embryo development in rats. In their study, female Sprague Dawley pregnant rats received 20 nm ZnONPs (100, 200 or 400 mg/kg/day) over the period

of GD5–19 by oral gavage. They demonstrated that ZnONPs did not show any harmful effects on embryo-fetal development.

Aluminum, molybdenum oxide and iron oxide nanoparticles

Aluminum nanoparticles (AlNPs) are being used in electronic circuits and military devices, as antimicrobials, new tissue-biopsy tools and to prevent scratches in plastic lenses (Braydich-Stolle *et al.*, 2010a; Ema *et al.*, 2010). Molybdenum oxide nanoparticles (MoO_3NPs) have applications as catalysts, gas sensors and display materials (Kim *et al.*, 2010a,b). Iron oxide nanoparticles (Fe_3O_4NPs) have several biomedical and diagnostic applications including targeted cellular therapy, magnetofection, magnetic imaging and drug delivery (Makhluf *et al.*, 2006).

Effects on male and female germ cells. The toxicological effects of these nanoparticles on male germ cells are limited. In only one study, Braydich-Stolle *et al.* (2005) assessed the cytotoxic effects of AlNPs (30 nm) and MoO_3NPs (30 nm) in a mouse SSC line (C18-4). The C18-4 line was treated with 5–100 µg/ml nanoparticles for 48 h. They demonstrated that both AlNPs- and MoO_3NP-induced dose-dependent cytotoxicity and apoptosis. However, MoO_3NPs are less cytotoxic as it induced around 6% apoptosis when applied at 50 µg/ml, whereas AlNPs induced around 13% apoptosis when applied at 5 µg/ml. In another study, Makhluf *et al.* (2006) evaluated the effects of Fe_3O_4NP on mammalian sperm functions. In their study, bovine sperm was treated with 5 nm Fe_3O_4NP (7.35 mM as Fe ions) for 6 h. They demonstrated that Fe_3O_4NP could spontaneously enter the spermatozoa without affecting sperm motility and acrosome reaction. The toxicological effect of these nanoparticles on female germ cells and embryo development has not been investigated yet.

Metal and metal oxide nanoparticles for gene delivery in germ cells and embryos

Among the class of metal and metal oxide nanoparticles, AuNPs (Sandhu *et al.*, 2002; Thomas *et al.*, 2003; Tsai *et al.*, 2004; Peng *et al.*, 2014; Du *et al.*, 2015), SiO_2NPs (Kneuer *et al.*, 2000; Csogor *et al.*, 2003; Ngamcherdtrakul *et al.*, 2015; Santo-Orihuela *et al.*, 2016) and Fe_3O_4NPs (Park *et al.*, 2014; Xiao *et al.*, 2015; Stephen *et al.*, 2016) are widely used as safe and efficient gene delivery vectors in mammalian cells and *in vivo*. AuNPs have been used in diverse applications in biology and medicine because of their facile synthesis, easy surface modification and bioconjugation, tunable size and shape, and tunable electronic and optical properties. Similarly, SiO_2NPs can load and release large amounts of nucleic acids depending on their pore size, pore morphology and surface properties that can be easily tuned. Super-paramagnetic iron oxide nanoparticles (Fe_3O_4NPs) can increase the uptake or internalization of nucleic acids complexed with Fe_3O_4NPs into cells under the influence of external magnetic fields (Park *et al.*, 2014; Xiao *et al.*, 2015; Stephen *et al.*, 2016). AuNPs and Fe_3O_4NPs did not show toxicity in male or female germ cells and developing embryos. On the other hand, SiO_2NPs show pregnancy complications and fetal growth restrictions, but these effects can be lessened via suitably modifying their surfaces (Yamashita *et al.*, 2011). Therefore, AuNPs, SiO_2NPs and Fe_3O_4NPs can be considered as safe carriers for gene delivery into gametes and embryos.

Several other researchers have also shown AgNPs (Brown *et al.*, 2013; Tao *et al.*, 2013; Peng *et al.*, 2014), TiO_2NPs (Levina *et al.*, 2012; Cho *et al.*, 2013) and ZnONPs (Zhang and Liu, 2010) could be used as efficient delivery vectors into mammalian cells. However, for gene delivery into germ cells and embryos, AgNPs and TiO_2NPs cannot be used as they cause severe toxicity in germ cells and decrease pregnancy rates in females. ZnONPs did not show any pregnancy complications and harmful effects on embryo-fetal development (Hong *et al.*, 2014), but affect spermatogenesis and induce sperm toxicity only when applied at very high doses (Talebi *et al.*, 2013; Moridian *et al.*, 2015). Therefore, ZnONPs can be used for gene delivery into germ cells and embryos with restrictions, such as avoidance of high concentrations. So far, AlNPs, MoO_3NPs and CeO_2NPs have not been reported as being used as gene delivery vectors. CeO_2NPs show genotoxic effects in both male and female germ cells, thereby affecting their fertilizing ability, whereas the embryotoxic effects are not yet known. Similarly, AlNPs and MoO_3NPs induce apoptosis in SSCs, but the effects on spermatozoa, female germ cells and embryo development have not been investigated. Therefore, at this stage we can assume that we cannot use those nanoparticles for gene delivery into germ cells, but it is difficult to predict whether these can be used for gene delivery into embryos.

Toxicological effects of carbon-based nanoparticles on germ cells and embryos

The *in vivo* and *in vitro* effects of carbon-based nanoparticles on male gametes/testes, female gametes/ovary and embryo development are shown in Table III.

Carbon nanotubes

CNTs have been classified as single-wall and multiwall carbon nanotubes (SWCNT and MWCNT) depending on the number of graphene layers. They attract extensive interest due to their unique physicochemical properties suitable for a variety of applications, such as in paper batteries, cables and wires, transistors and electrical circuits and biomedical imaging (Jerosz *et al.*, 2011; Cheng *et al.*, 2013a,b; Islam *et al.*, 2015; Bhattacharya *et al.*, 2016).

Effects on male and female germ cells. A few reports have been published on the toxicological effects of CNTs on male reproduction. The first report was published in 2010 by Bai *et al.*, who checked the effects of both amine ($-NH_2$) and carboxylate (COOH)-functionalized MWCNTs. In their study, male BALB/c mice were treated with either a single dose of 5 mg/kg MWCNT (diameter = 20–30 nm; length = 0.5–2 µm) or five doses of CNT every 3 days by intravenous injection at 5 mg/kg per dose. Reproductive toxicological assessments were conducted after both short (15 days), and long-term exposure (60 and 90 days). They checked the testicular accumulation of COOH-CNTs following a single intravenous injection and demonstrated that accumulation of CNTs increased for up to 24 h. Treatment with five intravenous injections of COOH-CNTs caused histopathologic changes in the seminiferous tubules after 15 days. In another short-term exposure study, Farombi *et al.* (2014) showed that intraperitoneally administered multiwall COOH-CNTs (diameter = 11.5 nm; length = 12 µm) in rats at a dose of 0.25–1.0 mg/kg for 5 days produced marked histopathological changes in both the testis and

epididymis. Bai et al. (2010) also demonstrated that repeated dose administration of COOH-CNTs caused a few necrotic and degenerative cells in seminiferous tubules, vasodilatation as well as hyperemia in the testes and partial disappearance or vacuolization of Sertoli cells in some of the seminiferous tubules. However, no changes were observed in Leydig cells and serum testosterone level. NH_2-CNTs also caused similar but less severe alterations in the testes and all of these pathologic changes had significantly recovered after 60 and 90 days. On the other hand, Farombi et al. (2014) reported decreased plasma testosterone levels after short-term exposure (5 days) of COOH-CNTs. Bai et al. (2010) also showed that repeated dose administration of COOH-CNTs caused a reduction in the thickness of the germinative layer and the number of spermatogonia; however, no changes were observed in spermatids.

COOH-CNTs also caused oxidative stress in the testis after 15 days, but not after 60 and 90 days. They demonstrated that CNTs neither affected the quantity and quality of the sperm cells nor the fertility of the treated male mice throughout the 90-day period. Farombi et al. (2014) demonstrated that short-term exposure of COOH-CNTs increased oxidative stress in both the testis and spermatozoa in a dose-dependent manner. They also showed that CNTs decreased epididymal sperm count, sperm progressive motility and daily sperm production with elevated levels of sperm abnormalities in pubertal male Wistar rats in a dose-dependent manner after short-term exposure. In another study, Hougaard et al. (2013) demonstrated that CNTs did not affect daily sperm production in 125-day-old male mice after maternal intratracheal instillation in C57BL/6J mice (with 67 μg MWCNT), followed by mating with mature males the next day. In one *in vitro* study, Rafeeqi and Kaul (2010) used CNTs as a scaffold for spermatogonial cell culture for 21 days. They showed good biocompatibility for CNTs with spermatogonial cells as compared to the positive control (Sertoli feeder layer). Therefore, CNTs show immediate negative effects on spermatogenesis only after short-term exposure.

Lim et al. (2011a) investigated the maternal toxic effects of MWCNTs (diameter = 10–15 nm; length = 20 μm) in pregnant female Sprague Dawley rats treated with CNTs (40, 200 or 1000 mg/kg/day) by oral gavage over the period of GD6–19. Upon evaluation on GD20, no significant differences in maternal weight gain, gravid uterine weight, food consumption, serum biochemistry parameters and pregnancy rates were observed among the groups. At the dose of 1000 mg/kg, only the thymus weights of the pregnant mice were decreased significantly. They demonstrated that CNTs did not show any harmful effects on the dam up to a 200 mg/kg dose.

In another study, Qi et al. (2014) checked the toxic effects of oxidized MWCNTs (diameter = 10–30 nm; length = 1–2 μm) administered at a dose of 20 mg/kg body weight intravenously (at GD7) in pregnant mice with different numbers of pregnancies. They showed that maternal body weight gain was inhibited until GD13, 10 and 11 for first-time, second-time and fourth-time pregnant mice, respectively, and then continued to increase until abortion or parturition. The abortion rate due to CNTs exposure in the first-time pregnant mice was higher than second-time and fourth-time pregnant mice. The serum progesterone levels were decreased, whereas serum estradiol levels were increased due to CNT exposure in the first-time pregnant mice compared with a control group. Furthermore, they checked the dose-dependent (4, 20 and 30 mg/kg) effects of CNTs on progesterone and estradiol levels in maternal serum at GD14. CNT treatment caused a dose-dependent increase of serum estradiol level compared with the control groups, whereas the serum progesterone level decreased to the minimum after 20 mg/kg administration. They also checked the effect of CNTs (20 mg/kg) on progesterone and estradiol levels in maternal serum at different gestational ages (7, 14 and 18 days). Compared to the control group, CNT treatment decreased the serum progesterone level at GD7, 14 and 18, whereas the serum estradiol levels were increased only at GD7 and 14. Finally, they checked the effect of exposure time, such as multiple exposure to a low dose (4 mg/kg/day, five doses) or single heavy exposure (20 mg/kg). The serum progesterone level decreased more in the group with multiple exposures to a low dose compared with the single heavy exposure, whereas the serum estrogen level in the single heavy exposure group was higher than in the multiple exposures to a low dose group. Therefore, the toxicity of CNTs is largely dependent on its surface composition, and oxidized CNTs can cause severe toxicity during pregnancy, whereas unmodified CNTs were considered relatively safe. However, the damaging effects of CNTs weakened with increasing gestational age and number of pregnancies.

Effects on embryo development. There have been several reports regarding the toxic effects of CNTs on placenta and embryo development. Darne et al. (2014) checked the *in vitro* cytotoxicity and genotoxicity of a series of CNTs in Syrian hamster embryos. SWCNTs did not induce cell viability loss after 24 h exposure in the concentration range of 0.23–3.75 μg/cm^2, whereas all the double-wall CNTs (DWCNTs), and MWCNTs showed a dose-dependent cytotoxicity. Neither the SWCNTs nor the DWCNTs induced oxidative stress after 24 h exposure in the concentration range 0.27–2.1 μg/cm^2, whereas only one kind of MWCNTs out of four significantly induced oxidative stress at the highest applied concentration. Furthermore, they checked the genotoxicity of the CNTs after 24 h exposure in the concentration range of 0.23–3.75 μg/cm^2. In the comet assay, SWCNTs did not show any DNA damage, whereas only one kind of DWCNTs (out of three) and two kinds of MWCNTs (out of four) showed DNA damage at the highest concentration. They proposed that MWCNTs showed more cytotoxic and genotoxic effects than SWCNTs or DWCNTs. However, they could not find any correlation between CNT-induced genotoxicity and metal impurities, surface area, length or oxidative stress, but genotoxicity has been seen to increase with CNT width.

The accumulation of both SWCNTs and MWCNTs in the placenta of pregnant mice has already been reported (Campagnolo et al., 2013; Huang et al., 2014; Qi et al., 2014). Campagnolo et al. (2013) checked the biodistribution of fluorescently labeled pegylated SWCNTs (length = 90 nm) in pregnant mice, which were exposed to 10 μg CNTs per mouse intravenously at either GD5.5 or GD14.5 and analyzed 24 h post-injection. They demonstrated that CNTs reached the conceptus when administered at GD5.5, whereas they were detected in the placenta and the yolk sac when administered at GD14.5. Huang et al. (2014) checked the biodistribution of amine-functionalized SWCNTs (diameter = 1–2 nm, length = 0.2–2 μm) and MWCNTs (diameter = 8, 20–30, 50 nm, length = 0.2–2 μm) in the placenta of pregnant p53 heterozygous (p53$^{+/-}$) mice. The pregnant mice were injected at a dose of

2 mg/kg intravenously at GD15.5 and the CNTs-biodistribution was analyzed 48 h post-injection. They showed accumulation of both SWCNTs and MWCNTs in the placenta; however, the accumulation of CNTs was independent of particle size. Qi *et al.* (2014) showed that oxidized MWCNTs (diameter = 10–30 nm; length = 1–2 μm) could accumulate in the placenta at 6 h after injection and then dramatically decreased from 6 to 16 h post-injection after administering 20 mg/kg body weight to the pregnant mice on GD17.

Campagnolo *et al.* (2013) also showed that a single intravenous injection into CD-1 pregnant mice at a dose of 30 μg/mouse at GD5.5, or three injections at a dose of 10 μg/mouse at GD5.5, 8.5 and 11.5, caused abnormal placental development accompanied by a dramatic reduction in the vascularization of the labyrinth layer. Pietroiusti *et al.* (2011) reported similar kinds of vascular lesions in the placenta derived from the malformed fetuses from CD-1 pregnant female mice, which were administered CNT (oxidized single-wall) injections (at a dose of 100 ng–30 μg/mouse) on GD5.5. CNTs also caused extensive oxidative stress in the placenta derived from the malformed fetuses. Later, Qi *et al.* (2014) also showed that oxidized MWCNTs (diameter = 10–30 nm; length = 1–2 μm) could increase ROS formation as well as decrease the content of vascular endothelial growth factor and number of blood vessels in placenta of first-time pregnant mice after injecting 20 mg/kg body weight CNTs. However, the damaging effects of CNTs weakened with increasing number of pregnancies.

Pietroiusti *et al.* (2011) investigated the effects of CNTs (diameter = 1.58–2.37 nm; length = 0.37–0.85 μm) on embryonic development in CD-1 female mice, which were administered CNT (pristine or oxidized single-wall) injections (at a dose of 10 ng–30 μg/mouse) on GD5.5. Ten days later, the mice were sacrificed and embryonic development was assessed. They demonstrated that CNTs induced early miscarriages and fetal malformations in a dose-dependent manner. However, the oxidized SWCNTs (lowest effective dose: 100 ng/mouse) showed enhanced toxicity compared to pristine SWCNTs. CNTs caused extensive oxidative stress in the malformed fetuses. Philbrook *et al.* (2011) also investigated the effects of hydroxyl functionalized SWCNTs (diameter = 1–2 nm; length = 5–30 μm) on reproduction and development in CD-1 female mice, which received a single dose of either 10 mg/kg or 100 mg/kg on GD9 by oral gavage. On GD19, mice were sacrificed, fetuses were recovered and evaluated. They demonstrated that CNT treatment did not affect the fetal length, weight and viability, whereas 10 mg/kg CNTs treatment significantly increased the percentage of resorptions and resulted in morphological defects and skeletal abnormalities in fetuses. However, exposure to 100 mg/kg CNTs did not produce any defects in fetuses, which may be due to concentration-dependent complex aggregate formation and its inefficiency to cross intestinal cells.

Campagnolo *et al.* (2013) also checked the embryotoxic effects of pegylated SWCNTs (length = 90 nm) after single intravenous injection into CD-1 pregnant mice at different doses (0.1, 10 or 30 μg/mouse) at GD5.5, or three injections at a dose of 10 μg/mouse at GD5.5, 8.5 and 11.5. All female mice were sacrificed at GD15.5 and analyzed. The 10 μg/mouse treated group did not show any adverse effects on embryos and dams. However, fetal abnormalities were observed in 1 out of 10 dams (1 malformed embryo) in the 30 μg/mouse treated group and 2 out of 10 dams (5 malformed embryos) in the group with three repeated doses of 10 μg/mouse. Qi *et al.* (2014) showed that oxidized MWCNTs (diameter = 10–30 nm; length = 1–2 μm) can pass through the maternal body into the fetus *in vivo* after injecting 20 mg/kg body weight CNTs to the pregnant mice on GD17. However, the accumulation of CNTs peaked in the fetuses at 6 h after injection and then dramatically decreased at 6–16 h post-injection. The abortion rate due to CNT exposure in the first-time pregnant mice was higher than second-time and fourth-time pregnant mice, which were given CNTs at a dose of 20 mg/kg body weight intravenously at GD7. Besides, the development of the dead fetus after abortion in the second-time or fourth-time pregnant mice was better than in the first-time pregnant mice.

In another study, Lim *et al.* (2011a,b) investigated the effects of MWCNTs (diameter = 10–15 nm; length = 20 μm) on embryo-fetal development in pregnant female Sprague Dawley rats treated with CNTs (40, 200 or 1000 mg/kg/day) over the period of GD6–19 by oral gavage. On GD20, all pregnant mice were subjected to cesarean section and the fetuses were examined. They demonstrated that CNTs did not show any harmful effects on embryo-fetal development up to the 1000 mg/kg dose. In their study, they used very high concentrations of CNTs that might cause concentration-dependent complex aggregate formation, therefore could not efficiently cross the intestinal cells and did not show toxicity.

Huang *et al.* (2014) showed genotype-dependent effects of amine-functionalized MWCNTs on fetal development. They checked the biodistribution of SWCNT (diameter = 1–2 nm), MWCNT-8 (diameter = 8 nm), MWCNT-20 (diameter = 20–30 nm) and MWCNT-50 (diameter = 50 nm) with similar length of 0.2–2 μm in fetuses. They injected each CNT at a dose of 2 mg/kg intravenously into pregnant $p53^{+/-}$ mice at GD15.5 and the CNT biodistribution in the fetuses was analyzed 48 h post-injection. They showed accumulation of SWCNT, MWCNT-8, MWCNT-20 and MWCNT-50 in the liver of fetuses. However, the accumulation of CNTs was independent of particle size. They also checked the effects of CNTs on fetal body weight after injecting the CNTs intravenously into different $p53^{+/-}$ pregnant mice at, variously, GD10.5, GD12.5 or GD15.5. MWCNT-20 and MWCNT-50 treatment decreased the fetal body weight significantly. MWCNT-50 showed significant brain deformity in fetuses, whereas SWCNT, MWCNT-8 and MWCNT-20 did not. These results clearly indicated that MWCNT-50 had more fetotoxicity than SWCNT, MWCNT-8 and MWCNT-20 at the same dose. MWCNT-50 also decreased the percentage offspring survival significantly (20%) after multiple administrations at a dose of 5 mg/kg compared to untreated control. To check the genotype-dependent toxicity, MWCNT-50 was injected intravenously (a single dose of 5 mg/kg) into different $p53^{+/-}$ pregnant mice at GD10.5 and GD15.5, and fetuses were isolated at GD17.5. MWCNT-50 induced significant brain deformity in $p53^{+/-}$ fetuses with injection at GD10.5, whereas the untreated and MWCNT-50-treated $p53^{+/+}$ and $p53^{+/-}$ fetuses did not show any malformation. However, abnormal development of fetuses was not seen at GD15.5 injection, but the fetal body weight significantly decreased compared with the untreated fetuses. They further showed that MWCNT-50 (administered at either GD10.5 or GD15.5) could induce similar nuclear DNA damage to all three genotypes in both fetal liver and placenta. However, MWCNT-50 caused more damage to $p53^{-/-}$ fetuses than the other two

genotypes ($p53^{+/+}$ and $p53^{+/-}$). MWCNT-50 also increased the expression of p21 and Bax in $p53^{+/+}$ fetuses during gestation, but not in $p53^{-/-}$ fetuses. They proposed that the higher percentage of brain deformity in $p53^{-/-}$ fetuses is due to the defects of induction of p21 and Bax. On the other hand, the slow body weight gain in $p53^{+/+}$ fetuses is due to the induction of p21 expression causing cell cycle arrest.

All these studies show that CNTs are able to reach the placenta and fetus, and induce structural/functional abnormalities of placenta and cause embryotoxicity.

Carbon black nanoparticles

Carbon black nanoparticles (CBNPs) are produced industrially by the incomplete thermal decomposition of heavy petroleum. The most common use of CBNPs is as a pigment, in automobile tires, radar-adsorbent materials and laser printer toner (Donaldson *et al.*, 2005; Ema *et al.*, 2010).

Effects on male and female germ cells. Yoshida *et al.* (2009) first checked the effects of CBNPs on the male reproductive system in mice. In their study, ICR male mice were intratracheally administered 14, 56 or 95 nm CBNPs at a dose of 0.1 mg/mouse 10 times at weekly intervals. In another group, mice were intratracheally administered a dose of 1.56 μg/mouse with 14 nm CBNPs (14 N group), having similar particle number per unit volume as the 56 nm CBNPs. They showed that all the 14, 56 and 95 nm CBNPs induced vacuolation of the seminiferous tubules, whereas no change was observed in the 14 N group. After that, Yoshida *et al.* (2010) also checked the effects of CBNPs on the reproductive function of male offspring after maternal gestational exposure to CBNPs. Female pregnant mice were given 200 μg of CBNPs (14 nm) intratracheally on GD7 and 14, and the reproductive toxicity parameters were measured in male mice at 5, 10 and 15 weeks after birth. CBNP administration did not affect testicle and epididymis weight. They demonstrated that CBNPs caused a reduction in the cellular adhesion of seminiferous epithelia and partial vacuolation of seminiferous tubules, although the percentage of damaged seminiferoud tubules was higher in 5-week-old mice. CBNPs did not affect serum testosterone concentration in male offspring. On the other hand, in their previous study, they reported that 14 and 56 nm CBNP treatment groups caused dysfunction of Leydig cells as indicated by increased serum testosterone levels (Yoshida *et al.*, 2009). Besides, both the 14 and 56 nm CBNP treatment groups decreased the daily sperm production more than 95 nm CBNPs or 14 N groups. Based on the experimental results, they proposed that the effects of CBNPs on male reproductive system not only depend on particle numbers but also on particle mass. CBNPs also caused a decrease in daily sperm production by 47%, 34% and 32% in 5-, 10- and 15-week-old male offspring, respectively, after maternal exposure (Yoshida *et al.*, 2009).

In another study, Kyjovska *et al.* (2013) investigated the effects of CBNPs (printex 90) on daily sperm production in male offspring after maternal exposure to CBNPs. In their study, time-mated C57BL/6J mice were exposed to CBNPs intratracheally four times during gestation (67 μg/animal). They showed that maternal exposure to CBNPs did not affect the daily sperm production in F1 male offspring, but F2 male offspring, born from a prenatally CBNP-exposed father, showed a lowered sperm production. All these studies suggest that CBNP exposure has adverse effects on male reproductive health. The toxicological effect of CBNP on female germ cells has not been investigated yet.

Effects on embryo development. Jackson *et al.* (2012) checked the toxicogenomic effects of carbon black on dams and offsprings after maternal exposure. In their study, C57BL/6 BomTac mice were exposed by intratracheal instillation to 2.75, 13.5 or 67 μg/animal of carbon black Printex 90 on GD7, 10, 15 and 18. Samples were collected from the offspring on PND2. Exposure to Printex 90 at the highest doses to dams caused significant changes in hepatic gene expression in both male and female newborn offspring on PND2, although the hepatic response was more pronounced in the female offspring. The majority of the altered genes in male offspring belonged to inflammatory, respiratory and nutritional diseases, whereas female offspring showed selective altered expression in genes related to metabolic disease and endocrine systems disorders. Therefore, maternal exposure to CBNPs during pregnancy impacts fetal development.

Fullerene (C_{60})

Fullerene (C_{60}), also known as buckminsterfullerene or bucky-ball, has a cage-like structure. Due to the unique physicochemical properties, it has been extensively used in lubricants, electronics, cosmetics, fuel cells and as dietary supplements (Loutfy *et al.*, 2002).

Effects on male and female germ cells. The toxicological effect of C_{60} on male germ cells has not been investigated yet. In only one study, Yamashita *et al.* (2011) checked the effects of C_{60} in pregnant mice, intravenously injected with 0.8 mg per mouse on two consecutive days, at GD16 and GD17. They did not find any change in maternal body weight and uterine weight at GD18.

Effects on embryo development. There are few reports regarding the measurement of embryotoxic effects of C_{60} in mice. Tsuchiya *et al.* (1996) checked the effects of C_{60} in embryonic midbrain cells (obtained from normal pregnant mice), which were cultured in the presence of C_{60} at doses of 10–1000 μg/ml for 6 days. They demonstrated that the IC_{50} value of C_{60} in cell proliferation and differentiation were 0.47 and 0.43 mg/ml, respectively, and treatment with antioxidant enzymes partly restored the inhibition of cell proliferation by C_{60}. They proposed that C_{60} affected cell proliferation *in vitro* partly via the formation of ROS. However, C_{60} shows *in vitro* embryotoxic effects only at doses more than 200 μg/ml.

Sumner *et al.* (2010) checked the distribution of C_{60} in the pregnant rat and their fetuses, and in the lactating rat and offspring. Pregnant rats were administered 0.3 mg/kg C_{60} via tail vein injection on GD15 and lactating rats were dosed on PND8. Tissues were collected at 24 and 48 h after dosing and analyzed. They demonstrated that C_{60} could cross the placenta and transmit to the offspring via the dam's milk and subsequently be systemically absorbed. Yamashita *et al.* (2011) checked the fetotoxicity of C_{60} in pregnant mice, intravenously injected with 0.8 mg per mouse on two consecutive days, at GD16 and GD17. C_{60} did not induce any change in fetal resorption rates and fetal weight at GD18.

On the other hand, Tsuchiya *et al.* (1996) reported the embryotoxic effects of C_{60} in SLC mice, which were intraperotoneally injected with very high doses of C_{60} (25–137 mg/kg) on GD10 and

GD11, and the embryos were evaluated 18 h after administration. All the embryos on the 137 mg/kg dose died 18 h after treatment. At the 50 mg/kg dose, C_{60} was found to be distributed into embryos and 50% of the embryos showed morphological abnormalities especially in the head region and tail. Treatment with 50 mg/kg had a seriously harmful effect on the yolk sac, such as a shrunken membrane and narrowed blood vessels. They also showed that C_{60} treatment affected cell proliferation and the differentiation of embryonic midbrain cells obtained from C_{60}-treated pregnant mice at the 25 mg/kg dose. Therefore, we can assume that fullerene does not induce pregnancy complications unless the used dose is very high.

Grapheme oxide

Grapheme oxide (GO) is obtained by oxidation of graphite and has a wide range of applications in the biomedical field, such as biological imaging, drug delivery, cancer photothermal therapy, biosensing and tissue engineering (Sun *et al.*, 2008; Artiles *et al.*, 2011; Zhang *et al.*, 2011a,b; Sheng *et al.*, 2013).

Effects on male and female germ cells. There is only one report regarding the toxicological study of GO on male fertility (Liang *et al.*, 2015). In their study, male mice were either intravenously administered with three repeated doses of small-sized GO (S-GO, 55 nm) at 6.25–25 mg/kg and large-sized GO (L-GO, 200–300 nm) at 25 mg/kg or intraperitoneally administered with five repeated doses of S-GO at 24–60 mg/kg and L-GO at 24 mg/kg. They reported that the testis and epididymis retained their normal histology even after the highest administration dose at post-injection 30 days. The sperm concentration, morphologies in the epididymis and the levels of endogenous sex hormones at 30 or even post-injection 60 days with different sizes of GO were similar with those of the control mice. The activities of several important epididymal enzymes, such as α-glucosidase, acid phosphatase, lactate dehydrogenase and glutathione peroxidase, were not affected at post-injection 30 days with GO. No significant differences were observed in pup numbers, weights, survival rates or growth compared to those of offspring obtained from control female mice mated with GO-treated male mice. Besides, the GO-treated male mice were able to produce a second, third, fourth and even fifth litter of healthy offspring after mating with the untreated female mice. Therefore, they proposed that that both S-GO and L-GO had nearly no or very low reproductive toxicity in male mice.

Xu *et al.* (2015a,b) checked the effects of reduced GO (RGO) on female mouse reproductive ability. In their study, female mice were intravenously administered with either small-sized RGO (20–150 nm) or large-sized RGO (200–1500 nm) at 6.25–25 mg/kg 1 or 30 days before mating or on GD6 or 20. They showed that RGO treatment did not cause any alterations in the sex hormone levels either at 1 or 30 days post-injection. Besides, the GO-treated (1 day before mating) female mice were able to produce second and third litters of healthy offspring after mating with the untreated male mice. Therefore, neither RGO caused any reproductive adverse effects after injection before mating or during early gestation. At late gestation stage, when small-RGO was administered at low doses (6.25 and 12.5 mg/kg), all mice had abortions and most of the mice died when administered the high dose. However, the surviving mothers administered with low or medium doses of small-RGO became pregnant again after mating with adult male mice and successfully gave birth to living pups. Therefore, reduced graphene oxides are toxic to late stage pregnant females, but the toxicity of RGO to parturient mice (if they survived) may not be long term.

Effects on embryo development. Xu *et al.* (2015a,b) also checked the toxic effects of RGO on offspring. They demonstrated that small-RGO could not be transferred from mother to fetuses. No significant differences were observed in pup weights, survival rates or growth in mice being treated with RGO either 1 or 30 days before mating or at an early stage of gestation compared with control groups. The serum biochemistry parameters related to liver and kidney functions and hematology parameters of 30-day-old offspring of dams injected with RGO (small or large) 1 day before mating or at GD6 were all similar to those of the control and their living offspring appeared healthy. However, in the large RGO (high dose) treated group (1 day before mating), one pregnant mouse had two abnormal fetuses and the corresponding placenta size was also smaller compared to control. At the early stage of gestation, in the medium dose group of small-RGO, one pregnant mouse had two abnormal fetuses, whereas in the high-dose group of large RGO, one abnormal fetus was found. All other fetuses and placentas produced by the small or large RGO-treated groups (GD6) appeared normal. Therefore, reduced graphene oxides are toxic to the progestational (drawing near pregnancy) and pregnant female and can cause severe embryotoxicity.

Carbon-based nanoparticles for gene delivery in germ cells and embryos

Recently carbon-based nanoparticles have attracted much attention as novel and versatile gene delivery vehicles, such as GO (Kim and Kim, 2014; Paul *et al.*, 2014; Imani *et al.*, 2015), CNTs (Liu *et al.*, 2005; Singh *et al.*, 2005; Klumpp *et al.*, 2006; Lo and Wang, 2008; Al-Jamal *et al.*, 2011; Karimi *et al.*, 2015; Nia *et al.*, 2016) and C_{60} (Maeda-Mamiya *et al.*, 2010; Montellano *et al.*, 2011; Sigwalt *et al.*, 2011). The most attractive candidate for gene delivery among the carbon-based nanoparticles is C_{60} because of its highly biocompatible nature and facile functionalization. After suitable chemical modification with hydrophilic side chains, amphipathic C_{60} offers great potential for gene delivery due to effective complex formation with DNA. Regarding its toxicity issue, it does not induce any pregnancy complications in females and fetotoxicity when administered at moderate doses. Besides, it shows *in vitro* embryotoxic effects only at very high doses (more than 200 μg/ml). Therefore, C_{60} can be considered a safe carrier for gene delivery into gametes and embryos. GO has recently been used by many researchers for gene delivery applications due to its multiple advantageous features, such as facile synthesis, easily tunable surface functionalization, high water dispersibility and biocompatibility. It has nearly no or very low reproductive toxicity in males. They also did not show reproductive adverse effects in pregnant females or fetotoxicity when administered in early gestation. Therefore, GO can be used for gene delivery into germ cells and embryos. The high length-to-diameter ratio and easy covalent functionalization with amine terminal groups (to increase the solubility and improve biocompatibility) make the CNTs ideal candidates for gene delivery. However, they show moderate cytotoxic effects on male spermatogenesis and pregnancy complications in females depending on the surface composition. Both SWCNTs and MTCNTs have potential fetotoxic effects in pregnant mothers, whereas

SWCNTs do not show *in vitro* embryotoxicity. Therefore, CNTs can be used for gene delivery into germ cells and embryos only after suitably selecting the type of SWCNTs or MTCNTs and modifying its surface in order to increase its biocompatibility.

Toxicological effects of luminescent and chitosan nanoparticles on germ cells and embryos

The *in vivo* and *in vitro* effects of luminescent and chitosan nanoparticles (CSNPs) on male gametes/testes, female gametes/ovary and embryo development are shown in Table III.

Quantum dots

QDs are a new class of fluorescent nanomaterials that are being used as an alternative to fluorescent dyes for biological imaging (Feugang *et al.*, 2012).

Effects on male and female germ cells. The toxic effects of QDs in mammalian sperm cells have not yet been reported. Feugang *et al.* (2012, 2015) evaluated the effects of CdSeQDs (5–8.2 nm) on boar spermatozoa (2×10^8 sperm/ml), which were incubated with 1–5 nM CdSeQDs for 30 min. They demonstrated that CdSeQDs did not show any harmful effects on sperm motility when treated with 1 nM concentration. Sperm viability was also not significantly affected with 1–5 nM concentrations of CdSeQDs. However, CdSeQDs could decrease the acrosome integrity of the sperm without affecting fertilization at 1 nM.

To date, two research groups have shown the toxic effects of QDs on female germ cells (Hsieh *et al.*, 2009; Xu *et al.*, 2012). Xu *et al.* (2012) investigated the effects of CdTe/ZnTeQD-transferrin (Tf) bioconjugates (4 nm) on follicle development and oocyte maturation. In their study, mouse preantral follicles were exposed to CdTe/ZnTeQD-Tf bioconjugates (0.0289–28.9 nM). QD-Tf bioconjugates could permeate into the cytoplasm of granulosa cells and theca cells, and accumulated in a dose-dependent manner when incubated for 8 days, but could not enter the oocyte. The rate of follicle survival and cumulus-oocyte-complex mucification was not altered significantly in treated groups; however, the rate of antrum cavities decreased when the QDs-Tf concentration was higher than 2.89 nM. QDs-Tf did not show any effects on progesterone production. Qds-Tf also decreased the number of matured oocytes with a first polar body as well as delay oocyte antrum cavity formation when the dose exceeds 2.89 nM. The authors further proposed that CdTe/ZnTeQDs-Tf bioconjugates disturbed oocyte cytoplasmic maturation but did not affect nuclear maturation. In another study, Hsieh *et al.* (2009) investigated the cytotoxic effects of CdSeQDs (3.5 nm) on the maturation of mouse oocytes (collected from ICR female mice) and their IVF rate following a 24 h incubation with CdSeQDs (125–500 nM). They demonstrated that ~98% of the oocytes reached the metaphase II (MII) stage of maturation after *in vitro* maturation, but the maturation rate decreased in the CdSeQDs-treated groups in a dose-dependent manner. The rate of fertilization also decreased in CdSeQDs-treated oocytes as compared to the controls in a dose-dependent manner. At 125 nM concentration, CdSeQDs did not show any toxicity. However, ZnS coating significantly reduced the CdSeQDs-induced cytotoxic effects on oocyte maturation and fertilization rates. These studies clearly showed that QDs have deleterious effects on oocyte maturation and fertility, which can be prevented by surface coating over QDs.

Effects on embryo development. There have been three reports on the toxic effects of QDs on embryo development (Chan and Shiao, 2008; Hsieh *et al.*, 2009; Chu *et al.*, 2010). In one study, Hsieh *et al.* (2009) investigated the cytotoxic effects of CdSeQDs on mouse embryo development in detail. In their study, oocytes (collected from ICR female mice) were treated with CdSeQDs (125–500 nM) for 24 h, then fertilized *in vitro*, and cultured *in vitro* to blastocyst-stage embryos. They demonstrated that CdSeQDs decreased *in vitro* embryo development to the two cells and blastocyst-stage in a dose-dependent manner. CdSeQDs also decreased the number of TE cells in a dose-dependent manner without affecting the number of ICM cells in blastocysts. In addition, CdSeQDs-induced apoptosis in a dose-dependent manner in developing embryos. However, at 125 nM concentration, CdSeQDs did not show any embryotoxicity. The embryotoxic effects of CdSeQD (3.5 nm) are further supported by the study from Chan and Shiao (2008). They treated the blastocysts (collected from ICR mice) with CdSeQDs (125–500 nM) for 24 h. At 125 nM concentration, CdSeQDs did not show any embryotoxicity. CdSeQDs-induced apoptosis and decreased the number of ICM in a dose-dependent manner (from 250 to 500 nM) without affecting the number of TE cells in blastocysts; however, this observation is opposite to Hsieh *et al.* (2009). Chan and Shiao (2008) checked the blastocyst cell numbers directly after treating the blastocysts (collected from ICR mice) with CdSeQDs (125–500 nM) for 24 h. On the other hand, Hsieh *et al.* (2009) first treated the oocytes (collected from ICR female mice) with CdSeQDs (125–500 nM) for 24 h, then fertilized *in vitro*, and cultured *in vitro* to blastocyst-stage embryos. After that they checked the blastocyst cell numbers. Therefore, these observations demonstrate that differences in exposure routes might produce different results.

Both these researchers further checked the effects of QDs on post-implantation embryo development after implanting the QDs-treated embryos into the right uterine horn in pseudopregnant mice on day 4. They demonstrated that CdSeQDS decreased the placental and fetal weight, fetal survival, implantation rates, as well as increased resorption of post-implantation embryos after treatment with 500 nM. These studies indicate that QDs have negative effects on both pre- and post-implantation embryo development and impose severe fetotoxic effects. However, ZnS coating significantly reduced all of these CdSeQDs-induced cytotoxic effects on embryo development. Hsieh *et al.* (2009) demonstrated that CdSeQD cytotoxicity is associated with the surface oxidation-mediated release of free Cd^{2+} from the CdSe lattice. They also showed that incubation of CdSe-core QDs (500 nM) in oocyte IVM medium for 24 h yielded 17.5–18.4 ppm free Cd^{2+} in the medium. They proposed that the ZnS coating prevented cytotoxicity of CdSeQDs by blocking surface oxidation and the subsequent release of Cd^{2+} ions.

Chu *et al.* (2010) checked the transfer of QDs across the placental barrier from pregnant mice to pups when QDs were injected 1–5 days before delivery. They showed that 3-mercaptopropionic acid (MPA)-modified QDs (1.67–3.21 nm) could be effectively transferred from pregnant mice (treated once with QDs containing 20–125 μg Cd via tail vein injection) to their fetuses across placental barrier in a

size-dependent and dose-dependent manner. Smaller QDs showed increased Cd accumulation in pups. However, coating with PEG or SiO_2 reduced the Cd concentration in pups compared to MPA-coated QDs after administering the same concentration. The reduced accumulation of Cd in PEG or SiO2-coated QDs could be due to increased size, i.e. 4.2 and 4.09 nm, respectively, compared to MPA-coated CDs (3.21 nm). Another possibility is the protective effects of PEG or SiO_2 that could reduce the release of Cd^{+2} from QDs. They also showed that administration of MPA-coated QDs into pregnant mice decreased the survival rate of the pups in a dose and size-dependent manner. All pups survived when the pregnant mice were injected with QDs containing 20 µg Cd and the survival rate decreased to 33.68% when treated with QDs containing 125 µg Cd. Smaller QDs showed more toxic effects to the fetuses. Coating with PEG or SiO_2 increased survival rate but still produced dead pups when administered with QDs having a very high Cd concentration. All these studies clearly indicate that although QDs induce cytotoxic effects on embryo development, surface coating can be effective in minimizing its toxic effects.

Chitosan nanoparticles

Chitosan is a natural polysaccharide obtained by N-deacetylation of chitin. Due to their biodegradable nature, CSNPs are being used as drug/gene delivery vectors (Tripathi *et al.*, 2012; Liu *et al.*, 2016). The other important applications of chitosan are in agriculture, textiles and cosmetics and as food preservatives (Li *et al.*, 1992; Ravi Kumar, 2000b).

Effects on male and female germ cells. The toxicological effect of CSNPs on germ cells has not been investigated yet.

Effects on embryo development. There is only one report regarding the adverse effects of CSNPs on pre- and post-implantation embryos (Park *et al.*, 2013). In their study, morulae-stage embryos (obtained from ICR mice) were treated with 100 nm CSNP (10–200 µg/ml), then cultured *in vitro* for 24 h or implanted into the right uterine horn on day 2.5 in pseudopregnant mice. They demonstrated that CSNPs caused a dose-dependent adverse effect on blastocyst development, which was evident from abnormal blastocoel formation in blastocyst-stage embryos. CSNPs decreased cell proliferation and enhanced apoptotic cell death in both TE and ICM cells in embryos in a dose-dependent manner. CSNPs-treated embryos showed lower expression levels for both TE-associated and pluripotent marker genes. They also demonstrated that CSNP-treated blastocyst-stage embryos having either no cavity or a small cavity were associated with reduced mitochondrial activity and a defect in TE cells. Furthermore, they checked the gene expression pattern associated with blastocyst developmental competence, such as B3gnt5 (cell differentiation and adhesion), Wnt3a (development) and Eomes (TE differentiation). CSNP-treated blastocysts that had either no cavity or a small cavity showed a significant decrease in the expression of B3gnt5, Wnt3a and Eomes genes. They also investigated the effects of CSNPs on blastocyst development *in vivo* and showed that at 18 days post-transfer, the CSNP-treated groups had fewer implantation sites and fetuses than those of the controls. All of these results clearly indicate that CSNPs cause both pre- and post-implantation embryo complications.

Quantum dots and chitosan for gene delivery in germ cells and embryos

QDs are a new class of fluorescent nanomaterials that are being used as an alternative to fluorescent dyes for biological imaging of mammalian gametes and potential *in vivo* targeted-imaging (Feugang *et al.*, 2012; 2015). Recently, they have also been proven as efficient gene carriers in mammalian cultured cells (Wisher *et al.*, 2006; Li *et al.*, 2012a,b; Yang *et al.*, 2014). QDs did not show any toxicity towards spermatozoa when administered up to 5 nM concentration. Although QDs induce cytotoxic effects on oocyte maturation and fertility as well as embryo development, surface coating can be effective in minimizing its toxic effects. Therefore, QDs can be used for effective labeling of mammalian gametes for *in vitro* monitoring as well as for gene delivery into germ cells and embryos only after suitably modifying its surface in order to increase its biocompatibility. CSNPs are widely used cationic polysaccharides in many gene delivery applications due to the stable electrostatic interactions with nucleic acids as well as their biodegradable nature (Tripathi *et al.*, 2012; Jeong *et al.*, 2015; Liu *et al.*, 2016). The toxicological effects of CSNPs on germ cells have not been investigated yet, but they show harmful effects in developing embryos. Therefore, at this stage it is difficult to predict whether these can be used for gene delivery into germ cells and embryos.

Factors affecting nanoparticle toxicity

Size and surface area-dependent toxicity

Nanoparticle toxicity is largely dependent on size and surface area. Small nanoparticles have a larger surface area as well as particle number per unit mass compared to larger particles, therefore, reactivity is increased and they can cause more damage to biological tissues compared to microparticles having similar mass (Gatoo *et al.*, 2014). Park *et al.* (2010) studied the testicular accumulation of 22, 42, 71 and 323 nm AgNPs after oral administration with a 1 mg/kg body weight dose for 2 weeks in mice and showed that only 22 and 42 nm AgNPs significantly accumulated in the testes compared with controls. Similarly Gromadzka-Ostrowska *et al.* (2012) showed that 20 nm AgNPs exhibited more decrease in epididymal sperm count than 200 nm AgNPs after intravenous injection in male rats. Huang *et al.* (2015) checked the ability of fluorescent polystyrene particles with diameters 20, 40, 100, 200 and 500 nm to cross the mouse placental. They showed that small-sized nanoparticles were significantly taken up by placental tissue and induced trophoblast cell apoptosis, with increased cleaved caspase-3 and reduced cell proliferation. Therefore, the enhanced cytotoxicity of small-sized nanoparticles can be attributed to easy internalization and increased interaction within the biological system.

Chemical composition, crystal structure, aggregation and surface-coating agents

Although size of the nanoparticles is more important than chemical composition while assessing nanoparticle toxicity, the particle chemistry is also critical. Cellular uptake, subcellular localization and reactivity of nanoparticles are largely dependent on their chemical nature. Asare *et al.* (2012) checked the effects of 20 nm AgNPs and 21 nm TiO_2NPs in testicular cells and showed that AgNPs exhibited more cytotoxicity compared to TiO_2NPs. In another study, Tiedemann *et al.* (2014) incubated porcine oocytes with 6, 8 and 20 nm AuNP

(10, 30 mg/ml) or 11 nm AgNP (10 mg/ml) for 46 h. They showed that AuNP did not have any impact on oocyte maturation, but AgNPs inhibited cumulus-oocyte maturation. Therefore, these studies clearly indicated that nanoparticle type, not solely size, may be the limiting factor in their exerted toxicity. The crystal structure of nanoparticles also influences their toxicity (Gatoo et al., 2014). Gurr et al. (2005) showed that rutile TiO_2NPs induced lipid peroxidation, oxidative DNA damage and micronuclei formation, whereas anatase TiO_2NPs of the same size did not. Nanoparticles may aggregate when they are placed in biological fluids of high ionic strength, which shields the repulsion among the nanoparticles (Kim et al., 2005; Lacerda et al., 2010; Maiorano et al., 2010; Rausch et al., 2010) and leads to bigger particles. Albanese and Chan (2011) checked the effect of Tf-coated gold nanoparticle aggregates of different sizes on cellular uptake in HeLa and A549 cells, and showed a 25% decrease in uptake of aggregated nanoparticles in comparison to single and monodisperse nanoparticles. Similarly, Tripathy et al. (2014) checked the effects of ZnONP aggregation on the toxicity of RAW 264.7 murine macrophage and showed that smaller secondary aggregates exhibited higher toxicity than the larger secondary aggregates. Therefore, the aggregation propensity of nanoparticles is important for defining their toxicological effects.

Nanoparticle dispersion stability in fluids depends on surface charge. High surface charge (both positive and negative) confers stability in dispersion media. Nanoparticles can be made either positively or negatively charged by the selective use of surface-coating agents. The nanoparticle uptake by the cells and its toxicity are dependent on the nature of the surface-coating agents too (Wang and Fan, 2014; Manshian et al., 2015; Maurizi et al., 2015; Misra et al., 2015; Pang et al., 2016). Hoshino et al. (2004) showed that the cytotoxicity of QDs was related to the surface-coating agents, but not to the QD-core materials. Zhang et al. (2015c) showed that cationic surface-coating agents are more toxic as compared to anionic and neutral surface-coating agents. Both positively and negatively charged nanoparticles can cross the biological barriers and cause damage to the tissues, although greater toxicity and bioaccumulation are observed with positively charged nanoparticles (Di Bona et al., 2014; Lee et al., 2015). Furthermore, low molecular weight surface-coating agents showed less toxicity as compared to their high molecular weight analogs when attached to the nanoparticle surface (Fischer et al., 2003; Forrest et al., 2003). Amin et al. (2015) demonstrated that nanoparticles with hydrophilic surfaces showed less toxicity due to the protective surface (from binding of different molecules).

Mechanisms of nanoparticle toxicity

Nanoparticle internalization

As we have discussed earlier, due to their very small-size nanoparticles can effectively cross biological barriers, which protect the reproductive tissues. In the case of the male reproductive organ, nanoparticle exposure increases proinflammatory responses that weaken the BTB (Lan and Yang, 2012; Han et al., 2016). Nanoparticles also reduce the expression of tight junction genes in Sertoli cells, which are necessary for BTB formation (Zhang et al., 2015c). As a result, the size of the BTB gap becomes larger and nanoparticles can easily penetrate the BTB and accumulate in spermatogenic cells. The adverse effects of nanoparticles on spermatogenesis are depicted in Fig. 1. Besides, several researchers showed that nanoparticles could bind to and enter the plasma membrane as well as the head of spermatozoa *in vitro* (Pawar and Kaul, 2014; Taylor et al., 2014a; Preaubert et al., 2015; Yoisungnern et al., 2015). In our laboratory, we checked the internalization of nanoparticles, such as citrate-coated AgNPs and polyethylenimine-coated AuNPs, in mouse spermatozoa (Fig. 2). We observed that AgNPs could spontaneously penetrate the plasma membrane of acrosome intact spermatozoa, whereas AuNPs could only bind to the plasma membrane surface of acrosome-reacted spermatozoa. Here, we also depicted the possible uptake mechanisms of nanoparticles in spermatozoa (Fig. 2). Several other researchers have also shown that nanoparticles can translocate to the ovaries and accumulate within ovarian cells (Austin et al., 2012; Gao et al., 2012; Zhao et al., 2013; Tassinari et al., 2014). The adverse effects of nanoparticles on oogenesis have been depicted in Fig. 1. However, there is no report regarding the nanoparticle distribution in oocytes *in vivo*. On the other hand, Tiedemann et al. (2014) reported that AuNPs are taken up by the oocytes *in vitro*, which is different from the *in vivo* cellular environment of the developing oocyte. Therefore, we can assume that nanoparticles exert their adverse effects on oocyte development (oogenesis) by damaging the surrounding cellular environment in developing follicles (Fig. 1). The transplacental crossing ability and distribution into fetal organs of several nanoparticles has also been reported. The direct and indirect pathways of effect in embryonic/fetal toxicity of nanoparticles are depicted in Fig. 3. Several factors such as size, shape, chemical composition, surface charge, coating with inorganic or organic molecules and biological cargo may cause nanoparticle toxicity.

Oxidative stress

We have already shown that nanoparticle-induced germ cell toxicity is mainly mediated via oxidative stress (Gao et al., 2012; Pawar and Kaul, 2014; Xu et al., 2014; Hong et al., 2015; Meena et al., 2015; Preaubert et al., 2015; Yoisungnern et al., 2015). The most important source of ROS in spermatozoa is mitochondrial electron leakage via the electron transport chain, and monovalent reduction of molecular oxygen (Aitken and Clarkson, 1987; Koppers et al., 2008). Due to their very small size, nanoparticles can gain access to the mitochondria and cause damage to the mitochondrial structure (Xu et al., 2014, 2015a,b; Yoisungnern et al., 2015). This damage can lead to a defective electron transport chain, thereby stimulating ROS formation. Transition metal and metal oxide nanoparticles can produce ROS via catalyzing Fenton-type reactions that involve a transition metal ion that reacts with hydrogen peroxide (H_2O_2) to produce a highly reactive hydroxyl radical (OH) (Thannickal and Fanburg, 2000). The other source of ROS in spermatogenic cells is cytoplasmic glucose-6-phosphate dehydrogenase. However, the majority of the cytoplasm extrudes during normal spermiogenesis from the maturing spermatozoa (Rengan et al., 2012). The residual cytoplasmic droplet contains enzymes, such as creatinine kinase and glucose-6-phosphate dehydrogenase, that produce essential ROS for sperm capacitation (Rengan et al., 2012). Glucose-6-phosphate dehydrogenase catalyzes the reduction of $NADP^+$ to NADPH, which can increase ROS production through the action of NADPH oxidase (Aitken et al., 1997; Dona et al., 2011). Gomez et al. (1996) demonstrated that defective

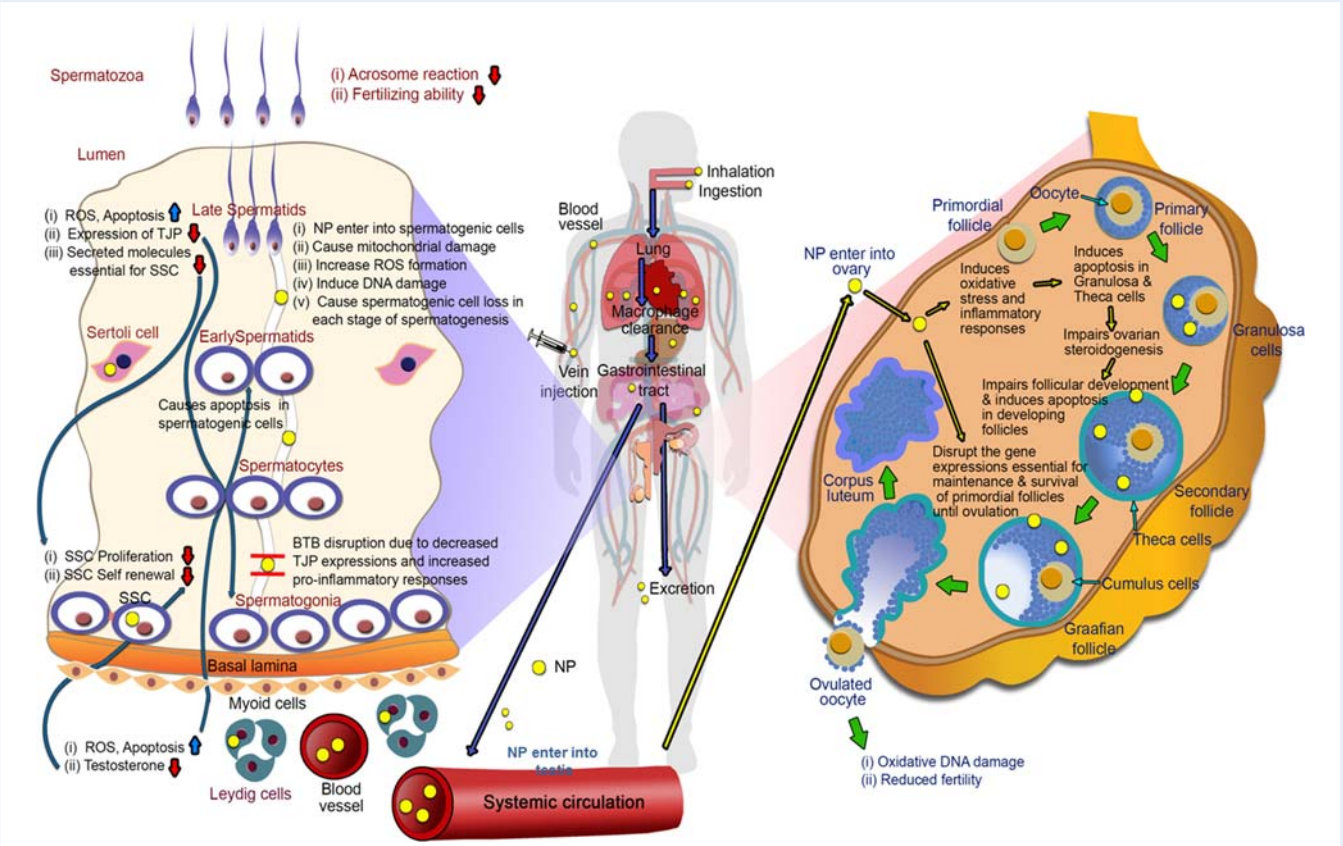

Figure 1 The adverse effects of nanoparticles on spermatogenesis and oogenesis. BTB, blood–testis barrier; NP, nanoparticle; ROS, reactive oxygen species; SSC, spermatogonial stem cell; TJP, tight junction protein.

spermiogenesis with excess residual cytoplasm in immature spermatozoa increases the amount of glucose-6-phosphate dehydrogenase, thereby increasing ROS production. Saleh *et al.* (2002) also demonstrated that immature spermatozoa could produce excess ROS in the presence of proinflammatory factors. As we have already discussed, nanoparticles can accumulate inside spermatogenic cells by crossing the BTB, and can affect spermiogenesis or sperm maturation, therefore, immature spermatozoa represent a potential source of ROS after nanoparticle exposure. Similarly in oocytes, nanoparticles can also produce ROS via direct interaction with oocytes *in vitro* or indirectly after the released ions from nanoparticles diffuse through the zona pellucida (Courbiere *et al.*, 2013). The induction of oxidative stress in embryos and malformed fetuses due to nanoparticle exposure has also been reported (Pietroiusti *et al.*, 2011; Darne *et al.*, 2014).

Inflammation

Inflammation is a part of the biological response of the body to injury. The purpose of inflammation is to stimulate the regeneration of healthy tissues; however, when generated in excess, it can lead to disease. Several earlier experiments demonstrated that nanoparticle-induced reproductive toxicity is associated with increased inflammatory responses (Zhao *et al.*, 2013; Meena *et al.*, 2015; Han *et al.*, 2016). Inflammation is normally controlled by a series of intracellular and extracellular events, such as oxidative stress, which result in the release of proinflammatory cytokines that act as intercellular chemical messengers to alert the body's immune system.

DNA damage

Sperm DNA damage is considered an important cause of male infertility due to nanoparticle exposure (Pawar and Kaul, 2014; Xu *et al.*, 2014; Meena *et al.*, 2015; Preaubert *et al.*, 2015; Smith *et al.*, 2015). In this regard, oxidative stress is assumed to be the main cause of DNA fragmentation in spermatozoa (Aitken and De Iuliis, 2010; Xu *et al.*, 2014; Meena *et al.*, 2015; Smith *et al.*, 2015). Badouard *et al.* (2008) demonstrated that ROS could induce DNA damage via the formation of oxidized DNA adducts, leading to the generation of abasic sites that subsequently cause destabilization of DNA structure and single-stranded breaks. In addition, a balance of ROS and antioxidants is very important for chromatin compaction in maturing spermatozoa. In spermatids, histones are replaced by protamines that lead to chromatin compaction in toroid structures (Gonzalez-Marin *et al.*, 2012). In the epididymis, further compaction occurs via the formation of disulfide bonds in maturing spermatozoa. Several glutathione peroxidase enzymes, such as sperm specific nuclear GPX4 and GPX5, bound to nearby acrosomal membranes to form disulfide bonds (Pfeifer *et al.*, 2001; Conrad *et al.*, 2005; Drevet, 2006). On the other hand, membrane-bound or free GPX5 acts as an antioxidant and

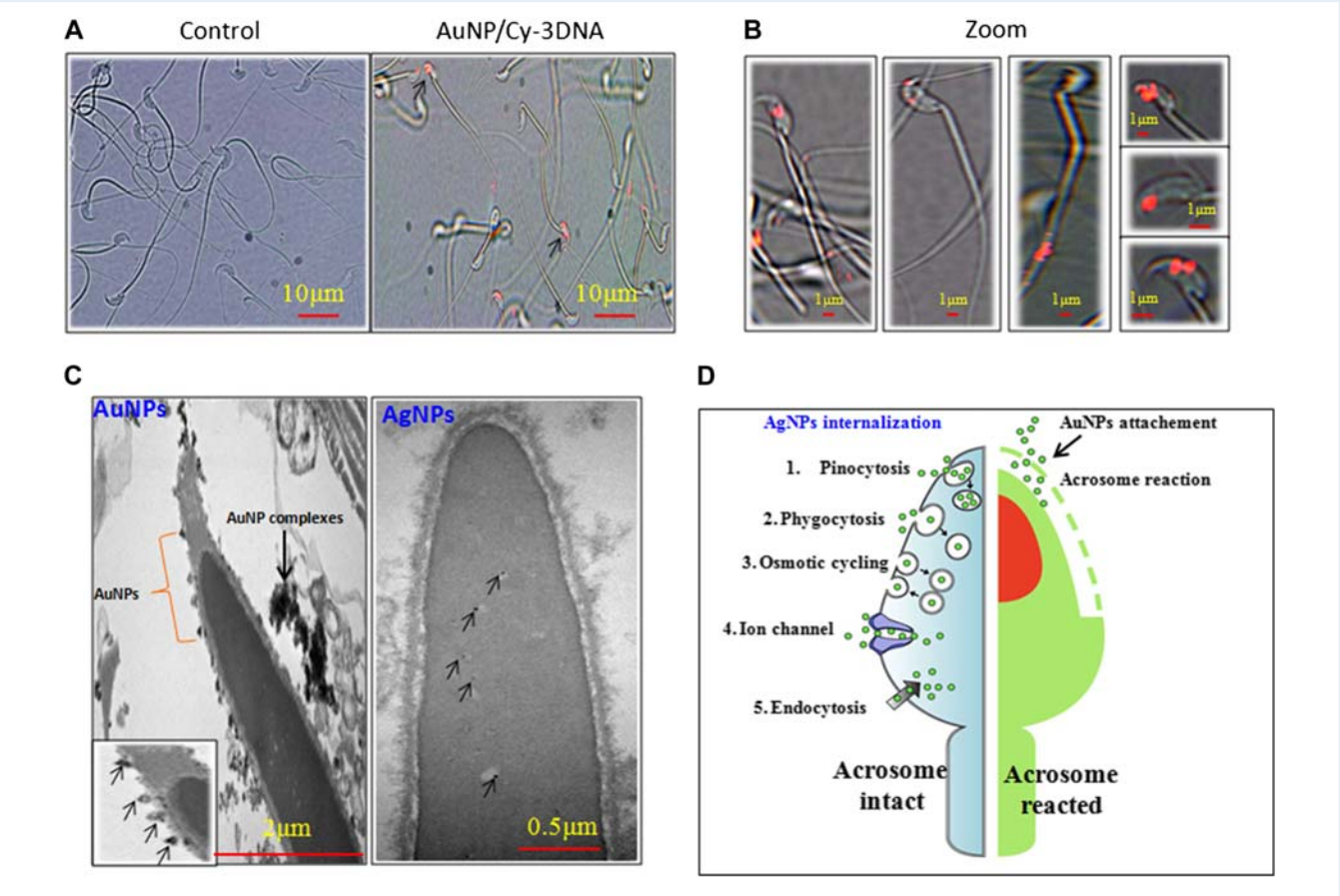

Figure 2 Internalization of nanoparticles in spermatozoa. (**A**) Internalization of polyethylenimine-coated AuNPs/Cy-3DNA complexes in murine spermatozoa observed by fluorescence microscope; (**B**) magnified image of (A); (**C**) internalization of citrate-coated AgNPs and polyethylenimine-coated AuNPs in murine spermatozoa observed by transmission electron microscope. We observed that AgNPs could spontaneously penetrate the plasma membrane of acrosome intact spermatozoa, whereas AuNPs could only bind to the plasma membrane surface of acrosome-reacted spermatozoa. (**D**) The possible uptake mechanisms of nanoparticles in spermatozoa. AgNPs, silver nanoparticles; AuNPs, gold nanoparticles.

tightly controls the H_2O_2 concentration (Drevet, 2006). Therefore, the concentration of these antioxidant enzymes decreases during sperm maturation due to increased utilization (Seligman *et al.*, 2005; Weir and Robaire, 2007). It has been reported that incorrect chromatin compaction is associated with male infertility (Hammadeh *et al.*, 2001; Molina *et al.*, 2001; Conrad *et al.*, 2005). Therefore, nanoparticle-induced oxidative stress can lead to incorrect chromatin compaction, causing fertility failure. Nanoparticles can also induce DNA damage via direct interactions, since they can also enter the nucleus of spermatogenic cells (Morishita *et al.*, 2012) and spermatozoa (Pawar and Kaul, 2014). Furthermore, nanoparticles can induce DNA damage in follicular cells and oocytes via oxidative stress *in vitro* (Courbiere *et al.*, 2013; Greco *et al.*, 2015, Preaubert *et al.*, 2015).

Therapeutic approaches

Nanoparticle-induced toxicity is mainly mediated through oxidative stress and oxidative stress-triggered inflammation as well as DNA damage, therefore antioxidant and anti-inflammatory drug treatment should be helpful in combating their adverse health effects. It has been shown by several researchers that treatment with agents having antioxidant or both antioxidant and anti-inflammatory properties (i.e. gallic acid, vitamin E, beta-carotene, green tea, resveratrol, taurine, curcumin, N-acetyl cysteine, propolis, quercetin, lycopene, etc.) have a protective effect against oxidative stress-induced reproductive toxicity and effects on embryo development (Das *et al.*, 2012; Liu *et al.*, 2013; El-Sharkawy *et al.*, 2014; Głombik *et al.*, 2014; Orazizadeh *et al.*, 2014; Sharma *et al.*, 2014; Yu *et al.*, 2014; Boeira *et al.*, 2015; Khanna *et al.*, 2015; Mosbah *et al.*, 2015; Oyagbemi *et al.*, 2015; Qin *et al.*, 2015; Santana *et al.*, 2015). Furthermore, treatment with selective metal chelators (Crapper McLachlan *et al.*, 1991; Casdorph, 2001; Ritchie *et al.*, 2003; Nguyen *et al.*, 2014; Liu and Guo, 2015; Ríha *et al.*, 2014; Santos and Chaves, 2015) can also be used to diminish the level of free intracellular metal ions released from the nanoparticles via complex formation, thereby reducing adverse health effects. The nanoparticle surface is a crucial determinant of its toxic response as it makes direct contact with cells and tissues. The effects of surface-coating agents have been discussed earlier. Thus, we can also control nanoparticle toxicity and its biocompatibility via the selective use of surface-coating agents.

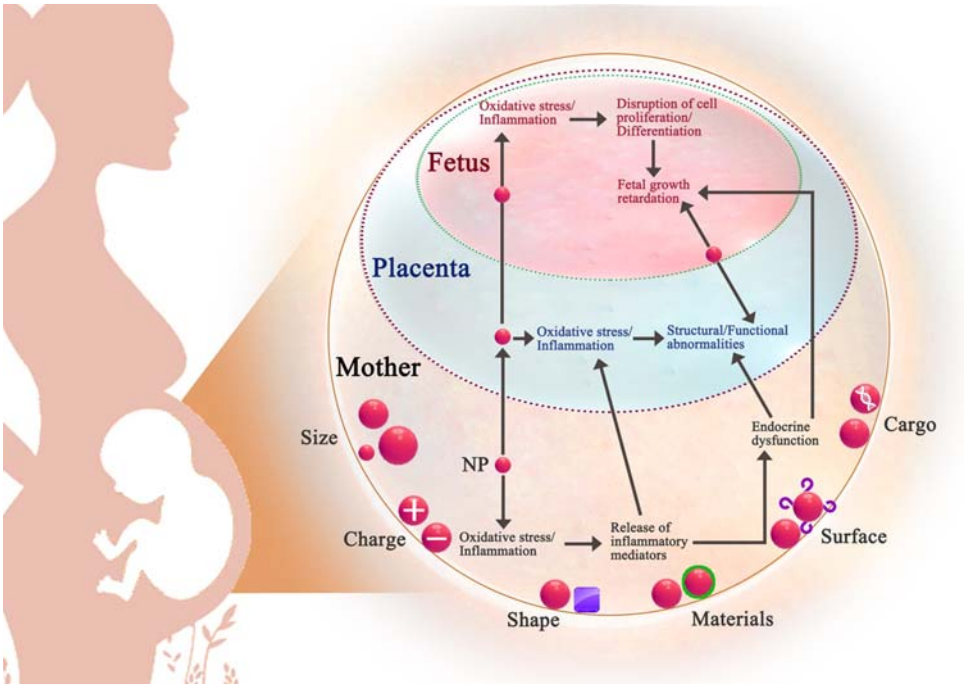

Figure 3 The pathways of the effects of nanoparticles in embryonic/fetal toxicity. Indirect effects occur due to maternal stress/toxicity and structural/functional abnormalities in placenta. Direct effects occur when nanoparticles reach embryonic cells/fetal tissues and induce inflammation and oxidative stress. Several factors affecting nanoparticle toxicity such as size, shape, chemical composition, surface charge, coating with inorganic or organic molecules or biological cargo have been depicted.

Applications of nanoparticles in transgenic animal production for the study of human disease

Transgenic animals are being used to generate models in order to investigate the mechanisms of human disease. In reproductive biology, one of the most anticipated applications of nanoparticles is gene delivery. Nanoparticles can be considered as a potential candidate for gene delivery into germ cells and early stage embryos to produce transgenic animals (discussed earlier). In this regard, nanoparticles having neither germ cell toxicity nor embryotoxicity should be taken into account because the impairment of germ cells or embryos may have pathological effects in the resulting offspring. We describe (below) several ways by which nanoparticles can be used for gene delivery into germ cells and early embryos to produce transgenic animals (Fig. 4).

SSC transfection

SSCs support spermatogenesis in adult males throughout life via self-renewal and differentiation as well as contributing genes to the next generation (Meistrich and van Beek, 1993; de Rooij and Russell, 2000). Therefore, genetic modification of SSCs can produce transgenic animals. So far, transgenic animal production via retrovirus- and adenovirus-mediated gene delivery into SSCs has been reported (Nagano *et al.*, 2001; Takehashi *et al.*, 2007). Braydich-Stolle *et al.* (2010b) demonstrated that AgNPs coated either with hydrocarbons or with polysaccharides could spontaneously enter SSCs. Therefore, we hypothesize that nanoparticles could also be used to transfect SSCs and produce transgenic animals.

Female germ-line stem cell transfection

Researchers have also isolated female germ-line stem cells (FGSCs) from neonatal as well as adult mice, which were cultured *in vitro* for a long time (Zou *et al.*, 2009; Pacchiarotti *et al.*, 2010; Zhang *et al.*, 2011a,b). These cultured FGSCs can produce normal oocytes and fertile offspring, following transplantation into ovaries. This FGSC-mediated transgenesis has important implications in the field of transgenic animal production. Therefore, we can also extrapolate our idea for nanoparticle-mediated gene delivery into FGSCs.

In vivo *injections into testis*

Intratesticular injections of exogenous DNA are also an efficient method for producing transgenic animals. Yonezawa *et al.* (2001) and Chen *et al.* (2013a,b) reported the production of transgenic offspring via direct injection of DNA/liposome complexes into the testes. Recently, nanoparticles are being used by many researchers for *in vivo* gene delivery (Tripathi *et al.*, 2012; Du *et al.*, 2015; Altangerel *et al.*, 2016; He *et al.*, 2016). Therefore, intratesticular injections of nanoparticles–DNA complexes can become a useful technique for transgenic animal production.

Sperm-mediated gene transfer via IVF or ICSI into oocytes

A number of researchers have shown that nanoparticles can bind to the sperm plasma membrane and enter the sperm plasma membrane

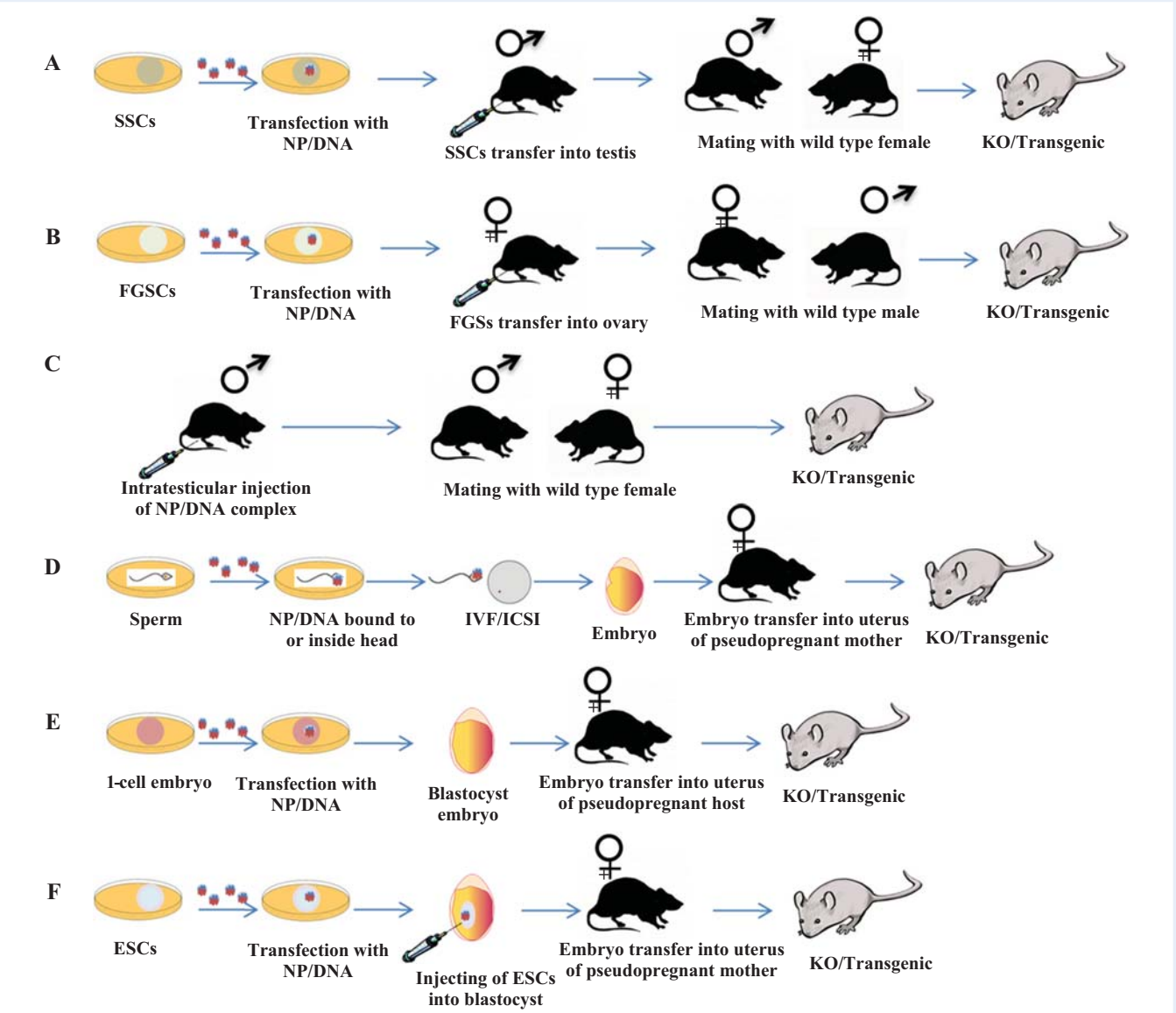

Figure 4 Possible ways by which nanoparticles can be used for gene delivery into germ cells and early embryos to produce transgenic animals. (**A**) SSC transfection, implantation into the testis of infertile male animals, followed by mating with female animals; (**B**) FGSC transfection, implantation into the ovary of infertile female animals, followed by mating; (**C**) *in vivo* injections into testis and mating; (**D**) sperm-mediated gene transfer (SMGT) via IVF or ICSI into oocytes, followed by transplantation of the embryos into the uterus of pseudopregnant host animals; (**E**) transfection of one-cell embryos followed by transplantation into the uterus of pseudopregnant host animals; (**F**) transfection of ESCs, followed by injection into embryos, and transplantation into the uterus of pseudopregnant host animals. ESCs, embryonic stem cells; FGSCs, female germ-line stem cells; KO, knockout.

as well as the head (Pawar and Kaul, 2014; Taylor *et al.*, 2014a; Preaubert *et al.*, 2015; Yoisungnern *et al.*, 2015). We also observed the association of polyethylenimine-coated AuNPs-cy-3DNA with mouse spermatozoa (Fig. 2). Similarly, Barkalina *et al.* (2014) also showed that mesoporous SiO_2NPs loaded with nucleic acids/proteins could form a strong association with boar sperm. So far, there are only two reports where the authors showed that nanoparticles could efficiently introduce exogenous DNA into embryos via spermatozoa (Kim *et al.*, 2010a,b; Campos *et al.*, 2011b). Therefore, more research is required in the field of nanoparticle-assisted sperm-mediated gene transfer **(**SMGT) in order to make it a useful technique for the production of transgenic animals.

Microinjection into male pronuclei

Transgenic animals can also be made via the introduction of exogenous DNA into the nucleus of a one-cell embryo (Gordon *et al.*, 1980). Nucleic acids are usually transferred into the embryos via microinjection or electroporation (Haraguchi *et al.*, 2004; Peng *et al.*,

2012). Recently, Selokar *et al.* (2015) reported the transfection of one-cell stage, zona-free buffalo embryos with commercial transfecting agents. Zona-free zygotes can also develop into blastocysts and be successfully implanted into the host in a similar manner as the zona-intact zygotes (Suzuki *et al.*, 1995). These observations lead us to hypothesize that nanoparticles can also be used to transfect zona-free, one-cell-stage embryos.

Transfection of embryonic stem cells

Embryonic stem cells (ESC) are a kind of pluripotent stem cell that can be derived from the blastocyst ICM and can maintain their self-renewal properties during *in vitro* culture (Evans and Kaufman, 1981). Transgenic chimeric animals can be produced from transgenic ESCs after microinjecting them into blastocyst embryos (Tokunaga and Tsunoda, 1992; Papaioannou and Johnson, 2000; De Repentigny and Kothary, 2010). Moreover, there are several reports demonstrating nanoparticle-mediated gene delivery into ESCs with a high efficiency (Lee *et al.*, 2008; Yang *et al.*, 2009). Thus, nanovectors can also be used as an efficient and reliable tool for the transgenesis of ESCs and chimeric animal production.

Advantages of nanoparticle-mediated gene delivery over the existing technologies for transgenic animal production

While the viral vectors have traditionally been used as effective delivery vehicles, their use in clinical application and basic research laboratories is limited due to safely problems associated with toxicity, immunogenicity and oncogenicity of the viral vectors. Other major limitations are small DNA load as well as expensive and complex production procedures (Luo and Saltzman, 2000; Kay *et al.*, 2001; Thomas *et al.*, 2003; Mancheño-Corvo and Martin-Duque, 2006). Electroporation techniques show high transfection efficiency of the exogenous genes but often results in extensive cell death (Aslan *et al.*, 2006; Green *et al.*, 2008). Therefore, non-viral vectors, such as nanoparticles, are considered as attractive gene delivery vectors because they can be easily functionalized or structurally varied, able to carry and incorporate huge and diverse genetic materials into cells, and are relatively safe.

Pronuclear microinjection of DNA into the early embryo is a popular method of germ-line gene transfer, but its use is limited because of high costs in terms of both money and the labor associated with it (Smith, 2004). In 2015, Selokar *et al.* showed that one-cell-stage, zona-free buffalo embryos can be transfected with a commercial transfecting agent, lipofectamine. Nanoparticle-mediated gene transfection requires simple incubation of cells with nanoparticle–DNA complex and many researchers have already shown that several ENPs have a higher transfection efficiency compared to commercially available liposomes in cultured cells (Tripathi *et al.*, 2012; Park *et al.*, 2014; Du *et al.*, 2015). Therefore, nanoparticles possess the potential to be used for gene delivery into early embryos and can be considered as an alternative to the highly expensive pronuclear microinjection methods.

Another advantage of nanoparticles is efficient binding and/or internalization to the sperm plasma membrane as well as the head (Barkalina *et al.*, 2014; Pawar and Kaul, 2014; Taylor *et al.*, 2014a; Preaubert *et al.*, 2015; Yoisungnern *et al.*, 2015). Kim *et al.* (2010a,b) and Campos *et al.* (2011b) reported that nanoparticles can efficiently introduce exogenous DNA into embryos via spermatozoa after IVF, which could become the alternative to the highly laborious ICSI into the oocyte cytoplasm. It has been reported previously that naked exogenous DNA used in SMGT is rapidly degraded by DNases present in sperm (Smith, 2002; Lanes *et al.*, 2009; Collares *et al.*, 2010; Campos *et al.*, 2011a). On the other hand, nanoparticles have the potential to protect the DNA from enzymatic degradation because of tight complexing with DNA on the nanoparticle surface (Tripathi *et al.*, 2012; Park *et al.*, 2014). Therefore, nanoparticles possess the potential to be used as highly promising gene delivery vectors in germ cells and developing embryos. However, the future success of nanoparticles as gene delivery vectors *in vivo* and in clinical application depends on attainment of efficient therapy with minimal or no adverse effects.

Conclusions

Based on the existing literature, we have discussed the toxicological effects of a few ENPs in mammalian germ cells and developing embryos by considering both *in vitro* and *in vivo* experimental models. Our review will provide mechanistic insights into the reprotoxicological aspects of ENPs to reliably estimate the potential impacts on human health. Due to the extremely small size of nanoparticles, they can readily cross biological barriers, such as the BTB, placental barrier and also easily gain access to the ovaries. However, there are controversial results related to nanoparticle biodistribution and the observed toxicities with the same chemical composition. These conflicting results could be related to the different routes of exposure, different exposure times, single or multiple exposure methods, as well as differences in size, concentration and surface-coating agents for the nanoparticles used in their studies. The nanoparticle-induced toxicities are mainly mediated via oxidative stress, inflammation and DNA damage. Antioxidant and anti-inflammatory drug treatments as well as use of selective metal chelators would be beneficial in combating nanoparticle-induced adverse health effects. Besides, we can also increase the biocompatibility of nanoparticles via the selective use of surface-coating agents.

So far, research in the field of ENP-mediated germ cell toxicity and embryo development is still quite preliminary and there is a lack of sufficient data on the reproductive toxicity of ENPs in humans. Besides, we cannot extrapolate the conclusions derived from animal models to humans directly for several reasons, such as differences in reproductive structures and endocrine functions, duration of gestation or spermatogenesis period, etc. In addition, most of the animal and *in vitro* studies were conducted at much higher doses that exceed the clinically relevant doses in humans. Regarding the protection against nanoparticle-mediated reproductive toxicity, only a few studies have been conducted. Therefore, all of these aspects should be considered in designing future experimental studies either to explore the mechanism of nanoparticle toxicity or to find protective agents combating nanoparticle-mediated reproductive toxicity. Finally, more research is required to make nanovectors a useful tool for the production of transgenic animals.

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Authors' roles

J.D. and J.H.K. designed and wrote the manuscript. J.D. and Y.J.C. performed the experiments for showing nanoparticle internalization into spermatozoa. J.D., Y.J.C., H.S., and J.H.K. analyzed the data, designed the pictures and critically revised the manuscript.

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Conflict of interest

The authors declare no conflicts of interest.

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