Reproductive Nanotoxicity of Carbon Nanoparticles

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Carbon nanoparticles have unique chemical and physical properties that make them an excellent material that can be applied in many fields of human activity, including industry, food processing, the pharmaceutical industry, or medicine. Although it has a high degree of biocompatibility, possible toxic effects on different tissue types must also be taken into account. Carbon nanoparticles are known to be toxic to the respiratory, cardiovascular, nervous, digestive system, etc., and they also have a negative effect on reproduction and offspring development.

Keywords: carbon nanoparticles ; graphene ; nanotoxicity

1. Introduction

Carbon is one of the most common and most important elements in universe. It is an essential component of macromolecules indispensable for life, such as proteins, lipids, nucleic acids, and carbohydrates with unique chemical and physical properties. These extraordinary chemical-physical properties are further enhanced in carbon-based nanomaterials. Carbon-based nanomaterials are characterized by excellent electrical and heat conductivity, extreme stiffness, strength, toughness, and high biocompatibility and low toxicity [1].

Carbon nanomaterials have found applications in various industries, including electronics, agriculture, food, pharmaceuticals, medicine, and cosmetics [2][3][4][5]. Among the most widely used and studied carbon nanoparticles are graphene, graphene oxide, carbon nanotubes, fullerenes, and nanodiamonds. Graphene is a two-dimensional (2D) material, a monolayer where carbon atoms are arranged in a honeycomb lattice structure. Graphene has unique properties, including high thermal and electrical conductivity and stability, high flexibility and elasticity, hardness and resistance, and large surface area [6].

Graphene oxide (GO) and reduced graphene oxide (rGO) are graphene derivates with different structural and chemical properties. GO is usually synthesized by chemical oxidation and exfoliation of graphite. It possesses various oxygen groups (hydroxyl, carboxyl, epoxy groups) that functionalize the surface and modify the properties of CNPs. To reduce GO, chemical, thermal, or photo-thermal reduction can be used; however, rGO did not reach the original structure of pristine graphene and still contains residual oxygen, even after strong reduction. The main functional differences between GO and rGO are in electrical conductivity. GO shows low electrical conductivity and lower mechanical strength compared to rGO and pristine graphene [7][8]. Carbon nanotubes are CNPs with tubular structure; graphene sheets rolled into a cylindrical shape. They are classified on the basis of the number of walls, such as single-walled, double-walled, or multi-walled. Like graphene, nanotubes have exceptional properties, especially high electrical and thermal conductivity, strength and elasticity, and large surface that can be easily functionalized [9][10].

Fullerenes (buckyballs) are a class of carbon allotropes with a spherical or ellipsoidal shape. Their size is dependent on the number of carbons—C_{60}, C_{70}, C_{80}, etc. The most common fullerene structure C_{60} is a molecule that consists of 60 carbon atoms arranged as 20 regular hexagons and 12 regular pentagons. Fullerenes are soluble in organic solvents, especially C_{60}, C_{70}, and easily functionalized [11]. Interestingly, fullerenes are great antioxidants. The antioxidant capacity can be enhanced by functionalization with hydroxyl molecules [12][13][14].

2. Reproductive Toxicity In Vitro Studies

Several in vitro toxicity studies evaluated the deleterious effect of CNP on cells of the reproductive system, especially sperm and oocytes.

First, researchers mention the negative effect of multi-walled carbon nanotubes on steroidogenesis and the production of sex hormones, which are essential for reproduction (gametogenesis, ovulation, and sexual behavior). Qu et al. showed that multi-walled carbon nanotubes (MWCNTs) inhibited progesterone production in preovulatory rat granulosa cells.

Production decreased significantly at a concentration of 10 and 50 µg/mL/48 h. MWCNTs altered the expression of steroidogenic proteins StAR that transfer cholesterol from the outer to the inner mitochondrial membrane, where cholesterol is metabolized by P450scc to pregnenolone. The inhibitory effect is reversible. Removal of MWCNTs restored progesterone production. Furthermore, MWCNTs induced ROS production and slightly altered mitochondrial membrane potential. These results suggest that steroidogenesis is compromitted by StAR inhibition and oxidative stress induced by MWCNTs, which act as an endocrine disruptor [15].

Male germ cells are susceptible to xenobiotics, including CNPs. Gurunathan et al. determined the toxic effect of graphene oxide (GOs) on Leydig and Sertoli cells (TM3 and TM4). They exposed cells to two types of GOs with different lengths (20 and 100 nm) and different zeta potential (electrokinetic potential in colloidal systems). Both GOs inhibited cell viability and proliferation in a dose-dependent manner, induced the release of lactate dehydrogenase (a marker of cellular damage or death), and altered mitochondrial membrane potential which was associated with elevated reactive oxygen species (ROS). Furthermore, GOs were responsible for DNA damage depending on nucleoside oxidation, and 8-oxo-dG formation, and suppression of pro-apoptotic gene expression (Bax, Bak, p53, p21, caspase-3), while the expression of genes coding anti-apoptotic proteins (Bcl-2) increased. Cell survival was also altered by suppression of EGFR (epidermal growth factor receptor) and AKT kinase phosphorylation. Interestingly, shorter GOs caused significantly greater damage in some measured parameters, and TM3 appeared to be more susceptible to the GOs exposure [16].

Ji et al. exposed TM4 and GC-2 spd (mouse testicular germ cell lines) to GO quantum dots (QD). Although graphene oxide quantum dots (GOQDs) did not affect cell viability, they induced apoptosis in both cell lines. Transmission electron microscopy images showed that GOQD treatment increased the number of autophagosomes, and thus autophagy. The degradation of sequestered material in autophagosomes depends on the fusion between autophagosomes and lysosomes. The researchers found that this process was not inhibited, but that undegraded cargo occurred. This phenomenon was due to a reduction in lysosomal activity and the ability to degrade the material. The accumulation of undegraded cargo is a hallmark of aging and senescent cells [17].

GC-2 spd cells are sensitive not only to GOQD but also to MWCNTs. The study by Xu et al. showed that while the dose of 0.5 µg/mL MWCNTs was not lethal to cells, the accumulation of MWCNTs in mitochondria was detected. The presence of MWCNTs in mitochondria was associated with a decrease in the expression of mitochondrial-related genes, the rate of oxygen consumption, and especially with decreased ATP production that is necessary to maintain cell function and survival [18].

The toxic effect of CNPs was evaluated not only on mouse germinal cells but also on the spermatozoa of buffalos or boars. The toxicity of MWCNTs was tested by Sanand et al. in buffalo spermatozoa and the half-maximum inhibitory concentration (IC_{50}) was determined. Buffalo sperm were exposed to different doses of MWCNT for 30, 60, and 120 min. MWCNTs time- and dose-dependently decreased cell viability, severely depressed the membrane integrity, increased malondialdehyde levels (a marker of oxidative stress), and decreased the activity of antioxidant enzymes (glutathione peroxidase /GTP/, superoxide dismutase /SOD/) [19].

The results confirming the toxicity of CNPs were obtained in a study by Bernabò et al. who exposed boar spermatozoa to GO at different concentrations. Although doses of 5, 10, and 50 µg/mL were toxic to cells (reduced viability, fertilization capacity, and impaired acrosome integrity and adhesion capacity), lower doses of 0.5 and 1 µg/mL promoted sperm fertilization capacity. Importantly, the researchers demonstrated that GO interacted with sperm membranes and altered membrane fluidity by extracting cholesterol from the membrane [20].

Li et al. observed quite different effects of CNPs when exposed boar spermatozoa to carboxylated fullerene (C_{60}–COOH). Sperm incubation with C_{60}–COOH at a dose of 2 µg/mL for 10 days increased sperm motility compared to the control group, improved acrosome integrity and mitochondrial activity, and reduced oxidative stress. C60 has been shown to have antioxidant effects [21].

The conclusions of studies on human sperm are still not concise. Asghar et al. described that carboxylated single-walled carbon nanotubes (SWCNT–COOH) induced ROS production of ROS in human spermatozoa at a concentration of 25 µg/mL, while the presence of reduced GOs did not increase oxidative stress. Importantly, neither GOs nor SWCNTs at a dose of 1–25 µg/mL affected sperm viability [22]. Human sperm, in the study by Aminzadeh et al., were exposed to SWCNT–COOH or MWCNT–COOH (0.1–100 µg/mL/5 h). Both CNPs did not attenuate sperm viability. However, sperm motility decreased in a dose-dependent manner, and even the lowest concentration of nanoparticles increased ROS production, which may be associated with mitochondrial and DNA damage [23].

As follows, exposure to female germ cells induces several serious changes. The results showed that the oocytes are sensitive to CNP treatment. Lin et al. documented that graphene QDs altered the maturation of mouse oocytes. Oocytes incubated with graphene QD doses failed to extrude polar bodies. This effect was dose-dependent and was accompanied by accumulation of intracellular ROS and DNA damage. Furthermore, graphene QDs were detected in oocytes, located primarily in the nucleus and near the mitochondria, whose morphology and functions were severely altered [24].

The resumption of rat oocyte meiosis was described by Lei et al. using an in vitro maturation culture model and exposing oocyte granulosa cells (OGCs) to fullerenols. Fullerenols reduced transzonal protrusions (TZPs), accelerating the retraction of TZPs from oocytes. Transzonal projection is the connection between granulosa cells (the cell layer surrounding the oocyte) and oocytes and forms a functional complex that is essential for the maintenance and development of oocytes. All doses reduced TZPs and only a few thin filaments of granulosa cells connected to the oocyte (in the control culture, the filaments were intact and abundant). Furthermore, fullerenols reduced the expression of connexin 43 in granulosa cells, which is a part of gap junctions. The two-hour treatment decreased expression by 56%. Retraction of TZPs and lower expression of connexin 43 altered gap junction channels and reduced mass transport, leading to a decrease in cyclic adenosine monophosphate in oocytes and an accelerated resumption of meiosis, which can lead to reduced oocyte quality [25].

While there is a possibility that fullerenols have cytotoxic potential, in the field of genotoxicity, they have a rather protective effect. Mrdanovic et al. found that fullerenols, even at high concentrations, reduced the frequency of micronuclei and aberrations of the chromosome in Chinese hamster's ovary (CHO-K1) compared to control cell culture. Surprisingly, lower doses and shorter exposures were more effective in reducing the levels of these genotoxicity markers [26].

Yaday et al. cultured CHO-K1 cells with different types of CNPs, including MWCNTs, which induced alteration of the cell cytoskeleton. Elongation, an increase in the number of cytoplasmic vacuoles, and the formation of lamellipodia via actin polymerization were observed. Cytoskeleton remodeling was associated with enhanced expression of the Dlc-1, cofilin, and Rac1 proteins that affect the cytoskeleton and cell motility [27]. CHO-K1 was also influenced by GOs. Batiuskaite et al. found that GO (alone or with bovine serum albumin) significantly reduced cell viability in a dose-dependent manner. Importantly, GO-BSA induced lower changes in viability than GOs [28].

3. Reproductive Toxicity In Vivo Studies

In vivo studies determining the toxicity of CNPs used different types of animal models, both non-mammal and mammal species. Nematodes, insects, mice, and rats are the most commonly used species in nanotoxicity research.

3.1. Experiments with Nonmammal Species

Nematodes are represented by *Caenorhabditis elegans* (roundworms, *C. elegans*) whose reproduction might be altered by various types of CNPs. Kim et al. exposed *C. elegans* to GOs at a dose of 10 mg/L. Two hours after exposure, GOs were detected throughout the body, including the reproductive system; however, at 48 h the GOs accumulated especially in the area around the germline and embryos. Furthermore, exposure to GOs altered spermatogenesis and decreased the number of sperm, thus inducing severe reproductive toxicity. It also altered fat metabolism and increased oxidative stress [29].

The reproductive toxicity of GOs was also described by Chatterjee et al. *C. elegans* was exposed to either GOs, reduced GOs, or both. GOs were uptaken by cells and accumulated in reproductive organs. A significant reduction in reproductive function was observed even after exposure to the low dose of 5 mg/L and the dose of 50 mg/L completely stopped reproduction. Exposure to GOs deregulated the MAPK (mitogen-activated protein kinase) and Wnt pathways. Interestingly, reduced GOs were more biocompatible and did not induce tissue to the damage of reproductive system [30].

Zhao et al. found that GO not only reduced reproductive capacity and altered gonad development in *C. elegans* but also induced germ cells. The signaling pathway involved in germ cell apoptosis was HUS-1/CLK-2-CEP-1-EGL-1-CED-4-CED-3, which reflects DNA damage and induction of apoptosis. GOs also activated miRNA 360 which interacts with the gene encoding CEP-1. In this way, GOs can alter the epigenetic signaling involved in the self-protection mechanism against GOs toxicity [31].

Kong et al. determined the toxicity of graphene and polylactic acid-functionalized graphene (PLA-G) on *C. elegans*. They exposed *C. elegans* to concentrations of 50–1000 µg/mL. In contrast to the results with GOs, graphene and PLA-graphene did not affect the reproductive capacity of *C. elegans*, indicating that the functionalization of CNPs may be beneficial and improve biocompatibility [32].

Other nonmammalian animal species for which the toxicity of CNPs was evaluated included insects (*Acheta domesticus*, *Spodoptera frugiperda, Drosophila melanogaster*, *Bombyx mori)* and aquatic animals (*Paracentrotus lividus*, *Anabas testudineus*). *Acheta domesticus*, cricket, was used in the study of Kapeta-Kaczmarek et al. who chronically exposed animals to different doses of nanodiamonds (NDs) in the diet of animals. Exposure to NDs decreased cricket survival (21 days of NDs vs. 28 days of control) and negatively influenced egg production and hatching success. The females in the higher NDs group laid an average of 15 eggs, the females in the lower dose 25 eggs, while the females in the control group laid 35 eggs in 48 h. The results indicate that exposure to NDs reduced fecundity [33].

Spodoptera frugiperda, fall armyworm, was exposed to different doses of oxidized MWCNTs and GOs in the diet. Martins et al. confirmed that, in a dose-dependent manner, both CNPs reduced fertility and fecundity [34]. Philbrook et al. exposed *Drosophila melanogaster* (*D. melanogaster*) fly, to hydroxylated single-walled carbon nanotubes that affected neither fertility nor fecundity [35]. However, a more recent study with *D. melanogaster* by Priyadarsiny et al. used GO nanosheets and revealed the toxic effect of CNPs. Especially teratotoxicity. In the hatching test, a significantly lower number of adult flies hatched from every vial. The decrease depended on the dose [36].

Fang et al. exposed *Bombyx mori* (*B. mori*) to GOs to evaluate its effect on reproduction. The dose of 25 mg/L of GOs induced oxidative stress and DNA damage in ovary cells and reduced the gonadosomatic index in *B. mori* larvae by 41%. GOs similarly increased the level of oxidative stress in silkworm ovary tissue, which was associated with a decrease in the number of both oogonia and oocytes in the ovary and increased vacuole formation in follicle cells. Transcription of genes related to ovarian development was also reduced [37].

Studies also evaluated the effect of CNPs on animals living near or in aquatic environments. Carbon black (CB) and GOs were tested for reproductive toxicity to *Paracentrotus lividus*, sea urchin. CB at doses of 0.0001–1.0 mg/L/h reduced egg fertilization by approximately 50%. On the other hand, GOs did not affect fertilization [38].

Sumi et al. focused on the effect of two sublethal doses of Buckminsterfullerene (BCF; 5 mg/L and 10 mg/L) for short- and long-term duration on *Anabas testudineus*, a freshwater fish. BCF reduced the weight of both the ovary and testes, the activity of antioxidant enzymes (SOD, GTP), and increased the production of ROS. Prolonged exposure was associated with histological alterations in the ovary and testes. In the ovary, atresia, vacuole formation, thickening of the vitellogenic oocyte membrane, or completely degenerated oocytes were detected. In the testes, the formation of vacuoles, the decrease in the number of sperm and spermatocytes, the distortion of the seminiferous epithelium, and atresia were found. The results indicate that BCF leads to reproductive toxicity [39].

The toxic effect of MWCNT–COOH was determined in adult *Danio rerio* (zebrafish). Arrillo et al. showed that doses of 0.5 and 1.0 ppm of MWCNT–COOH significantly increased oxidative stress and lipid peroxidation in their ovary and testicular tissue [40]. Zhao et al. used *Xenopus tropicalis* that was exposed to either 0.5 or 2.5 mg/L MWCNTs for 56 days. The presence of MWCNTs inhibited body growth, including gonads (testes and ovaries), and histopathological sample analysis revealed that spermatogonia and oocyte formation was negatively affected [41]. A pair of *Oryzias latipes* was injected once intraperitoneally with GO (25–200 µg/g) and the pair continued to breed for another 21 days (Dasmahapatra et al.). The dose-dependent reduction in fecundity was documented during the early days after injection. Furthermore, embryo hatchability was significantly reduced in the 200 µg/g group; but embryo mortality was not altered. Interestingly, folliculogenesis in the ovary and the morphology of granulosa and Leydig cells did not change significantly, although the researchers identified GO agglomerates in the gonads [42].

3.2. Experiments with Mammals

Studies on mammals were conducted mainly in mice and rats.

Zhang et al. showed that graphene QD (GQD) administered by oral gavage or intravenously injected did not change sexual behavior, sperm quality, or testosterone levels in male mice. Even high doses are rapidly excreted from the body and did not accumulate in tissues. Female mice housed with males had first, second, and subsequent litters of healthy pups with no apparent differences from females housed with buffer-treated males [43]. Similar results were described by Skovmand et al. who intratracheally instilled GOs, amorphous CB (Flammruss 101), CB (Printex 90), and diesel particle matter (SRM1650b) in mice. Any changes in sperm parameters, sperm production, and testosterone levels were detected [44]. Liang et al., who administered GOs intravenously (25 mg/kg/d) to male mice, described that GO-treated mice did not show abnormalities in reproductive activity, hormonal levels, and sperm quality. Their offspring were also healthy and their survival rate and growth were similar to those of the control group. Furthermore, even a high intra-abdominal injection dose of 300 mg/kg in male mice (60 mg/kg/d for 5 days) did not cause damage to reproductive organs [45].

However, in contrast to the findings of the above studies, some studies show the opposite and describe a negative effect of CNPs on the reproduction of mice. For example, the study by Farshad et al. showed the toxic effect of SWCNTs and MWCNTs. BALB/c mice were orally administered 10 and 50 mg/kg/d for 5 weeks. Higher doses of SWCNTs significantly reduce body and testis, epididymis, and vas deferens weight, whereas MWCNT reduced only body weight. Both CNTs dose-dependently decreased sperm count, viability, and motility, and increased oxidative stress. Importantly, exposure to CNTs disrupted the mitochondrial functions of the sperm (elevated mitochondrial membrane depolarization and decreased dehydrogenase activity and ATP production. Histological analysis revealed testicular tissue injuries (tubular injury, tubular desquamation) and lowered spermatogenic index [46].

The toxic effect of nanoscale GO (NGOs) was evaluated by Akhavan et al. BALB/c mice were injected intravenously with different doses of NGOs (2, 20, 200, or 2000 µg/mL) and after 8 weeks were sacrificed. The NGOs were taken up by various types of tissue, including the thyroid and testes. Importantly, sperm viability and motility decreased dramatically in a dose-dependent manner from 75 to 40% and morphological abnormalities of the sperm tail and head were observed, especially at the highest dose. At doses of 200 µg/mL and 2000 µg/mL, ROS production in semen also increased and DNA fragmentation and aberrations occurred. Female mice inseminated by males treated with NGOs showed lower levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), progesterone, and prolactin during pregnancy. NGO concentration of 2000 µg/mL reduced the secretion of FSH, LH, progesterone, and prolactin by 38, 57, 31, and 37%. It was associated with altered fetus growth and a 15% reduction in postnatal viability of delivered pups. The results indicate that the NGOs altered reproductive health and even the next generations [47].

It seems that CNPs can negatively affect not only male mice, but also female mice. Hougaard et al. pre-conceptually administered MWCNTs (intratracheal instillation of 67 µg NM-400 MWCNT) to adult female mice. Subsequently, mice were bred together with adult males. The time to birth of the first litter was delayed by an average of 5 days. Interestingly, exposure to MWCNT caused lung and liver damage that lasted almost 4 months [48].

Johansson et al. evaluated the effect of intratracheally instilled MWCNTs on estrous cycle regularity and reproductive function. The administration of MWCNTs prolonged the estrous cycle during which exposure occurred. Before exposure, the estrous cycle lasted 3–5 days, after exposure, the estrous cycle increased by two days. In contrast, the cycle beginning after administration was shorter and lasted 4.3 days. Exposure to MWCNTs also affected delivery time. Mice exposed to the dose of 2 µg gave birth earlier compared to the control group, while the doses of 18 µg and 67 µg delayed delivery [49].

A similar effect was also documented for C_{60} (Buckminsterfullerene). The toxicity of C_{60} 1 µm diameter (micro-C_{60}) and 50 nm diameter (nano-C_{60}) in mice and rats was described in NTP Technical Report. Animals inhaled (nose only) C_{60} for 3 months. Micro-C_{60} at doses of 2, 15, or 30 mg/m^3 decreased sperm motility in male mice and rats and increased the likelihood of a prolonged estrous cycle in female mice. Nano-C_{60} at concentrations of 0.5 or 2 mg/m^3 lowered sperm motility in male rats and in female mice elevated the probability of an extended estrous cycle [50].

Nirmal et al. conducted two studies in which they intraperitoneally exposed rats to either three increasing doses of nanoscale GOs (NGOs; 0.4, 2.0, or 10.0 mg/kg) for 7, 15, or 30 days or hydroxylated MWCNTs at doses of 0.4, 2.0, and 10.0 mg/kg (15 doses). NGOs caused a dose-dependent reduction in sperm, spermatogonia, and spermatids. Furthermore, a decrease in sperm motility and morphological abnormalities (atrophy of seminiferous tubules with reduction in germinal epithelium, germ cells, and vacuolization) was detected in animals treated with the highest doses of NGOs [51]. OH–MWCNTs decreased sperm count and motility in a dose-dependent manner, viability was not affected; however, a significant increase in sperm abnormalities (headless sperms, absence of normal hook, amorphous head, bent tail, folded tails) was documented. Histological analysis revealed severe damage to testicular tissue. A dose of 2.0 mg/kg damaged the seminiferous tubules and induced vacuolization, caused interstitial engorgement and edema, and reduced the thickness of the germinal epithelium. More severe damage occurred after administration of the highest dose [52]. In summary, NGOs and MWCNTs had a similar destructive effect on the male reproductive system of rats.

Farombi et al. tested the response of pubertal rat organs to exposure to MWCNT–COOH. Rats were administered different doses of MWCNT–COOH suspension intraperitoneally (0.25, 0.5, 0.75, and 1.0 mg/kg/d) for 5 days. After treatment, the activity of antioxidant enzymes (superoxide dismutase and glutamate pyruvate transaminase) increased in the testes, epididymis, and sperm, as well as peroxide and malondialdehyde levels, while glutathione-S-transferase and glutathione levels decreased. Thus, oxidative stress increased significantly. Furthermore, MWCNT–COOH caused a decrease in the number and motility of sperm in the epididymis and the level of testosterone and an increase in sperm abnormalities and morphological changes in the testes and epididymis. The results indicate that MWCNT–COOH is toxic to the reproductive system of rats [53].

Both in vitro and in vivo studies show the toxic potential of CNPs, including grapheme, GO, MWCNTs, SWCNTs, QD, nanodiamonds, and fullerenes. They can alter spermatogenesis, sperm morphology and functions, hormonal balance, damaged ovary, and testicular tissue by inducing the production of reactive oxygen species, and DNA damage. However, in vivo studies also provided results that do not support reproductive toxicity of CNPs. The toxic effect depends on the type of CNPs, dose, and time of exposure.

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