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Physico-Chemical Properties Mediating Reproductive and Developmental Toxicity of Engineered Nanomaterials

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Abstract: With the increasing production of engineered nanomaterials (ENMs) exploited in many consumer products, a wider number of people is expected to be exposed to such materials in the near future, both in occupational and environmental settings. This has raised concerns about the possible implications on public health. In particular, very recently the scientific community has focused on the effect that ENMs might exert on the reproductive apparatus and on embryonic development. Indications that ENMs might have adverse effects on cells of the germ line and on the developing embryos have been reported. In the present minireview we will perform a critical analysis of the published work on reproductive and developmental toxicity of the most commonly used nanoparticles with a major focus on mammalian models. We will place emphasis on the main physico-chemical characteristics that can affect NP behaviour in biological systems, i.e. presence of contaminants and nanoparticle destabilization, size, dosage, presence of functional groups, influence of the solvent used for their suspension in biological media, aggregation/agglomeration, intrinsic chemical composition and protein corona/opsonisation.

The importance of this specific field of nanotoxicology is documented by the rapidly increasing number of published papers registered in the last three years, which might be a consequence of the growing concerns on the possible interference of ENMs with reproductive ability and pregnancy outcome, in a time in which reproductive age has increased and the possibility to bear children appears reduced.

Keywords: Nanoparticles, engineered nanomaterials, reproductive toxicology, nanotoxicology, embryo, embryonic development, silica, carbon nanotubes, silver, gold, placenta, placental barrier

INTRODUCTION

In the last twenty years, the increasing biomedical and industrial interest on engineered nanomaterials (ENMs) has posed concerns on their potential toxicity for public health and the environment. The clear identification of possible adverse effects of ENMs appears pivotal, since their outstanding physico-chemical properties make them great candidates for many applications, which could be demonstrated to be advantageous for the community. Indeed, several ENMs have been already tested for drug delivery and used in many consumer products. To cite few examples, binding of the anti-cancer drug doxorubicin to poly-alkylcyanoacrylate nanoparticles (NPs) has been shown to bypass multidrug resistance [1], and polysorbate 80 coated poly-butylcyanoacrylate NPs delivered doxorubicin to brain tumours, crossing the otherwise impermeable blood-brain barrier [2]; TiO_2 and ZnO nanoparticles are used as additives in commercial products, including sunscreen lotions, due to their ability in blocking the UV light [3]. As a consequence of the reduced size (with at least one dimension below 100nm) and high reactivity, NPs differ substantially from their micrometric counterpart, and their interaction with biological systems cannot be predicted based on what is known for the material of origin, raising concerns about the risks for human health and the environment [4]. For such reason in the last two decades a great deal of data have been published, reporting on the effects of several ENMs in living organisms, and in 2004 (fourteen years after the first two reports on nanoparticle impact on human health were published [5,6]), the term "nanotoxicology" was coined, to comprise all data, past and future, regarding the interaction of NPs with biological systems *in vitro* and *in vivo*. To date much information have been accumulated suggesting adverse effects of NPs in several cells and organs, and most studies have focused on the lung, liver and immune system, the targets that most likely would be affected by environmental exposure to ENMs. However, attention should be paid when interpreting some of these results, as adverse effects have been sometimes observed using incredibly high amounts of NPs, a situation that unlikely would occur in reality [7]. In addition, many of the physico-chemical parameters that might contribute to the biological effect, such as agglomeration/dispersion in the media, release of toxic ions, aging of the preparation and coating, have been often overlooked. In spite of these limitations, some rules on the toxicological behaviour of ENMs can be drawn from the available data. In particular, some peculiar chemical and physical properties of engineered nanoparticles have been demonstrated to play a major role in determining their adverse effect in cells and animal models. For example, it has been recently shown that length is crucial in inducing toxic effects by carbon nanotubes, as only multi-walled carbon nanotubes (MWCNTs) with a length higher than 20 μm show an asbestos-like pathogenic behaviour when intraperitoneally administered to mice [8]. Interestingly, in other model systems, same size particles of different chemical composition exerted different effects, indicating that chemistry may also play a pivotal role [9].

These observations pose the problem that the effects following the interaction of NPs with a biological system are often hard to be predicted.

The increasing interest in nano-bio interactions has stimulated the birth of a new area of research focusing on reproductive systems and embryonic development. This fairly new branch of nanotoxicology could be named "nanoreprotoxicology". The potential relevance of such topic resides in the increasing infertility affecting the population in the developed countries, which in Europe only has risen from 16% of 1976 to 20% of 2004 (data from the WHO. See: http://www.euro.who.int/__data/assets/pdf_file/0010/73954/EN63.pdf). A dependence of such increase on several factors, among which environmental and occupational issues, is widely recognized. Interestingly, in recent years the number of published paper on the role of ENMs in reproductive functions and embryonic development has increased rapidly (Fig. **1**), indicating that in this respect the potential importance of intentional or accidental exposure to ENMs is emerging.

Proliferation, maturation and apoptosis of the gametes are finely regulated processes, whose perturbation by toxicants, including ENMs, might lead to reduced or, in the worst case scenario, abolished fertility. Despite the apparent sectoriality of such a topic, it has to be considered that any event that might induce DNA mutations in the germ line affects not only the individual itself but also its progeny, thus exerting a wider spectrum effect. In addition, beside a direct action on germ cells, indirect effects through the dysregulation of the endocrine system might as well interfere with

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proliferation and differentiation of the germ line. Regarding the possible interference of ENMs with the intra-uterine development of the embryo, the reproductive age in the developed countries is rapidly rising due to social reasons, limiting the chances to bear children; for such reason any threat that might interfere with the progression of pregnancy has to be avoided to a greater extent.

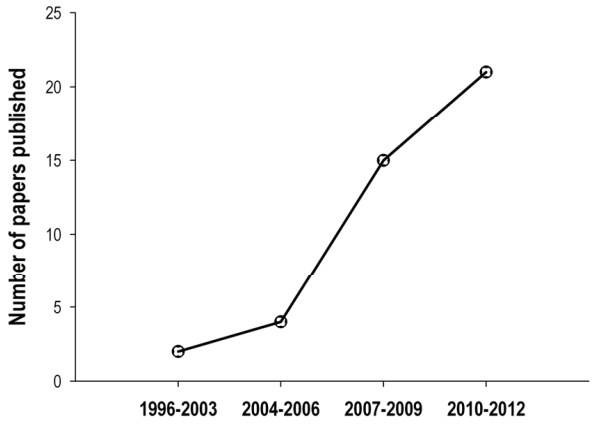

Fig. (1). The graph shows the sharp increase in the number of published papers reporting on reproductive and developmental effects of engineered nanoparticles occurred in recent years.

In the present review we will perform a critical analysis of the published work on reproductive and developmental toxicity of the most commonly used nanoparticles with a major focus on mammalian models. We will place emphasis on the main characteristics that can affect NP behaviour in biological systems, i.e. presence of contaminants and nanoparticle destabilization, size, dosage, presence of functional groups, influence of the solvent used for their suspension in biological media, aggregation/agglomeration, intrinsic chemical composition and protein corona/opsonisation.

THE ROLE OF SIZE

There is general agreement that ENMs are biologically more reactive than the corresponding bulk form; however size related effects within the nanometric range (1-100 nm) are less certain. In nanoreprotoxicology, studies in which a direct comparison of different size particles has been performed generally support the size-dependent effect both when comparing NPs with the bulk form and within the nanoscale range. However, alternative hypotheses to the obvious explanation that smaller size is associated with better penetration in cells and tissues have been successfully tested as discussed below.

In a study on the biodistribution of spherical-shaped gold nanoparticles, 4 different size ENMs (10, 50, 100 and 250 nm) were intravenously administered to male rats, and all distributed to liver and spleen, but only the smaller ones were able to access other tissues, including the testis [10]. Similarly, after repeated oral administration, bigger silver nanoparticles (72 and 323 nm) were detected in brain, lung, liver and kidney, while only smaller size nanoparticles (22 and 42 nm) were detected in testis as well [11]. Due to the analysis performed in both studies (ICP-MS), the localization of the particles within the tissues was not assessed, giving no indication on the ability of the particles to cross the blood-testis barrier, reaching the seminiferous epithelium; in addition possible alteration of fertility of the treated animals was not assessed.

Very recently, size-dependent effect of silica NPs on embryonic development has been also reported [12]. In this study the biodistribution of doped silica NPs was shown to vary according to the size of the particles, so that only the smaller particles (of about 70 nm), beside accumulating in the liver, were able to reach the placenta and the fetus, inducing resorptions and in utero growth restriction. At variance, bigger particles (300 and 1000 nm) accumulated exclusively in the liver. Size-related crossing of the placenta have been also shown for quantum dots of three different sizes (3.21±0.32 nm, 2.59±0.43 nm, 1.67±0.29 nm), which accumulated in fetal tissues, with the smaller accumulating more [13]. Similarly, crossing of the placental barrier was demonstrated for fluorescently labelled polystyrene beads up to 240 nm, while crossing of bigger size NPs was negligible (Fig. **2**) [14]. Altogether these results indicate that size alone cannot account for the ability of NPs to cross biological barriers, since the crossing threshold varies for the different NPs, thus suggesting that chemical composition and, perhaps, other physical properties may also play a role.

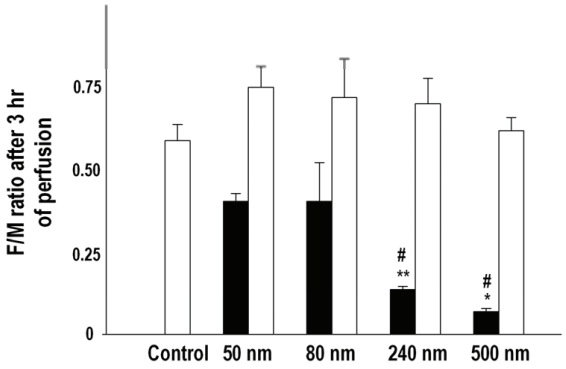

Fig. (2). Size-dependent ability of PS nanoparticles to cross the blood-placental barrier, reported as the ratio between fetal and maternal concentrations, after 180 min of perfusion. White bars: 14C-antipyrine, used as a control. Blue bars: PS beads. Data represent mean ± SE of at least four independent experiments. *p < 0.05 compared with 240-nm ratio value; **p <0.05 compared with 80-nm ratio value; #p <0.05 compared with 50-nm ratio value (Reprinted with permission from Wick P *et al.*, Environ Health Perspect, 2010).

Interestingly, the idea that size-related factors and not size "per se" are actually responsible for the apparent higher toxicity of low size NPs is discussed in a pioneeristic work on transplacental distribution of colloidal gold particles (5 and 30 nm) [15] in near term pregnant rats. The authors observed a higher amount of the smaller particles reaching the placental and fetal tissues, seemingly in line with the idea that smaller particles can more easily penetrate barriers. However, they calculated that the estimated blood clearance of the particles from the maternal blood was slower for the smaller particles, so that a higher amount of smaller particles is available in the maternal circulation and can reach the utero-placental area. Thus, these results suggest that the average concentration of NPs in maternal blood also affects the possibility of blood-placental barrier crossing, and might contribute to toxicity.

The hypothesis that other factors, beside size, might have a more relevant role, has been demonstrated by Yoshida *et al.*, who compared the effect of different size carbon black (CB) NPs (14, 56 and 95 nm) on the male reproductive system, and observed similar toxicity of 14 and 56 nm particles [16]. However, when treating male mice with a suspension of 14 nm particles with the same particle number per volume unit as the 56 nm suspension, no testicular damage or reduction in testosterone production where observed. These data suggest that total mass, and not particle size, is the key factor underlying the observed higher toxicity of low size NPs on the male reproductive apparatus. Nevertheless, further studies are needed to confirm this observation.

In vitro, size dependent toxicity of NPs has been less explored. Smaller particles appeared to be more toxic to embryonic systems

using a modified version of the Embryonic Stem Cell test (EST) [17], a test that allows classifying compounds as strong, weak or non-embryotoxic [18]. The presence of silica NPs of 10 and 30 nm in the culture medium strongly inhibited the differentiation of mouse embryonic stem cells (mES), which are derived from the inner cell mass of the peri-implantation embryo, the blastocyst, and represent a nice *in vitro* model to test for embryotoxicity. These cells can recapitulate *in vitro* the differentiation of the three embryonic germ layers and give contracting cardiomyocytes, that can be easily visualized under a microscope and are considered a feature of differentiation . Bigger silica particles of 80 and 400 nm had no effect on the differentiation of cardiomyocytes even at concentrations as high as 100 μg/ml [17].

Comparison of the effect of different size silver NPs (between 10 and 80 nm) on the viability of spermatogonial stem cells *in vitro* also shows that only smaller particles greatly affect cell survival through the disruption of the Glial-cell-line Derived Neurotrophic Factor (GDNF) signalling cascade, probably due to their higher penetrance of the plasma membrane [19]. Altogether, these results support the hypothesis that smaller nanoparticles have a higher penetrance in cells and tissues.

THE IMPORTANCE OF DOSAGE

The amount of nanoparticles administered to biological systems represents one of the main parameters to control when studying the toxicity of nanoparticles, and a dosage labelled as toxic should fall into a reasonable interval of concentrations, as even poison-less substances can become toxic if abused. As an example, in an old study, the harmful effect of fullerene C60 has been demonstrated in cells of the midbrain of mouse embryos *in vitro*, and in embryos *in vivo* by maternal parenteral exposure to extremely high concentrations of the material (between 25 and 137 mg/kg) [20] corresponding, in the highest range, to an exposure of about 10 grams as a single bolus for a man weighing 70 kg, a clearly unrealistic event. Not surprisingly, quite different findings have been obtained with lower doses. In fact, fullerenes have been indicated as potent anti-oxidants, behaving as "radical sponges" [21], and treatment of streptozotocin-induced diabetic rats with hydrated fullerenes in drinking water has been shown to ameliorate reproductive disfuctions [22]. Similarly, pre-treatment of male rats with fullerenol ameliorated the nephro-testicular phenotype induced by doxorubicin [23]. On the other hand, fullerenes have been reported to induce embryo malformations and death in the zebrafish model [24]. Whether fullerenes behave as embryotoxic agents in mammals needs to be further investigated, as they might behave as embryotoxic agents at high plausible dosage (such as that possibly occurring in the event of accidental exposure), but have anti-oxidant properties at lower doses.

As a general assumption, by increasing the concentration, the adverse effect of most nanoparticles appears exacerbated, and several studies have demonstrated a dose-dependent toxic response to nanoparticles of different composition. It is important to stress that for some NPs embryotoxic effects have been reported using dosages that might be expected for humans [25]. A concentration dependent toxicity of Multi-Walled Carbon Nanotubes (MWCNTs) has been demonstrated *in vitro* in embryonic stem cells [26]. Zhu and colleagues show that culturing the cells in the presence of nanotubes induced phosphorilation of p53, a marker of DNA damage, and such effect proportionally increased in an exposure range from 5 to 100 μg/ml. A direct dose-dependent effect of different types of nanoparticles on mouse spermatogonial stem cells has been also described [27]. These cells represent the stem reservoir of the male germ line and their proliferation and differentiation ability accounts for the progression of a normal spermatogenesis, so that any perturbation of such processes might harm fertility.

A concentration dependent role of Single-Wall Carbon Nanotubes (SWCNTs) in the development of the mouse embryo has been recently reported [25]. Early loss of embryos was observed when pregnant females received 30 μg/mouse. Such effect was less prominent by decreasing the concentration of the nanotubes, and disappeared below 3 μg/mouse; a similar behaviour was observed with respect to the presence of fetal malformations, the highest percentage being observed at 3 μg/mouse, with no malformed fetuses detected below 0.1 μg/mouse. A dose-related embryotoxic effect of SWCNTs has been further confirmed using the EST (Fig. **3**) [25].

Focusing on the dosage, evaluation of reproductive and developmental toxicity exerted by one time or repeated exposures to nanomaterials should also be considered, as accumulation in cells and tissues occur. Such aspect is important not only from an occupational point of view, but also in light of exploiting nanoparticles for therapeutic purposes, when repetitive administration of a medication is often required.

Repeated administration of fluorescent magnetic NPs to mice by nose-only exposure showed accumulation in various organs, with liver and testis showing the higher amount [28]. Within the testis, nanoparticles appeared localized mainly in the intertubular compartment, where they might interact with Leydig cells, which are devoted to the production of testosterone; however, in this study interference of nanoparticles with fertility was not investigated. In a similar approach, repeated intra venous administration of 5 mg/kg of multi walled carbon nanotubes to male mice, over a period of 13 days, caused a more marked damage in the testes than a single injection. In these experiments alterations were generally repaired after 60 days [29]. However, whether a more prolonged exposure may have led to irreversible damage was not investigated. Very recently, increasing doses of similar particles (40, 200 and 1000 mg/kg/day) were administered every day for 13 days to pregnant rats (starting from day 6 of gestation), and their ability to interfere with fetal development studied [30]. No significant changes between control and three groups of treated females in terms of number of corpora lutea, implantations, pre and post-implantation loss rates, fetal death, litter size, gender ratio of live fetuses, fetal body weight, and placental weight were observed. In the experimental procedures, particles were administered by gavage, but occurred adsorption was not assessed, making unclear whether particles actually crossed the intestinal epithelial layer. Further studies assessing intestinal adsorption of MWCNTs are needed, since there are indications that if in contact with fetal tissues, MWCNTs might be toxic [26].

Indeed, indication that repeated exposure to some nanoparticles may exert negative effects on pregnancy has been recently pointed by a study on maternal administration of cadmium oxide nanoparticles [31]. Treatment of female mice every day with 230 $\mu g/m^3$ of CdO NPs (between 4.5 and 16.5 days of gestation) showed a significant decrease in the incidence of pregnancy, together with a delay in maternal weight gain, alteration in placental weight and a decrease in fetal length. Such effects were not observed when mice received 100 $\mu g/m^3$ of NPs every other day, strongly indicating the importance of dosage.

Very recently biodistribution of Silver nanoparticles in maternal organs and their effects on early embryonic development after repeated administration has been studied [32]. After intravenous administration of relatively high doses for three consecutive days during early organogenesis (7-9 days post coitum; total dosage 35 and 66 μg/mouse), Ag NPs distributed in most maternal organs, extra-embryonic tissues and embryo. No evident embryonic malformations were induced by the treatment, although the analysis was performed one day after the last injection only. Whether analysis at later stages (after the completion of organogenesis) would have revealed abnormalities remain unanswered.

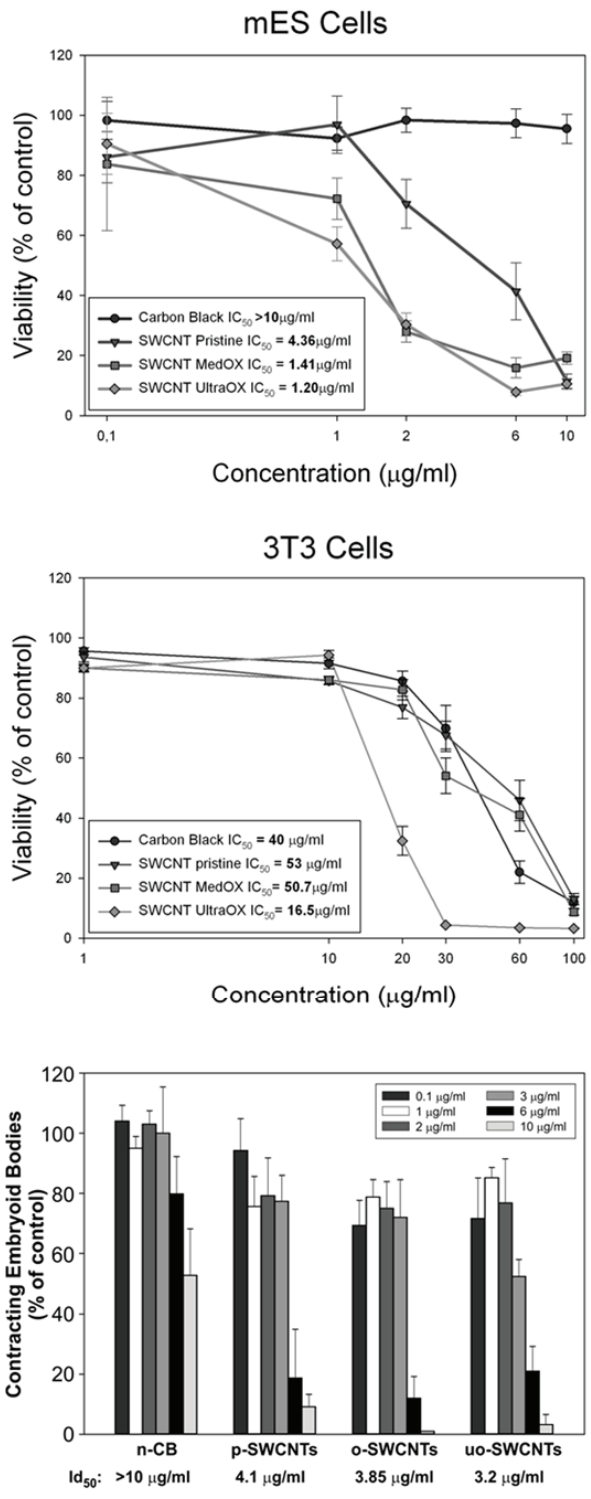

Fig. (3). Concentration-dependent effect of SWCNTs in the Embryonic Stem cell Test. Viability of mES (A) and NIH3T3 (B) cells in the presence of increasing concentrations of SWCNTs and CB used as control nanosized carbonaceous material, measured using the WST-1 assay. The calculated IC_{50} values are reported in boxes at the lower left corner. The ability of SWCNTs to impair mES differentiation into contracting EBs was evaluated by direct visualization of beating areas under a light microscope. (C) The calculated ID_{50} for each material is reported at the bottom of the graph. Values are means ± standard error of at least 5 independent experiments. (Reprinted with permission from Pietroiusti *et al.*, ACS Nano 2011)

Further studies on repeated administration of low doses of nanoparticles are needed in order to better mimic possible occupational and environmental exposure of humans. This might represent a hot topic for non-bio-degradable, bio-persistent nanomaterials, whose permanence in gonadal tissues might go way beyond exposure, potentially influencing reproductive ability even years later.

TOXICITY AS A FUNCTION OF CHEMICAL COMPOSITION

Several studies report on reproductive and developmental toxicity comparing the effect of nanoparticles with different chemistry, showing different degrees of toxicity probably depending on chemical composition. Most of the *in vivo* studies comparing the embryotoxic effect of different nanoparticles have been carried using the zebrafish model, due to several advantageous characteristics compared to Mammals (e.g., rapid development outside the mother and short life cycle) [9, 33, 34]. However, translation of results from fish to humans is not always applicable, therefore results obtained mainly in mammals will be reported.

The possibility of using nanoparticles to tag cells of the mouse embryo has been investigated *ex vivo*, comparing polystyrene and polyacrylonitrile particles (particle size ranging from 40 to 120 nm) [35]. When applied either externally or injected into the cytoplasm of a one-cell stage embryo, both types of materials impaired preimplantation development and inhibited hatching, although polystyrene displayed a lower degree of toxicity. *In vitro*, the effect of aluminium, silver and molybdenum trioxide nanoparticles was compared using spermatogonial stem cells [27]. Exposure of a spermatogonial stem cell line to the three different nanoparticles was evaluated in terms of metabolic activity, membrane integrity and apoptotic rate. Silver NPs appeared to be the most toxic, while molybdenum NPs were the least toxic. It should be however noticed that silver NPs were the smallest NPs used (15 nm in diameter against 30 nm). This might in part contribute to the higher toxicity observed, as already discussed above. The effect of NPs with different chemistry has been also studied on a Leydig cell line [36]. TiO_2 and CB were administered to the Leydig cultures and cellular uptake, inhibition of cell survival and proliferation and modulation of gene expression analyzed. TiO_2 showed a more marked inhibition of cell viability and proliferation than CB, while only small differences were detected in gene expression experiments.

The embryotoxic potential of same size particles (12-17 nm) of different chemical composition (gold and cobalt ferrite) has been studied *in vitro* using the EST [37]. Through the evaluation of proliferation and differentiation parameters, hyaluronan coated gold and silanes coated cobalt ferrite (CoFe NPs) NPs were defined as weakly embryotoxic. Interestingly, coating CoFe NPs with gold completely abolished the embryotoxic effect, suggesting a higher biocompatibility of gold. Biocompatibility of gold NPs has been also reported in several *in vivo* studies on the zebrafish [9, 33, 38, 39], but no data on mammalian species are available. Recently, one report has shown a 25% inhibition of sperm motility after incubation of human semen with 9 nm gold NPs compared to a 5% reduction in the control [40], however only one semen sample was used in this study, so that no clear significance of the study can be speculated.

IMPLICATIONS OF SOLVENTS AND CONTAMINANTS

Although the solvent-dependent structure and the presence of contaminants in nanoparticle preparation plays a major role in determining toxicity, not many papers have directly addressed such topic, with respect to reproductive and developmental toxicity, especially in mammalian systems. Some information can be extrapolated from a study on the Japanese medaka embryo, in which the effect of three fullerene suspensions, differing for the type of

solvent used for their preparation, were compared [41]. Fullerene C60 suspensions were alternatively produced by toluene exchange (Tol/nC60), DMSO dissolving (DMSO/nC60) or stirring overtime (Aqu/nC60). Interestingly, Tol/nC60 induced the highest rate of mortality, and Aqu/nC60 had the lowest effect, while with respect to the induction of embryo malformations the highest effect was exerted by DMSO/nC60. Such differential behaviour was explained by the closely packed structure of Tol/nC60 and the possible presence of residual solvent in the DMSO/nC60 preparation. The importance of contaminants in raw preparations of SWCNTs has been also speculated in a study on the zebrafish, in which hatching delay of the embryos was observed in the presence of SWCNTs agglomerates that could not penetrate the chorion pore, thus indicating that most likely toxicity was induced by the cobalt and nickel contaminants used for nanoparticle synthesis [42].

FUNCTIONALIZATION, SURFACE CHARGE AND SURFACE COATING

The addition of chemical functional groups at the surface of nanoparticles is generally used to improve their biocompatibility. This has been demonstrated in zebrafish embryos [43, 44] and recently proven true also in mammalian species. In fact, the administration of 70 nm silica NPs to pregnant mouse females have been shown to induce *in utero* growth restriction (IUGR) and fetal resorptions [12]. However, when nanoparticle surface was modified with amino or carboxyl functional groups the negative effects on the fetuses was abolished, clearly indicating that surface modification of silica nanoparticles can prevent fetal toxicity. Nevertheless, decreased toxicity as a function of functionalization cannot be considered a general rule, as for example oxidation of SWCNTs exacerbates their embryotoxicity [25]. In addition, the type of functionalization is also an important parameter that can influences NP biocompatibility. With respect to the male reproductive apparatus, repeated administrations of amine- and carboxyl-functionalized MWCNTs of comparable size, shape and density of functional groups were demonstrated to similarly affect the integrity of the seminiferous epithelium, although amine-functionalized nanotubes produced slightly less severe testicular alterations [29]. Surface modification also affects the biodistribution of nanoparticles in extra-embryonic and embryonic tissues [45]. Carboxyl modified polystyrene nanoparticles have been shown to access embryonic tissues in a size dependent manner (20 versus 100 nm), but the size dependency was abolished if particles were modified with amino groups, as even 200 nm NH_2-polystyrene particles were able to translocate to the embryonic tissues. In an *ex vivo* perfused human placenta model, PEGylated gold NPs of 10-30 nm were shown not to cross the placental barrier [46], differently from what had been previously reported for non PEGylated ones [15]. These findings point towards the role of PEGylation in modifying the ability of gold NPs in crossing the placental barrier.

This observation is in line with the above mentioned data on the non-size-dependent ability of nanoparticles with different chemistry to cross biological barrier.

THE AGGREGATION/AGGLOMERATION STATUS

The aggregation status of nanoparticles appears pivotal in determining embryotoxicity when the zebrafish embryo is used as a model system. In fact, embryos in the zebrafish are protected by the chorion, an acellular envelope with nanosized pores of about 0.5-0.7 μm, which represents a barrier for agglomerates of nanoparticles exceeding the pore dimension [33, 42].

The role of agglomeration has been also shown in mammalian models, in a mesothelioma cell line, for which it has been demonstrated that well suspended carbon nanotubes appear less cytotoxic than agglomerated rope-like CNT [47]. Thus, size distribution in biological fluids might play a major role in eliciting the adverse effects; however, no indication on the role of agglomeration in mediating reproductive and developmental toxicity in mammalian species is so far available, suggesting that such aspect should be rapidly investigated.

NANOPARTICLE INTERACTION WITH BIOLOGICAL FLUIDS

Concerning the effect on the reproductive apparatus and on embryonic development of nanoparticle surface modifications following the interaction with proteins of biological fluids (i.e. the protein corona formation) no data have been so far published. Such data are, however, urgently needed, since the nanoparticle itself is hardly seen by the cell machinery, while it is the coated form, which mediates the biological effect. Elucidating this aspect might be of great help in designing ENMs with tailored properties, aimed, for example, to avoid crossing of the placental barrier. In addition, the performance of *in vitro* experiments in appropriate biological fluids might give better predictive value to the *in vivo* situation, thus increasing the reliability of these experiments

POTENTIAL PATHOGENETIC MECHANISMS

The possible mechanisms through which NPs exert their toxic effect have been widely explored in several *in vitro* and *in vivo* systems [48], suggesting that NPs might induce inflammation, apoptosis and oxidative stress. Whether and how these events are linked together remains to be clarified. With respect to nanoreprotoxicology, recently some indication on the potential role of oxidative stress has been reported [12, 25]. Non-functionalized silica nanoparticles have been demonstrated to induce oxidative stress and activate the coagulation cascade in the near term mouse placenta [12]. Similar results were obtained after administration of SWCNTs to pregnant mouse females early during pregnancy, with increased oxidative stress detected in both abnormal placentas and fetuses [25]. Alternative mechanisms of NPs toxicity might be linked to the possible interference and/or mimicking of cellular structures, as for example the cytoskeleton. Some NPs, in fact, have been shown to modulate the actin cytoskeleton [49] and to interfere with microtubule assembling [50]. This latter effect might be very important during germ cell differentiation and embryonic development. Crucial steps of these processes, like chromosome segregation and massive cell proliferation of the growing organism, require in fact the full integrity and function of the cytoskeleton. The relative weight of oxidative and non oxidative related mechanisms of damage needs to be systematically checked for individual NPs in nanoreprotoxicology.

CONCLUSIONS

Taken together all the above reported studies indicate that assessment of reproductive and developmental toxicity of engineered nanoparticles is at its start, and the knowledge of the physicochemical parameters is of major importance for a correct interpretation of experimental results. While for size and concentration some data are available, for other characteristics information are still sparse, indicating that nanoreprotoxicology needs more future studies, also in consideration of the lack of legislative measures aimed to control the exposure of pregnant women, as well as of women and men of childbearing age to ENMs. In particular, with regards to exposure during pregnancy it is important to consider that human placental development is a complex process in which structural modifications occur mainly during the first trimester, which is considered the most critical for the embryo. However during this period the placenta shows the highest barrier ability since three layers of cells separate the fetal circulation and the maternal blood, while in the following two trimesters the barrier reduces to a two cell layer,

which might allow higher permeability. In this respect, adverse effects of ENMs during pregnancy might also depend on the exposure window.

Other nanoparticles, commonly used in consumer products (such as ZnO, CeO_2 and Al_2O_3), for which adverse effects have been reported in several biological systems, should be investigated with respect to their ability to interfere with the reproductive functions and with embryonic development.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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