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Review Article

Perspectives of nanotechnology in male fertility and sperm function

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ABSTRACT

Recent advances in nanotechnology have tremendously expanded its possible applications in biomedicine. Although, the effects of nanoparticles (NPs) at cellular and tissue levels have not been fully understood, some of these biological effects might be employed in assisted reproduction to improve male fertility particularly by enhancing sperm cell quality either *in vivo* or *in vitro*. This review summarises the available literature regarding the potential applications of nanomaterials in farm animal reproduction, with a specific focus on the male gamete and on different strategies to improve breeding performances, transgenesis and targeted delivery of substances to a sperm cell. Antioxidant, antimicrobial properties and special surface binding ligand functionalization and their applications for sperm processing and cryopreservation have been reviewed. In addition, nanotoxicity and detrimental effects of NPs on sperm cells are also discussed due to the increasing concerns regarding the environmental impact of the expanding use of nanotechnologies on reproduction.

1. Introduction

Nanoparticles (NPs) are particles that are synthetized with extremely small size, at the nanometer scale, with flexible fabrication and high surface-area ratio. Nanoparticles can be made from a variety of materials including metals, polysaccharides, and proteins. Recent advances in nanotechnology have tremendously expanded its possible applications in several scientific branches including medicine. This is mainly attributed to the engineering of nanoparticles with various physical and chemical properties that make them more stable, soluble and more biologically effective compared to their corresponding unengineered homologues. In addition, NPs have been increasingly employed in the field of drug delivery for generating therapeutic formulations either for lipophilic or unstable hydrophilic substances. Systemic delivery of poorly water-soluble (hydrophobic) drugs remains in fact a major problem in clinical pharmacology. In the WHO (World Health Organization) Model List of Essential Medicines, about 25% of the drugs are considered poorly water-soluble [1] for which the application of nanotechnology could prove highly valuable.

Nanoparticles or nanoparticle-loaded drug formulations are currently being developed to increase drug efficiency through e.g.; 1) protecting against digestion and degradation in the gastrointestinal tract and thus maximizing intestinal absorption and increased oral bioavailability [2]; 2) prolongation of the half-life of drugs in circulation; 3) bypassing blood-tissue barriers and delivery to specific target tissues, or even at cellular level; 4) rapid onset and prolonged therapeutic action; and 5) reduced effective dose and side effects or toxicity. Several reviews of literature provide comprehensive overviews on the expanding applications of nanotechnology in the field of drug delivery [3–5].

However, a better knowledge of the effects of nano-compounds at cellular and tissue levels is necessary, and little information is available about their activity in the reproductive system. The capacity of nanocarriers to cross the hemato-testicular barrier [6] have been demonstrated raising concerns about their distribution and biocompatibility at systemic level and suggesting that *in vivo* effects may be the result of systemic alterations but also a consequence of direct effects at the testicular level.

This review will focus on the reported *in vitro* and *in vivo* effects of different types of nanoparticles, either beneficial or detrimental, on male fertility and sperm cell function in farm animals, keeping in mind that these effects strictly depend on the chemical nature of the compound (size, surface charge, coating etc.) but also on the biological system involved (animal species, tissue, cell variability). A special

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attention was given to the anti-oxidant properties of some nano-compounds and to the possibility to build nano-cargos, promising tools for improving transgenesis and targeted delivery in assisted reproductive techniques.

2. Effects of NPs as antioxidants on sperm functions

Recent advances in nanoparticle technologies resulted in the development of several NP formulations with potent antioxidant, anti-inflammatory, and antimicrobial properties [7–9]. This obviously opens great potentials in the area of improvement of reproductive functions either *in vitro* or *in vivo* [10].

Applications of NPs based on their antioxidative properties can be particularly valuable for sperm functions and male fertility. Semen cooling and cryopreservation are known to increase oxidative stress in spermatozoa leading to a significant reduction in their fertilization capacity [11]. We have recently tested the efficiency of cerium oxide (CeO_2) NPs, which are able to store oxygen and act as ROS (reactive oxygen species) scavengers, to protect ram sperm cell viability during cooling [12]. Supplementation of CeO_2 in semen extender improved motility parameters after 48 h and up to 96 h of incubation and increased sperm velocity of sperm cells. This was associated with protecting the integrity of plasma membranes and DNA of spermatozoa [12]. These effects could not be linked with any reduction in intracellular ROS as detected by H2DCFDA staining [12], however, the sensitivity of this cytoplasmic stain may not be sufficient to detect minor differences in sperm cells due to the cellular structure of the sperm head which is mostly occupied by a nucleus. These beneficial effects on sperm preservation by cooling can be very useful to increase productivity of small sheep farms where routine artificial insemination is done using liquid cooled semen prepared in house.

Nano-selenium (SeNPs) has also been used in several studies as a scavenger of ROS to protect against oxidative damage in sperm cells. Addition of Nano-Se to semen extender improved the post-thawing quality and oxidative variables of rooster semen [13]. In addition, oral supplementation of SeNPs also protected the quality of spermatozoa (motility, DNA integrity) and spermatogenesis against oxidative damage induced by Cisplatin, an anticancer agent with male reproductive toxicant properties [14]. When supplemented in the diet of bucks, SeNPs proved to be more effective in increasing antioxidant enzyme activity (GSH-Px, SOD, CAT) and sperm quality when compared with other selenium forms (sodium selenite or selenized yeast) [15,16].

Nano-zinc is also among the metal NPs that were efficiently used to improve semen quality. In young rams, supplementation of the diet with 50 mg/kg or 100 mg/kg ZnNPs in diet improved epididymal semen quality, seminal plasma anti-oxidase activities and the expression of copper-zinc superoxide dismutase (Cu-Zn SOD) [17]. Similarly, supplementation of bull semen extender with zinc nano-complex decreased the levels of malondialdehyde (MDA) and improved mitochondrial activity [18]. Moreover, Zn nano-complex improved the functionality of sperm plasma membranes in a dose dependent manner without any deleterious effect on motility parameters [19]. In streptozotocin-induced diabetic rats, ZnO NPs have been shown to enhance the activity of antioxidant enzymes in the testicular tissue, to increase sperm count and improve sperm characteristics by protecting against oxidative stress [20].

3. Nanotechnologies for sperm purification and sorting in farm animals

Modern breeding systems require, nowadays, the diffusion of superior genotypes and thus the selection of high quality gametes and embryos. In male reproduction, many factors such as routine procedures of handling and storage, might affect negatively the quality of spermatozoa, and several strategies have been designed to overcome these limitations in order to achieve high *in vivo* and *in vitro* fertility rates [21,22]. Among others, sperm purification and sorting represent a valid tool for the selection of sub-populations of the best spermatozoa (in terms of motility and morphology) within an ejaculate and the discard of defective cells, contaminations and debris. These techniques also allow to recover spermatozoa from sub-optimal ejaculates from animals of high genetic values. In literature, several techniques have been developed to achieve this purpose and have been tested in farm animals: among others, swim up assay [23–25]; density gradient centrifugation [23,25–27], filtration through columns [28–32] and single layer centrifugation (SLC) [33–35]. Although these procedures greatly improve motility and functional parameters especially in post-thaw semen, on the other side they lead to a reduction in the concentration of the insemination doses due to their variable recovery rates, they are time consuming and add costs and labour to the standard procedures of semen handling and storage [36].

A novel approach for semen selection is represented by the development of techniques that target specific negative biomarkers of sperm quality, such as for example, defects in the acrosome and cytoplasm membrane or the presence of ubiquitin on sperm surfaces [as reviewed by [37]. Magnetic beads coated with probes that specifically bind to these biomarkers (peanut and pisum sativum agglutinin (PNA/PSA) lectins, annexin V, anti-ubiquitin antibodies) have been successfully used both in humans [38] and in bulls [39] for removing defective spermatozoa by exposure to magnetic field (MACS, magnetic activated cells sorting).

In this context, nanoparticles, with their peculiar characteristics of tuneable size, surface charge and binding properties, have recently contributed to optimise MACS protocols. In particular, Fe_3O_4 NPs, known for their magnetization, bio-compatibility and bio-functionalisation properties [40], have been conjugated either with PNA/PSA lectin, to selectively bind to glycans expressed on reacted acrosomes, or with anti-ubiquitin antibodies. With the specific binding to defective spermatozoa, they have been employed to nanopurify frozen-thawed bull spermatozoa, resulting in an increase in conception rates following artificial insemination [41]. Similarly, Fe_3O_4 NPs coated with PNA/PSA lectins were used for nanopurification of boar semen, efficiently selecting a subpopulation of highly motile and viable spermatozoa [42]. Interestingly, the authors of these studies reported that the procedure of sperm nanopurification did not require extensive semen manipulation, labour and additional costs, as it takes less than 1 h to prepare a purified semen sample without expensive equipment [36]. Removal of dead spermatozoa is indeed of critical importance as it negatively affects the motion characteristics and membrane integrity of live spermatozoa [43,44]. These encouraging results suggest that nanotechnologies could be easily included in the routine procedures of selection of high quality sperm population to enhance fertility rates in farm animals.

Another promising application of nanotechnologies was suggested by Farini et al. [45] for bovine sperm selection using the Cell-SELEX (systematic evolution of ligands by exponential enrichment) technique. In this method, avidin-coated superparamagnetic Fe_3O_4 NPs (SPION) selectively bound to synthetic DNA aptamers specific for spermatozoa with damaged membranes. The authors reported that the removal of damaged spermatozoa with the aptamer/SPION system significantly improved semen quality, in terms of viable cells, and did not affect *in vitro* embryo development [45]. Those magnetic NPs have been previously shown not to affect sperm motility or acrosome reaction [46].

NPs have also been recently suggested as valid alternatives to fluorochromes and flow cytometry for sperm sorting, and targeting specific sperm DNA sequences. The use of NPs as DNA tags has been investigated with encouraging results by Barchanski et al. [47], proposing bio-conjugated AuNPs as tools for genetic labelling in bull sperm. Recently, in the bovine, Gamrad et al. reported successful labelling of specific sites of the Y chromosome by oligonucleotide functionalised AuNPs [48]. In future, the development of specific nanoprobes will provide precious tools to sex sort sperm cells in routine reproductive management of farm animals, replacing flow cytometry and its limitations [49–51].

4. Sperm transport of NPs functionalised substances

In the past years, nanotechnologies have been gaining increasing interest in the development of nanoplatforms for delivery of biological compounds to specific targets within tissues or cells. These applications would provide great advantages in the progress of assisted reproductive technologies in farm animals and the potential of these nanocarriers interacting with gametes is yet to be fully understood, opening new perspectives but also new controversies. The majority of the reports on transport of nanocargos and reproduction focused on the spermatozoa for the peculiar characteristics of this cell: motility, scarce intracellular uptake, great capacity to interact through its membranes with several compounds. These properties, combined with the great plasticity, targeted action, binding attitude and small size of NPs have been increasingly investigated in the past decades to achieve successful loading of sperm cells with nucleic acids or proteins. Makhluf et al. in a series of experiments described the spontaneous loading of polyvinyl alcohol (PVA) -Fe_3O_4 and PVA-Eu_2O_3NPs by bovine sperm cells, without detrimental effects on motility and acrosome integrity [46]. Following these observations the same research group designed PVA-Fe_3O_4 NPs conjugated with an antibody raised against protein kinase C. They further described up-take of the antibody/NPs cargo by sperm cells (mainly in the post-acrosomal and upper regions of the head) and functional activity of the antibody in binding its specific antigen. These results suggested a strong ability of nanocarriers to deliver functional cargos into spermatozoa representing a promising tool for protein identification in target tissues or cells [52]. Similarly, in boar spermatozoa, mesoporous silica NPs (MSNPs) loaded with either a fluorescent nucleic acid (lamin siRNA) or a fluorescent protein (mCherry) interacted with cell membranes without compromising sperm function, indicating these NPs as potential candidates of biological cargos for intragamete delivery [10]. These encouraging results pave the way for important applications in the field of animal reproduction. Loading sperms with specific nucleic or protein markers may help understanding a number of mechanisms that regulate physiological processes such as fertilization and embryo development. NPs might also be conjugated with target nutrients or treatments for direct supplementation to sperm cells. Moreover, the use of nanocarriers conjugated with specific labels of sperm quality, might help the investigation of the processes of oxidative stress and membrane damage during semen storage at low temperatures (refrigeration or cryopreservation) that greatly impair sperm function, especially in those species in which handling and storing semen is sub-optimal (for example ovine, endangered species, etc) [11].

Mesoporous silica NPs (MSNPs), functionalised with aminopropyltriethoxysilane, polyethileneimine and optionally loaded with two common types of cargo (nucleic acid/protein) were designed to form strong associations with boar sperm without negatively affecting sperm functions such as sperm viability, acrosomal reaction and DNA fragmentation [10,53]. Together with the high affinity of these MSNPs to sperm cells [53], they are suggested to serve as promising nanomaterial to develop or increase the efficiency of existing diagnostics and therapeutic compounds related to sperm quality and male fertility.

5. Treatment of genital infections

In literature, several reports described the antibacterial, antiviral and antifungal properties of metal NPs or dendrimers [54–56]. These characteristics derived either by their actions of lysis on the microbial cell walls, inhibition of viral adhesiveness and penetration into cells, pro-oxidant action with consequent induction of apoptosis and death of the microbial cell [57] or by their drug delivery capacity, enhancing the target release of specific antimicrobial substances [58]. In the field of human reproduction, nanotechnology has been applied for potential treatment of a variety of genital infections raised by different pathogens: among others *Clamydia trachomatis* [59], *Candida albicans* [60], vaginal *Herpes-virus* [61]. On the other hand, a very limited number of reports on this topic has been published on farm animals. Recently, the antimicrobial effects of AgNPs have been tested against Gram negative *Prevotella Melaninogenica* and Gram + *Arcanobacterium pyogenes,* two of the multiple drug resistant and most frequently isolated pathogens responsible for bovine clinical endometritis. The results showed that NPs were able to induce metabolic disturbance increasing intracellular production of free radicals, and decrease in biofilm activity [62], representing a valid alternative to traditional antibiotic treatments. No trials to counteract male genital tract infections in farm animals using NPs have been reported. A great deal of information still need to be collected in order to develop therapies *in vivo* and to introduce nanotechnologies in clinical practice and in farming routine. However, this novel approach to contrast infectious diseases in farm animals will provide great health and economic benefits, leading to targeted therapies and contrasting drug resistance.

6. Other recent applications of NPs in semen preparations

An assay using heparin AuNPs (HAuNPs) has been developed as a biomarker for human fertility [63]. This assay utilize the polycationic properties of protamine, the most abundant nuclear protein in the sperm. The electrostatic interactions between polycationic protamine and polyanionic heparins result in visibly observable color change that can be detected by naked eye [63]. Development and optimization of similar "lab-free" tests can be used in the future as animal-side tests to assist in the selection of males for breeding in pets, rabbits and farm animals.

7. Controversies in the use of nanotechnology in reproduction: Environmental impact of NPs dispersion and reprotoxicity

Nanoparticles, particularly the metal oxide NPs, are increasingly used in many fields of every-day life e.g. in cosmetics, electronics and food packaging. Therefore, there are environmental concerns about the dispersion of these NPs and their biological effects on living organisms. Controversial reports about the reprotoxic effects of NPs has been addressed in several recent articles and particular attention has been given to the specific effects on male gametes.

Zinc oxide NPs were shown to exert a cytotoxic action on murine testicular germ cells in a dose dependent manner [64]. Xu et al. [65] found that SNPs primarily affect the maturation process of spermatozoa in the epididymis that turned out to be more sensitive to NPs toxicity compared to both spermatogonia and spermatocytes. ZnO NPs lead to the reversible damage of epididymal sperm without affecting fertility. The NPs have detrimental effects on the quantity and quality of sperm in the epididymis by causing oxidative stress and by damaging the structure of mitochondria, resulting in energy metabolism dysfunction [65].

Silver NPs are a potential cytotoxic agent for sperm cells and exert adverse effects, possibly through the induction of oxidative stress. Furthermore, exposure of murine sperm cells to AgNPs reduces the success rate of *in vitro* fertilization, delays subsequent blastocyst formation, and downregulates gene expression responsible for embryonic development [66]. Lafuente et al. [67] showed that in rats, exposure to oral sub-chronic doses of 50–200 mg/kg/day PVP-AgNPs possesses slight negative influence on sperm morphology.

Hong et al. [68] reported that dose-dependent exposure to TiO_2NPs caused testicular toxicity, reduced sperm production, and induced sperm lesions in mouse testes, which were closely related to reductions of daily food and water intake, biochemical dysfunctions and oxidative stress.

Gold and AgNPs are also widly used metal NPs. Adminstration of AuNPs for 35 days in mice resulted in a significant reduction in sperm motility and normal morphology, as well as sperm chromatin remodeling, lower chromatin stability and increased DNA damage [69]. Similar effects could be detected in epidydimal sperms in adult rats after exposure to AgNPs [67], even if the exposure occurs during the prepubertal stage [70]. These effects are also evident in bull sperms [71] and are likely to be due to direct interaction with the sperm cells; since in bulls, AuNPs were shown to impair sperm functions and reduce sperm fertilizing ability, solely by interaction with the sperm surface membrane, and without cellular penetration [72].

Similarly, administration of TiO_2NPs caused functional defects and DNA damage in male mice [73], and affected the fertility of male offsrpring after maternal exposure [74]. It was shown to exert cytotoxic and genetoxic damage in the testicular tissue in rats [75] and increased heat-shock protein 70 expression [76] suggesting high levels of cellular stress that may significantly affect subsequnt embryo development if these spermatozoa are used for fertilization. Titanium oxide was also toxic for buffalo sperm *in vitro* [77].

Other metal NPs such as dimercaptosuccinic acid (DMSA)-coated maghemite and CeO_2NPs did not influence ram or bull sperm structure or function when added *in vitro* [78,79]. Although CeO_2 NPs were shown to enhance ram semen preservation by chilling [12], it may also have detrimental effects if orally supplemented, since in mice, induction of oxidative stress and lower sperm characteristics were reported [80].

8. Other limitations for practical applications of NPs

Major limitations for practical applications of NPs *in vivo* include overcoming the biological barriers to drug delivery, avoiding nonspecific distribution and targeting, as well as minimizing opsonization and clearance by the immune system after oral or intravenous administration [81]. Recently, several strategies have been developed to create multifunctional NPs with incorporation of active targeting moieties to enhance uptake in specific cells [82]. Functionalization with protein corona and PEGylation, modification of surface charge, and attachment of specific ligands for target tissue, among other strategies have been successfully developed to avoid the above mentioned obstacles and enhance targeting [81]. This has not been widely used in treatment of male infertility, especially in farm animals. However, there are indications that this approach can be successfully used to enhance testicular functions; SOD-loaded biodegradable NPs targeted to *in vitro*-cultured Sertoli cells using FSH-hormone peptide have been developed and reported to have higher protective effects against oxidative stress [83]. Understanding of the mechanisms by which these NPs are taken up by the target cells and their intracellular fate is of primary importance to optimize their intended functions [84]. Further research is still needed before such technology can be translated into effective products for *in vivo* use.

9. Conclusions

The above mentioned reports show evidence that different types of NPs have different effects on sperm cell functions either upon direct exposure under *in vitro* conditions or if administered *in vivo*. The antioxidant properties of some NPs are among the most promising applications to protect sperm cell functions during cryopreservation. Other surface ligand functionalizations were proven efficient in sperm cell purification and selection. Fewer reports considered utilizing the antimicrobial properties of NPs on sperm preparations or treatment of genital infections. Nevertheless, toxic effects of NPs on male reproductive performance are evident, which increase the environmental concerns about the dispersion of these NPs and their biological effects on the fertility in both humans and animals.

Competing interests

The authors have no conflict of interest to declare.

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