Development of nanoparticle-mediated delivery tools to investigate the role of molecular genetic mechanisms underlying male infertility

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Received 29 May 2015; Accepted 06 Jul 2015; Available Online 06 Jul 2015

Abstract

In 15 – 25% of human male infertility, there is no known cause. Such idiopathic, or unexplained, cases clearly represent a major problem for diagnosis/treatment. However, mounting evidence indicates that impairment of testicular gene expression may play a crucial role. To help elucidate the molecular mechanisms underlying idiopathic male infertility there is a need to manipulate gene function in the testis and sperm. Sperm-mediated gene transfer (SMGT), testis-mediated gene transfer (TMGT), *in vivo* gene transfer (IVTG), and viral-mediated gene transfer were all developed to achieve these aims, but remain limited by inefficient transgene delivery and expression. There is an urgency, therefore, to devise new methods to engineer and manipulate sperm. Nanoparticles are a promising candidate with clear potential to enhance techniques such as conventional SMGT. By delivering a variety of biological compounds into sperm, nanoparticles could provide a powerful means of investigating the molecular pathways that might underlie idiopathic infertility.

Keywords: Male infertility; Sperm-mediated gene transfer; Nanoparticle

1. Introduction

Male factor infertility is a complex disorder, affecting approximately 10-15% of couples worldwide, however many of its aetiologies are unknown [1,2]. With the advent of assisted reproductive technology (ART), men with sub-optimal sperm quality are now able to overcome natural selection mechanisms and produce viable offspring [3]. Since ART is a relatively new phenomenon, the impact of inheritance and the frequency of mutations or altered gene expression is not yet fully understood, and there are concerns that ART may conceal reproductive defects that could have negative consequences at the epigenetic level [4-6]. Comprehending the genetic foundation of male infertility has large implications for both understanding the underlying cause of infertility as well as determining the prognosis for patients. Mounting evidence from investigations into the specific mechanisms underlying reproduction suggests an association between abnormal gene expression/genetic polymorphisms and various forms of previously unexplained states of infertility, such as gonadal insufficiency [7] and fertilization failure [8]. Consequently, efficient targeted gene transfer into gametes, embryos, and reproductive tissues would be a powerful tool with which to manipulate and study specific mechanisms underlying infertility. With the increased prevalence of ART treatments worldwide, it is necessary to develop efficient tools for research into idiopathic infertility. Elucidating the underlying genetic causes of infertility will aid in discovering their origin and determine effective treatments for patients. In this review, we discuss important aspects of genetic male infertility, describe research advances in the development of engineered sperm constructs as research tools and to combat genetic abnormalities, as well as highlight the promising development of mesoporous silica nanoparticles as a powerful non-invasive tool to engineer sperm.

2. Testicular gene problems contributing to male infertility

Male infertility is a complex and multifactorial disorder, with genetic abnormalities responsible for 15 - 30% of cases, and 15 - 25% of cases remaining idiopathic in nature [9-11]. Large-scale molecular investigations suggest that over 3000 genes are associated with male reproduction, and it is likely that most cases of idiopathic infertility could be accounted for by underlying genetic causes [12,13]. The most commonly known causes of genetic male infertility include chromosomal abnormalities, single gene point mutations, polygenic genetic defects, Y chromosome deletion or micro deletion, and genetic endocrine disorders (Table 1) [14].

Genetic and molecular defects leading to infertility disrupt physiological processes such as hormonal homeostasis and thus, spermatogenesis and sperm quality [7,15]. Spermatogenesis is a complex process regulated by many genes, and the molecular mechanisms involved are beginning to be discovered and better understood. The formation of spermatozoa is a sequential process controlled by an intricate genetic system governing phases of mitotic, meiotic and post-meiotic differentiation. The complex series of events in spermatogenesis are vulnerable to the accumulation of errors that can severely affect spermatozoa production [6]. Although the majority of children conceived through ART appear normal, it is unknown whether the artificial procedures deployed in this technology may transmit molecular genetic abnormalities to the offspring. Consequently, it is imperative to evaluate male infertility and investigate the underlying causes utilizing a multidisciplinary approach to develop appropriate tests for abnormal phenotypes and effective treatments. It is therefore of the utmost importance to understand the genetic basis of infertility in order to provide optimal therapeutic options for couples undergoing assisted reproduction.

Table 1. Commonly known genetic causes associated with male infertility and their prevalence in the overall population.

Genetic dysfunction	Range of physical manifestation	Overall prevalence	Reference
Chromosomal aberration	Azoospermia* to normozoospermia#	2-10%	[1]
Y chromosome deletions/microdeletions	Azoospermia to severe oligospermia†	5-10%	[9,98]
AZFa	Azoospermia to Sertoli cell only syndrome	0.5-1%	[99]
AZFb	Azoospermia to severe oligospermia	0.5-1%	[99]
AZFc	Azoospermia to severe oligospermia	0.5-1%	[99]
Klinefelter's syndrome	Azoospermia to severe oligospermia	5-10%	[1]
Robertsonian translocations	Azoospermia to severe oligospermia	0.5-1%	[3]
Reciprocal translations	Azoospermia to severe oligospermia	0.5-1%	[100]
Androgen receptor gene	Azoospermia to oligospermia	2-3%	[17]
CFTR gene	Obstructive azoospermia	4-5%	[3]
Kallman's syndrome	Hypogonadotropic hypogonadism	5%	[15]

* no measurable level of sperm in the semen
# normal sperm
† semen with a low concentration of sperm

2.1. Gene mutations

Chromosomal abnormalities and genetic defects in sex or autosomal chromosomes are common in infertile men [3]. The severity of the phenotype depends on which chromosomes are affected. Genetic analysis of infertile male patients has shown that a large proportion of male infertility is linked to disruptions in gene expression, such as mutations in the genes encoding azoospermia factor (AZF), androgen receptor, sex hormone-binding globulin (SHBG), and transcription initiation factor (TAF7L).

All men with discordant sex chromosomal patterns are azoospermic since the long arm of the Y chromosome containing the AZF gene is missing. The Y chromosome locus is divided into four sections, (AZFa, AZFb, AZFc, AZFd). These regions contain important genes for germ cell development, and gene deletions in these regions are pathogenically implicated in azoospermia and severe oligozoospermia [16]. Androgens (testosterone and 5α-dihydrotestosterone) are critical steroid hormones in the development and maintenance of spermatogenesis. Mutations in the androgen receptor gene is an X-linked genetic condition with a frequency of 1:60,000 and is known to affect 2% of infertile men [17]. Mutations and polymorphisms in the androgen receptor gene, and its expressed protein, may also result in reduced spermatogenesis and idiopathic male infertility without any abnormal phenotype in secondary sexual characteristics [18].

SHBG is expressed in testicular germ cells and contributes to the concentration of androgens in the testis by binding or increasing the bioavailability of androgens [19]. SHBG accumulates between the outer acrosomal membrane and sperm plasma membrane and is being investigated by researchers with regard to its role in spermatogenesis and potential contribution to male infertility [20]. TAF7L (transcription initiation factor TFIID subunit7-like) plays an important role in transcription and is an X-linked single-copy testis-specific gene essential for maintaining spermatogenesis and thus represents a possible contributor to male infertility [21]. It is highly likely, however, that many cases of idiopathic male infertility may represent the result of multiple genetic defects. As a consequence, understanding the precise factors involved, and the development of potential novel treatments, is likely to be far more complex than originally perceived.

2.2. RNAs in sperm

The highly condensed sperm nucleus is transcriptionally inert but contains mRNA, antisense RNA and miRNAs that have been transcribed prior to inactivation. These diverse RNA populations are produced during spermiogenesis or early spermatogenesis, and evidence has shown that there are different RNA populations in mature sperm from infertile compared to fertile men [22]. Currently, there is significant debate over whether such RNA populations merely represent biological remnants of transcriptional processes occurring previously during spermatogenesis, or whether they play a functional role within the early embryo post-fertilization [23]. Despite their inert status, sperm RNAs are indeed delivered into the oocyte during gamete fusion and may therefore be important in development of the early embryo [13, 24, 25]. miRNAs regulate approximately one third of human genes by down-regulation through interactions with the 3' untranslated region, and are expressed in various testicular cell populations and the epididymis, which suggests a critical role in different stages of the highly organized processes of spermatogenesis, sperm maturation and morphogenesis [26]. Another specialized type of RNA known as piRNAs have been detected in round spermatids and pachytene spermatocytes during spermatogenesis. It is thought that piRNAs may regulate spermatogenesis and male infertility by protecting developing male germ cells from invasive transposable elements [27]. Development of methodology which would permit the in-depth spatial and temporal analysis of gene and RNA expression in sperm could be very useful in identifying genes that are involved in different stages of development and in the molecular pathways associated with infertility.

2.3. Proteomics in male infertility

Post-translational modifications of proteins during spermatogenesis, sperm maturation and capacitation are essential and can alter the functional properties of spermatozoa and seminal plasma proteins [28]. The identification of differentially expressed proteins, and changes in modification status, between fertile and infertile men could uncover new target proteins for investigation, and elucidate deleterious mutations in the encoding genes. For example, phospholipase C zeta (PLCζ) is a sperm-specific protein responsible for activating the oocyte upon gamete fusion [8]. Genetic

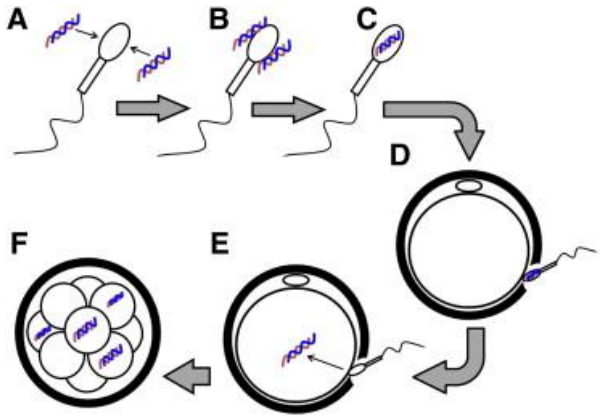

Figure 1. Sperm-mediated gene transfer to oocytes and embryos. Sperm spontaneously bind, internalise and incorporate an exogenous DNA target construct upon simple incubation *in vitro* (A-C). The target construct can then be delivered into oocytes at the time of fertilization (D, E) to create transgenic/mosaic embryos (F). (Reproduced with permission from [88]).

mutations resulting in the perturbation of this enzyme have been strongly linked to human infertility via oocyte activation deficiency or total fertilisation failure [29-31]. The identification of differences in gene expression, and thus protein translation, between normal and abnormal sperm populations could allow for the identification of novel biomarkers for prognostic, diagnostic, or therapeutic purposes in infertile men.

3. Development of systems to engineer sperm expression constructs

It has become very evident that there are clear differences between the sperm of fertile and infertile men in relation to gene expression, protein translation, and residual RNA population post-transcription. The potential effect of such differences upon spermatogenesis, fertility and embryogenesis, has led to an increased research focus on the precise functional implications involved. Adopting a comprehensive approach to examine novel genes may allow for a deeper understanding of the complex interactions between genetics and infertility and potentially uncover genes associated with infertility but without any known cause or role [7]. In order to achieve these aims, a series of laboratory methods have been explored over time, with varying levels of success.

3.1. Sperm-mediated gene transfer

Transgenic model organisms have revolutionized the study of gene function in whole organisms, and the pursuit of new methods to create transgenic animals was a driving force behind the development of sperm mediated gene transfer (SMGT). During SMGT, sperm spontaneously incorporates exogenous DNA and acts as a natural transporter to deliver a target construct into the oocyte at the time of fertilization (Figure 1) [32-34]. In 1989, Lavitrano and colleagues demonstrated for the first time that mouse epididymal sperm that was simply incubated with plasmid DNA could deliver this exogenous DNA into the oocyte and successfully produce transgenic offspring [33]. While there have been some reports of successful SMGT in chicken, mouse, xenopus, zebrafish, and mouse models, there is still controversy regarding the efficiency of this method and its mechanism of action [35]. It was discovered that mammalian seminal fluid contains inhibitory factor IF-1 which appears to block the binding of exogenous DNA to binding proteins on the surface of sperm, potentially explaining the inconsistent results regarding SMGT success, and the resistance of mammalian sperm to uptake exogenous DNA under normal physiological conditions [36,37].

Different methods to improve the efficiency of SMGT include the use of liposomes to enhance the passage of exogenous DNA across the sperm cell membrane, electroporation, and combination with intra-cytoplasmic sperm injection (ICSI) [38-41]. While SMGT, along with the different methods used to enhance its success, has proven to be a viable method for generating transgenic embryos in a variety of animal models, there are still formidable issues regarding its efficiency, repeatability, and our understanding of the underlying molecular basis of the procedure. Despite the numerous modifications made to increase the capacity of sperm to take up exogenous DNA, the efficiency rate of successful gene transfer remains low and varies greatly among species. Studies continue to report highly variable levels of transgene expression. Consequently, SMGT has yet to become an established and reliable method for gene transfer [42,43].

3.2. Testis-mediated gene transfer

Testis-mediated gene transfer (TMGT) is another method of introducing transgenes into sperm by genetically modifying spermatogenic cells in the testis, and can be used to investigate the role of specific genes in testicular and sperm function [44,45]. This method involves the *in vivo* transfer of genes, or engineered DNA constructs, directly into the testes in order to deliver target DNA into oocytes via the sperm itself [44]. Studies have shown that plasmid DNA injected directly into mouse testis remained in the testis for 7 days and could be detected in ejaculated sperm [46]. In another experiment, testicular injections of DNA plasmids that had been encapsulated in liposomes were utilized in male mice, and the mice subsequently used in breeding experiments. The delivered transgene was detectable in embryos at the blastocyst stage [47]. Injection into the rete testis, and retrograde filling of the seminiferous tubules with a solution containing the target transgene, has been reported as the optimum route for expression with minimal damage to the testis [48]. In general however, the efficiency of TMGT to achieve transgenesis appears to be low and transgene expression appears to be very transient [44]. Reports have also indicated that this approach was not successful in integrating transgenes into the sperm genome [49,50].

3.3. In vivo gene transfer

A variety of other methods have been further explored in order to improve the efficiency of TMGT to introduce transgenes directly into the testis, such as *in vivo* electroporation of testis [51-53]. This technique, which has the advantage of being quick and straightforward, involves the injection of a DNA expression construct into the testis, followed by the application of a series of electrical pulses to the testes to disrupt cell membranes thereby allowing the construct to enter cells (Figure 2) [44]. TMGT using electroporation has shown potential as a method for studying gene function in the testis and sperm by introducing reporter genes driven by germ cell-specific promoters [53-55], to induce specific gene knockdown using small hairpin RNA

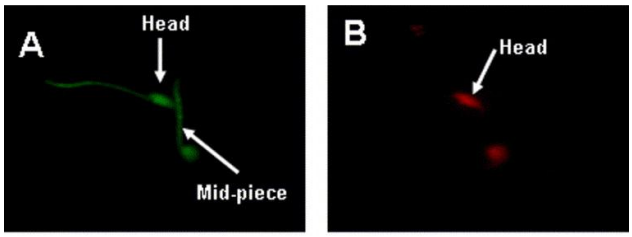

Figure 2. Early *in vivo* gene transfer (IVGT) methodology applied to mouse testis. Fluorescent images of epididymal sperm from mice subjected to *in vivo* gene transfer by electroporation and maintained for 40 days. (A) represents EYFP expression in the sperm head and midpiece. (B) represents a control with the sperm head stained. Original magnification ×400. (Adapted with permission from [57]).

[56], or to use tagged sperm proteins to study their localization patterns and mediating factors [57]. While electroporation was proved not to be detrimental to testicular integrity and sperm quality, the proportion of sperm expressing the delivered transgenes was still very low (5-10%), thus limiting its potential as an efficient approach to create transgenic organisms [52]. Moreover, there are concerns that the electrical pulses applied during electroporation could generate heat within the testicular tissues, a factor known to disrupt spermatogenesis, and also potentially induce apoptotic mechanisms [58].

3.4. Viral-mediated gene transfer

Viral-mediated gene transfer is being investigated as a more refined method for introducing transgenes into sperm or testicular cells. This is due to the inherent ability of a virus to specifically enter host cells in order to deliver their genetic information for replication [59]. Current viral gene transfer investigations tend to focus on adenoviruses, retroviruses, and lentiviruses. However, research findings have sometimes been contradictory and generally disappointing. Adenovirus-mediated gene transfer has shown successful expression in Sertoli and Leydig cells but not germ cells [60,61]. Retroviruses have been shown to deliver transgenes into spermatogonial stem cell *in vitro* [62,63], and to generate transgenic offspring following injection into the seminiferous tubules of mice [64]. Lentiviruses have been used to produce transgenic offspring by infection *in vitro* followed by subsequent transfer of infected cells into testes with the transgene transmitted into the F2 generation [65]. Injection of lentiviral vectors *in vivo* into the testis of hamsters [66], and into seminiferous tubules of mice [67], showed evidence of strong transgene expression in what appeared to be various types of male germ cells of different stages, however there was no evidence that this approach could lead to germline transmission.

A large concern about using viruses for human gene therapy is that viral vectors integrate their own DNA into the host genome. There have been reports of the successful use of viruses to incorporate exogenous genes, however endogenous proto-oncogenes were consequently activated in response to viral incorporation, resulting in the contraction of leukemia [68]. Viruses unfortunately pose significant problems as a vector for gene therapy because they can result in adverse effects upon the cells and tissues into which they are introduced. The use of viruses also pose concerns with regard to safety, since the integration of viral DNA may disrupt the expression of endogenous genes in the genome, or, if protective host mechanisms are employed to silence the integrated transgenes, this silencing effect could also reduce effectiveness and thus limit the efficiency of the technique [35]. Data regarding the safety of viral vectors for gene transfer remain highly contradictory [69,70]. While transgene delivery and expression appears to be rather efficient when using viral vectors, particularly those based upon lentiviruses, it appears that safety concerns unfortunately dominate the future of this approach.

3.5. Spermatogonial stem cell transfer

Spermatogonial stem cells (SSCs) are undifferentiated cells which can self-renew and differentiate into mature spermatozoa; they maintain spermatogenesis throughout the whole reproductive life in mammals [71]. SSCs have been involved in investigations for fertility restoration in patients who have undergone gonadotoxic treatments, such as chemo- and radiotherapy, and experiments using rodent models appear to be reasonably encouraging [72]. SSCs are further being investigated as a novel method to produce transgenic organisms [73,74]. Studies have reported the successful generation of transgenic mammalian embryos following the transfer of transfected SSCs [75,76]. One study using chicken embryos transplanted SSCs transfected with GFP into recipients and showed that GFP expression was subsequently detectable in these tissues. The authors further showed that male chickens produced sperm that carried GFP, and that transgenic offspring could be produced, although only 2 of the 18 embryos tested were transgenic [77]. SSCs have further been studied as a target for lentiviral infection or DNA injection into the testis using DNA containing a transgene downstream of an appropriate promoter [78]. Although SSCs clearly have potential as a successful tool with which to investigate male infertility, they remain difficult to study and manipulate due to their small number in the testis, and pose challenges with respect to identifying, culturing, and assaying their biological activity [79].

3.6. The need for new methodology

Targeted transgenesis is a crucial component of research in the reproductive sciences. Unfortunately, the laboratory methods developed thus far remain inefficient and costly in terms of equipment, time, and labour. There is a very real need to develop new approaches to engineer spermatozoa as efficient vectors for transgenesis. Safe and efficient gene transfer could be used to precisely manipulate gene expression in testes and sperm in order to investigate the role of specific proteins and molecular pathways during gametogenesis, fertilization and early embryogenesis. Establishing a reliable and efficient method of transgene delivery may enable the discovery of genetic causes and mechanisms underlying idiopathic infertility. Moreover, the availability of a technique devoid of the biological risks associated with traditional gene therapy could support research into reproductive gene transfer as a possible treatment for specific forms of infertility in the future [80]. Therefore, the development of an alternative and more efficient method than conventional gene transfer techniques is highly desirable.

4. Development of nanoparticle systems to engineer sperm

Biomedical nanotechnology has revolutionized existing approaches for research, diagnosis, and the treatment

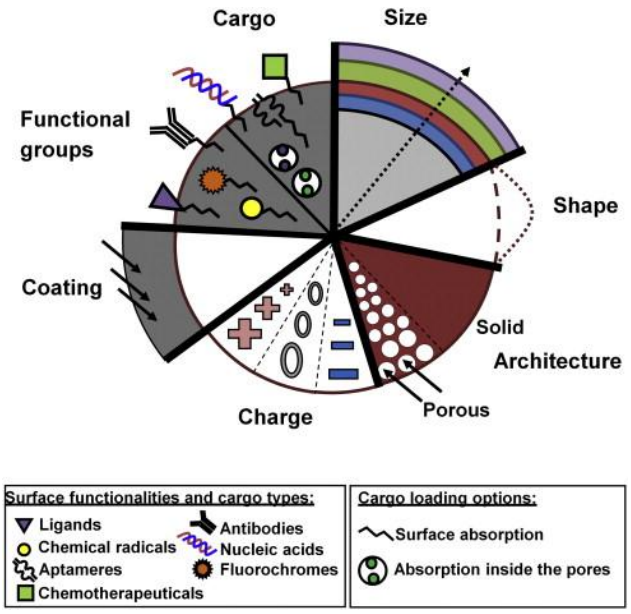

Figure 3. Nanomaterials are highly versatile research tools with adjustable physical and chemical properties. Changes in the size, shape, architecture, surface charge and coating allow researchers to manipulate interaction with cells. Surface groups (for example, ligands, chemical radicals, aptameres, and antibodies) can further act as targeting moieties and/or independent functional cargo. Nanoparticles can also be optionally loaded with biological cargo (chemotherapeutics, proteins, nucleic acids) (Reproduced with permission from [88]).

of various medical conditions, by fostering the development of sensitive tools and methods to investigate the fine pathophysiological mechanisms underlying such conditions. Targeted nanovectors for biological delivery can be tailored to facilitate the precise transport of large amounts of molecular cargo to specific cellular destinations (Figure 3), and have already been used to deliver compounds into sperm to assess effects upon molecular pathways [81,82], and to facilitate SMGT [67,83].

4.1. Nanoparticles to improve SMGT

A promising application of nanomaterials is to enhance the efficacy of SMGT. The overall reproducibility and efficiency of SMGT exhibits large variation, even when exogenous DNA has been successfully taken up by sperm [84,85]. Various studies report that the adsorption of exogenous DNA upon nanocarriers improves the efficacy of construct internalization into sperm for SMGT [67,83]. Kim et al reported that more than double the amount of plasmid DNA was taken up by boar sperm when the DNA was simultaneously exposed to magnetic iron nanoparticles, compared to exposed only to lipofectamine. When a magnetic field was applied, DNA uptake was increased by almost three fold. The construct was then successfully transferred via SMGT into oocytes with gene expression evident in the developing morula and blastocysts [67]. Campos et al reported that using halloysite clay nanotubes to deliver an EGFP construct into boar sperm resulted in a 4-fold increase in DNA uptake compared to lipofectamine, and a 5-fold increase compared to free plasmid DNA. Following successful SMGT into oocytes, EGFP could be detected by polymerase chain reaction (PCR) in the resultant embryo, although the embryos did not express EGFP [83].

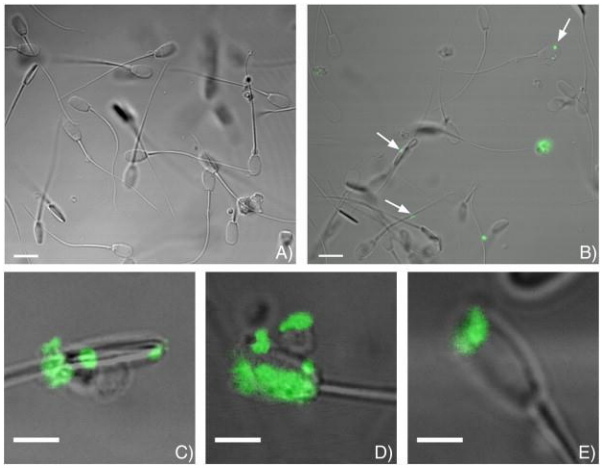

Figure 4. Association of naked (unloaded) MSNPs with boar sperm. (A) Control; (B) Association of naked MSNPs with sperm. Nanoparticles associated with boar sperm produced fluorescent signals in discrete regions of physiological interest (white arrows indicate association). Scalebar = 10 µm. (C-E) Association of unloaded MSNPs with the mid-piece and head of boar sperm. Scalebar = 5 µm. (Reproduced with permission from [93]).

The use of nanovectors and their spontaneous internalization into target cells conveys numerous benefits compared to conventional electro- and viral transfer. Gene transfer using viral vectors has shown the capacity to restore production of functional gametes in mouse models of genetic gonadal failure, however data concerning the safety of this methodology is highly contradictory [61,86,87]. Nanomaterials combine the main advantages of viral vectors, namely high specificity and non-invasiveness of delivery, while fully avoiding any viral integration into the host genome. Indeed the uptake of nanoparticles, and their biological cargo, occurs via normal endogenous cellular processes, such as endocytosis.

4.2. Nanoparticles to improve sperm-mediated cargo delivery

In theory, sperm could be used to deliver various forms of biological cargo into oocytes, in a manner similar to SMGT, and could be enhanced by coupling cargo with nanocarriers. Sperm-mediated nanoparticle delivery could transport cargo such as proteins, peptides, antibodies, fluorescent markers, or any agents designed to enhance, suppress or detect endogenous biological activity [88]. The development of such a powerful system could provide invaluable insight into the molecular pathways associated with fertilization and early embryo development.

Several studies have emerged indicating the use of nanoparticles to enhance sperm-mediated cargo delivery. Makhluf et al demonstrated the successful delivery of an antibody raised against protein kinase C into bovine sperm using polyvynylalcohol-coated magnetic iron oxide nanoparticles, without the antibody losing any functional activity. It has also been demonstrated that sperm loaded with nanoparticles do not lose the ability to undergo the acrosome reaction [81]. Barkalina et al [89] demonstrated that mesoporous silica nanoparticles (MSNPs), functionalized with polyethileneimine (PEI) and aminopropyltriethoxysilane (APTES), and optionally loaded with common types of nucleic acid or protein cargo, form strong associations with boar sperm following incubation *in vitro* (Figure 4). The rate of association between sperm and APTES-coated MSNPs loaded

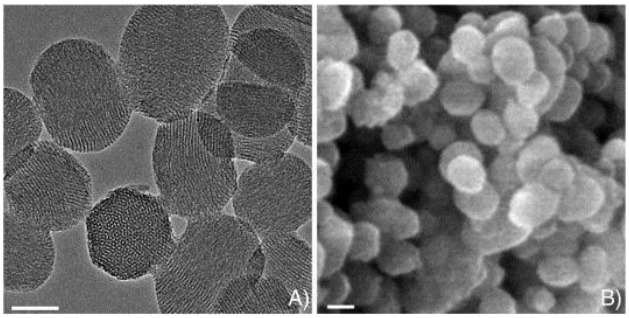

Figure 5. Mesoporous silica nanoparticles (MSNPs) used routinely in our laboratory. (A) and (B) represent unmodified MSNPs imaged by transmission and scanning electron microscopy, respectively. (Scalebar = 0.05 µm for A, and 0.1 µm for B). MSNPs exhibited homogenous size with slightly non-spherical shape and nanometre-sized pores with hexagonal symmetry. (Reproduced with permission from [93]).

with mCherry cargo was 25.3% in a typical boar semen sample [89]. This high level of association between MSNPs and sperm suggests the ability of nanoparticles to deliver cargo via sperm to a much more efficient degree than conventional SMGT, which only boasted maximum rates of just 5-10% [52]. Barkalina and colleagues further demonstrated that these associations do not exert any negative effects upon the main parameters of sperm function such as motility, viability, acrosomal status, and DNA fragmentation index [89].

A significant benefit of using nanovectors to assist in sperm cargo delivery is the ability to tailor the nanoparticles to specific objectives or scenarios. Using different functionalization strategies, nanoparticles can be customized to carry a specific type of cargo or to deliver cargo to specific target destinations, thus further increasing their efficiency. Another recent study by Barkalina et al. (2015) investigated the effects of actively targeting MSNPs towards mammalian sperm with a cell penetrating peptide (C105Y), and found that C105Y-functionalised MSNPs exhibited a 4-fold increase in affinity towards boar sperm in the early stages of incubation, while also preserving their biocompatibility [90]. After only one hour of incubation with 30µg MSNP per 10^7 sperm, 38.3% of sperm were shown to be associated with C105Y-functionalised MSNPs, whereas unmodified MSNPs showed an average association of 41% only after 2 hours of incubation. The novel features of C105Y-functionalised MSNPs could permit the reduction of MSNP-sperm exposure time *in vitro*, resulting in a better overall environment [90]. The promising high level of cargo association to sperm observed with MSNPs, and even further enhanced with functionalization of MSNPS, will hopefully work to improve the efficiency of transgene delivery and expression, compared to the low transgene expression observed with traditional SMGT and IVGT, and provides a rationale for the use of MSNPs for the transfer of investigative, diagnostic, and/or therapeutic compounds into mammalian sperm.

4.3. Mesoporous silica nanoparticles as candidates for sperm delivery

MSNPs are universally recognized as a powerful biomedical nanomaterial distinguished for their low cytotoxicity across a variety of cell types [91]. MSNPs are synthetically modified colloidal silica with highly ordered meso-scale sized pores (Figure 5) (2-50nm)[92], and exhibit many favourable characteristics for use as a targeted delivery vector for reproductive biology. MSNPs are robust with a high surface-area to volume ratio, possess an increased likelihood to persist in target cell populations following internalization, exhibit high loading capacity, and therefore require reduced doses of nanovector. Moreover, MSNPs and their degradation products are chemically inert and not prone to induce free radical formation. They are also versatile and easily customizable, allowing for specific loading and targeted delivery [93].

Mesoporous silica has been extensively studied in somatic cell types, and data is now emerging concerning its biocompatibility and potential with gametes and embryos. The use of non-toxic MSNPs for delivery into gametes is highly promising, compared to other types of nanoparticle, for example gold or silver, which are widely accepted for use in somatic cells but exhibit toxicity when applied to gametes [89,94]. Barkalina et al., reported that the exposure of mammalian sperm to MSNPs under conditions similar to those used during routine sperm processing for *in vitro* fertilization (IVF) resulted in the binding of MSNPs to sperm without any deleterious effects upon motility, viability, acrosome morphology, or levels of DNA fragmentation [89]. MSNPs have also been investigated as a delivery system in zebrafish embryos, a common and powerful model organism for reproductive biology investigations, with eminently positive reports of safety and efficacy [95].

The development of an efficient active targeting and internalization strategy for the transport of biological compounds into gametes could represent a milestone in enhancing the efficacy of current research methodologies associated with gene transfer. Mesoporous silica, with its inherent high loading capacity, low toxicity, and tunable affinity, appears to represent a very promising candidate for these advancements in reproductive biology.

5. Future perspectives for nanoparticle delivery systems

5.1. Optimising and improving research using animal models

The use of nanomaterials for reproductive biology has great potential for expansion in the coming years. An in-depth spatial and temporal analysis of gene expression would be very useful in order to determine the genes that are involved in specific stages of development and disease [7]. Animal models form the cornerstone of research, and the welfare of the animals used is of upmost importance for ethical, scientific, legal, and economic reasons. One particular advantage of nanoparticle-mediated delivery tools for molecular genetic investigations is the obvious improvement in which animal models are used for these investigations. The three main principles underlying the humane use of animals in scientific research are: 1) replace the animals with alternative techniques, or avoid the use of animals altogether, 2) reduce the number of animals used, to obtain information from fewer animals, and 3) refine the way experiments are carried out, to make sure animals suffer as little as possible [96]. The ability to engineer sperm *in vitro* using nanoparticle technology represents a most elegant refinement of conventional sperm-mediated gene transfer methods, and could eliminate the need for animal sacrifice, microsurgery, and invasive techniques such as electroporation. Without the need for animal surgery, not only is overall animal welfare improved, but a significant reduction in overall procedure costs will be highly evident to the agencies funding such research since analgesics,

anesthetics, surgery tools, and the housing/care of animals will be totally unnecessary. Nano-mediated delivery systems only require samples of the target semen, which can be readily obtained from certain animal models (e.g. the boar, or stallion) using established veterinary collection techniques. Robust nanoparticle platforms could therefore represent a significant milestone in reproductive research and lead to a revolution in the research methodologies used to transport biological compounds into gametes for investigative research purposes.

5.2. Clinical implications and options for gene therapy

A large proportion of infertile males are diagnosed as idiopathic ('unexplained'), which reflects our poor understanding of the underlying mechanisms regulating spermatogenesis and sperm function. Presently, the diagnosis of male infertility has been primarily based on physical examinations, blood tests, and traditional semen analysis using rudimentary criteria. However, mounting evidence indicates that conventional semen evaluation (which ordinarily investigates concentration, motility, volume, pH, and morphology) is highly insufficient and does not reflect the overall biological quality of the sperm [97]. Development of technologies at the proteomic and transcriptomic level are now allowing researchers to identify thousands of proteins and RNAs as biomarkers of fertility. This rapid expansion of research will help prepare clinicians to affirmably diagnose and treat infertile patients. Delivery vectors involving reproductive cells, such as gametes, and perhaps embryos, should undergo extensive nanotoxicological studies in order to ensure their safety and ability to preserve the viability and function of such specialized cells before clinical implementation. It is possible that in the future, with appropriate regulatory legislation, that nanoparticle-mediated delivery into gametes and embryos may one day be used clinically as a non-invasive method to supplement molecular deficiencies associated with aberrant gametogenesis, fertilization, and embryo development.

Acknowledgements

LMD and NB are supported by Clarendon Scholarships from the University of Oxford and by the Nuffield Department of Obstetrics and Gynaecology, University of Oxford. NB is additionally supported by Scatcherd European and Cyril & Phillis Long Scholarships. Some of the nanobiology work described herein was supported by an EPSRC Pathways to Impact grant awarded to KC. The authors would like to acknowledge the assistance of several colleagues who have made significant contributions to our nanodelivery project over the last few years, notably Celine Jones, Junaid Kashir, and Helen Townley.

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Cite this article as:

Lien M. Davidson *et al.*: **Development of nanoparticle-mediated delivery tools to investigate the role of molecular genetic mechanisms underlying male infertility.** Sci. Lett. J. 2016, **5**: 222