OXFORD UNIVERSITY PRESS | Human Reproduction Update

Extracellular vesicle-mediated delivery of molecular compounds into gametes and embryos: learning from nature

Journal:	*Human Reproduction Update*
Manuscript ID:	HRU-15-0019
Manuscript Type:	Review
Date Submitted by the Author:	24-Mar-2015
Complete List of Authors:	Barkalina, Natalia; University of Oxford, Nuffield Department of Obstetrics and Gynaecology Jones, Celine; University of Oxford, Nuffield Department of Obstetrics and Gynaecology Wood, Matthew; University of Oxford, Department of Physiology, Anatomy and Genetics Coward, Kevin; University of Oxford, Nuffield Department of Obstetrics and Gynaecology
Keywords:	extracellular vesicles, nanomaterials, delivery, gametes, ART

COMPREHENSIVE REVIEW

EXTRACELLULAR VESICLE-MEDIATED DELIVERY OF MOLECULAR COMPOUNDS INTO GAMETES AND EMBRYOS: LEARNING FROM NATURE

Running title: Extracellular vesicle for intra-gamete delivery in ART

Authors:

Natalia Barkalina, MD, MSc[1] (natalia.barkalina@obs-gyn.ox.ac.uk)

Celine Jones[1] (celine.jones@obs-gyn.ox.ac.uk)

Matthew J.A. Wood, PhD[2] (matthew.wood@dpag.ox.ac.uk)

Kevin Coward, PhD[1] (kevin.coward@obs-gyn.ox.ac.uk)

[1]*Nuffield Department of Obstetrics and Gynaecology, University of Oxford, Level 3, Women's Centre, John Radcliffe Hospital, Headington, Oxford, OX3 9DU, UK;*

[2]*Department of Physiology, Anatomy and Genetics, University of Oxford, Le Gros Clark Building, South Parks Road, Oxford, OX1 3QX, UK.*

Corresponding author (address for reprints): Kevin Coward, PhD, Nuffield Department of Obstetrics and Gynaecology, Level 3, Women's Centre, John Radcliffe Hospital, Headington, Oxford, OX3 9DU, United Kingdom (Telephone: +441865 782878; E-mail: kevin.coward@obs-gyn.ox.ac.uk).

TABLE OF CONTENTS

ABSTRACT

Background: Currently, even the most sophisticated methods of ART allow us to achieve live births in only approximately 30% of patients, indicating that our understanding of the fine mechanisms underlying reproduction is far from ideal. One of the main challenges associated with studies of gamete structure and function is that these cells are remarkably resistant towards the uptake of exogenous substances, including 'molecular research tools' such as drugs, biomolecules, and intracellular markers. This phenomenon can affect not only the performance of reproductive biology research techniques, but also the outcomes of the *in vitro* handling of gametes, which forms the cornerstone of ART. Improvement of intra-gamete delivery in a non-aggressive fashion is vital for the investigation of gamete physiology, and the advancement of infertility treatment. In this review, we outline the current state of nanomaterial-mediated delivery into gametes and embryos *in vitro*, and discuss the potential of a novel exciting drug delivery technology, based upon the use of targeted 'natural' nanoparticles, known as extracellular vesicles (EVs), for reproductive science and ART, given the promising emerging data from other fields.

Methods: A comprehensive electronic search of PubMed and Web of Science databases was performed using the following keywords: 'nanoparticles', 'nanomaterials', 'cell-penetrating peptides', 'sperm', 'oocyte', 'egg', 'embryo', 'exosomes', 'microvesicles', 'extracellular vesicles', 'delivery', 'reproduction' to identify the relevant research and review articles, published in English up to January 2015. The reference lists of identified publication were then scanned to extract additional relevant publications.

Results: Biocompatible engineered nanomaterials with high loading capacity, stability and selective affinity represent a potential versatile tool for the minimally-invasive internalisation of molecular cargo into gametes and embryos. However, it is becoming increasingly clear that the translation of these experimental tools into clinical applications is likely to be limited by their non-biodegradable nature. To allow the subsequent use of these methodologies for clinical ART, studies should utilise biodegradable delivery platforms which mimic natural mechanisms of molecular cargo trafficking as closely as possible. Currently, EVs represent the most physiological intracellular delivery tools for reproductive science and medicine. These natural mediators of cell communication combine the benefits of engineered nanomaterials, such as the potential for *in vitro* production, targeting and loading, with the essential feature of biodegradability.

Conclusion: We anticipate that future investigations into the possibility of applying EVs for the intentional intracellular delivery of molecular compounds into gametes and embryos will open new horizons for reproductive science and clinical ART, ultimately leading to improvements in patient care.

Key words: extracellular vesicles, nanomaterials, delivery, gametes, ART

INTRODUCTION

BACKGROUND

Assisted reproduction technology (ART) has revolutionised the field of infertility treatment, resulting in the birth of more than 5 million children worldwide ever since its first successful use in humans in 1978 (Adamson et al. 2013). Over the last four decades, pregnancy rates following ART have increased by nearly six-fold, from 6% (Wang and Sauer 2006) to approximately 35% (ESHRE, 2014, HFEA, 2014). Although ART has expanded and improved, perhaps even more than anticipated in its early days, and transformed from a controversial experimental procedure to a routine medical treatment, its success rates, from the modern perspective, remain remarkably insufficient to consider this technique a reliable solution to the problem of infertility. According to recent estimations, the average live birth rate after ART globally still remains reasonably low, and does not exceed 30% per started cycle (ESHRE, 2014). At the same time, the demands for successful ART, especially in post-industrial economies, are continuously growing under the pressure of an increasing prevalence of age-related infertility due to the postponement of parenthood, and expansion of assisted reproduction into 'non-infertility' indications, such as the pre-implantation genetic diagnosis of hereditary diseases and fertility preservation for medical or social reasons (reviewed in Barkalina, Charalambous, et al. 2014).

The sub-optimal success rates of ART are generally attributed to two key factors: firstly, the inherent limitations of conventional techniques for the selection of embryos to be transferred back into the patient's uterus, which are based exclusively upon the morphological assessment of embryos, and not the evaluation of their chromosomal status and, therefore, developmental potential in the long-term (Fragouli et al. 2014). Secondly, *in vitro* handling of gametes and embryos, which forms an integral part of ART, has been reported to induce microstructural and functional damage in these delicate structures, with consequential reduction in developmental competency. There is mounting evidence that gamete processing *in vitro* during ART also promotes the fragmentation of sperm DNA (Toro et al. 2009; Matsuura et al. 2010; Rougier et al. 2013), reduces the levels of sperm-borne oocyte-

activating factor phospholipase C zeta (Kashir et al. 2011; Yelumalai et al. 2013), and facilitates oxidative stress in unfertilised oocytes (Martin-Romero et al. 2008; Otsuki et al. 2009), all of which, in the case of gametes with already compromised fertility, can have profound negative effects. Optimisation of *in vitro* culture conditions, such as the supplementation of culture media with antioxidants, small molecules and growth factors (Kawamura et al. 2012; Yun et al. 2013; Tardif et al. 2014), or embryo incubation in time-lapse monitoring systems, which do not require repeated interruptions of culture for morphology assessment (Meseguer et al. 2012), has been reported to increase gamete/embryo survival and improve developmental potential. These observations elegantly indicate that the potential to improve ART success rates via the wider adoption of such approaches is both exciting and necessary. Nevertheless, substantial breakthroughs in the field of clinical ART can only be achieved via ongoing fundamental reproductive biology studies into the physiological mechanisms underlying reproduction, enabling the discovery of targeted molecular tools to investigate or manipulate these fine mechanisms at the cellular level.

RESEARCH INTO THE MECHANISMS OF GAMETE FUNCTION: CURRENT CHALLENGES

The use of molecular research tools, including oligonucleotides, nucleic acids, peptides, antibodies, fluorescent markers and small molecules, forms the cornerstone of experimental studies in developmental and reproductive biology. These tools allow the precise mapping of specific cellular structures and molecular pathways, and tracking of their activity and fate at the different stages of gamete/embryo development. However, this seemingly straightforward approach, which is universally applied for experiments in biology, is associated with substantial challenges when used for studies of gamete structure and function *in vitro*. These highly specialised cells, especially in their mature form and after isolation from the natural microenvironment, acquire remarkable resistance towards the uptake of exogenous compounds. The specific molecular structure of the sperm membrane, characterised by an increased proportion of polyunsaturated fatty acids and the presence of rare ether-linked phospholipids, plasmalogens (Lenzi et al. 2000; Tapia et al. 2012), along with high structural

and functional compartmentalisation (James et al. 2004) and low activity of endocytotic processes (Jones et al. 2013), render sperm a particularly difficult target for the intracellular delivery of investigative compounds *in vitro*. Similarly, the oocyte, throughout its maturation *in vivo*, maintains an intimate relationship with the surrounding cumulus cells which deliver essential nutrients into the oocyte via a system of gap junctions between the long cumulus cell processes penetrating the *zona pellucida* and the oocyte plasma membrane (Eppig et al. 2005). Studies of oocyte structure and function *in vitro* often require the mechanical removal of these surrounding nurturing cells to facilitate visualisation of the female gamete, and, subsequently, compromise the physiological mechanisms of cargo internalisation.

In its current form, the *in vitro* intracellular delivery of research compounds into gametes, and particularly into sperm, often requires the use of powerful membrane-disrupting agents, such as cholamidopropyl-dimethylammoniopropanesulfonate hydrate (CHAPS), Tween 20 and Triton X-100 (Jakop et al. 2009) with subsequent fixation, which renders the gametes entirely unsuitable for further use (Garcia-Vazquez et al. 2009; Yamauchi et al. 2012). Consequently, this approach does not allow for the evaluation of how the differences in gamete structure relate to their functionality, especially the ability to initiate and sustain normal embryo development. Improvement of intracellular delivery into gametes in a non-aggressive fashion and without effects upon developmental potential is, therefore, pre-exquisite for the improvement of our existing knowledge of reproductive biology, and, consequently, the advancement of ART.

From a rather more applied perspective, tools for efficient and non-damaging intra-gamete delivery could hold a therapeutic promise for patients with infertility caused by specific molecular deficiencies in gametes, for example deficiency of the sperm-borne oocyte activating factor phospholipase C zeta (PLCζ), resulting in oocyte activation failure post-fertilization, even following the intracytoplasmic injection of sperm into oocytes (ICSI) (Amdani et al. 2013). Similarly, these tools could be used in applied ART to supplement gametes with fertility-enhancing compounds, either

promoting sperm motility or protecting gametes from deterioration during long-term culture *in vitro* (Kawamura et al. 2012; Yun et al. 2013; Tardif et al. 2014), especially for such indications as the *in vitro* maturation of oocytes or the *in vitro* culture of oocytes from primordial follicles for experimental fertility preservation programmes (Telfer and McLaughlin 2012).

In this review, we outline the current state of nanomaterial-mediated delivery into gametes and embryos *in vitro*, and discuss the potential of a novel exciting drug delivery technology, based upon the use of targeted 'natural' nanoparticles, known as extracellular vesicles (EVs), for reproductive science and ART, given the promising emerging data from other fields.

METHODS

A comprehensive electronic search of PubMed (US National Library of Medicine, National Institute of Health; http://www.ncbi.nlm.nih.gov/pubmed/) and Web of Science (Thomson Reuters, http://webofknowledge.com/) databases was performed using the following keywords: 'nanoparticles', 'nanomaterials', 'cell-penetrating peptides', 'sperm', 'oocyte', 'egg', 'embryo', 'exosomes', 'microvesicles', 'extracellular vesicles', 'delivery', 'reproduction' to identify the relevant research and review articles published in English up to January 2015. The reference lists of identified publication were then scanned to extract potential additional relevant publications.

RESULTS

NANOPARTICLE-MEDIATED DELIVERY FOR REPRODUCTIVE BIOLOGY: A POTENTIAL STRATEGY TO IMPROVE UPTAKE INTO GAMETES

Nanotechnology is a novel and rapidly developing field of science, positioned at the interface of physical, chemical, biological, materials and computer sciences, which investigates and manipulates physical matter at the nanoscale (1-100nm). From the biomedical perspective, the revolutionary nature of nanotechnology lies in its ability to design a customizable small-scale biocompatible delivery platform with large loading capacity, stability and highly specific affinity towards selected cell

populations (Riehemann et al. 2009; Lehner et al. 2013; Tsai et al. 2014). Biomedical nanomaterials offer enormous targeting options, which can be achieved either via the modification of the physicochemical properties of nanomaterials, such as size, shape, surface charge and chemistry ('passive' targeting), or intentional functionalization of the surface of nanomaterials with specific affinity moieties, for example peptides, antibodies and aptamers ('active' targeting), which selectively bind with the complimentary ligands on the surface of target cells (Riehemann et al. 2009; Petros and DeSimone 2010; Albanese et al. 2012). Nanomaterials are universally characterised by their small size, comparable to the size of biological molecules and/or intracellular organelles, and their vast surface area, allowing the nanocarrier to be loaded with large amounts of almost any type of biological cargo, including a combination of contrast and therapeutic agents for the simultaneous detection and targeted treatment of pathologic lesions ('nanotheranostic') (Lammers et al. 2011). The small size enables straightforward internalisation of nanomaterials inside the cells using the innate physiological mechanisms of uptake, with subsequent intracellular transport and metabolism (Petros and DeSimone 2010; Kunzmann et al. 2011). Furthermore, nanomaterials are robust, and, therefore, capable of carrying payloads to distant target locations following systemic administration. Collectively, these features, summarised in Table 1, render biomedical nanomaterials as strong candidates for the targeted delivery of diagnostic and therapeutic agents, including those with poor bioavailability after systemic use or high non-specific cytotoxicity.

Over the last decade, the use of nanomaterials for diagnostic imaging and drug delivery into pathological lesions has consistently proven advantageous in such fields as oncology, infectious and chronic internal diseases (Ulbrich and Lamprecht 2010; Brakmane et al. 2012; Psarros et al. 2012; Holmes 2013; Tsai et al. 2014). This success has triggered an expansion of nanobiotechnological 'vision' to other scientific disciplines, including reproductive biology (Barkalina, Charalambous, et al. 2014). Indeed, universal evidence that nanomaterials improve the selectivity and efficacy of cargo delivery across a variety of cell types (Ryan and Brayden 2014; Tsai et al. 2014) and do not

compromise cell function, render them particularly attractive candidates for intracellular delivery into gametes and embryos.

The number of studies, utilizing nanomaterials for the transfer of molecular compounds into gametes, has been steadily growing since the mid-2000s, however the total number of publications still remains relatively low (Table 2). Currently, the spectrum of nanomaterials with favourable biocompatibility with gametes/embryos includes polyvinylalcohol-functionalised iron oxide (Ben-David Makhluf et al. 2006; Makhluf et al. 2008), magnetic (Kim et al. 2010) and polystyrene (Fynewever et al. 2007) nanoparticles (NPs), mesoporous silica (Barkalina, Jones, et al. 2014), cerium dioxide (Falchi et al. 2014), perfluorocarbon (Jallouk et al. 2014), halloysite clay nanotubes and commercial polymeric nanotransfectants (Campos, de Leon, et al. 2011; Campos, Komninou, et al. 2011), specialised CdSe/ZnS quantum dots (Feugang et al. 2012), and nanogold (Taylor, Garrels, et al. 2014; Tiedemann et al. 2014). Most of these studies have consistently demonstrated that the use of nanomaterials improves the efficacy of research techniques, based upon the internalisation of molecular compounds into gametes. These techniques primarily involved loading sperm with exogenous genetic constructs for subsequent sperm-mediated gene transfer (SMGT) into the oocyte at the time of fertilisation (Kim et al. 2010; Campos, de Leon, et al. 2011; Campos, Komninou, et al. 2011), proof-of-principle transfer of proteins into sperm (Makhluf et al. 2008), labelling of pre-implantation embryos during *in vitro* culture (Fynewever et al. 2007), sperm bioimaging (Feugang et al. 2012), and sorting into sub-populations (Odhiambo et al. 2014; Barchanski et al. 2015) – all with positive outcomes.

However, even in view of these encouraging findings, substantial concerns associated with the use of nanomaterials for intra-gamete delivery remain. Thus far, most studies, evaluating the potential effects of nanomaterials in gametes, have focused specifically on sperm, since this cell represents the main target for loading with exogenous compounds, either for sorting purposes or for sperm-mediated gene/protein delivery into the oocyte. Secondly, the nanomaterials which have been tested for safety in

sperm almost exclusively belong to the non-biodegradable category, which raises legitimate concerns about their potential long-term effects in the case of stable integration into embryonic cells. Although the studied nanomaterial-based intra-gamete delivery platforms have been reported to exert their transport function primarily via the anchoring to the surface of gamete plasma membrane or intra-membrane sequestration, rather than cytoplasmic internalisation, a small proportion of nanomaterial has been reported to reach the intracellular compartment in most cases (Kim et al. 2010; Feugang et al. 2012; Courbiere et al. 2013; Barkalina, Jones, et al. 2014; Taylor, Barchanski, et al. 2014; Barchanski et al. 2015). These observations, along with the contradictory nature of data regarding the long-term effects of non-biodegradable nanomaterials upon the embryo/foetal development, which arise from the methodologically diverse studies utilizing different protocols and animal models (Celá et al. 2014), form the main reasons for general caution towards the application of non-biodegradable nanomaterials for intra-gamete delivery outside the experimental setting. Therefore, discovery of an alternative small-scale versatile delivery platform, which would interact with gametes and transport molecular compounds inside these cells in a fashion similar to previously studied inorganic nanomaterials, but, at the same time, undergo biodegradation, would be highly advantageous for reproductive biology and ART.

In recent years, the search for a targeted biodegradable delivery tool, capable of transporting biological cargo into gametes and embryos, prompted an investigation into the potential benefits of cell-penetrating peptides. Cell-penetrating peptides (CPPs) are a specific class of short cationic/amphipathic peptides (<30 amino acids), capable of undergoing the energy- and receptor-independent translocation across the plasma membrane and transporting a considerably larger molecular cargo inside the cell, bypassing the traditional internalisation pathways (Patel et al. 2007; Jones and Sayers 2012). To date, a number of CPPs have been described as possessing affinity towards mammalian gametes and embryos, with several CPPs also demonstrating a promising delivery capacity (Table 3). Apart from their innate delivery potential, these CPPs can be applied as

functionalization tools for active targeting of nanomaterials towards a particular cell population. For example, in a recent study, Dr Coward's group have identified that functionalization of mesoporous silica nanoparticles with a cell-penetrating peptide C105Y results in an increase of their binding rate with mammalian sperm *in vitro* and changes in binding profiles, which start to mimic the previously described for free C105Y (Jones et al. 2013; Barkalina et al. 2015). Other authors have utilised the functionalization with nona-arginine R9 for CdSe/ZnS quantum dots (Feugang et al. 2012), and deca-arginine (R10), transactivator of transcription and simian-virus 40 large T antigen nuclear localization signal peptide for gold nanoparticles (Barchanski et al. 2015) to target mammalian sperm, although the efficacy of these CPPs in improving the sperm-particle interaction has not been steadily demonstrated.

Although CPPs are biodegradable and demonstrate a cargo delivery capacity, the high cost of production, need for specialised peptide synthesis equipment, and dependence upon a third-party manufacturer, along with a lower loading capacity and limited potential for additional functionalization restrict their use as delivery tools outside a large research laboratory setting. An ideal delivery vehicle for reproductive science and medicine would represent a nano-sized multifunctional biodegradable cargo carrier, which can be produced in most laboratories in a straightforward fashion, targeted towards gametes/embryos and loaded with different types of cargo. In fact, a prototype of this platform exists in nature already, and is known to cell biologists as extracellular vesicles (EVs). There is increasing understanding that these natural nanoparticles, universally secreted by pro- and eukaryotic cells, are involved in the crucial processes underlying gamete development, maturation and acquisition of fertilisation potential. Moreover, these processes appear to be highly conserved across a variety of biological species (Sohel et al. 2013; Sullivan and Saez 2013). These observations form another justification for studies into the feasibility of manipulating these key processes using similar 'recombinant' nanoplatforms.

EXOSOMES AND MICROVESICLES IN REPRODUCTIVE BIOLOGY: NATURAL DELIVERY VECTORS AND MEDIATORS OF CELL FUNCTION

Exosomes and microvesicles (MVs), collectively referred to as EVs, are nanoscale-sized phospholipid bilayer-enclosed particles, naturally released by a variety of cell types into their microenvironment. According to the most recent views, the main difference between exosomes and MVs lies not in their size, from 40nm to ~100nm for exosomes and up to 1μm for MVs, as it was assumed previously, but in the mechanism of production (Raposo and Stoorvogel 2013). Exosomes represent derivatives of multi-vesicular bodies (MVBs), and are first formed as intra-luminal vesicles (ILVs) inside these enclosed intracellular compartments via inward budding of the MVB membrane. These ILVs undergo release from cells upon the fusion of MVBs with the plasma membrane, and form exosomes. In contrast, MVs originate via direct budding from the plasma membrane (Akers et al. 2013). Ever since exosomes were first described in the 1980s as reticulocyte-secreted vesicles, involved in the process of transferrin receptor recycling (Harding et al. 1983; Pan and Johnstone 1983), our understanding of the fundamental role of these natural organic nanoparticles in cellular communication has evolved enormously. Exosomes have been demonstrated to have a complex tissue-specific organic content, including bioactive lipids (Record et al. 2014), proteins (Fontana et al. 2013), cytokines, growth factors, messenger RNAs (mRNAs) and non-coding transcripts, such as microRNAs (RNAs) (Braicu et al. 2015). However, the composition of MVs has been studied to a far lesser extent (Raposo and Stoorvogel 2013). Today, EVs are universally recognised as powerful paracrine and long-range mediators of cellular communication, playing an essential role in stem cell maintenance, cell proliferation and apoptosis, tissue repair, angiogenesis and immune response (El-Andaloussi et al. 2012).

Interest in the potential role of EVs in the key processes underlying reproduction began with the elucidation of their function in mammalian pregnancy, both as mediators of maternal immunosuppression, preventing the rejection of the semi-allogenic foetus by the mother's immune system, but also as vasoactive messengers involved in endothelial dysfunction during pre-eclampsia

(Knight et al. 1998; Taylor et al. 2006). Such seemingly conflicting roles were later attributed to the contrasting functions of two distinct pregnancy-specific EV subpopulations: placental exosomes with multiple immunomodulatory properties, favouring successful pregnancy, and pro-inflammatory syncytiotrophoblast-derived microvesicles/microparticles (STBMs), closely involved in pre-eclampsia (Redman et al. 2012; Mincheva-Nilsson and Baranov 2014). Today, pregnancy-specific EVs are recognized as paramount mediators of feto-maternal crosstalk, responsible for the orchestration of a series of events, leading to the establishment and maintenance of mammalian pregnancy. In particular, placental exosomes have been reported to promote vascular smooth muscle cell and endothelial migration, which is essential for the remodeling of uterine spiral arteries and the establishment of physiological foeto-maternal placental circulation (Salomon et al. 2013; Salomon et al. 2014), and triggering apoptosis in activated immune cells (Stenqvist et al. 2013). STBMs, in contrast, are characterized by their proinflammatory, anti-endothelial, and procoagulant effects, and the total concentration and levels of expression of 'endogenous danger molecules', such as heat shock protein 70 (HSP70) and high mobility group box 1 (HMGB1), have been reported to directly correlate with the severity of pre-eclampsia (Redman et al. 2012).

Over recent years, and in addition to their role in mammalian pregnancy, the significant contribution of EVs in the fine processes of gamete maturation and the acquisition of fertilisation potential are beginning to be elucidated (Table 4), along with the fact that these processes are highly conserved across many species (Corrigan et al. 2014). Although prostate-derived EVs ('prostasomes') were first discovered in human semen in 1978, the highly important contribution of EVs, produced in various portions of the male reproductive tract, to post-testicular sperm maturation across a variety of mammalian species, was not recognised until the 1990s (reviewed in Saez et al. 2003; Sullivan et al. 2005). Post-testicular sperm maturation involves structural and functional reorganisation of the sperm membrane during its passage through the epididymis, and is essential for the acquisition of motility and fertilisation ability. At this stage, the sperm have already lost the capacity for active protein

synthesis, and therefore, the modification of sperm surface structure with protein targets for the recognition of *zona pellucida* is largely dependent upon the direct transfer of essential compounds from the epididymal microenvironment. The limited endocytotic activity of sperm at this stage necessitates the non-conventional molecular translocation mechanisms, which are currently considered to be mediated by the EVs of epididymal origin, also referred to as epididymosomes. Epididymosomes are involved in the enrichment of the sperm surface membrane with a wide range of proteins: glycosyl-phosphatidylinositol (GPI)-anchored proteins, including the proteins P26h, P25b, and P34H, essential for fertilisation in hamster, bull, and human, respectively (Legare et al. 1999; Frenette et al. 2002); a disintegrin and metalloprotease (ADAM) 7 (Oh et al. 2009) and glioma pathogenesis-related 1-like protein 1 (GliPr1L1) (Caballero et al. 2012), both of which are involved in the interaction of sperm with *zona pellucida*; tyrosine kinase cSrc, playing a role in sperm capacitation (Krapf et al. 2012); epididymal sperm binding protein 1 (ELSPBP1), serving as a 'molecular tag' for dead epididymal sperm in certain animals (D'Amours et al. 2012); plasma membrane Ca^{2+}-ATPase (PMCA) with important functions for male fertility (Schwarz et al. 2013). In addition, recent observations show that epididymosomes are also involved in the regulation of post-transcriptional gene expression within the epididymal epithelium via the intercellular transport of micro (mi)RNAs between the portions of male reproductive tract (Belleannee et al. 2013). The mechanisms of protein transfer between epididymosomes has not been yet elucidated in detail, however it has been shown that epididymosomes, similarly to sperm, demonstrate the compartmentalization of proteins on the surface membrane and contain similar detergent-resistant domains, or lipid rafts, enriched in cholesterol and sphingomyelin, which can directly exchange proteins with the corresponding domains on the sperm surface (Girouard et al. 2009). Another study has shown that epididymosomes can fuse with the sperm membrane, and this fusion changes both the protein and lipid composition of sperm (Schwarz et al. 2013).

Prostasomes, secreted by the prostate gland epithelium and rich in sphyngomielin and cholesterol, have also been reported to 'supplement' sperm with complement-inhibitory molecules (CD59), shielding the sperm from immune recognition in the female genital tract (Rooney et al. 1993), via the pH-dependent fusion mechanism (Arienti et al. 1997), interact with polymorphonuclear and mononuclear leucocytes (Arienti et al. 1998) and reduce the overall reactive oxygen species production by polymorphonuclear neutrophils via the inhibition of NADPH oxidase activity by lipid transfer (Saez et al. 2000). Prostasomes enhance sperm motility (Fabiani, Johansson, Lundkvist, and Ronquist 1994; Fabiani, Johansson, Lundkvist, Ulmsten, et al. 1994; Arienti et al. 1999; Wang et al. 2001), and influence sperm capacitation (Cross and Mahasreshti 1997; Pons-Rejraji et al. 2011; Piehl et al. 2013) and acrosome reaction (Cross and Mahasreshti 1997; Palmerini et al. 2003; Siciliano et al. 2008). These EVs play an essential role in supplying sperm with the Ca^{2+}-signalling machinery required for the maintenance of sperm motility, capacitation and acquisition of its specific patterns, associated with fertilisation. The Ca^{2+}-signalling tools, currently considered to be provided provided to sperm by prostasomes, include the free Ca^{2+} (Palmerini et al. 1999) and the enzyme adenosine diphosphate (ADP) ribosyl cyclase (CD38), required for the production of cyclic adenosine diphosphoribose (cADPR), serving as the second messenger in Ca^{2+}-signaling pathway in sperm (Park et al. 2011). The mechanism prostasome-sperm transport appears to be similar to the epididymosome-sperm exchange of molecular compounds. Similarly to epididymosomes, the prostasomes have been shown to fuse with sperm at acidic or low pH (Arienti et al. 1997; Palmerini et al. 1999), and deliver the molecular cargo. Apart from the proteins, prostasomes have been recently demonstrated to contain fragments of DNA, randomly selected from the genome, and rapidly, within 15 minutes of co-incubation, transfer these fragments into the sperm head, neck and tail under the physiological vaginal pH – an important observation, highlighting yet another potential biological role of these mediators of cell communication (Ronquist et al. 2011).

More recently, EVs have been identified in the follicular fluid of ovarian follicles, where they have been shown to harbour a wide array of granulosa and cumulus cell-derived miRNAs, and common exosomal and cell-type-specific proteins, suggesting a role in cell communication within mammalian ovarian follicles and in the regulation of follicular maturation (da Silveira et al. 2012; Sohel et al. 2013; da Silveira et al. 2014; Santonocito et al. 2014). Exosomes have also been isolated from mammalian oviducts (Al-Dossary et al. 2013; Alminana et al. 2014) and uterine cavity fluid (Ng et al. 2013; Ruiz-Gonzalez et al. 2015), and in all cases have been shown to transport molecular cargo (miRNAs/proteins) with important functions for fertilisation, implantation and early pregnancy or the induction of specific sperm motility patterns, required for penetration of the *zona pellucida* ('hyperactivation'). In the same way as pregnancy-specific exosomes and STBMs, EVs in the male and female reproductive tract are structurally heterogenous and form multiple sub-populations (Poliakov et al. 2009; Aalberts et al. 2012; Brouwers et al. 2013; Caballero et al. 2013). It is hypothesised that different sub-populations of these EVs have different functional roles, however the exact mechanisms involved remain to be characterised.

Interestingly, the powerful effects of natural EV-mediated paracrine regulation were intentionally exploited by Saadeldin et al. (2014), who reported that the co-culture of cloned porcine embryos, produced using the nuclear transfer technique, improved substantially during co-culture with parthenogenetic embryos, releasing EVs containing multiple pluripotency gene mRNAs, which could be internalised into the cloned embryos. This promising observation further strengthens the hypothesis that extracellular vesicles could represent an attractive candidate for intra-cellular delivery in reproductive biology and medicine, and improve the efficacy of existing investigative and therapeutic techniques.

EXTRACELLULAR VESICLE-MEDIATED DELIVERY: BRIDGING THE GAP BETWEEN NATURE AND NANOTECHNOLOGY

The concept of using EVs therapeutically is the focus of intense research in the fields of cancer treatment, regenerative medicine, infectious, inflammatory and neurodegenerative diseases (El-Andaloussi et al. 2013). The beneficial effects of EV-mediated paracrine signalling form the cornerstone of mesenchymal stromal cell (MSC)-based therapies for the treatment of neural, cardiac or acute generalised tissue damage (Cashman et al. 2013; Monsel et al. 2014; Xin et al. 2014). These cells are characterised by strong paracrine activity rather than differentiation potential, and produce large amounts of EVs, potentiating cell proliferation, regenerative reprogramming, angiogenesis and immunomodulation in affected areas (El-Andaloussi et al. 2013). In addition to these approaches involving the indirect uses of EVs, an increasing number of studies describe the intentional production of EVs for subsequent use as drug delivery platforms.

There is mounting evidence that EVs are naturally produced by a variety of cell types (El-Andaloussi et al. 2012), and can be purified from culture media using relatively straightforward ultracentrifugation protocols labelled with fluorescent probes (Nazarenko et al. 2013; Takahashi et al. 2013) and loaded with molecular cargo via co-incubation (Sun et al. 2010) or electroporation (Alvarez-Erviti et al. 2011; El-Andaloussi et al. 2012; Tian et al. 2014). Furthermore, cells can be engineered through transfection to secrete modified EVs, either loaded with specific cargo (Akao et al. 2011; Mizrak et al. 2013) or expressing targeting moieties on their surface, which allows users to direct EVs towards a particular cell population in order to improve selectivity and range of action (Alvarez-Erviti et al. 2011; Rountree et al. 2011; El-Andaloussi et al. 2012; Ohno et al. 2013; Tian et al. 2014). The latter could be extensively explored in reproductive science, especially considering emerging evidence that certain cell-penetrating peptides promote the internalisation of compounds into gametes and embryos and improve the outcome of *in vitro* culture (Jones et al. 2013; Kwon et al. 2013; Yang, Seol, et al. 2014; Barkalina et al. 2015). Interestingly, in response to growing interest in EV-mediated drug

delivery, several research groups have proposed alternative approaches for EV production, involving the passage of source cells through a series of filters (Jang et al. 2013; Jo, Kim, et al. 2014) or microfluidic channels (Jo, Jeong, et al. 2014) to create artificial exosome-mimetic nanovesicles. These nanovesicles have been demonstrated to have a similar composition and delivery capacity to secreted EVs, and contain mRNAs, intracellular and plasma membrane proteins.

Thus far, purified and loaded EVs have been successfully applied for the delivery of anti-inflammatory compounds into activated myeloid cells and microglial brain cells as prototype treatments for autoimmune/inflammatory diseases (Sun et al. 2010; Zhuang et al. 2011), targeting of chemotherapeutics, suicide gene mRNAs/proteins, microRNAs and investigative therapeutic cancer vaccines towards malignant cells (Rountree et al. 2011; Mizrak et al. 2013; Ohno et al. 2013; Tian et al. 2014), and the targeted systemic delivery of small interfering (si)RNAs into the brain as an experimental therapy for Alzheimer's and Parkinson's disease (Alvarez-Erviti et al. 2011; El-Andaloussi et al. 2012; Cooper et al. 2014). In these studies, EVs have been consistently shown to have highly promising features for intracellular delivery, including high specificity and selectivity, and significant potential for the systemic delivery of experimental agents with otherwise unfavourable biodistribution profiles.

Collectively, the relative ease of production, biodegradability, possibility for targeting and loading with a variety of compounds, relevant for reproductive biology and science, including small molecules, nucleic acids and proteins, along with the enormous physiological role of EVs in cell communication processes underlying reproduction, and encouraging evidence from another fields, render these natural 'nanoplatforms' as particularly attractive candidates for compound delivery into gametes and embryos (Figure 1).

CONCLUSIONS

Today, assisted reproductive technology is viewed upon not only as a routine approach for infertility treatment, but, progressively, as a state-of-art guarantee of successful conception and childbirth at any chosen time in an individual's life. However, despite the ever-growing success of ART in the field of infertility treatment, this technique still only results in the live birth of healthy children in approximately 30% of couples starting treatment. The increasing reliance of medical practitioners and the general public upon ART justifies further extensive investigation into the fundamental mechanisms of reproduction to improve our existing levels of knowledge and facilitate the continuous improvement of current medical technology. However, it is highly unlikely that a substantial breakthrough in the field of reproductive science and medicine can be achieved without the discovery of novel research tools that allow us to systematically study and manipulate gamete and embryo function in a real-time setting, while fully preserving their viability. In recent years, biomedical nanotechnology has offered potent solutions to the problem of delivering molecular compounds into gametes. Nevertheless, the predominant use of non-biodegradable nanoparticles to promote the uptake of DNA and proteins into gametes continues to raise concerns, which limits the use of these techniques to a purely investigative platform. To allow the subsequent translation of these methodologies to clinical ART, studies should utilise biodegradable delivery platforms, which mimic natural mechanisms of molecular cargo trafficking as closely as possible. In this view, the field of reproductive science could be substantially advanced by actively developing EV-mediated delivery technology.

Currently, EVs represent the most physiological intracellular delivery tools available for reproductive science and medicine. These natural universal mediators of cell communication combine the benefits of engineered nanomaterials, such as the potential for *in vitro* production, targeting and loading, with the essential feature of biodegradability. Furthermore, the high degree of involvement of EVs in the essential processes underlying gamete maturation, the acquisition of fertilisation potential and the establishment/maintenance of pregnancy, renders the use of similar 'modified' nanoplatforms

particularly exciting. We anticipate that future investigations into the possibility of applying EVs for the intentional intracellular delivery of molecular compounds into gametes and embryos will open new horizons for reproductive science and clinical ART, ultimately leading to significant improvements in patient care.

AUTHOR'S ROLES

NB has designed the manuscript, performed the literature search and manuscript drafting. CJ participated in manuscript drafting, performed revisions and critically discussed the manuscript. MJAW performed revisions and critically discussed the manuscript. CB intellectually conceived the manuscript, and performed revisions and critically discussed the manuscript. KC revisions and critically discussed the manuscript.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Prof Christopher Barratt (Division of Cardiovascular & Diabetes Medicines, University of Dundee) for a critical discussion of the original draft of this manuscript.

FUNDING

NB, CJ and KC have a patent pending related to the work discussed in this article entitled 'Delivery Method' (PCT Patent Application Number PCT/GB13/053394 filed on the 20th December 2013). NB is funded by the Clarendon, Scatcherd European and Cyril & Phillis Long Schemes, the Nuffield Department of Obstetrics and Gynaecology and an EPSRC Pathways to Impact Award (University of Oxford). No specific funding was sought for the preparation of this manuscript. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This

includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties. No writing assistance was utilized in the production of this manuscript.

TABLES

***Table 1* – Key features of nanomaterials which favour their use in biomedicine [reproduced from Barkalina, Charalambous, et al. (2014)]**

Feature	Relevance for biomedical applications
Small size	Comparability with the size of biological molecules Potential for a straightforward integration into cellular processes and physiological pathways
Large surface area	Capacity to carry large amounts of biological cargo, including simultaneous transport of various types of cargo on one nanocarrier
Versatility	Adjustable physicochemical properties (size, shape, surface charge and architecture) for increased efficacy of targeting Adjustable surface chemistry (addition of specific functional groups and/or coatings) for the covalent or non-covalent absorption of a particular type of payload Options for the 'fine-tuning' of surface chemistry through the addition of highly-specific ligands for molecular recognition and further enhanced selectivity of targeting
Targeted action	High sensitivity and specificity Decreased 'off-target' effects of cargo Improved accuracy of detection profiles for diagnostic agents Increased bioavailability and/or decreased toxicity for therapeutic agents
Stability	Distance of action Options for systemic administration Protection of 'sensitive' payloads and optimised biodistribution

***Table 2* – Nanoparticle-mediated delivery into gametes and cell-labelling *in vitro*: experimental studies in animal models**

Study	Nanomaterial	Application
Fynewever et al. (2007)	Polystyrene and polyacrylonitrile NPs	External and intracytoplasmic labelling of pre-implantation embryos during *in vitro* culture
Makhluf et al. (2008)	Polyvinylalcohol-coated magnetic iron oxide NPs (Fe_3O_4)	Proof-of-principle transfer of anti-protein kinase C-antibody into sperm
Kim et al. (2010)	Magnetic NPs (commercial agent)	Facilitation of SMGT
Campos, de Leon, et al. (2011)	Nanopolymer (commercial agent)	Facilitation of SMGT ('NanoSMGT')
Campos, Komninou, et al. (2011)	Nanopolymer (commercial agent) and halloysite clay nanotubes	Facilitation of SMGT ('NanoSMGT')
Feugang et al. (2012)	CdSe/ZnS quantum dots	'Live' bioimaging of sperm
Odhiambo et al. (2014)	Magnetic iron oxide NPs (Fe_2O_3)	Magnetic removal of defective sperm subpopulation from the ejaculate ('nanopurification')
Barchanski et al. (2015)	Nanogold	Proof-of-principle investigation of the potential to label the specific DNA sequences in viable sperm

Table 3 – Cell-penentrating peptides with affinity towards reproductive tissues and gametes and their delivery potential

Author	Peptide	Target	Intracellular translocation	Delivery potential
Jones et al. (2013)	Penetratin	Bovine sperm	Yes	No
	Tat		Yes	No
	C105Y		Yes	N/A
	Mitoparan		Yes	N/A
	Inverso mitoparan		Yes	N/A
	Inverso mastoparan		Yes	N/A
	Transportan 10		Yes	N/A
Yang, Hou, et al. (2014)	Poly-arginine 11R	Fish (*Takifugu rubripes*) spermary cells	Yes	Yes (Biologically active Oct4)
Kwon et al. (2013)	Human papillomavirus L1 capsid protein (LDP12)	Mouse oocytes and embryos	Yes	Yes (EGFP)
Yang, Seol, et al. (2014)	7X-arginine (R7)	Mouse oocytes and embryos	Yes	Yes (Estrogen related receptor β)
Campelo et al. (2014)	Crotamine	Bovine embryos	Yes	N/A

509 ***Table 4*** **– Physiological roles of extracellular vesicles in the mechanisms underlying reproduction**

Type of EVs	Origin	Physiological role
Placental exosomes	Secretion from the endosomal compartment of STB	Promotion of maternal immunotolerance: suppression of NK- and T-cell-induced cytotoxicity, impairment of T-cell response, activation of apoptosis in cytotoxic immune cells at the foeto-maternal surface, transport of mRNAs and miRNAs
Syncytiotrophoblast-derived microvesicles/microparticles (STBMs)	Shedding of the apical plasma membrane of the STB	Pro-inflammatory, pro-coagulant and anti-endothelial effects, activation of immune response
Epididymosomes	Epididymal epithelial cells	Direct transfer of proteins, essential for sperm function and fertilization, to sperm membrane and cytoplasm as the component of post-testicular sperm maturation; transfer or miRNAs in the male reproductive tract
Prostasomes	Prostate epithelial cells	Direct transfer of proteins, including the Ca^{2+}-signalling tools, lipids and cholesterol to sperm for sperm surface remodeling as the component of post-testicular sperm maturation; regulation of sperm motility, capacitation, acrosome reaction and immune recognition in the female reproductive tract
Granulosa cell microvesicles	Follicular granulosa and cumulus cells	Transport of miRNAs and proteins for regulation of follicular and oocyte maturation
Oviductal exosomes	Oviductal epithelium	Transfer of Ca^{2+}-signalling tools to sperm; possible role in fertilization and early pregnancy
Endometrial exosomes	Endometrium	Possible role in regulation of endometrial receptivity and embryo implantation

510

511

FIGURE LEGENDS

Figure 1* – Extracellular vesicle-mediated delivery into gametes and embryos *in vitro. Extracellular vesicles (EVs) are produced during cell culture and released into the culture medium. Exosomes are released from the multivesicular bodies (MVBs) upon their fusion with the cell plasma membrane. Microvesicles (MVs) arise from the direct budding of cell plasma membrane. Exosomes and MVs can be isolated by centrifugation and loaded with molecular cargo (nucleic acids (NAs), proteins, small molecules etc.) through co-incubation or electroporation. Loaded EVs and MVs can then be applied to sperm, oocytes and embryos *in vitro* to promote the internalisation of molecular cargo. 'Loaded' sperm can be subsequently used for downstream applications, including sorting, fertilisation, or sperm-mediated delivery of genetic constructs, proteins or small molecules into the oocyte at the time of gamete fusion. Addition of loaded EVs to *in vitro* culture systems could promote the maturation of oocytes, protect oocytes from degradation and improve the developmental parameters of early-stage embryos.

REFERENCES

Aalberts M, van Dissel-Emiliani FM, van Adrichem NP, van Wijnen M, Wauben MH, Stout TA, and Stoorvogel W. Identification of distinct populations of prostasomes that differentially express prostate stem cell antigen, annexin A1, and GLIPR2 in humans. *Biol Reprod* 2012: **86**; 82.

Adamson G, Tabangin M, Macaluso M, and de Mouzon J. The number of babies born globally after treatment with the assisted reproductive technologies (ART). *Fertil Steril* 2013: **100**; S42.

Akao Y, Iio A, Itoh T, Noguchi S, Itoh Y, Ohtsuki Y, and Naoe T. Microvesicle-mediated RNA Molecule Delivery System Using Monocytes/Macrophages. *Mol Ther* 2011: **19**; 395-399.

Akers JC, Gonda D, Kim R, Carter BS, and Chen CC. Biogenesis of extracellular vesicles (EV): exosomes, microvesicles, retrovirus-like vesicles, and apoptotic bodies. *J Neurooncol* 2013: **113**; 1-11.

Al-Dossary AA, Strehler EE, and Martin-Deleon PA. Expression and secretion of plasma membrane Ca2+-ATPase 4a (PMCA4a) during murine estrus: association with oviductal exosomes and uptake in sperm. *PLoS One* 2013: **8**; e80181.

Albanese A, Tang PS, and Chan WC. The effect of nanoparticle size, shape, and surface chemistry on biological systems. *Annu Rev Biomed Eng* 2012: **14**; 1-16.

Alminana C, Corbin E, Tsikis G, Soleilhavoup C, Galio L, Sandra O, and Mermillod P. 108 characterization of bovine oviductal exosomes from in vivo and in vitro origin. *Reprod Fertil Dev* 2014: **27**; 147.

Alvarez-Erviti L, Seow Y, Yin H, Betts C, Lakhal S, and Wood MJ. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat Biotechnol* 2011: **29**; 341-345.

Amdani SN, Jones C, and Coward K. Phospholipase C zeta (PLCzeta): oocyte activation and clinical links to male factor infertility. *Adv Biol Regul* 2013: **53**; 292-308.

Arienti G, Carlini E, Nicolucci A, Cosmi EV, Santi F, and Palmerini CA. The motility of human spermatozoa as influenced by prostasomes at various pH levels. *Biol Cell* 1999: **91**; 51-54.

Arienti G, Carlini E, and Palmerini CA. Fusion of human sperm to prostasomes at acidic pH. *J Membrane Biol* 1997: **155**; 89-94.

Arienti G, Carlini E, Saccardi C, and Palmerini CA. Interactions between prostasomes and leukocytes. *Biochim Biophys Acta* 1998: **1425**; 36-40.

Barchanski A, Taylor U, Sajti CL, Gamrad L, Kues WA, Rath D, and Barcikowski S. Bioconjugated gold nanoparticles penetrate into spermatozoa depending on plasma membrane status. *J Biomed Nanotechnol* 2015: **11**.

Barkalina N, Charalambous C, Jones C, and Coward K. Nanotechnology in reproductive medicine: emerging applications of nanomaterials. *Nanomedicine* 2014: **10**; 921-938.

Barkalina N, Jones C, Kashir J, Coote S, Huang X, Morrison R, Townley H, and Coward K. Effects of mesoporous silica nanoparticles upon the function of mammalian sperm in vitro. *Nanomedicine* 2014: **10**; 859-870.

Barkalina N, Jones C, Townley H, and Coward K. Functionalisation of mesoporous silica nanoparticles with a cell-penetrating peptide to target mammalian sperm in vitro. *Nanomedicine (Lond)* 2015: **[In press]**.

Belleannee C, Calvo E, Caballero J, and Sullivan R. Epididymosomes convey different repertoires of microRNAs throughout the bovine epididymis. *Biol Reprod* 2013: **89**; 30.

Ben-David Makhluf S, Qasem R, Rubinstein S, Gedanken A, and Breitbart H. Loading magnetic nanoparticles into sperm cells does not affect their functionality. *Langmuir* 2006: **22**; 9480-9482.

Braicu C, Tomuleasa C, Monroig P, Cucuianu A, Berindan-Neagoe I, and Calin GA. Exosomes as divine messengers: are they the Hermes of modern molecular oncology? *Cell Death Differ* 2015: **22**; 34-45.

Brakmane G, Winslet M, and Seifalian AM. Systematic review: the applications of nanotechnology in gastroenterology. *Aliment Pharmacol Ther* 2012: **36**; 213-221.

Brouwers JF, Aalberts M, Jansen JW, van Niel G, Wauben MH, Stout TA, Helms JB, and Stoorvogel W. Distinct lipid compositions of two types of human prostasomes. *Proteomics* 2013: **13**; 1660-1666.

Caballero J, Frenette G, D'Amours O, Belleannee C, Lacroix-Pepin N, Robert C, and Sullivan R. Bovine sperm raft membrane associated Glioma Pathogenesis-Related 1-like protein 1 (GliPr1L1) is modified during the epididymal transit and is potentially involved in sperm binding to the zona pellucida. *J Cell Physiol* 2012: **227**; 3876-3886.

Caballero JN, Frenette G, Belleannee C, and Sullivan R. CD9-positive microvesicles mediate the transfer of molecules to bovine spermatozoa during epididymal maturation. *PLoS One* 2013: **8**; e65364.

Campelo IS, Pereira AF, Alcantara-Neto AS, Canel NG, Souza-Fabjan JM, Teixeira DI, Camargo LS, Melo LM, Radis-Baptista G, Salamone DF*, et al.* Effect of crotamine, a cell-penetrating peptide, on blastocyst production and gene expression of in vitro fertilized bovine embryos. *Zygote* 2014; 1-10.

Campos VF, de Leon PMM, Komninou ER, Dellagostin OA, Deschamps JC, Seixas FK, and Collares T. NanoSMGT: Transgene transmission into bovine embryos using halloysite clay nanotubes or nanopolymer to improve transfection efficiency. *Theriogenology* 2011: **76**; 1552-1560.

Campos VF, Komninou ER, Urtiaga G, de Leon PM, Seixas FK, Dellagostin OA, Deschamps JC, and Collares T. NanoSMGT: transfection of exogenous DNA on sex-sorted bovine sperm using nanopolymer. *Theriogenology* 2011: **75**; 1476-1481.

Cashman T, Gouon-Evans V, and Costa K. Mesenchymal Stem Cells for Cardiac Therapy: Practical Challenges and Potential Mechanisms. *Stem Cell Rev and Rep* 2013: **9**; 254-265.

Celá P, Veselá B, Matalová E, Večeřa Z, and Buchtová M. Embryonic Toxicity of Nanoparticles. *Cells Tissues Organs* 2014: **199**; 1-23.

Cooper JM, Wiklander PB, Nordin JZ, Al-Shawi R, Wood MJ, Vithlani M, Schapira AH, Simons JP, El-Andaloussi S, and Alvarez-Erviti L. Systemic exosomal siRNA delivery reduced alpha-synuclein aggregates in brains of transgenic mice. *Mov Disord* 2014: **29**; 1476-1485.

Corrigan L, Redhai S, Leiblich A, Fan SJ, Perera SM, Patel R, Gandy C, Wainwright SM, Morris JF, Hamdy F*, et al.* BMP-regulated exosomes from Drosophila male reproductive glands reprogram female behavior. *J Cell Biol* 2014: **206**; 671-688.

Courbiere B, Auffan M, Rollais R, Tassistro V, Bonnefoy A, Botta A, Rose J, Orsiere T, and Perrin J. Ultrastructural interactions and genotoxicity assay of cerium dioxide nanoparticles on mouse oocytes. *Int J Mol Sci* 2013: **14**; 21613-21628.

Cross NL and Mahasreshti P. Prostasome fraction of human seminal plasma prevents sperm from becoming acrosomally responsive to the agonist progesterone. *Arch Androl* 1997: **39**; 39-44.

D'Amours O, Frenette G, Bordeleau LJ, Allard N, Leclerc P, Blondin P, and Sullivan R. Epididymosomes transfer epididymal sperm binding protein 1 (ELSPBP1) to dead spermatozoa during epididymal transit in bovine. *Biol Reprod* 2012: **87**; 94.

da Silveira JC, Carnevale EM, Winger QA, and Bouma GJ. Regulation of ACVR1 and ID2 by cell-secreted exosomes during follicle maturation in the mare. *Reprod Biol Endocrinol* 2014: **12**; 44.

da Silveira JC, Veeramachaneni DN, Winger QA, Carnevale EM, and Bouma GJ. Cell-secreted vesicles in equine ovarian follicular fluid contain miRNAs and proteins: a possible new form of cell communication within the ovarian follicle. *Biol Reprod* 2012: **86**; 71.

El-Andaloussi S, Lee Y, Lakhal-Littleton S, Li J, Seow Y, Gardiner C, Alvarez-Erviti L, Sargent IL, and Wood MJ. Exosome-mediated delivery of siRNA in vitro and in vivo. *Nat Protoc* 2012: **7**; 2112-2126.

El-Andaloussi S, Mager I, Breakefield XO, and Wood MJ. Extracellular vesicles: biology and emerging therapeutic opportunities. *Nat Rev Drug Discov* 2013: **12**; 347-357.

Eppig JJ, Pendola FL, Wigglesworth K, and Pendola JK. Mouse oocytes regulate metabolic cooperativity between granulosa cells and oocytes: Amino acid transport. *Biol Reprod* 2005: **73**; 351-357.

European Society of Human Reproduction and Embryology (ESHRE). ART fact sheet (June 2014). 2014. http://www.eshre.eu/Guidelines-and-Legal/ART-fact-sheet.aspx (accessed on 02 March 2015).

Fabiani R, Johansson L, Lundkvist O, and Ronquist G. Enhanced recruitment of motile spermatozoa by prostasome inclusion in swim-up medium. *Hum Reprod* 1994: **9**; 1485-1489.

Fabiani R, Johansson L, Lundkvist O, Ulmsten U, and Ronquist G. Promotive effect by prostasomes on normal human spermatozoa exhibiting no forward motility due to buffer washings. *Eur J Obstet Gynecol Reprod Biol* 1994: **57**; 181-188.

Falchi L, Bogliolo L, Galleri G, Vlachopoulou G, Murrone O, Epifani G, Pinna A, Innocenzi P, and Ledda S. 266 biocompatibility of nanoceria in ram sperm during 24 hours of incubation. *Reprod Fertil Dev* 2014: **27**; 222.

Feugang JM, Youngblood RC, Greene JM, Fahad AS, Monroe WA, Willard ST, and Ryan PL. Application of quantum dot nanoparticles for potential non-invasive bio-imaging of mammalian spermatozoa. *J Nanobiotechnology* 2012: **10**; 45.

Fontana S, Saieva L, Taverna S, and Alessandro R. Contribution of proteomics to understanding the role of tumor-derived exosomes in cancer progression: state of the art and new perspectives. *Proteomics* 2013: **13**; 1581-1594.

Fragouli E, Alfarawati S, Spath K, and Wells D. Morphological and cytogenetic assessment of cleavage and blastocyst stage embryos. *Mol Hum Reprod* 2014: **20**; 117-126.

Frenette G, Lessard C, and Sullivan R. Selected proteins of "prostasome-like particles" from epididymal cauda fluid are transferred to epididymal caput spermatozoa in bull. *Biol Reprod* 2002: **67**; 308-313.

Fynewever TL, Agcaoili ES, Jacobson JD, Patton WC, and Chan PJ. In vitro tagging of embryos with nanoparticles. *J Assist Reprod Genet* 2007: **24**; 61-65.

Garcia-Vazquez FA, Garcia-Rosello E, Gutierrez-Adan A, and Gadea J. Effect of sperm treatment on efficiency of EGFP-expressing porcine embryos produced by ICSI-SMGT. *Theriogenology* 2009: **72**; 506-518.

Girouard J, Frenette G, and Sullivan R. Compartmentalization of proteins in epididymosomes coordinates the association of epididymal proteins with the different functional structures of bovine spermatozoa. *Biol Reprod* 2009: **80**; 965-972.

Harding C, Heuser J, and Stahl P. Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *J Cell Biol* 1983: **97**; 329-339.

Holmes D. The next big things are tiny. *The Lancet Neurology* 2013: **12**; 31-32.

Human Fertilisation and Embryology Authority (HFEA). Fertility treatment in 2013: trends and figures. 2014. http://www.hfea.gov.uk/docs/HFEA_Fertility_Trends_and_Figures_2013.pdf (accessed on 02 March 2015).

Jakop U, Fuchs B, SuSZ R, Wibbelt G, Braun B, Muller K, and Schiller J. The solubilisation of boar sperm membranes by different detergents - a microscopic, MALDI-TOF MS, 31P NMR and PAGE study on membrane lysis, extraction efficiency, lipid and protein composition. *Lipids Health Dis* 2009: **8**; 49.

Jallouk AP, Moley KH, Omurtag K, Hu G, Lanza GM, Wickline SA, and Hood JL. Nanoparticle incorporation of melittin reduces sperm and vaginal epithelium cytotoxicity. *PLoS One* 2014: **9**; e95411.

James PS, Hennessy C, Berge T, and Jones R. Compartmentalisation of the sperm plasma membrane: a FRAP, FLIP and SPFI analysis of putative diffusion barriers on the sperm head. *J Cell Sci* 2004: **117**; 6485-6495.

Jang SC, Kim OY, Yoon CM, Choi DS, Roh TY, Park J, Nilsson J, Lotvall J, Kim YK, and Gho YS. Bioinspired exosome-mimetic nanovesicles for targeted delivery of chemotherapeutics to malignant tumors. *ACS Nano* 2013: **7**; 7698-7710.

Jo W, Jeong D, Kim J, Cho S, Jang SC, Han C, Kang JY, Gho YS, and Park J. Microfluidic fabrication of cell-derived nanovesicles as endogenous RNA carriers. *Lab Chip* 2014: **14**; 1261-1269.

Jo W, Kim J, Yoon J, Jeong D, Cho S, Jeong H, Yoon YJ, Kim SC, Gho YS, and Park J. Large-scale generation of cell-derived nanovesicles. *Nanoscale* 2014: **6**; 12056-12064.

Jones AT and Sayers EJ. Cell entry of cell penetrating peptides: tales of tails wagging dogs. *J Control Release* 2012: **161**; 582-591.

Jones S, Lukanowska M, Suhorutsenko J, Oxenham S, Barratt C, Publicover S, Copolovici DM, Langel Ü, and Howl J. Intracellular translocation and differential accumulation of cell-penetrating peptides in bovine spermatozoa: evaluation of efficient delivery vectors that do not compromise human sperm motility. *Hum Reprod* 2013: **28**; 1874-1889.

Kashir J, Heynen A, Jones C, Durrans C, Craig J, Gadea J, Turner K, Parrington J, and Coward K. Effects of cryopreservation and density-gradient washing on phospholipase C zeta concentrations in human spermatozoa. *Reprod Biomed Online* 2011: **23**; 263-267.

Kawamura K, Chen Y, Shu Y, Cheng Y, Qiao J, Behr B, Pera RAR, and Hsueh AJW. Promotion of Human Early Embryonic Development and Blastocyst Outgrowth In Vitro Using Autocrine/Paracrine Growth Factors. *PLoS One* 2012: **7**; e49328.

Kim TS, Lee SH, Gang GT, Lee YS, Kim SU, Koo DB, Shin MY, Park CK, and Lee DS. Exogenous DNA uptake of boar spermatozoa by a magnetic nanoparticle vector system. *Reprod Domest Anim* 2010: **45**; e201-206.

Knight M, Redman CWG, Linton EA, and Sargent IL. Shedding of syncytiotrophoblast microvilli into the maternal circulation in pre-eclamptic pregnancies. *BJOG* 1998: **105**; 632-640.

Krapf D, Ruan YC, Wertheimer EV, Battistone MA, Pawlak JB, Sanjay A, Pilder SH, Cuasnicu P, Breton S, and Visconti PE. cSrc is necessary for epididymal development and is incorporated into sperm during epididymal transit. *Dev Biol* 2012: **369**; 43-53.

Kunzmann A, Andersson B, Thurnherr T, Krug H, Scheynius A, and Fadeel B. Toxicology of engineered nanomaterials: focus on biocompatibility, biodistribution and biodegradation. *Biochim Biophys Acta* 2011: **1810**; 361-373.

Kwon S, Kwak A, Shin H, Choi S, Kim S, and Lim HJ. Application of a novel cell-permeable peptide-driven protein delivery in mouse blastocysts. *Reproduction* 2013: **146**; 145-153.

Lammers T, Aime S, Hennink WE, Storm G, and Kiessling F. Theranostic nanomedicine. *Acc Chem Res* 2011: **44**; 1029-1038.

Legare C, Berube B, Boue F, Lefievre L, Morales CR, El-Alfy M, and Sullivan R. Hamster sperm antigen P26h is a phosphatidylinositol-anchored protein. *Mol Reprod Dev* 1999: **52**; 225-233.

Lehner R, Wang X, Marsch S, and Hunziker P. Intelligent nanomaterials for medicine: Carrier platforms and targeting strategies in the context of clinical application. *Nanomedicine* 2013: **9**; 742-757.

Lenzi A, Gandini L, Picardo M, Tramer F, Sandri G, and Panfili E. Lipoperoxidation damage of spermatozoa polyunsaturated fatty acids (PUFA): scavenger mechanisms and possible scavenger therapies. *Front Biosci* 2000: **5**; E1-E15.

Makhluf SB, Abu-Mukh R, Rubinstein S, Breitbart H, and Gedanken A. Modified PVA-Fe3O4 nanoparticles as protein carriers into sperm cells. *Small* 2008: **4**; 1453-1458.

Martin-Romero FJ, Miguel-Lasobras EM, Dominguez-Arroyo JA, Gonzalez-Carrera E, and Alvarez IS. Contribution of culture media to oxidative stress and its effect on human oocytes. *Reprod Biomed Online* 2008: **17**; 652-661.

Matsuura R, Takeuchi T, and Yoshida A. Preparation and incubation conditions affect the DNA integrity of ejaculated human spermatozoa. *Asian J Androl* 2010: **12**; 753-759.

Meseguer M, Rubio I, Cruz M, Basile N, Marcos J, and Requena A. Embryo incubation and selection in a time-lapse monitoring system improves pregnancy outcome compared with a standard incubator: a retrospective cohort study. *Fertil Steril* 2012: **98**; 1481-1489 e1410.

Mincheva-Nilsson L and Baranov V. Placenta-Derived Exosomes and Syncytiotrophoblast Microparticles and their Role in Human Reproduction: Immune Modulation for Pregnancy Success. *Am J Reprod Immunol* 2014: **72**; 440-457.

Mizrak A, Bolukbasi MF, Ozdener GB, Brenner GJ, Madlener S, Erkan EP, Strobel T, Breakefield XO, and Saydam O. Genetically Engineered Microvesicles Carrying Suicide mRNA/Protein Inhibit Schwannoma Tumor Growth. *Mol Ther* 2013: **21**; 101-108.

Monsel A, Zhu YG, Gennai S, Hao Q, Liu J, and Lee JW. Cell-based therapy for acute organ injury: preclinical evidence and ongoing clinical trials using mesenchymal stem cells. *Anesthesiology* 2014: **121**; 1099-1121.

Nazarenko I, Rupp AK, and Altevogt P. Exosomes as a Potential Tool for a Specific Delivery of Functional Molecules. *Methods Mol Biol* 2013: **1049**; 495-511.

Ng YH, Rome S, Jalabert A, Forterre A, Singh H, Hincks CL, and Salamonsen LA. Endometrial exosomes/microvesicles in the uterine microenvironment: a new paradigm for embryo-endometrial cross talk at implantation. *PLoS One* 2013: **8**; e58502.

Odhiambo JF, DeJarnette JM, Geary TW, Kennedy CE, Suarez SS, Sutovsky M, and Sutovsky P. Increased conception rates in beef cattle inseminated with nanopurified bull semen. *Biol Reprod* 2014: **91**; 97.

Oh JS, Han C, and Cho C. ADAM7 is associated with epididymosomes and integrated into sperm plasma membrane. *Mol Cells* 2009: **28**; 441-446.

Ohno S, Takanashi M, Sudo K, Ueda S, Ishikawa A, Matsuyama N, Fujita K, Mizutani T, Ohgi T, Ochiya T, *et al.* Systemically Injected Exosomes Targeted to EGFR Deliver Antitumor MicroRNA to Breast Cancer Cells. *Mol Ther* 2013: **21**; 185-191.

Otsuki J, Nagai Y, and Chiba K. Damage of embryo development caused by peroxidized mineral oil and its association with albumin in culture. *Fertil Steril* 2009: **91**; 1745-1749.

Palmerini CA, Carlini E, Nicolucci A, and Arienti G. Increase of human spermatozoa intracellular Ca2+ concentration after fusion with prostasomes. *Cell Calcium* 1999: **25**; 291-296.

Palmerini CA, Saccardi C, Carlini E, Fabiani R, and Arienti G. Fusion of prostasomes to human spermatozoa stimulates the acrosome reaction. *Fertil Steril* 2003: **80**; 1181-1184.

Pan BT and Johnstone RM. Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. *Cell* 1983: **33**; 967-978.

Park KH, Kim BJ, Kang J, Nam TS, Lim JM, Kim HT, Park JK, Kim YG, Chae SW, and Kim UH. Ca2+ signaling tools acquired from prostasomes are required for progesterone-induced sperm motility. *Sci Signal* 2011: **4**; ra31.

Patel LN, Zaro JL, and Shen WC. Cell penetrating peptides: intracellular pathways and pharmaceutical perspectives. *Pharm Res* 2007: **24**; 1977-1992.

Petros RA and DeSimone JM. Strategies in the design of nanoparticles for therapeutic applications. *Nat Rev Drug Discov* 2010: **9**; 615-627.

Piehl LL, Fischman ML, Hellman U, Cisale H, and Miranda PV. Boar seminal plasma exosomes: effect on sperm function and protein identification by sequencing. *Theriogenology* 2013: **79**; 1071-1082.

Poliakov A, Spilman M, Dokland T, Amling CL, and Mobley JA. Structural heterogeneity and protein composition of exosome-like vesicles (prostasomes) in human semen. *Prostate* 2009: **69**; 159-167.

Pons-Rejraji H, Artonne C, Sion B, Brugnon F, Canis M, Janny L, and Grizard G. Prostasomes: inhibitors of capacitation and modulators of cellular signalling in human sperm. *Int J Androl* 2011: **34**; 568-580.

Psarros C, Lee R, Margaritis M, and Antoniades C. Nanomedicine for the prevention, treatment and imaging of atherosclerosis. *Nanomedicine* 2012: **8, Supplement 1**; S59-S68.

Raposo G and Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol* 2013: **200**; 373-383.

Record M, Carayon K, Poirot M, and Silvente-Poirot S. Exosomes as new vesicular lipid transporters involved in cell-cell communication and various pathophysiologies. *Biochim Biophys Acta* 2014: **1841**; 108-120.

Redman CW, Tannetta DS, Dragovic RA, Gardiner C, Southcombe JH, Collett GP, and Sargent IL. Review: Does size matter? Placental debris and the pathophysiology of pre-eclampsia. *Placenta* 2012: **33 Suppl**; S48-54.

Riehemann K, Schneider SW, Luger TA, Godin B, Ferrari M, and Fuchs H. Nanomedicine--challenge and perspectives. *Angew Chem Int Ed Engl* 2009: **48**; 872-897.

Ronquist GK, Larsson A, Ronquist G, Isaksson A, Hreinsson J, Carlsson L, and Stavreus-Evers A. Prostasomal DNA Characterization and Transfer Into Human Sperm. *Mol Reprod Dev* 2011: **78**; 467-476.

Rooney IA, Atkinson JP, Krul ES, Schonfeld G, Polakoski K, Saffitz JE, and Morgan BP. Physiologic relevance of the membrane attack complex inhibitory protein CD59 in human seminal plasma: CD59 is present on extracellular organelles (prostasomes), binds cell membranes, and inhibits complement-mediated lysis. *J Exp Med* 1993: **177**; 1409-1420.

Rougier N, Uriondo H, Papier S, Checa MA, Sueldo C, and Alvarez Sedo C. Changes in DNA fragmentation during sperm preparation for intracytoplasmic sperm injection over time. *Fertil Steril* 2013: **100**; 69-74.

Rountree RB, Mandl SJ, Nachtwey JM, Dalpozzo K, Do L, Lombardo JR, Schoonmaker PL, Brinkmann K, Dirmeier U, Laus R, *et al.* Exosome Targeting of Tumor Antigens Expressed by Cancer Vaccines Can Improve Antigen Immunogenicity and Therapeutic Efficacy. *Cancer Research* 2011: **71**; 5235-5244.

Ruiz-Gonzalez I, Xu J, Wang X, Burghardt RC, Dunlap KA, and Bazer FW. Exosomes, endogenous retroviruses and toll-like receptors: pregnancy recognition in ewes. *Reproduction* 2015: **149**; 281-291.

Ryan SM and Brayden DJ. Progress in the delivery of nanoparticle constructs: towards clinical translation. *Curr Opin Pharmacol* 2014: **18**; 120-128.

Saadeldin IM, Kim SJ, Choi YB, and Lee BC. Improvement of cloned embryos development by co-culturing with parthenotes: a possible role of exosomes/microvesicles for embryos paracrine communication. *Cell Reprogram* 2014: **16**; 223-234.

Saez F, Frenette G, and Sullivan R. Epididymosomes and prostasomes: Their roles in posttesticular maturation of the sperm cells. *J Androl* 2003: **24**; 149-154.

Saez F, Motta C, Boucher D, and Grizard G. Prostasomes inhibit the NADPH oxidase activity of human neutrophils. *Mol Hum Reprod* 2000: **6**; 883-891.

Salomon C, Ryan J, Sobrevia L, Kobayashi M, Ashman K, Mitchell M, and Rice GE. Exosomal signaling during hypoxia mediates microvascular endothelial cell migration and vasculogenesis. *PLoS One* 2013: **8**; e68451.

Salomon C, Yee S, Scholz-Romero K, Kobayashi M, Vaswani K, Kvaskoff D, Illanes SE, Mitchell MD, and Rice GE. Extravillous trophoblast cells-derived exosomes promote vascular smooth muscle cell migration. *Front Pharmacol* 2014: **5**; 175.

Santonocito M, Vento M, Guglielmino MR, Battaglia R, Wahlgren J, Ragusa M, Barbagallo D, Borzi P, Rizzari S, Maugeri M*, et al.* Molecular characterization of exosomes and their microRNA cargo in human follicular fluid: bioinformatic analysis reveals that exosomal microRNAs control pathways involved in follicular maturation. *Fertil Steril* 2014: **102**; 1751-U1590.

Schwarz A, Wennemuth G, Post H, Brandenburger T, Aumuller G, and Wilhelm B. Vesicular transfer of membrane components to bovine epididymal spermatozoa. *Cell Tissue Res* 2013: **353**; 549-561.

Siciliano L, Marciano V, and Carpino A. Prostasome-like vesicles stimulate acrosome reaction of pig spermatozoa. *Reprod Biol Endocrinol* 2008: **6**; 5.

Sohel MM, Hoelker M, Noferesti SS, Salilew-Wondim D, Tholen E, Looft C, Rings F, Uddin MJ, Spencer TE, Schellander K*, et al.* Exosomal and non-exosomal transport of extra-cellular microRNAs in follicular fluid: implications for bovine oocyte developmental competence. *PLoS One* 2013: **8**; e78505.

Stenqvist AC, Nagaeva O, Baranov V, and Mincheva-Nilsson L. Exosomes secreted by human placenta carry functional Fas ligand and TRAIL molecules and convey apoptosis in activated immune cells, suggesting exosome-mediated immune privilege of the fetus. *J Immunol* 2013: **191**; 5515-5523.

Sullivan R and Saez F. Epididymosomes, prostasomes, and liposomes: their roles in mammalian male reproductive physiology. *Reproduction* 2013: **146**; R21-35.

Sullivan R, Saez F, Girouard J, and Frenette G. Role of exosomes in sperm maturation during the transit along the male reproductive tract. *Blood Cells Mol Dis* 2005: **35**; 1-10.

Sun D, Zhuang X, Xiang X, Liu Y, Zhang S, Liu C, Barnes S, Grizzle W, Miller D, and Zhang H-G. A Novel Nanoparticle Drug Delivery System: The Anti-inflammatory Activity of Curcumin Is Enhanced When Encapsulated in Exosomes. *Mol Ther* 2010: **18**; 1606-1614.

Takahashi Y, Nishikawa M, Shinotsuka H, Matsui Y, Ohara S, Imai T, and Takakura Y. Visualization and in vivo tracking of the exosomes of murine melanoma B16-BL6 cells in mice after intravenous injection. *J Biotechnol* 2013: **165**; 77-84.

Tapia J, Macias-Garcia B, Miro-Moran A, Ortega-Ferrusola C, Salido G, Pena F, and Aparicio I. The membrane of the Mammalian spermatozoa: much more than an inert envelope. *Reprod Domest Anim* 2012: **47 Suppl 3**; 65-75.

Tardif S, Madamidola OA, Brown SG, Frame L, Lefievre L, Wyatt PG, Barratt CL, and Martins Da Silva SJ. Clinically relevant enhancement of human sperm motility using compounds with reported phosphodiesterase inhibitor activity. *Hum Reprod* 2014: **29**; 2123-2135.

Taylor DD, Akyol S, and Gercel-Taylor C. Pregnancy-associated exosomes and their modulation of T cell signaling. *J Immunol* 2006: **176**; 1534-1542.

Taylor U, Barchanski A, Petersen S, Kues WA, Baulain U, Gamrad L, Sajti L, Barcikowski S, and Rath D. Gold nanoparticles interfere with sperm functionality by membrane adsorption without penetration. *Nanotoxicology* 2014: **8 Suppl 1**; 118-127.

Taylor U, Garrels W, Barchanski A, Peterson S, Sajti L, Lucas-Hahn A, Gamrad L, Baulain U, Klein S, Kues WA*, et al.* Injection of ligand-free gold and silver nanoparticles into murine embryos does not impact pre-implantation development. *Beilstein J Nanotech* 2014: **5**; 677-688.

Telfer EE and McLaughlin M. Strategies to support human oocyte development in vitro. *Int J Dev Biol* 2012: **56**; 901-907.

Tian YH, Li SP, Song J, Ji TJ, Zhu MT, Anderson GJ, Wei JY, and Nie GJ. A doxorubicin delivery platform using engineered natural membrane vesicle exosomes for targeted tumor therapy. *Biomaterials* 2014: **35**; 2383-2390.

Tiedemann D, Taylor U, Rehbock C, Jakobi J, Klein S, Kues WA, Barcikowski S, and Rath D. Reprotoxicity of gold, silver, and gold-silver alloy nanoparticles on mammalian gametes. *Analyst* 2014: **139**; 931-942.

Toro E, Fernández S, Colomar A, Casanovas A, Álvarez JG, López-Teijón M, and Velilla E. Processing of semen can result in increased sperm DNA fragmentation. *Fertil Steril* 2009: **92**; 2109-2112.

Tsai N, Lee B, Kim A, Yang R, Pan R, Lee D-K, Chow EK, and Ho D. Nanomedicine for global health. *JALA* 2014; pii: 2211068214538263.

Ulbrich W and Lamprecht A. Targeted drug-delivery approaches by nanoparticulate carriers in the therapy of inflammatory diseases. *J R Soc Interface* 2010: **7 Suppl 1**; S55-66.

Wang J, Lundqvist M, Carlsson L, Nilsson O, Lundkvist O, and Ronquist G. Prostasome-like granules from the PC-3 prostate cancer cell line increase the motility of washed human spermatozoa and adhere to the sperm. *Eur J Obstet Gynecol Reprod Biol* 2001: **96**; 88-97.

Wang J and Sauer MV. In vitro fertilization (IVF): a review of 3 decades of clinical innovation and technological advancement. *Ther Clin Risk Manag* 2006: **2**; 355-364.

Xin H, Li Y, and Chopp M. Exosomes/miRNAs as mediating cell-based therapy of stroke. *Front Cell Neurosci* 2014: **8**; 377.

Yamauchi Y, Riel JM, and Ward MA. Paternal DNA damage resulting from various sperm treatments persists after fertilization and is similar before and after DNA replication. *J Androl* 2012: **33**; 229-238.

Yang NJ, Seol DW, Jo J, Jang HM, Yoon SY, and Lee DR. Effect of cell-penetrating peptide-conjugated estrogen-related receptor beta on the development of mouse embryos cultured in vitro. *Clin Exp Reprod Med* 2014: **41**; 1-8.

Yang XX, Hou XN, Xu B, Hao X, Jiang GJ, and Fan TJ. Cell-penetrating peptide delivery of biologically active oct4 protein into cultured Takifugu rubripes spermary cells. *J Fish Biol* 2014: **85**; 1369-1380.

Yelumalai S, Jones C, Mounce G, McVeigh E, Fatum M, and Coward K. Levels of the oocyte activation factor, phospholipase c zeta, in human sperm are reduced following incubation with polyvinylpyrrolidone (PVP). *Fertil Steril* 2013: **100**; S88.

Yun JI, Gong SP, Song YH, and Lee ST. Effects of combined antioxidant supplementation on human sperm motility and morphology during sperm manipulation in vitro. *Fertil Steril* 2013: **100**; 373-378.

Zhuang X, Xiang X, Grizzle W, Sun D, Zhang S, Axtell RC, Ju S, Mu J, Zhang L, Steinman L*, et al.* Treatment of brain inflammatory diseases by delivering exosome encapsulated anti-inflammatory drugs from the nasal region to the brain. *Mol Ther* 2011: **19**; 1769-1779.

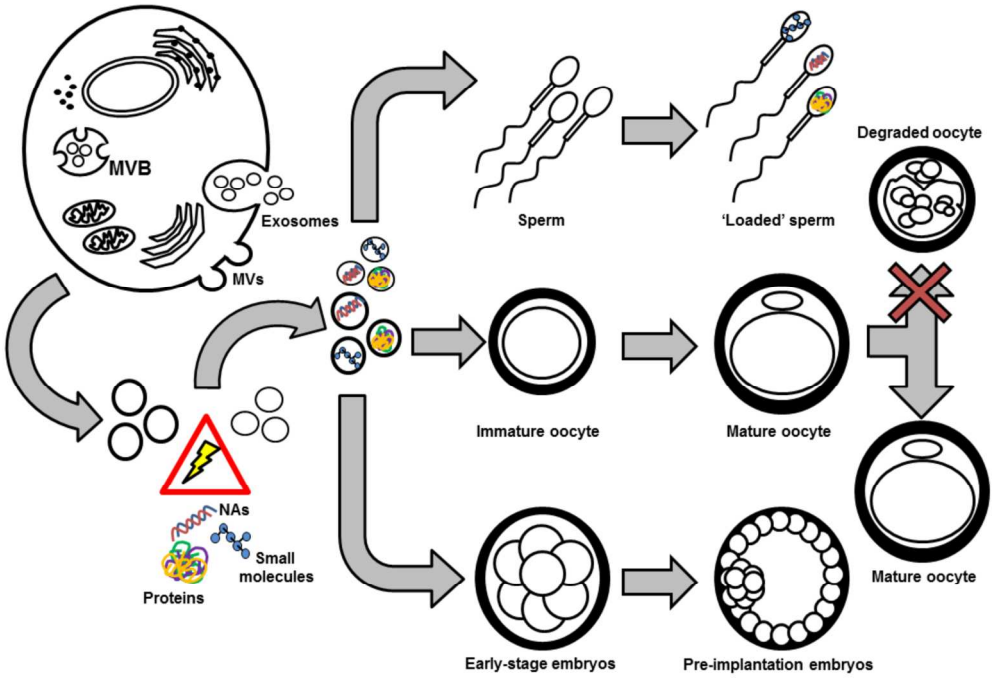

Figure 1
190x131mm (300 x 300 DPI)