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Nanotechnology in Reproductive Biotechnologies: Colloid Centrifugation for the Diagnosis and Treatment of Fertility in Animals

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ABSTRACT

Nanotechnology is employed in sperm preparation for assisted reproduction in animals, in the form of colloid centrifugation using coated-silica nanoparticles. The sperm sample is layered on top of the colloid; robust spermatozoa pass through the colloid whereas less robust spermatozoa are retained the colloid/semen interface or within the colloid layer. This method appears to be one of the most suitable sperm preparation techniques for animal semen since it is possible to process large volumes of semen without losing too many spermatozoa, producing highly fertile samples. Robust spermatozoa, with good motility, intact membranes, an unreacted acrosome, and intact chromatin, can be separated from the rest of the ejaculate, thus enhancing fertility when used in reproductive biotechnologies. These spermatozoa tend to produce less hydrogen peroxide than unselected samples, thus enhancing their quality and extending their shelf-life in storage. Furthermore, sperm cryopreservation is enhanced by selection prior to freezing. Sperm samples for artificial insemination, *in vitro* fertilization or intracytoplasmic sperm injection can be prepared with this method. The method can also be used therapeutically, as an indicator of fertility, to treat fertility issues and for conservation breeding. Furthermore, spermatozoa can be separated from viruses and bacteria in the ejaculate, thus improving biosecurity and potentially reducing the use of antibiotics. The latter can contribute to slowing the development of antimicrobial resistance.

INTRODUCTION

Nanotechnology has been applied in the field of assisted reproduction for several decades as a technique for selecting the most robust spermatozoa from a semen sample using a colloid, e.g. Bolton & Braude [1]. The evidence suggests that the spermatozoa selected in this way have the best chance of fertilizing and activating an oocyte, and of contributing to the subsequent development of the embryo. The history of nanotechnology in assisted reproduction has been covered in previous articles, e.g. Morrell & Rodriguez-Martinez [2], Morrell et al. [3]; the purpose of the current review is to focus on the diagnosis and treatment of fertility issues in livestock and horses, with some extrapolations to exotic animals. First there will be an explanation of the reproductive biotechnologies concerned, followed by a brief description of sperm selection in the animal and the various selection methods that are available *in vitro*, as an introduction to how nanoparticles function in reproductive biotechnologies. The remainder of the review will focus on how colloids can be used therapeutically in this context, namely for diagnosis and treatment of reproductive issues, as well as in

conservation biology. In addition, the final section provides some thoughts on the role of colloids in pathogen removal and in helping to combat antimicrobial resistance.

Reproductive biotechnologies in animals

Reproductive biotechnologies are various technologies that are utilised in living animals to facilitate conception and establishment of a pregnancy. By far the most frequently used biotechnology in animal breeding is Artificial Insemination (AI) [4]; in Europe and North America, the majority of dairy cattle, pigs and turkeys are bred by AI, with smaller number of beef cattle bred by this technique, principally in South America, and also sheep [5]. Standard bred horses are also bred by AI but, at the present time, it is not permitted to register thoroughbreds for racing if they have been produced by AI according to the Jockey Club registry [6]. This biotechnology may be used occasionally in other species, such as goats, deer, rabbits, dogs, cats and camelids [5], although details such as the timing of AI relative to ovulation, optimal sperm numbers per AI dose, number of AIs needed per cycle, have yet to be established in some of these species.

Artificial insemination is not the only reproductive biotechnology to be used in animals. Production of embryos outside the body – *In Vitro* Fertilization (IVF) – followed by transfer to a recipient female, is practiced in the cattle breeding industry [7] for the production of genetically elite offspring i.e. those with desirable production traits in terms of milk production or carcass composition [8]. The oocytes are either recovered from a donor animal or from slaughterhouse material. After maturation, fertilization and development, the resulting embryos are transferred to suitable recipients or frozen for later transfer [8]. As yet, it has not been possible to develop a technique for equine IVF that is repeatable and reliable [9]. However, it is possible to produce embryos by Intra Cytoplasmic Sperm Injection (ICSI), where a single spermatozoon is injected into the cytoplasm of an oocyte [5]. This is a time-consuming and technically demanding procedure but commercial companies exist to provide such a service. On the other hand, ICSI is not very successful in cattle, possibly due to the size of the bovine sperm head [10], or lack of activation of the injected oocyte by the spermatozoon [11].

All of these "embryo" technologies require a source of functional spermatozoa. The ability of the spermatozoa to reach the oocyte, fertilize and activate it [12], and to direct further development is crucial to the success of the procedure. Cryopreserved sperm stocks are ideal since spermatozoa are always available when required, but unfortunately, with the exception of bull spermatozoa, the spermatozoa of other species are highly susceptible to damage during freezing and thawing, thereby reducing their survival and functional capacity [13]. Whichever reproductive biotechnology is chosen, it is not possible to obtain good quality embryos successfully without good quality gametes [14]. Therefore, a method of sperm selection is required to improve the chances of success.

Sperm selection

The ejaculate typically consists of a heterogeneous population of spermatozoa of different ages and stages of maturity [3]. This population may include some damaged spermatozoa. After deposition in the female, the spermatozoa have to reach the uterotubal junction where they enter the epithelial crypts and form the sperm reservoir, where they undergo maturational changes to be able to (eventually) fertilize an oocyte [15]. On ovulation, some spermatozoa are released from these reservoirs and travel onwards to meet the oocyte. Here, the few spermatozoa that are ready to bind to the zona pellucida at exactly the right moment compete for a chance to fertilize the oocyte [16]. There are various hindrances within the female reproductive tract that must be circumvented in order to reach the site of fertilization [17]. These include the anatomy of the female reproductive tract itself [18], the ability of the spermatozoa to interact with it, the changing physical environment, and the timing of sperm maturation relative to ovulation [19]. These various aspects result in selection of spermatozoa possessing certain physical characteristics at a particular moment in time, so that only the "best" spermatozoon will fertilize the oocyte [15].

Various studies have shown that the proportion of spermatozoa with certain characteristics is correlated with the fertility of that ejaculate after insemination [20]. Thus, normal morphology is positively correlated with pregnancy rate after AI [21,22],

whereas the proportion of spermatozoa with DNA strand breaks is negatively correlated with pregnancy rate after AI [23]. Other parameters that are associated with fertility include membrane integrity, acrosome status and mitochondrial membrane potential [24].

When reproductive biotechnologies are used, some or all of the natural sperm selection mechanisms are avoided [25]. This increases the chance that any spermatozoon could fertilize an oocyte, regardless of their ability to direct further development of the fertilized oocyte [16]. Thus, to minimise the chance of fertilization by a spermatozoon that will be unable to fulfill its ultimate objective, undesirable spermatozoa can be removed using a variety of sperm selection techniques that mimic the selection techniques occurring within the female-so-called biomimetic techniques [2]. These involve identifying spermatozoa with desirable characteristics and separating them from the rest of the ejaculate.

Biomimetic methods of sperm selection

Biomimicry is the use of a technique that mimics a process taking place in a living animal [2]. Thus, biomimetic methods of sperm selection mimic the selection process occurring in the female. The idea of selecting sub-populations of spermatozoa possessing certain characteristics is not new [2]. The earliest method attempted was migration, where spermatozoa that are motile are able to move away from the rest of the ejaculate into a new layer of medium [26]. However, motile spermatozoa may possess other defects, such as abnormal morphology or DNA strand breaks, which will adversely affect fertility [23]. In addition, the method is time consuming and only approximately 10% of the sperm population is recovered [27]. Therefore, other methods that select for other characteristics may be better suited to sperm selection from poor quality ejaculates. However, if the desired outcome is to separate some motile spermatozoa from seminal plasma (which contains decapacitation factors) and cryopreservation medium, then a migration method might suffice. Some IVF laboratories use this method when preparing bull spermatozoa for IVF [28]. Another method of sperm selection is filtration e.g. through sephadex gel [29] or glass wool [30]. In this case, spermatozoa with defective membranes or reacted acrosomes cannot pass through a column of the material, so that the subpopulation that does pass through is enriched for spermatozoa with intact membranes and unreacted acrosomes. Although several studies have been published using this method for sperm selection in a variety of species in the laboratory [31], there are few reports of actual field use. Again, some IVF laboratories use glass wool when preparing their sperm samples [32].

In contrast to filtration through Sephadex gel or glass wool, colloid centrifugation has been used to prepare sperm samples both for IVF and for AI [3]. In this method, the sperm sample is pipetted on top of one or more layers of colloid (single layer centrifugation or density gradient centrifugation, respectively). During gentle centrifugation, some spermatozoa pass through the colloid and form a sperm pellet. Depending on the centrifugation conditions, the sperm pellet typically contains a sub-population that is enriched for motile, morphologically normal spermatozoa with intact membranes, unreacted acrosomes and intact chromatin compared to the unselected sample [2]. Selection is due to the ease with which the spermatozoa can pass between the colloid particles, with less robust spermatozoa being retained at the colloid/semen interface or trapped within the colloid layer. Increasing the centrifugation force or time, or using a low density colloid, permits more of the spermatozoa to pass through [33] but there is less selection for the best spermatozoa. Although the selected samples obtained are highly enriched for spermatozoa with desirable characteristics, there will be some loss of good spermatozoa, depending on the quality of the original ejaculate [34].

The colloids that are commonly used in reproductive biotechnologies consist of silane-coated silica nanoparticles suspended in a buffered salt solution. Examples of these are polyvinyl pyrrolidone-coated silica and silane coated silica [25]. It is important that the buffered salt solution should have the correct physical properties for the specific semen sample (e.g. osmolarity and pH), since semen has different physical characteristics depending on the species from which it originates [35]. Changes in the osmolarity and pH of the surrounding medium can cause sperm capacitation-like changes to occur. Magnetic activated cell sorting can be used to select

spermatozoa with intact membranes [36]. Sperm membrane disruption allows certain binding sites to be exposed which can then bind to antibodies such as Annexin V. If these antibodies are coated on to magnetic beads, passing the sperm suspension through a magnetic field will allow the spermatozoa that are bound to Annexin V to be retained in the column whereas those with intact membranes pass through. Although there have been several reports of this technique being used for human spermatozoa [37], it does not appear to have found a niche in the animal breeding industry.

The ability of spermatozoa to swim against fluid flow (rheotaxis) has been utilized as a method of selection [38]. However, although differences in sperm velocity were seen between spermatozoa that exhibited rheotaxic flow compared to those that did not, no differences in sperm morphology or in hyaluronic acid binding ability were seen between the different groups.

Human spermatozoa have been selected for ICSI using a microfluidic chamber [39]. The selected spermatozoa were reported to have better morphology and DNA integrity than unselected spermatozoa. However, at present the method is probably of limited use for animal semen since only 560 µL of semen at a low sperm concentration could be processed. Animal semen generally has a larger volume and much higher sperm concentration than human semen [2].

Use of colloid centrifugation as a diagnostic tool

The preceding section dealt briefly with the natural selection of sperm populations occurring in the female and the mechanism of action of nanoparticles when used for biomimetic sperm selection. The next section will deal with how nanoparticles can aid in the diagnosis of sperm fertility.

The ability to identify sub-fertile males at an early stage would be of considerable benefit to breeding companies [40]. Although laboratory assays have been developed as an indirect assessment of sperm function, the ability to predict pregnancy outcomes remains elusive [41], partly because of the multifactorial nature of the establishment of pregnancy but also because sperm function itself depends on many aspects [3]. Currently subfertile individual animals are usually identified by observing a lower than expected pregnancy rate in inseminated females, resulting in an economic loss to the animal owner and increased cost for the consumer [40]. Identifying potentially subfertile stud animals allows such an animal to be replaced with another one, if appropriate. Sustainable livestock production for meat and milk requires that non-productive animals are removed from the breeding herd to reduce consumption of natural resources and possible production of greenhouse gases [42].

As already mentioned, sperm characteristics such as normal morphology and chromatin integrity are closely linked to fertility after AI [23]. It was observed previously that the size of the sperm pellet after colloid centrifugation of stallion semen varied considerably between individual ejaculates, and seemed to be related to the quality of the original ejaculate [43]. Thus, sperm samples that were poorly motile or contained a high proportion of morphologically abnormal spermatozoa would produce a smaller sperm pellet than sperm sample that were highly motile and had a high proportion of morphologically normal spermatozoa. Therefore, an experiment was conducted to determine whether the number of stallion spermatozoa passing through the colloid was related to pregnancy rate after AI using the original (unselected) semen. The results indicated that the number of spermatozoa in the pellet was indeed linked to the fertility of the original ejaculate, and was actually more closely linked to fertility than any of the indirect assays on their own [43]. Therefore, using a standard protocol for colloid centrifugation, it is possible to predict the likely fertility of a stallion or a given semen sample, provided that the mare to be inseminated is of normal fertility.

If a potential fertility issue is identified with a given sperm samples or male, one has the option to investigate further to try to identify the likely cause. If it is a so-called “compensable” defect [44], one can increase the insemination dose in an effort to provide more spermatozoa that have a chance to reach the site of fertilization. Alternatively, having identified a male with a fertility issue, one could choose a reproductive biotechnology that is likely to circumvent the problem, depending on the species. Thus, for example, if a stallion has few motile spermatozoa, the chances of a pregnancy after insemination are low. The chances could be increased by resorting to ICSI [45]. However, the owner should be informed that the chances of producing another subfertile individual could be increased,

depending on the nature of the fertility problem. This may not be a problem if the horse is destined to be a sport horse but could be a problem for a stud animal. Such a solution is not practical in livestock species, where males are usually kept for long periods only as stud animals; hence keeping a subfertile animal or breeding more animals with the same fertility issue are not viable options.

A preliminary experiment with boar semen indicated that the number of spermatozoa in the sperm pellet was correlated with litter size after insemination with the unselected ejaculate, although there was no correlation with farrowing rate [46]. However, more studies are needed to determine the usefulness of colloid centrifugation, in particular centrifugation through a single layer of colloid (SLC) as an indicator of boar fertility.

Treatment of fertility issues

As previously mentioned, fertility is associated with various sperm quality parameters, e.g. pregnancy rate is positively correlated with the proportion of morphologically normal spermatozoa in a sample, and negatively correlated with the proportion of spermatozoa with fragmented chromatin [23]. Therefore, any sperm selection method that enriches for spermatozoa with normal morphology and intact chromatin should enhance fertility rates. Colloid centrifugation has been shown to select for stallion spermatozoa with both of these traits, and the number of females was increased compared to control samples when selected sperm samples were inseminated [47]. This result is interesting since the trial used stallions that were considered to have "normal" fertility, i.e. no fertility issue was apparent when their semen was used for insemination in previous breeding seasons. Thus, pregnancy rates can be improved by colloid centrifugation, even when semen from normal stallions is used. Previous results had shown an improvement in pregnancy rates when ejaculates from five stallions known to have poor sperm quality were prepared by colloid centrifugation [48].

The most likely explanation for the enhanced pregnancy rate when semen from normal stallions is prepared by colloid centrifugation is due to the considerable reduction in hydrogen peroxide in the selected samples [49]. Hydrogen peroxide is known to damage sperm membranes and DNA, and levels can be increased in stored semen samples. Selected stallion sperm samples were shown to survive much longer than controls (at least 5 days compared to 1.0-1.5 days), and to have "normal" fertility when used for insemination after storage [50].

Exotic animals and Conservation breeding

Fertility issues can arise when attempting to breed exotic animals, or rare breeds and endangered species. These individuals will not have been selected for good sperm quality, and the choice of males is limited [7]. The animals may be very old or very young, both of which are known to affect sperm quality, and they may be geographically distant from suitable females [2]. Sperm cryopreservation techniques may not have been optimised for the species concerned, and post-thaw sperm quality may be poor. Colloid centrifugation can help to improve sperm quality arising from all of these issues.

Poor quality sperm samples: Males of the more common livestock species have been selected for good sperm quality over many decades. This is not the case for rare breeds, where sperm quality may be poor. Males with poor sperm quality do not tend to produce many offspring, which is a problem for conservation breeding [7]. Colloid centrifugation can help to improve sperm quality in these cases and thus increase the chance of producing offspring. Tipkantha et al. [51] showed an improvement in sperm quality from clouded leopards when colloid centrifugation was used to prepare the semen, and sperm quality was also better than when a sperm washing procedure was used.

A different problem is encountered in camelids. Dromedaries are kept as dairy animals by many pastoralists in the Middle East and Africa [52], whereas alpacas are kept for their fleece and meat in South American countries [53]. Artificial insemination is not commonly used when breeding these animals because the seminal plasma is extremely viscous, creating difficulties in handling the semen [54]. Since it is difficult to produce representative sperm doses for AI, the details of this technique have not been optimised, such as the number of spermatozoa needed and the timing of sperm deposition relative to ovulation [55]. Although some researchers claim that using enzymes to break up the viscous seminal plasma liberates the spermatozoa, others consider that these enzymes can damage the spermatozoa [56]. It has been possible to prepare dromedary camel spermatozoa for AI using a

combination of gentle repeated aspiration with a plastic pipette to liberate the spermatozoa followed immediately by colloid centrifugation to separate the spermatozoa from the seminal plasma, which otherwise tends to coagulate again [57]. It has even been possible to freeze spermatozoa prepared in this way and to obtain offspring after insemination of thawed samples [58]. It remains to be seen whether alpaca semen can be prepared in a similar manner.

Sperm cryopreservation: Apart from bull spermatozoa, which generally show good cryosurvival, spermatozoa of most other species are difficult to cryopreserve successfully with maintenance of sperm function. Colloid selection of robust spermatozoa prior to cryopreservation improves post-thaw sperm quality [34,46].

To improve post-thaw sperm quality: Sperm quality in thawed sperm samples can be improved by colloid centrifugation, in a similar manner to fresh sperm samples. Jiminez-Rabadan et al. [59] improved the quality of thawed ram semen from an rare goat breed, while Cai et al. [60] improved the quality of thawed Giant panda semen.

Removal of microorganisms

Although AI was first developed as a means of controlling disease transmission, by minimising contact between animals during breeding, it is still possible to spread viruses or bacteria in insemination doses [61]. Males for semen collection are usually tested for a variety of viral diseases before entering a semen collection programme and at regular intervals during the collection period, so that seropositive males can be identified and removed [5]. However, a newly infected male may shed virus in semen before an antibody response can be detected e.g. porcine, respiratory and reproductive syndrome virus, or the male may shed virus in semen for a prolonged period e.g. equine arteritis virus [62]. It was shown to be possible to reduce the level of virus in semen drastically by colloid centrifugation [62,63]. However, it is not certain whether residual virus would be capable of causing infection in an inseminated female since the threshold level for infection depends on many factors in conjunction with the number of virus particles, such as the immune status of the female, number of previous pregnancies etc.

Ejaculates from healthy animals contain some bacteria as contaminants [64]. These bacteria, arising from the environment or from the animal itself, colonise the lower reproductive tract and are transferred to semen during ejaculation [65]. Further contamination can occur during collection and handling, from personnel and from the environment. Currently the growth of these bacteria is controlled by adding antibiotics to the semen extender when preparing semen doses but this represents a non-therapeutic use of antimicrobial agents that may contribute to the development of antimicrobial resistance. Therefore, it is questionable whether the addition of antibiotics represents "prudent use". Colloid centrifugation was shown to be effective in separating spermatozoa from seminal plasma, in which most of the bacteria are found initially [64,66]. Other studies have shown similar reductions in bacterial numbers [67] but this may depend on the g force and centrifugation time employed [68]. Although it was not always possible to remove all bacteria, the method shows considerable promise as an alternative to antibiotics.

A further development in the fight against antimicrobial resistance involves the separation of sperm cells from seminal plasma using a low density colloid for SLC [65]. With this method, all the spermatozoa pass through the low density colloid, regardless of their quality. The purpose of this modification is solely to separate the spermatozoa from seminal plasma and it bacterial load. The advantage is that no spermatozoa are lost during the procedure, but there is no selection for good quality spermatozoa either. In this preliminary experiment, a considerable reduction in bacterial load was achieved, with no difference in sperm quality between the control and SLC treatment groups [65]. This result indicates that the centrifugation does not adversely affect sperm quality during subsequent storage.

CONCLUDING REMARKS

Colloid centrifugation has proved to be a useful technique when preparing spermatozoa for reproductive biotechnologies. Thus, nanoparticles can be utilised in both diagnosis and treatment of fertility issues in livestock, and in conservation breeding of rare breeds and endangered species. It has proved to be more useful than other sperm selection techniques, both in its ability to select robust spermatozoa that survive

storage or cryopreservation well, and in the numbers of spermatozoa recovered.

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CONFLICT OF INTEREST

The author is the developer and one of the patent holders of the colloids reported in this review.

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