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Practical implications of sperm selection techniques for improving reproduction

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Abstract

Sperm selection techniques are needed to separate spermatozoa from seminal plasma and extender for *in vitro* fertilization (IVF) and to improve sperm quality for a range of assisted reproduction techniques. Apart from sperm washing, which removes some but not all of the seminal plasma, the selection techniques that are currently used are mainly swim-up and colloid centrifugation; filtration through Sephadex columns or glass wool is seldom used in the field. Although swim-up can be used to prepare sperm samples for IVF, the low recovery rate and lack of selection for sperm quality other than motility make this technique ineffective for routine use. Colloid centrifugation is used to prepare semen for all types of assisted reproduction. The method has been scaled-up for voluminous ejaculates e.g. from stallion and boar, and scaled-down to accommodate small volumes of thawed semen (e.g. from bull). Sperm quality and fertility are improved, as shown in laboratory assays and in various fertility trials. Some normal spermatozoa are lost during the selection process but overall the advantages of improved longevity and fertility in the selected spermatozoa outweigh the disadvantages. Since spermatozoa are separated from bacteria in the ejaculate, it may be possible to reduce antibiotic usage in semen extenders. New applications of colloid centrifugation include extracting camelid spermatozoa from viscous seminal plasma, selecting spermatozoa with condensed chromatin (i.e. with fewer free thiols), and using the number of spermatozoa passing through the colloid as a diagnostic tool to indicate male fertility.

Keywords: chromatin integrity, colloid centrifugation, extended longevity, fertility, single layer centrifugation.

Introduction

Fertility in some species e.g. dairy cattle, has been declining in recent decades (Rodriguez-Martinez *et al*., 2007; López-Gatius, 2013). This decline may be partly due to negative energy balance in high yielding dairy cows (Diskin and Morris, 2008), but other factors may also be involved. For example, the number of bull spermatozoa included in an insemination dose has decreased, especially for sexed sperm samples (Seidel, 2014); two million spermatozoa may not be sufficient to give good pregnancy rates for all bulls (Andersson *et al*., 2004). In addition, changes have been made to freezing protocols, such as the avoidance of extenders containing egg yolk (Leite *et al*., 2010; Röpke *et al*., 2011) and increasing pre-freezing equilibration times (Leite *et al*., 2010; Shahverdi *et al*., 2014). In horse breeding, there has been a decrease in the foaling rate after artificial insemination (AI) to approximately 65% (Rota *et al*., 2004) but this could be due to the more widespread use of cooled shipped semen nowadays, or to a real decrease in fertility. The porcine AI industry still uses liquid (fresh) semen for AI in preference to cryopreserved semen (unless for export), because pregnancy rates and litter sizes are perceived to be better with fresh semen, despite considerable progress in developing cryopreservation protocols that result in more consistent sperm survival (Roca *et al*., 2006.)

One point is certain, however: good quality gametes are needed to produce good quality blastocysts (Vandaele and Van Soom, 2011; Morrell *et al*., 2016). Embryo quality, assessed using the guidelines established by the International Embryo Transfer Society (Stringfellow and Seidel, 1998), was linked with oocyte quality following embryo production *in vitro* in pigs (Chen *et al.,* 2012), cattle (O´Hara *et al*., 2014; Saini *et al*., 2015), stallions (Colleoni *et al*., 2011 and human patients (Kurosawa *et al*., 2016).

Many studies have attempted to define the factors contributing to "good quality" spermatozoa: *in vivo*, bull sperm morphology was correlated with pregnancy rate (Attia *et al*., 2016; boar sperm fertility was linked independently with morphology, active mitochondria, beat cross frequency and oscillatory frequency (Schultze *et al*., 2013). The DNA integrity of stallion spermatozoa was correlated with pregnancy rate (Cuervo-Arango *et al*., 2009; Morrell *et al*., 2014c; Barrier Battut *et al*., 2016), whereas Underwood *et al*. (2010) linked the stresses of sex-sorting and re-freezing bull spermatozoa with low pregnancy rate. Ohlweiler *et al*. (2013) showed that there was no difference in fertilization rate when semen from bulls of low vigor was used to fertilize good and poor quality oocytes, whereas for semen of high vigor, the blastocyst rate was higher for good quality oocytes than for poor quality oocytes.

Sperm selection can be used to improve sperm quality (Morrell and Rodriguez-Martinez, 2009), which is the subject of this review, although oocyte quality and sperm/uterine interaction are vital components in fertilization and embryo production. In this review, the impact of sperm quality on fertility will be described briefly, followed by a discussion of sperm selection

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techniques, especially colloid centrifugation. After describing the practical uses of colloid centrifugation, the implications of sperm selection by this method for animal breeding will be presented.

Relationship between sperm quality and fertility

Sperm quality is a term used to define the predicted functionality of a sperm population. It incorporates a number of components that affect the ability of the spermatozoa to move through the reproductive tract to locate and interact with the oocyte. These factors include sperm motility, morphology, membrane integrity, degree of apoptosis, acrosome integrity, capacitation, mitochondrial membrane potential, chromatin integrity, chromatin maturity, production of reactive oxygen species, and ability to bind to the zona pellucida. Relationships have been found between a number of these parameters and fertility, either *in vitro* or following AI; examples have been described in detail in another review (Morrell and Rodriguez-Martinez, 2009) and are summarized in Table 1.

Table 1. Examples of the relationship between parameters of sperm quality and fertility in various species.

Parameter	Reference
Normal morphology (cattle)	Wiltbank and Parrish, 1986; Menon *et al*., 2011
Normal morphology (pigs)	Tsakmakidis *et al*., 2010
Normal morphology (horses)	Parlevliet *et al*., 1999; Kavak *et al*., 2004
Chromatin integrity (boars)	Lopez Fernandez *et al*., 2008
Chromatin integrity (horses)	Love and Kenny, 1998
Chroamtin integrity (bulls)	Karoui *et al*., 2012
Acrosome integrity (buffalo)	Ahmed *et al*., 2003
Acrosome integrity (bulls)	Al-Mahkzoomi *et al*., 2008
Plasma membrane integrity (bulls)	Christiansen *et al*., 2011
Mitochondrial membrane potential	Pena *et al*., 2009
Reactive oxygen species	Aitken *et al*., 2012

Sperm selection techniques

Since sperm quality appears to affect fertility and therefore reproduction, selecting sperm sub-populations with certain characteristics known to be associated with fertility should lead to increased reproductive efficiency. Several sperm selection techniques are available, as reviewed previously (Morrell *et al*., 2016). The purpose of sperm selection techniques is to separate specific sperm sub-populations from the ejaculate and to separate them from seminal plasma. In this respect, laboratory procedures mimic events occurring in the female reproductive tract, whereby normal spermatozoa interact with parts of the female reproductive tract whereas abnormal spermatozoa do not (Suarez, 2007). The hypothesis is that this interaction with the female reproductive tract enables unsuitable spermatozoa to be removed, thereby prevented them from reaching the oocyte. Since laboratory selection techniques mimic a biological activity, they are known as "biomimetic".

Selection can be done by migration e.g. swim-up, by filtration e.g. through Sephadex or glass wool, or by colloid centrifugation. Cell sorting can be done in a flow cytometer or magnetic cell sorter, but these techniques are mostly too time-consuming to be used routinely, except on the larger semen collection stations. Swim-up is used mostly to prepare spermatozoa for *in vitro* fertilization or ICSI; colloid centrifugation is used for these applications and also to prepare semen for AI, especially the scaled-up versions (Morrell *et al*., 2009b), and for cryopreservation (Hoogewijs *et al*., 2011).

In the swim-up technique, collection medium is pipetted on top of extended semen in a centrifuge tube, which is then placed in an incubator. Motile spermatozoa move away from the rest of the sample and can be harvested from the collection medium. However, selection is only for sperm motility; there is no selection for normal morphology or chromatin integrity (Somfai *et al*., 2002). The procedure takes 45-60 min and results in the recovery of approximately 10% of the spermatozoa. Whilst such small sperm numbers may be adequate for IVF, they are clearly impractical when preparing spermatozoa for AI.

In filtration, spermatozoa pass through a column of Sephadex or glass wool; spermatozoa with damaged membranes or defective acrosomes bind to the Sephadex and are retained on the column (Bussalleu *et al*., 2008). Although a higher recovery rate is achieved with this technique than with swim-up, sperm quality may only be improved if there is a high proportion of damaged spermatozoa in the original sperm sample. This technique is used mainly in the laboratory or for research: there are no reports of its routine use under field conditions.

Colloid centrifugation involves layering extended semen over colloid e.g. silane-coated silica, in a centrifuge tube, followed by centrifugation for 20 min at a low g force, typically 300 g. After centrifugation, the sperm pellet contains mostly highly motile, morphologically normal spermatozoa with intact membranes and good chromatin integrity (Johannisson *et al*., 2009) that are not producing hydrogen peroxide (Morrell *et al*., 2017). Immotile or damaged spermatozoa are retained at the semen:colloid interface while seminal plasma remains above the colloid (boar semen; Kruse *et al*., 2011). This technique has been used to prepare stallion spermatozoa in the field for AI (Morrell *et al*., 2011a, 2014b) as well as bull and boar spermatozoa for IVF (Thys *et al*., 2009; Sjunnesson *et al*., 2013) or stallion spermatozoa for ICSI (Colleoni *et al*., 2011. Single Layer centrifugation (SLC) is a

modification of density gradient centrifugation (DGC) in which only one layer of colloid is used i.e. no density gradient is present. The advantage of SLC is that it is easier to use than DGC, requiring fewer steps in the preparation, and it can be scaled-up to process large volumes of semen e.g. 15-20 ml per 50 ml tube for stallion semen (Morrell *et al*., 2009b) or up to 150 ml per 500 ml tube for boar semen (Morrell *et al*., 2011b). It can also be scaled-down to process only 250 µl thawed bull semen on 1 ml colloid in a 15 ml tube (Abraham *et al*., 2016); a higher sperm yield is obtained if a 15 ml centrifuge tube is used rather than a small (1.5 ml) tube.

Practical uses of colloid centrifugation

For in vitro *fertilization or intracytoplasmic sperm injection*

When preparing spermatozoa for IVF or ICSI it is important to remove seminal plasma, which contains decapacitating factors, and to replace the cryopreservation medium with capacitating medium or fertilization medium. The simplest way to do this is by centrifugation to pellet the spermatozoa, followed by resuspension of the sperm pellet in the new medium. However, all the spermatozoa, including the dead and dying or abnormal spermatozoa, will appear in the pellet since there is no selection for normal spermatozoa with this method. Such centrifugation can also cause damage to intact spermatozoa and may result in the release of reactive oxygen species that attack sperm membranes and DNA.

An important consideration for IVF or ICSI is that the selection of normal spermatozoa that occurs in the female reproductive tract is not present (Suarez, 2007) potentially allowing abnormal spermatozoa or those with damaged chromatin to fertilize the oocyte (Rath *et al*., 2008). Although the oocyte is fertilized and activated, there may be problems due to the damaged chromatin during embryo development or even after implantation (Katari *et al*., 2009; Evenson, 2016). It can be speculated that this may be one of the reasons why so many conceptuses or early pregnancies are lost in cattle, since approximately 90% of dairy cow oocytes are fertilized but less than half of these result in the birth of a calf (Diskin and Morris, 2008; Lopez-Gatius, 2013).

Karoui *et al*. (2012 observed that sperm DNA fragmentation (chromatin dispersion test) could be used to identify the least fertile bulls in a cohort study. Evenson (2016) reviewed many studies on the association between %DFI (evaluated by the Sperm Chromatin Structure Assay) and fertility; he reported threshold values for %DFI in various species above which there was likely to be an impact on fertility as: pigs 6%, bulls 10-20%, horses 28%, humans 25-30%.

For artificial insemination

The ejaculate contains a heterogeneous population of spermatozoa at different stages of maturity, some of which may be abnormal. These abnormal spermatozoa are included in the insemination dose and thus may reduce the number of normal spermatozoa reaching the oviducts to below the threshold required in order to ensure fertilization. This is particularly important for cattle where the number of spermatozoa in the insemination dose has been reduced to a small fraction of those in the original ejaculate. Alternatively, spermatozoa with damaged chromatin may compete with those with intact chromatin to fertilize the oocyte. Selecting normal spermatozoa with good chromatin integrity would be an obvious way of circumventing this problem.

Does colloid centrifugation really overcome this problem? This question can be answered using stallion spermatozoa as an example. Usually stallion semen is inseminated within 24-36 h after semen collection, being stored in the meantime at approximately 6°C (Varner *et al*., 1987). There are occasional reports of pregnancies being achieved after longer periods of cooled storage but this is not usual within the equine breeding industry and not feasible for some stallions. For some stallion semen, it is not possible to cool it at all; it must be inseminated within a short time of collection in order to obtain pregnancies. Preparing stallion semen by SLC allows even spermatozoa from these so-called “poor coolers” to be cooled and transported to other stud farms for insemination after 24 h (Morrell *et al*., 2011a). Chromatin integrity is higher in SLC-selected samples, and this integrity is maintained during storage (Table 2). Semen from normal stallions, processed by SLC, survive for at least 96 h after semen collection (Richter *et al*., 2016) and have good fertilizing ability (Lindahl *et al*., 2012. In a controlled trial inseminating mares with cooled sperm samples 24 h after semen, a higher number of mares were pregnant with the SLC-selected spermatozoa (54/78) than with control (unselected; 37/82) sperm samples (Morrell *et al*., 2014b). The SLC-selected samples showed an increased proportion of morphologically normal, membrane-intact spermatozoa with good chromatin integrity than control samples (Morrell *et al*., 2009a; 2010). Interestingly, production of hydrogen peroxide is markedly decreased in these SLC-selected samples (Morrell *et al*., 2017), which may contribute to the longer survival and retention of fertilizing capacity compared to control sperm samples. *In vitro* fertilizing capacity is also enhanced in SLC-selected sperm samples; the number of SLC-selected boar spermatozoa used for IVF had to be drastically reduced to avoid polyspermy (Sjunnesson *et al*., 2013). The SLC preparation technique has also been used to prepare stallion spermatozoa for ICSI (Table 3).

Table 2. DNA fragmentation index (%DFI) in stallion sperm samples prepared by Single Layer Centrifugation compared to uncentrifuged controls immediately after preparation and again after 24 h storage at 6ºC.

Time after preparation (number)	Uncentrifuged	SLC
0 h (n = 144)	15.1 ± 9.3	10.2 ± 8.9
24 h (n = 77)	26.0 ± 14.0	13.9 ± 14.0

Modified from Morrell *et al*. (2010).

Table 3. Blastocyst development after intracytoplasmic sperm injection of equine oocytes, using spermatozoa prepared by colloid centrifugation.

Treatment	No. injected oocytes	No. blastocysts	Development to blastocyst (%)
DGC fertile (control)	17	4	21
SLC fertile (control)	21	4	19
DGC infertile	104	15	21
SLC infertile	107	21	30

Note: DGC = density gradient centrifugation, SLC = Single Layer Centrifugation; fertile and infertile refer to the fertility status of the two stallions used as a source of spermatozoa. From Colleoni *et al*. (2011).

In cryopreservation

Stallion spermatozoa selected by SLC show better cryosurvival than non-selected sperm samples (Hoogewijs *et al*., 2011) and may have longer post-thaw survival (Hoogewijs *et al*., 2012). This latter attribute may also be a result of decreased hydrogen peroxide production in the thawed sperm samples, although reactive oxygen species production was not measured in their study. However, in studies with boar spermatozoa, SLC-selected spermatozoa were found to produce less hydrogen peroxide than non-selected spermatozoa (Martinez-Alborcia *et al*., 2012); and showed increased cryosurvival (Martinez-Alborcia *et al*., 2012, 2013). A higher mitochondrial membrane potential was observed in frozen-thawed SLC-selected bull spermatozoa compared to controls (Nongbua *et al*., 2017). In a previous study with fresh bull spermatozoa, chromatin integrity was significantly better in SLC-selected samples compared to controls (Goodla *et al*., 2014).

Removal of bacteria

When considering semen quality, one factor that is often overlooked is the bacterial content of the semen (Morrell and Wallgren, 2014). Almost all ejaculates become contaminated with bacteria during semen collection and subsequent handling (Maes *et al*., 2008); a negative correlation has been found between bacterial contamination above a certain threshold and litter size in pigs (Maroto Martin *et al*., 2010). Therefore, antibiotics are added to semen extenders to control microbial growth. Nowadays, there is increasing awareness of antibiotic resistance arising from the widespread use of antibiotics, both in human and veterinary medicine (Catry *et al*., 2010). Genes for resistance spread between bacteria in different host species (Johansson *et al*., 2004). There are anecdotal accounts that many bacteria found in semen are resistant to the antibiotics that are added to semen extenders; therefore the widespread practice of adding antibiotics to semen extenders may contribute to the problem of antibiotic resistance. An alternative to antibiotics would be to physically separate spermatozoa from bacteria in semen. Colloid centrifugation removes, or substantially reduces, bacterial contamination in boar (Morrell and Wallgren 2011) and stallion semen (Morrell *et al*., 2014a). Such an alternative would seem to be an excellent solution to avoid the further development of antimicrobial resistance, particularly when one considers the scale of usage of antibiotic-containing extenders in the semen production industry.

Recent developments with colloid centrifugation

Camelid semen

Processing camelid semen is known to be a problem due to the high viscosity of the seminal plasma, which also prevents penetration of cryoprotectants into spermatozoa during freezing. One solution to this problem has been to use enzymes to break down the viscous seminal plasma, although there is concern that the enzymes might damage the spermatozoa. Llama semen processed SLC after treatment with enzymes resulted in the production of hatched blastocysts in IVF (Trassoras *et al*., 2012). Obviously it would be preferable to avoid enzymes completely, if at all possible, but separating the spermatozoa from the enzyme-containing medium after a short exposure appears to be an option. A further development is that dromedary camel semen, processed by SLC after mechanical breakdown of the viscous seminal plasma by gentle pipetting, survived freezing and thawing (Malo *et al*., 2017), and pregnancies have been obtained.

Indicators of fertility

It was reported previously that the number of spermatozoa passing through the colloid could be used as an indicator of potential fertility of the original ejaculate. This was found to be the case for stallions where the yield of spermatozoa after SLC was found to be related to the pregnancy rate of mares inseminated with cooled semen from the same ejaculates (Morrell *et al*., 2014b). In a similar study with boar semen, the number of spermatozoa passing through the colloid was

related to the pregnancy rate in inseminated sows (Martinez-Alborcia *et al*., 2017). Thus, SLC could be used to provide a rapid means of identifying less highly fertile boars without the necessity of waiting for the outcome of AI trials, to allow their removal from the breeding pool at an early stage.

Additional marker of chromatin integrity

Chromatin integrity can be evaluated either by means of the Sperm Chromatin Structure Assay or by the Chromatin Dispersion Assay. However, there are other methods of evaluating chromatin structure, e.g. by measuring free thiols (as an indicator of lack of disulfide bond formation). Preliminary studies with bull spermatozoa indicate that SLC-selected sperm samples have fewer free thiols than control samples (Fig. 1). If this result is confirmed in a larger sample size, it could indicate that SLC selects for mature spermatozoa with highly condensed chromatin.

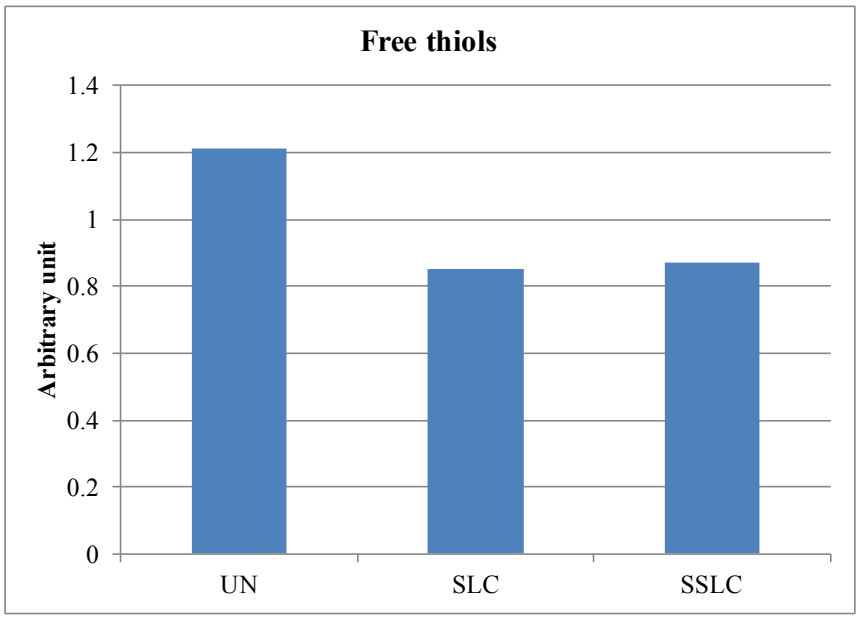

Figure 1. free thiols in SLC and control bull sperm samples. Note: un = control (uncentrifuged); SLC = single layer centrifugation; SSLC = stored for 24 h befor SLC.

Implications of colloid centrifugation to animal breeding

As described in the preceding section, colloid centrifugation has a number of practical uses; there are many advantages and some disadvantages to the technique, as summarized in Table 4. The main advantages are the improved sperm quality and fertilizing ability, which are retained for longer in the selected sperm samples than in unselected ones. However, since only good quality spermatozoa are selected, there can be a considerable reduction in sperm numbers during processing, depending on the quality of the original ejaculate (Hoogewijs *et al*., 2011; Martinez-Alborcia *et al*., 2013), and some good quality spermatozoa may be lost. The advantages outweigh the disadvantages: the vast majority of the spermatozoa recovered are functional and retain this functionality because they are not in the presence of damaged spermatozoa and hydrogen peroxide. Since the spermatozoa are highly fertile, it may be possible to reduce the number in the insemination dose. The small volume of colloid needed for the scaled-down version is economically more attractive than the larger volume when preparing spermatozoa for IVF or ICSI.

The ability to separate spermatozoa from seminal plasma without causing damage opens up the possibility of manipulating semen samples to obtain desirable attributes (de Graaf *et al*., 2008). This could involve substituting seminal plasma from an animal of low fertility (or poor freezability) with that from a high fertility (or good freezability) male (Morrell *et al*., 2014d; Nongbua *et al*., 2016a).

The possibility of separating spermatozoa from bacteria without the use of antibiotics has the potential to be of considerable importance, to slow the development of antimicrobial resistance. The animal breeding industry uses very large amounts of antibiotics at present, and there is a need to reduce this non-therapeutic use, which should be possible with SLC. The industry would also like to have more reliable indicators of a male´s potential fertility; by extrapolating from the results with stallion semen, SLC appears to offer such an indicator

Table 4. Advantages and disadvantages of colloid centrifugation for sperm preparation.

Advantages	Disadvantages
Species-specific colloid formulations are available commercially	Colloid is expensive
Technique is not complicated; little training is required	Centrifuge required; 20 minute centrifugation time
Highly fertile spermatozoa recovered	Some good quality spermatozoa are lost
Motile, morphologically normal spermatozoa with intact membranes are selected	
Spermatozoa retain fertilizing ability for longer than unselected spermatozoa	
Spermatozoa with damaged DNA are removed → embryo development not impeded	
Fewer spermatozoa may be needed in insemination dose	
Reduction in bacterial contamination can obviate the need for antibiotics	
May be used as an indicator of fertility	
Can be used to extract camel spermatozoa from viscous seminal plasma	
May select spermatozoa with highly condensed chromatin (fewer free thiols)	

Conclusions

Sperm selection techniques are used when preparing sperm samples for assisted reproduction. Single layer centrifugation is especially beneficial since it selects the best quality spermatozoa from the rest of the ejaculate and also separates them from contaminating bacteria. The technique is simple to use, even in the field, although some good quality spermatozoa are also lost during processing. The technique shows promise for handling semen from unusual species such as camelids, and may be used in other contexts such as to differentiate between elite males and less highly fertile ones. It has advantages over migration or filtration techniques in selecting for sperm chromatin integrity.

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