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Biomimetic Techniques for Improving Sperm Quality in Animal Breeding: A Review

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Abstract: Sperm quality in insemination doses is known to affect pregnancy rates following artificial insemination (AI) in a number of animal species. Furthermore, biotechnologies such as *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) require the spermatozoa to be removed from seminal plasma, which contains inhibitory substances, cellular debris and even pathogens. Several methods have been advocated to separate sperm cells from the rest of the ejaculate, and even to select the subpopulation of better quality spermatozoa by providing a physical barrier to the passage of abnormal, dead or dying spermatozoa. In this article, these methods are critically reviewed in the light of their suitability for use in the animal breeding industry, where requirements for efficiency, practicality and, above all, economy of the selection method are paramount. Colloid centrifugation, particularly the new "Single Layer Centrifugation" (SLC) technique, appears to be the most effective method both for separating spermatozoa from seminal plasma and for selecting the most robust spermatozoa. Furthermore, SLC can be scaled-up to enable large ejaculates to be processed easily.

1. INTRODUCTION

Much has been written on the topic of sperm quality in different species, including how it can be defined and measured. There have also been numerous reviews on the merits and demerits of various sperm selection techniques used to improve sperm quality when preparing human spermatozoa [1] or animal spermatozoa [2] for assisted reproduction (AR). However, animal breeders have different requirements to clinicians in human fertility clinics; first, there can be large differences in the number of spermatozoa needed for AR in humans (one to one hundred thousand motile spermatozoa for ICSI and IVF respectively, or $50x10^6$ motile spermatozoa for IUI) and those required for AI in animals (20×10^6 to several billion motile spermatozoa, depending on the species; [3]). Moreover, the volume of ejaculate to be processed is much larger in some animal species than in humans, e.g. 20-50 mL for stallion semen, 100-250 mL for the sperm-rich fraction of boar semen, compared to 1-3 mL in human ejaculates [4]. In addition, there is a difference in the cost of the technique which is appropriate in relation to the economic value of the insemination dose for animal semen. Therefore, a sperm preparation technique which is used in human AR may not be suitable for animal semen.

Rather than repeat previous reviews, the purpose of the current overview is to provide a brief description of the techniques available for selecting the best quality animal spermatozoa, concentrating on the usefulness and practicalities of these techniques as applied to animal breeding. First, however, it is relevant to define sperm quality, its potential impact on reproductive efficiency in animals, and why it is necessary to remove seminal plasma and select the best quality spermatozoa. Then the different methods available to achieve these aims will be described and discussed.

1.1. What is Sperm Quality?

Although there are different opinions on the concept of sperm quality, and possibly species differences in the relative importance of individual parameters, it is considered that sperm quality can be described in terms of sperm number, motility and morphological normality [5]. Others have added additional parameters, such as sperm membrane integrity and sperm chromatin integrity [6, 7]. However, while poor semen quality is usually linked to sub-fertility, the converse is not necessarily true (stallion: [5]; bull: [8]). In general, it is assumed that fertilisation *in vivo* should be achieved by highly motile spermatozoa with normal morphology, intact membranes and intact chromatin (represented by the area of overlap of all four circles in Fig. (**1**)). Therefore, we hypothesise that the higher the proportion of motile, viable, morphologically normal spermatozoa with intact chromatin in the insemination dose, the better the chances of pregnancy following AI. However, on semen collecting stations, insemination doses are usually calculated only on the basis of sperm motility (assessed subjectively) and sperm number [9]. Since there are many extraneous factors apart from sperm motility which influence fertility, highly variable pregnancy rates can result following insemination, particularly with stallion spermatozoa [10]. The use of sperm motility as the sole parameter for predicting sperm fertilising ability is not ideal or even reliable [3] but, in the absence of other reliable, rapid and cheap tests for more predictive parameters of sperm quality in the field, it remains the only feasible method of assessment for most commercial semen collection enterprises.

1.2. What is the Impact of Sperm Quality on Reproductive Efficiency?

Correlations have been found between a number of parameters of sperm quality, especially computer assisted

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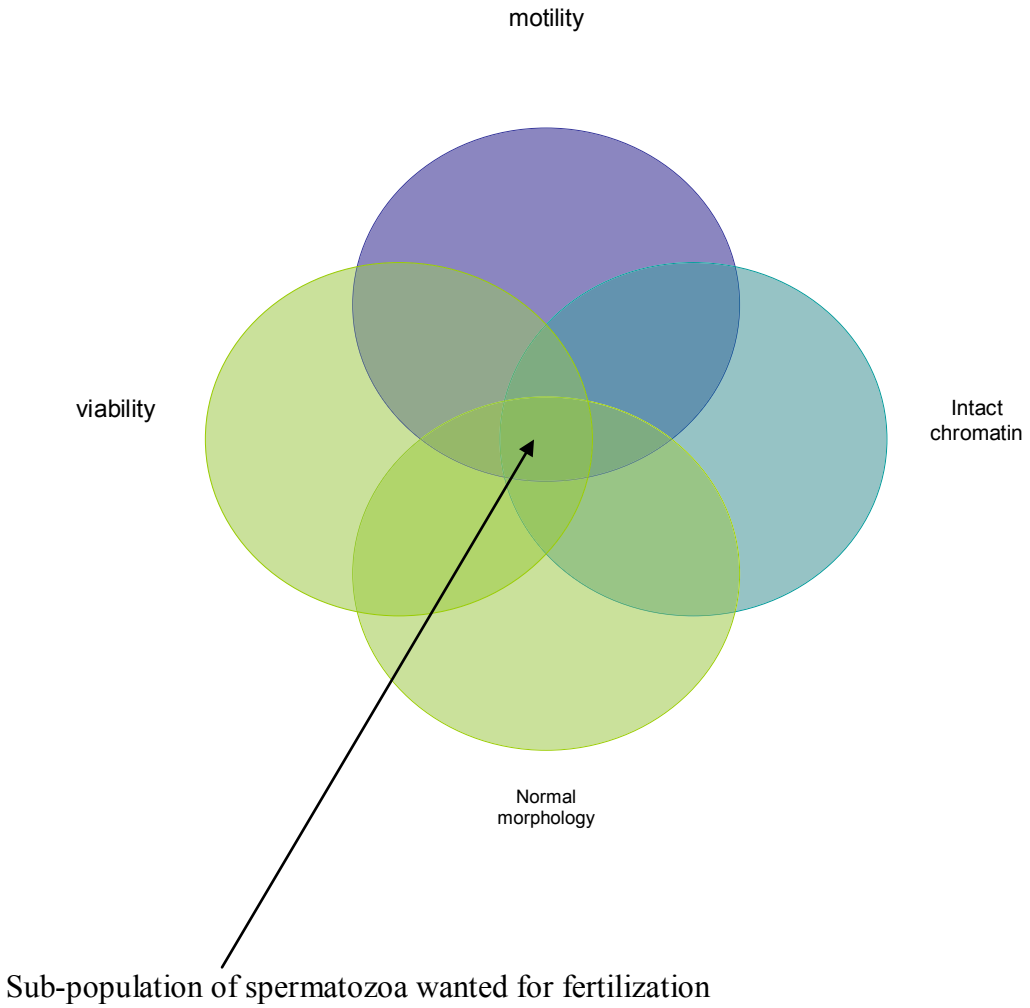

Fig. (1). Venn diagram to show different sub-populations of spermatozoa in the ejaculate based on parameters of sperm quality. Note: each colored circle represents a parameter of sperm quality. However, not all motile spermatozoa have normal morphology or intact chromatin or may be viable. Therefore, it is only the sub-population of spermatozoa which are motile AND viable AND have normal morphology AND intact chromatin which are wanted for fertilisation, represented by the area of overlap of all four circles (arrow). Thus for a semen dose which contained one billion motile spermatozoa when prepared shortly after collection, but which had motility of 50%, normal morphology of 60%, viability of 52.5% and chromatin integrity of 70.5% after 24 h, i.e. at the time of insemination, there would only be 177.7 million spermatozoa which fitted the above criteria.

sperm analysis (CASA) kinematics, and *in vitro* fertilization [11], and also between some parameters of sperm quality (normal morphology, chromatin integrity) and pregnancy rates following AI (boars: [12], stallion: [13, 14]). However, there are more challenges to be overcome by spermatozoa before fertilisation can occur *in vivo* than *in vitro* [15]. Therefore, the same parameters of sperm quality should not be used to predict fertilisation rates for the two situations.

There is a strong relationship between fertility and post-thaw motility, proportion of normal acrosomes, intact plasma membranes and sperm abnormalities in a number of species e.g. buffalo [16], bulls [17], and between morphology, chromatin integrity and pregnancy rates in stallions [14]. However, the relationship between sperm motility and pregnancy rates seems to be particularly poor for stallion spermatozoa, although whether this is due to sperm factors or female factors is unknown.

Males used as semen producers in the AI industry do not always produce semen of the desired quality [18]. There may be a transient loss of quality at any age, for example in sperm numbers, morphology or motility [19], but eventually there is a progressive and irreversible decline in semen quality as the male ages [20, 21].

1.3. Why Remove Seminal Plasma and Select Good Quality Spermatozoa?

The presence of seminal plasma is considered to be beneficial to sperm function but detrimental to sperm survival. Seminal plasma is known to contain many factors which help to retain sperm function e.g. decapacitation factors [22, 23], but also other substances e.g. sperm motility inhibiting factor [24] which has a restraining effect on sperm motility *in vitro*, and reactive oxygen species (ROS), which are detrimental to long-term sperm survival [25]. ROS are produced by leukocytes and cellular debris and, more importantly for most animal sires, cytoplasmic remnants in immature sper-

matozoa, or by damaged and dead spermatozoa. However, it is not known which seminal plasma fractions contain these antioxidants, nor which accessory glands produce them. High levels of ROS are associated with infertility in humans [26], although a low level of ROS is considered to be a prerequisite for the sperm membrane changes occurring prior to fertilisation [27, 28]. In contrast, fertilisation in porcine IVF may be improved by reducing ROS levels with superoxide dismutase or catalase [29].

It has been shown that removal of most, but not all, of the seminal plasma can improve sperm survival during cool storage and cryopreservation in some species, e.g. stallion [27, 30] and boar [31]. The effects of seminal plasma on ruminant spermatozoa [32] and on boar spermatozoa [33] have been reported, including the effects of adding back seminal plasma to processed spermatozoa just prior to AI.

Seminal plasma may also transfer a variety of pathogens to the female reproductive tract [34]. Important animal viruses transmitted in this way, as well as by other routes, include porcine respiratory and reproductive syndrome virus, bovine viral diarrhoea virus and equine viral arteritis virus, among others. Pathogenic bacteria include *Ureaplasma urealyticum, Klebsiella* spp. and *Taylorella equigenitalis*, the causative agent of contagious equine metritis, among others.

In vivo, spermatozoa rapidly migrate away from seminal plasma at the site of semen deposition and make their way to the site of fertilization over a restricted period of time, during which they undergo a series of changes to prepare the membranes for eventual binding to the zona pellucida (capacitation and eventually the acrosome reaction) [35]. Since there are inhibiting factors in seminal plasma, such as decapacitating proteins, it is essential that the spermatozoa are removed from their influence before they reach the oocyte. In addition, some parts of the female reproductive tract are thought to represent a barrier to the passage of spermatozoa, thus creating a passive filtering system beyond which poorly viable spermatozoa cannot proceed [36]. In this way, poor quality spermatozoa do not progress further up the female reproductive tract, whereas spermatozoa which are more motile, and remain viable longer, move onwards. Thus the cervix presents the first barrier to sperm progress in species where there is vaginal deposition of semen during natural mating, e.g. ruminants, primates. However, this selection site is bypassed in species where semen is naturally deposited directly into the uterus (such as pigs, horses, camels and dogs, among others), or where AI is used to deposit sperm doses in the cervix or uterus (pigs, sheep, cattle, horses). In these situations there is greater emphasis on the uterotubal junction as a barrier for filtering out poor-quality spermatozoa [37].

A more active selection process may occur *via* interaction of spermatozoa with the oviductal epithelial cells, resulting in a sub-population of highly motile, viable spermatozoa arriving at the site of fertilisation, ready to acrosome-react and bind with the zona pellucida [38]. Thus the female reproductive tract can be considered to have a dual filtering action, first in allowing spermatozoa to be separated from seminal plasma and subsequently in providing various barriers to the progress of abnormal spermatozoa [39]. However, it is unknown whether sperm samples of poor quality result in low pregnancy rates because (i) the natural filtering mechanisms take out all the abnormal spermatozoa, leaving insufficient sperm numbers for fertilization to occur, or (ii) the filtering mechanism is overwhelmed by the numbers of abnormal spermatozoa, resulting in fertilization of the oocyte by abnormal spermatozoa and subsequent failure of zygotic development.

On the other hand, in IVF the natural selection processes occurring in the female reproductive tract are, of course, completely absent [40]. Thus there is a requirement for both the removal of seminal plasma and selection of good quality spermatozoa prior to adding the sperm sample to the oocytes in fertilisation drops. Therefore, sperm selection techniques have been used more frequently in IVF using animal spermatozoa than in the preparation of semen doses for AI.

2. HOW CAN ONE REMOVES EMINAL PLASMA AND SELECT THE "BEST" ANIMAL SPERMATOZOA *IN VITRO*?

Biomimetics is the use of technologies and/or processes that mimic a naturally occurring event. Several mechanisms have been suggested that could be used to mimic selection of good quality spermatozoa in the female reproductive tract and thus fit the definition of biomimetics. These mechanisms either filter spermatozoa from seminal plasma actively or passively, thus mimicking the effect of spermatozoa migrating away from the site of semen deposition, or also permit selection of the better quality spermatozoa from the rest of the ejaculate, as may occur at the uterotubal junction and in the oviducts *in vivo*. Thus it is convenient to classify the biomimetic techniques into "separation" and "selection" categories (Table **1**). The following provides a brief description of each of these categories and their advantages and disadvantages are summarized in Table **2**.

Table 1. Classification of Sperm Separation Techniques Depending on the Presence or Absence of Sperm Selection Based on Quality

Separation of Spermatozoa from Seminal Plasma	Separation from Seminal Plasma and Selection Based on Sperm Quality
Washing	Migration (swim-up, underlay, migration-sedimentation); Migration into hyaluronic acid
	Filtration (glass wool, Sephadex beads, membranes)
	Centrifugation on a colloid (density gradient, single layer)

Note: sperm washing = semen is extended and centrifuged, with the result that the spermatozoa are pelleted, allowing most of the seminal plasma and extender in the supernatant to be removed; Migration = spermatozoa that are motile move from the extended semen into fresh extender; filtration = passage of the spermatozoa through a filter, for example glass fibres, Sephadex beads, or membrane pores, either by their motility alone or with the help of centrifugation; centrifugation through a colloid = centrifugation on colloids consisting of either PVP-coated silica particles or silane-coated silica particles.

2.1. Separation Technique: Washing

A warmed semen extender, suitable for the species, is added to the semen immediately after collection. The mixture is then centrifuged gently, the supernatant (consisting of seminal plasma and extender) removed and the sperm pellet resuspended in a suitable volume of the extender. The sper-

Table 2. Properties of Different Sperm Separation and Selection Methods

	Washing	Migration §	Filtration	Colloid Centrifugation
Ease of use	Simple	Simple	Simple	Requires some attention to detail
Equipment required	Centrifuge	Special tubes needed for swim-through	Centrifuge may be required	Centrifuge
Consumables	Centrifuge tubes	Special tubes needed for migration /sedimentation	Glass wool, Sephadex, filters	Colloids
Cost per sample	Lowest	Low, unless media contains hyaluronate	Higher	Highest
Sperm selection	None	Based only on motility	Based on motility, morphology, intact acrosomes	Based on motility, morphology, viability, chromatin quality, possibly intact acrosomes.
Seminal plasma removed	Mostly	Yes	Some removed	Yes
Pathogens removed	No	Data not available	Data not available	Yes
Removal of ROS	No	Yes	Data not available	Yes
Debris	May be present	Absent	May be present	Absent
Yield of motile spermatozoa	Data not available	10-20%	ca. 60-85%	>50%
Leukocytes	Present	Removed	Removed	Removed
Sperm chromatin	Can be poor	Can be poor	Data conflicting	Good
Acrosome	Unknown effect	May be damaged	Increased % intact	Increased % intact
Other		Hyaluronate-containing media may induce acrosome reaction	Contamination by e.g. glass fibres	Possible problem of endotoxins & PVP with Percoll™ *
Animal	Buffalo [78]; ram [79]; bull [80]; stallion [81, 82] boar [83]	Bull [42, 44, 62, 80]; ram [79, 84]; boar [43] buffalo [79]; stallion	Ram [79], boar [20] buffalo [16, 78] bull [47, 49, 81, 85] stallion [59, 86, 87]	Bull [62, 79, 88], ram [80]; boar [83, 89]; stallion [63], turkey [60]; dog [90].

Note: §Migration (swim-up, swim-down, swim-through).
* Percoll™ is not registered for clinical use.

matozoa are thus effectively separated from most of the seminal plasma component of the ejaculate [23, 41]. However, there is no selection from potential sources of ROS in seminal plasma, which are thought to be detrimental to sperm viability. There have been reports of chromatin damage due to this technique, at least for human spermatozoa [41], although this may be due to centrifuging human semen in the absence of antioxidants, rather than direct damage due to the technique itself. Extenders for animal semen, particularly milk- or egg yolk-based extenders, typically contain some antioxidants which may mitigate the effect of increase release of ROS during centrifugation of semen. However, centrifugation results in a sperm pellet containing dead, moribund and abnormal cells as well as viable spermatozoa [42], since all of the spermatozoa from the original sample are concentrated in the sperm pellet.

In a modification of the washing technique, low-molecular weight components of seminal plasma have been removed from boar ejaculates by dialysis [43].

2.1.1. Selection Techniques: Migration

Several variations on this theme exist, all relying on the ability of motile spermatozoa to move from one suspension, e.g. the extended ejaculate or washed sperm pellet, into a medium or extender of a different composition [41]. The spermatozoa effectively remove themselves from the seminal plasma environment. The original sperm population is either underneath, on top of, or to one side of the migration medium [41]. Thus the selection is based on the capability of spermatozoa to be motile and, as such, does not provide any selection based on normal head morphology, chromatin integrity (spermatozoa with intact chromatin), or viability and acrosome integrity [44]. Spermatozoa with tail abnormalities, which hinder their ability to swim, will not migrate into the swim-up medium and some studies show significantly better midpiece- and tail- morphology after swim-up than after washing (e.g. [42]). Moreover, migration into, or through, media containing hyaluronic acid may also select for spermatozoa with intact membranes [45] and, therefore, migration should be considered as a selection technique rather than a separation technique. The main disadvantage of any migration method is the low recovery rate, e.g. 10-20% [42] thus making it impractical for preparing AI doses in most animal species.

2.1.2. Selection Techniques: Filtration

The filtration effect is provided by interaction of the spermatozoa with the filter substance, which can be for example, glass fibres, Sephadex beads, or membrane pores, and also by the ability of the spermatozoa to move [46]. Non-

viable spermatozoa tend to adhere to the matrix more than motile and presumably functional spermatozoa [20], although the mechanism of action is unclear [47]. It is speculated that Sephadex, for example, either allows immotile and dead spermatozoa to agglomerate because of changes in surface charges [16], or a protein present on capacitated sperm binds to the Sephadex particles [48]. Experiments with canine semen showed that filtration through Sephadex G-15 improved the proportion of viable spermatozoa while decreasing the proportion of altered acrosomes compared to the untreated ejaculate [46]. The method has also been used to improve the freezability of bull spermatozoa [49].

These filtration methods are useful for eliminating leukocytes (and thus ROS) and selecting motile spermatozoa: they may also aid selection for morphologically normal [50] and possibly acrosome-intact spermatozoa [47]. In contrast, Januskauskas *et al.*. found no effect of these methods on the proportion of spermatozoa with intact acrosomes [49]. Fewer spermatozoa are lost than with other methods, with a recovery rate of approximately 63% being reported [49]. However, the filtrate is not considered to be as clean as for other sperm separation methods [1], presumably because not all of the seminal plasma and cellular debris is removed.

2.1.3. Selection Techniques: Colloid Centrifugation

In this method, extended semen is centrifuged through layers of colloid, which effectively separates spermatozoa from seminal plasma and also selects the sub-population of spermatozoa with good motility, viability and chromatin integrity. During centrifugation through a density gradient, cells move to the point in the gradient which matches their own density - the isopycnic point [51]. By altering the centrifugation conditions (g force and time) and the physical properties of the colloid, a sperm pellet is formed containing the most robust, good quality spermatozoa.

Oocyte penetration in IVF is considered to be better when spermatozoa are prepared by density gradient centrifugation than by swim-up [52]. Until recently, the technique was confined solely to density gradient centrifugation (DGC), but a new method has now been developed by the current authors at the Swedish University for Agricultural Sciences, so-called Single Layer Centrifugation (SLC) through a colloid [15, 53]. A summary of the advantages of SLC over DGC is provided in Table **3**. Since this technique is simpler to use than DGC (Fig. **2**), while apparently being equally effective, the two techniques will be discussed separately in greater detail in the following section.

3. CE NTRIFUGATION THROUGH A CO LLOID I N DETAIL

3.1. Density Gradient Centrifugation

DGC separates motile, morphologically normal, chromatin-intact spermatozoa from the rest of the ejaculate [34]. Furthermore, the technique has been reported to separate human spermatozoa from bacteria [54] and viruses [55-57] in the ejaculate. However, it is only possible to process small volumes of ejaculate by this method [58] and the recovery rate (yield) is often low [59, 60].

The most widely-used colloid used for animal semen to date is Percoll™, consisting of polyvinylpyrrolidone (PVP)-coated silica particles in a salt solution, used as two or more layers of different densities. During the 1990s, Percoll™ was re-classified by its manufacturer as being "for research purposes only" [41], probably because of problems with variable endotoxin levels in different batches of the colloid, although there have also been reports of alleged toxicity from free PVP in the colloid [61]. In electron microscopy studies [62], Cesari *et al.* showed a higher percentage of bovine spermatozoa with lost acrosomes after Percoll™ treatment compared to swim-up, although other studies in this species have shown the reverse [44]. More recently "apoptotic-like" changes were identified on equine spermatozoa after DGC with Percoll [63].

In the last decade, Percoll™ has been superseded by silane-coated silica colloids for use in human AR [41], and some species-specific formulations have been used for animal AR [34, 45, 57, 64, 65]. Silane-coated silica colloids have the advantages over PVP-coated colloids of being autoclavable, thus reducing the endotoxin levels, and of being stable for long periods in salt solutions, thus permitting standardised ready-to-use formulations to be sold commercially. These include products for human spermatozoa e.g. Isolate, Irvine Scientific, Irvine, USA; Silselect, Fertipro, Belgium; Suprasperm, Medicult, Denmark; PureSperm®, Nidacon International; and also BoviPure™ for bull, EquiPure™ for stallion and PorciPure™ for boar semen (all from Nidacon International AB, Gothenburg, Sweden). These commercial products have been used inter-species (for instance bull spermatozoa on preparations designed for human), with acceptable results, but the studies were restricted to diagnostic tests for sperm function only, e.g. consecutively frozen-thawed for diagnostic or flow-sorting purposes [66-68]. In other cases, species-specific preparations (e.g. BoviPure™) have been used to examine to value of the use of density gradient sperm selection procedure for IVF in an effort to

Table 3. Advantages of Single Layer Centrifugation Over Density Gradient Centrifugation

	Single Layer Centrifugation	Density Gradient Centrifugation
Ease of layering	Semen on top of one layer of colloid	Semen on top of several layers of colloid
Time for preparation	Less than DGC	Longer because more layers
Quality of preparation	Equal to DGC	Good
Scale-up	Easier than for DGC; shown to be possible for stallion spermatozoa	Difficult because of layering several different densities of colloid
Recovery rate	May be higher than DGC	May be lower than SLC

Note: DGC = density gradient centrifugation; SLC = Single layer centrifugation.

improve the efficiency of *in vitro* embryo production [65, 69].

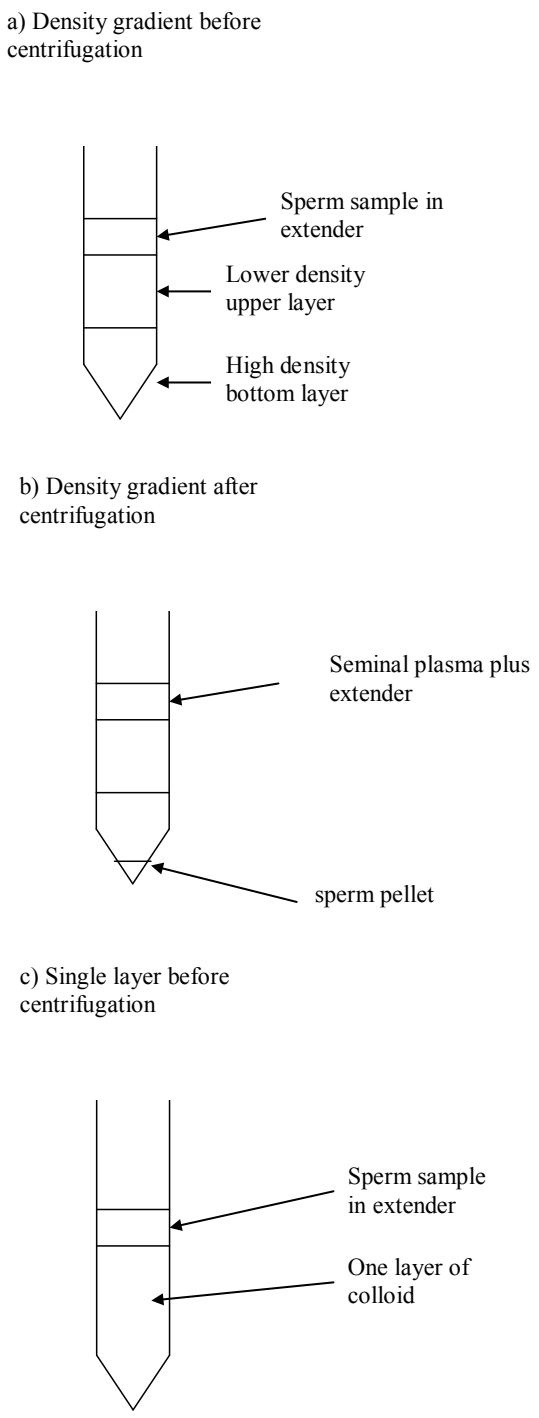

Fig. (2). Colloid centrifugation: (a) density gradient before centrifugation; (b) density gradient after centrifugation; (c) single layer centrifugation before centrifugation. Note: (**a**) the density gradient is composed of at least two layers of colloid of different densities. The sperm suspension is layered on the top of the uppermost layer of colloid. (**b**) After gentle centrifugation (typically 300 g for 20 min), a pellet of spermatozoa is seen in the bottom of the tube, while the supernatant consists of seminal plasma and semen extender. (**c**) The sperm suspension is pipetted directly on to a single layer of colloid, thus saving preparation time.

Comparisons of some density gradient products have been made with standard sperm washing procedures [70], with Percoll™ [65] or with swim-up [69], including recovery and selection of frozen-thawed bull spermatozoa. The results were variable, since BoviPure™-recovered spermatozoa did not lead to better production of bovine embryos than washing in modified Brackett-Oliphant medium [70]. Moreover, better cleavage rates but not blastocyst-development rates were observed for BoviPure™- compared to Percoll™-recovered spermatozoa [65], whereas the converse was seen when BoviPure™ preparation was compared to Swim-up sperm selection [69].

3.2. Single Layer Centrifugation

This method, a simplification of DGC, employs only one layer of colloid, thus obviating the need for preparing and layering several colloids of different densities (Fig. **2**). The seminal plasma is retained on top of the colloid while the spermatozoa move to the bottom of the conical centrifuge tube during centrifugation. An initial report using a single layer of Percoll™ for human spermatozoa, concluded that sperm quality from SLC was inferior to DGC preparations [71]. Subsequently, it was reported that 10 human sperm samples prepared by DGC (PureSperm® 40 + PureSperm® 80) and SLC (PureSperm® 80) were comparable in sperm motility and survival, although the SLC samples did not survive cryopreservation as well as the DGC samples [34]. Using the new, species-specific colloid formulations for animal spermatozoa developed at SLU, it was possible to produce sperm samples of equivalent quality from DGC and SLC using fresh stallion semen [53, 72, 73], frozen-thawed bull semen [15], fresh and stored boar semen [74]. The results for the comparison of DGC and SLC for fresh stallion spermatozoa are summarised in Table **4**. Furthermore, SLC has been used successfully to obtain robust good-quality equine spermatozoa from approximately 250 fresh stallion ejaculates (Morrell *et al*., unpublished data) and from frozen-thawed stallion semen [75, 76]. When various parameters of sperm quality were followed over 48 h in cooled, stored, stallion semen, there was a rapid deterioration in sperm motility, sperm viability, membrane integrity and chromatin integrity in unselected samples. In contrast, sperm quality was better in the SLC-prepared samples than in the uncentrifuged samples and was retained over the 48 h of the study [77]. The mean recovery rate of motile spermatozoa was 53.4%.

In studies with frozen-thawed stallion semen, prepared by SLC after thawing, there was an increase in progressive motility in SLC-selected samples (unselected 26.5± 8%, selected 71.3± 12.3%), viable spermatozoa measured by YO-PRO-1 (Quinolinium, 4-0(((3-methyl-2(3H)-benzoxazolylidene) methyl)-1-03-(trimethylammonio) propyl)-, di-iodide) (unselected 18.3±11.3%, selected 60.8±16.1%, $P<0.001$), and spermatozoa with intact mitochondrial function (unselected 8.9%; selected 42.2%) [75]. Abnormal head morphometry was significantly decreased ($P<0.001$) in the selected samples [76]. The authors concluded that SLC offers an alternative, more practical method than DGC for selection of viable, potentially fertile, frozen-thawed animal spermatozoa.

The SLC-technique is versatile and convenient, selecting good quality spermatozoa effectively and without damage. A

Table 4. Comparison of Stallion Sperm Quality Before and After Colloidal Centrifugation (Single Layer Centrifugation and Density Gradient Centrifugation)

Parameter	Before Centrifugation (Mean ± SD)	After Centrifugation (Mean ± SD)	
		Single Layer	Density Gradient
Motility (%)	68 ± 9.21	84.7 ± 5.45 [a b]	84.3 ± 5.87 [a b]
No. spermatozoa (x10^6)	Not relevant	32.08 ± 17.15 [b]	39.45 ± 20.23 [b]
Survival 4° (motility ≥ 35%)	2.46 ± 1.3 days (1.0-5.0 days)	5.6 ± 1.78 days [a b] (Range 3-9 days)	5.7 ± 1.86 days [a b] (Range 3-10 days)
Survival RT (motility ≥ 35%)	2.1 ± 0.66 days (1-3 days)	3.03 ± 0.91 [a b] (Range 2-6 days)	3.07 ± 0.91 [a b] (Range 2-5 days)
Normal Morphology (%)	67.5 ± 13.06	77.1 ± 9.3 [a b]	76.7 ± 8.7 [a b]
Chromatin damage (DNA fragmentation index, %)	11.01 ± 4.59	4.79 ± 2.63 [b c]	4.77 ± 2.78 [b c]

[a] Value significantly higher after centrifugation ($P < 0.001$).
[b] Not significantly different between the two centrifugation methods.
[c] Value significantly lower after centrifugation ($P < 0.001$).
RT = Room temperature 22-30 °C

further advantage of SLC is that it allows the process to be scaled-up to prepare whole ejaculates in a reasonable number of tubes. Use of the original DGC method enables only 1.5 mL ejaculate to be prepared per tube, which would mean that whole ejaculates from stallions or boars, which are characteristically voluminous [3], would require more than 70 tubes for centrifugation. This number of small tubes is totally impractical. However, with SLC, the technique can be scaled-up to allow processing of whole stallion ejaculates in 4-8 tubes (Morrell *et al*., unpublished data). A comparison of the sperm quality in scaled-up version of SLC and in the normal version using only 1.5 mL ejaculate, showed that there was no difference between the scaled-up SLC method and the original SLC method (Morrell *et al.*, unpublished data).

Further advantages of SLC lie in the removal of pathogens contained in the semen sample. It has been shown that SLC-prepared boar spermatozoa survive longer than unselected spermatozoa, at room temperature (20-22°C) and in the absence of antibiotics in the semen extender (Morrell *et al*., unpublished data). These results are interesting, suggesting alternatives both to the conventional storage of boar semen (usually at 16-18°C) and to the inclusion of antibiotics in semen extenders, since antibiotics are known to have a deleterious effect on sperm survival [34]. Moreover, initial studies with boar semen spiked with porcine circovirus, have shown that SLC-selection can substantially reduce the levels of virus in the sperm sample (Morrell *et al.*, unpublished data). This result is similar to earlier findings that equine arteritis virus (EAV) could be removed from naturally infected stallion semen by a double processing technique of DGC followed by swim-up [57]. An alternative density gradient method, using Percoll with trypsin added, has been shown to remove HIV-1 and hepatitis-C virus from spiked human semen samples [56]. It remains to be seen whether the trypsin-method is effective against a variety of field viruses without adversely affecting sperm fertilising capability.

4. CONCLUSION

Of the different types of selection methods discussed here, centrifugation through a colloid offers the best possibility for selecting good quality spermatozoa and removing cellular debris and pathogens which may be present in seminal plasma. The new technique of SLC using species-specific, optimised, colloid formulations based on silane-coated silica, is currently the most practical technique for selecting spermatozoa to be used in animal breeding. Since there is only one layer of colloid in the tube, preparation time is shorter and the process is less complicated than for the density gradient, which requires at least two densities of colloid to be layered in the tube. Care is required in the layering process for the gradient, since mixing of the different densities due to careless layering destroys the integrity of the interface between the two layers, thus reducing the efficiency of the sperm selection process. Other factors, such as the sperm concentration in the suspension applied to the top of the colloid, the proportion of morphologically normal spermatozoa in the original sample, and the time between collection and processing, affect the yield of spermatozoa. Importantly, use of SLC rather than DGC facilitates scaling-up the volumes of colloid and ejaculate used in order to process the large number of spermatozoa required for insemination doses in some animal species such as boar or stallion; it would be time consuming and tedious to process such large volumes using only 1.5 mL aliquots of extended ejaculate on small density gradients.

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