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Colloids: Applications in Sperm Preparation for Assisted Reproduction

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Abstract

Colloids have been used for several decades to prepare spermatozoa for assisted reproduction, initially for *in vitro* fertilization but, with the development of scaled-up techniques, increasingly for artificial insemination and cryopreservation as well. The colloids usually consist of coated silica particles. Using colloid centrifugation, it is possible to select sperm subpopulations consisting of motile spermatozoa with intact membranes, stable DNA and normal morphology and to separate them from the rest of the ejaculate. This review explains why different protocols for colloid centrifugation are needed for different species, as well as species-specific colloid formulations, to match the physical characteristics of the semen. The advantages and disadvantages of sperm preparation by this technique will be outlined. An emerging area of interest is the ability to separate spermatozoa from the bacteria that contaminate semen during collection. Thus, colloid centrifugation represents an alternative to using antibiotics in semen extenders. Since there is a worldwide movement to restrict the use of antibiotics, the possibility of physically removing the bacteria is of considerable interest. Moreover, it may be possible to use colloids to reduce viruses in semen. Transmission of viruses through semen is an emerging problem as more and more viruses are being identified that can potentially be spread in this manner.

Keywords: sperm quality, single layer centrifugation, antimicrobial resistance

1. Introduction

Assisted reproduction is the creation of a pregnancy by a means other than natural mating [1]. The most widely used form of assisted reproduction in animals is artificial insemination (AI); *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are confined to specialist

laboratories, for example, for bovine IVF, or are used occasionally for research or for conservation breeding. In contrast, human fertility treatments usually involve either IVF or ICSI. However, there are many differences between species regarding sperm numbers needed for AI, the type of semen used (fresh, stored or cryopreserved), the timing relative to ovulation, and site of deposition of the semen. These differences create a range of challenges for sperm preparation according to the species concerned. Despite the differences, one feature common to all species is that the chance of a successful outcome, that is, a pregnancy, is related to the quality of the sperm sample used. Colloid centrifugation can be used to select good quality spermatozoa for assisted reproduction [2].

The purpose of this review is to describe colloid centrifugation through species-specific colloids, particularly single layer centrifugation (SLC), to select functional spermatozoa. In this review, the history of single layer centrifugation (SLC) will be presented, the method of preparing semen will be explained and its applications in the semen industry will be proposed. Apart from describing the necessity for species-specific protocols, this review will also look at species-specific applications, particularly for the equine, porcine and bovine breeding industries, and the possibilities for germ-plasm conservation.

2. Why is it necessary to select spermatozoa for assisted reproduction?

Spermatozoa to be used for assisted reproduction technologies (ART) must be able to function normally, that is, to find and fertilize an oocyte and to direct the future development of the zygote [2]. Whichever form of assisted reproduction is used, good quality gametes are needed to ensure a successful outcome. Semen samples usually contain a heterogeneous population of spermatozoa of different stages of maturity and functional ability [3]. It is possible to select the subpopulations consisting of motile spermatozoa with intact membranes, stable DNA and normal morphology and to separate them from the rest of the ejaculate, using colloid centrifugation. These spermatozoa are most likely to be functional, to be able to fertilize and activate oocytes and to contribute to the zygote's future development.

3. Different types of semen

Semen for ART is available either fresh or cryopreserved, depending on the species [1]. Most bull spermatozoa are cryopreserved in small quantities, for example, 0.25 mL. However, the semen of some species or individuals may not freeze well; thus, boar and stallion semen is usually used fresh or after cooled storage. As a result, different protocols are needed to deal with the vast range of semen volumes and sperm concentration seen in different species, ranging from a few microliters (cat ejaculate or frozen semen of many species), to several hundred mL for a fresh boar ejaculate. Human semen in fertility clinics is used either fresh or frozen, if from the patient; if the semen has come from a sperm bank, it is usually frozen.

4. Colloid centrifugation

Colloid centrifugation is a sperm preparation technique whereby a sub-population of motile spermatozoa with normal morphology, intact membranes and good chromatin integrity are separated from the rest of the ejaculate, including the seminal plasma, by centrifugation through a colloid [2]. These robust spermatozoa pass through the colloid during gentle centrifugation and form a pellet in the bottom of the conical centrifuge tube. The seminal plasma, with its content of non-sperm cells, bacteria, etc., and semen extender are retained above the colloid. At the interface between the colloid and the seminal plasma, spermatozoa that have not been able to pass into the colloid are retained [4]. Typically, the spermatozoa at the interface are immotile spermatozoa, abnormal spermatozoa and those with damaged chromatin. If too many spermatozoa are loaded on to the colloid or there are very high numbers of abnormal spermatozoa, some normal spermatozoa may be prevented from entering the colloid and will be found in the interface population. Typically, the sperm pellet will have better sperm quality than the unselected samples [5, 6] and the resulting sperm sample will retain motility [7] and fertilizing ability [8] for longer than the unselected samples.

The colloids typically used to prepare spermatozoa are coated silica colloids in a mixture of salts, sugars and buffer. The composition of the mixture, and the pH, osmolarity and density of the colloid all affect the result in terms of number of spermatozoa in the pellet and their quality [9]. Although commercial colloid products are sterile, they do not contain antibiotics. Care should be taken to use aseptic techniques when opening the bottle to avoid contaminating the contents.

5. History of colloid centrifugation

Colloid centrifugation is a technique for separating different types of cells. The first application of this technique was as a density gradient, involving two or more layers of colloids of different densities [10]. During centrifugation on the density gradient, the cells move to a point in the gradient that matches their own density, which is called the isopycnic point. The degree of separation achieved depends on the number of layers of different densities used in the preparation of the density gradient and the differences in densities of the cell populations to be separated. The first and most widely used colloid was Percoll™ (now sold by GE Healthcare, Uppsala, Sweden), based on polyvinylpyrrolidone-coated silica particles. Since the mid-1990s, other coated silica colloids have been used, for example, silane-coated silica such as Redigrad® (originally sold by Amersham Biosciences, now GE Healthcare, Uppsala, Sweden) and Iodixanol (OptiPrep™, Progen, Heidelberg, Germany).

Semen samples consist of a heterogeneous population of spermatozoa of different ages and stages of maturity. Before spermatozoa can fertilize an oocyte, they must undergo a series of membrane changes over a period of time, known as capacitation, followed by the acrosome reaction just before they bind to the oocyte. If they do not bind to an oocyte after undergoing the acrosome reaction, they will die. The purpose of proving a mixture of spermatozoa of

different ages is to ensure that spermatozoa capable of fertilization will be available in sequence over a period of time. However, if the sperm sample is to be stored prior to being inseminated into a female, the presence of dead or dying spermatozoa, or cellular debris, may adversely affect the remaining spermatozoa. Separation of the viable spermatozoa from the rest of the ejaculate therefore enables them to remain functional over a longer period of time. A further advantage is that spermatozoa can be removed from seminal plasma (which contains decapacitating factors) and semen extender before use [11].

Early in the 1980s, researchers began to use density gradients for selecting viable spermatozoa [12, 13]. The spermatozoa that passed through the colloid and could be retrieved from the sperm pellet were mostly those that were motile, with normal morphology, intact sperm membranes and good chromatin integrity. Since these parameters of sperm quality have been correlated with pregnancy rate after artificial insemination in a number of species, it was proposed that the spermatozoa in the pellet were more likely to generate pregnancies when used for assisted reproduction than the uncentrifuged sample. Since the seminal plasma and extender were retained on the top of the uppermost layer of colloid, the method was ideally suited for preparing frozen spermatozoa for IVF or ICSI, where separation of the spermatozoa from seminal plasma was necessary. Immotile and immature spermatozoa or morphologically abnormal spermatozoa and those with damaged chromatin are mostly retained at the interface between the seminal plasma/extender and the colloid.

Density gradient centrifugation (DGC) was used for preparing human semen in fertility clinics, since the volume of semen to be processed was generally small and the sperm concentration low. Typically, the volume of colloid in each layer was 1–2 mL, depending on the number of layers used, and the volume of semen to be processed was up to approximately 1.5 mL. Thus, the technique had severe limitations when trying to process animal semen, which typically has much larger volumes and higher sperm concentrations than human semen. Nevertheless, frozen animal semen (mostly bovine) was processed for IVF; there were some reports of animal semen being processed by density gradient in the laboratory to improve sperm quality, but the consensus of opinion was that it was too impractical for processing whole ejaculates for AI in the field. Since AI is by far the most widely used biotechnology in animal breeding, there was little use for sperm preparation by DGC in this area.

In the mid-1990s, there was some debate as to whether Percoll™ itself was detrimental to spermatozoa, with some researchers claiming adverse effects from the use of the PVP-coated silica (e.g. [14]). Other researchers reported conflicting results with bovine and murine samples [15, 16]. This issue has never been fully resolved but factors other than the type of colloid may also be implicated, such as the centrifugation force and time used in the protocol [17], the osmolarity and chemical composition of the buffered salt solution used to dilute the colloid [9], the semen extender used for the semen [7], and even the sperm concentration of the sperm suspension [7].

Towards the end of the 1990s, a modification of DCG was developed in which only one layer of Percoll™ was used to prepare human spermatozoa [18]. These authors concluded that the single layer gave good results with oligospermic ejaculates but did not improve the sperm quality of normospermic semen. Thus, the technique was not universally adopted in fertility

clinics at that time, since there was a preference to have one standard protocol for all sperm preparations regardless of the quality of the original semen sample. Similar findings were later reported using one layer of Percoll™ for stallion semen [19], in that sperm quality was only improved in poor ejaculates.

Commercially available colloid formulations for use as a density gradient became available early in the twenty-first century. The first of these colloids for density gradients, EquiPure (Nidacon International AB, Mölndal, Sweden) was reported to improve stallion sperm quality [17], and it was used to prepare small numbers of spermatozoa from problem ejaculates for low dose insemination [20]. However, sperm yields were too low and the method too impractical for routine application when preparing spermatozoa for conventional insemination in the field.

Single layer centrifugation (SLC) using only one layer of a species-specific colloid formulation (Androcoll-E, now known as Equicoll) for preparing stallion spermatozoa was reported by Morrell et al. [21]. They described studies in which "normal" stallion semen, that is, from stallions with no known fertility problems, was split and processed by DGC and SLC. Sperm quality was improved in both types of selected samples compared to the uncentrifuged samples, and there was no difference in sperm quality between the two colloid centrifugation groups [5, 6]. The results from 250 ejaculates processed by SLC in three breeding seasons showed that the technique was repeatable and reliable under a variety of field conditions for stallions at commercial stud farms [7]. In addition, the development of a different colloid formulation (Androcoll-E Large) for use in 50-mL tubes enabled SLC to be scaled up to process whole stallion ejaculates [22].

It is also possible to use SLC process 25 mL of extended semen in 100-mL tubes or even 150-mL extended semen in 500-mL tubes [23]. The latter is particularly useful for processing boar ejaculates, which tend to be voluminous. However, the centrifuges commonly found on stud farms have rotors for 50-mL tubes; most stallion ejaculates can be processed in 4–6 of these 50-mL tubes.

Recently, the original protocol (4-mL colloid in a 15-mL centrifuge tube—designated Small SLC— was compared with protocols using 1 mL colloid, either in a 15-mL centrifuge tube (Mini-SLC) or in a microcentrifuge tube (Mini-EP), for preparing bovine spermatozoa for IVF [24]. The highest sperm yield was obtained from the Mini-SLC, that is, from 1 mL colloid in a 15-mL tube, although sperm quality was good in all three variants. An IVF trial using spermatozoa processed by Mini-SLC compared to swim-up did not show significant differences between the swim-up control and Mini-SLC, but since the chromatin integrity of the Mini SLC sperm preparation was significantly higher than the control, it would be expected that differences in embryo development might occur at a later stage in vivo than studied in vitro.

Thus, protocols have been developed for a range of tube sizes, different types of semen and various species as shown in **Table 1**. For optimum results, the specific colloid formulation for a particular size of tube should be used. However, the formulation developed for use in 50-mL tubes has also given good results when used in 15-mL centrifuge tubes (L Barros, personal

communication). Protocols based on other colloids have also been tried, such as Iodixanol (OptiPrep™, Progen, Heidelberg, Germany) [25, 26].

Type of semen/species	Tube size (mL)	Volume colloid (mL)	Volume extended semen* (mL)
Thawed: Red deer [64] Bull [24]	12–15	1.0	0.25
Fresh or thawed: Stallion [21] Bull [48] Boar [65]	12–15	4.0	4.5
Fresh: Stallion [22] Boar [42]	50	15	15–18
Fresh: Stallion [23]	100	20	25
Fresh: Stallion [22] Boar [23]	200	60	60
Fresh: Boar [23, 43]	500	150	150

Table 1. Modifications of single layer centrifugation according to volume of semen to be processed.

6. The method of single layer centrifugation

Preparing spermatozoa by SLC is a straightforward procedure (**Figure 1**). The following instructions refer to SLC in a 15 mL tube; the modifications required for other sizes of tube are shown below.

1. Equilibrate colloid and extended semen to room temperature to avoid cold-shocking the spermatozoa.

2. Adjust sperm concentration to not more than 100×10^6/mL for stallions and boars and not more than 50×10^6/mL for bulls.

3. Carefully layer up to 4.5 mL of extended semen on top of 4-mL Equicoll using a Pasteur pipette.

4. Use a centrifuge with a swing-out rotor; centrifuge at 300 g for 20 min.

5. Remove supernatant (seminal plasma, semen extender and most of the colloid) using a Pasteur pipette, taking care not to agitate the sperm pellet. Leave approximately 2 mm colloid above the sperm pellet.

6. Using a clean pipette, aspirate the sperm pellet from underneath the colloid.

7. Add extender to the sperm pellet to achieve the desired sperm concentration.

The same procedure is used for the other variants of SLC, differing only in the size of the tube, the type and volume of colloid, and the volume of semen to be used. For 50-mL tubes, 15-mL colloid and up to 20-mL extended semen are used. For 500-mL tubes, 150-mL colloid and 150-mL extended semen are used.
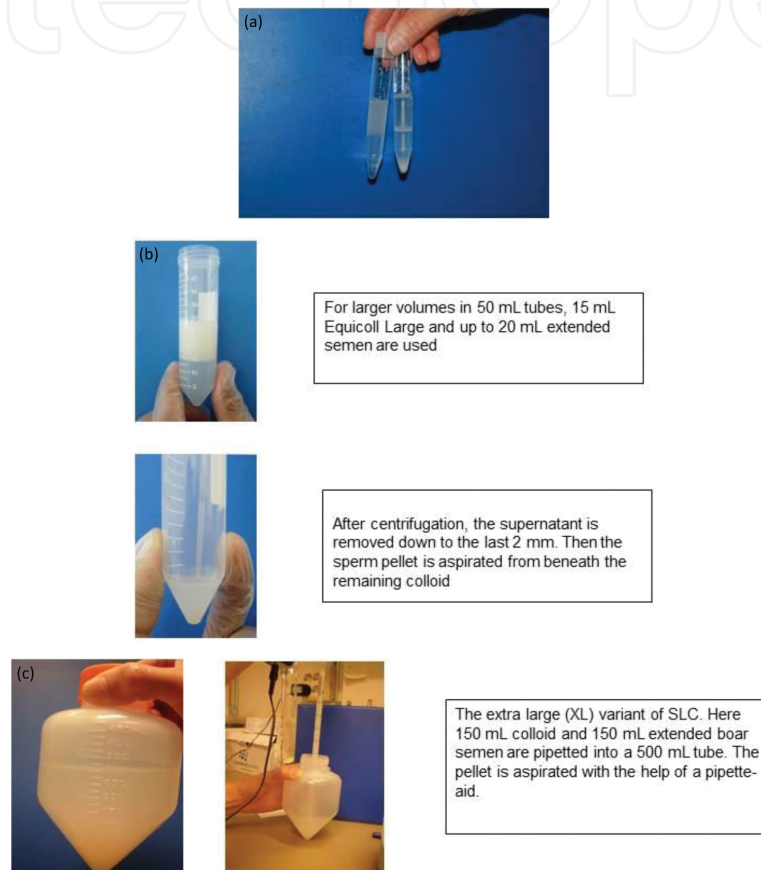

For larger volumes in 50 mL tubes, 15 mL Equicoll Large and up to 20 mL extended semen are used

After centrifugation, the supernatant is removed down to the last 2 mm. Then the sperm pellet is aspirated from beneath the remaining colloid

The extra large (XL) variant of SLC. Here 150 mL colloid and 150 mL extended boar semen are pipetted into a 500 mL tube. The pellet is aspirated with the help of a pipette-aid.

Figure 1. (a) Single layer centrifugation to process up to 4.5-mL semen ("Small"). (b) Single layer centrifugation to process up to 20-mL semen ("Large"). (c) Single layer centrifugation to process up to 150-mL semen ("XL") [23].

7. Specific applications of SLC

7.1. Applications in equine breeding

The equine breeding industry is extremely diverse ranging from studs with one stallion and a few mares to complex operations sending out hundreds of semen doses all over the country and beyond, and also large-scale embryo transfer facilities, not to mention stud farms with natural mating. The scale of the industry and the geographical distribution of the stud farms

present specific challenges in the distribution of good quality semen doses, which is where SLC could be of particular benefit.

7.1.1. Improving sperm quality for artificial insemination

Sometimes sperm quality is not as good in some ejaculates as desired. Since sperm morphology and chromatin integrity are correlated with pregnancy rates after AI, improving these parameters can lead to an increase in the pregnancy rate [27, 28]. Both normal morphology and chromatin integrity are higher in SLC-selected samples than in uncentrifuged control semen, indicating that pregnancy rates can be improved using SLC to select the spermatozoa. This has been shown to be the case both for problem stallions [27] and for those with no known fertility problem [28]. However, since the poor quality spermatozoa are removed, the number of spermatozoa remaining after SLC is less than in the original sample.

7.1.2. Maintaining sperm quality during transport

The sperm quality of the semen doses must be maintained during transport so that good pregnancy rates can be obtained after AI. Colloid centrifugation is ideal for this purpose, since the robust spermatozoa are separated from the dead and dying spermatozoa and also from seminal plasma and any bacteria contaminating the ejaculate at collection. Dead and dying spermatozoa can release intracellular contents, such as reactive oxygen species, that are detrimental to the survival of healthy spermatozoa. Thus, separating the robust spermatozoa from sources of reactive oxygen species helps to maintain them in good condition. Separating spermatozoa from seminal plasma and bacteria also increases the length of time for which the spermatozoa remain viable [20]. The spermatozoa are not damaged by SLC, whereas other centrifugation protocols to remove seminal plasma, such as cushion centrifugation, may cause damage [29]. The SLC-selected sperm samples are capable of fertilization when inseminated at least up to 96 h after semen collection [8], considerably longer than the 24–36 usually chosen by stud personnel.

7.1.3. Dealing with ejaculates that do not tolerate cooling

Some sperm samples cannot be cooled because the spermatozoa die. Previously, the only chance to use such ejaculates was to inseminate the mare immediately after semen collection at the same stud farm. However, the risk of injury and disease transmission make long-distance transport of mares for breeding undesirable. Removing all the seminal plasma from the spermatozoa and selecting the robust spermatozoa by SLC allows them to survive being cooled, and their fertilizing capability is retained [27]. Alternatively, SLC-selected sperm samples can be maintained at around 15° for at least 48 h and still retain their motility [30] and fertilizing capacity (J. Newcombe, personal communication)

7.1.4. Extending the shelf life of a conventional semen dose

If a conventional stored semen dose cannot be used when anticipated (usually 24–36 h after semen collection), it can be processed by SLC to extract the robust spermatozoa, thus extending

the time for which these spermatozoa can be stored prior to insemination [31]. The resulting sperm sample will have better sperm quality and a longer "shelf-life" than the stored semen dose, although sperm quality will not be quite as good or the shelf-life as long as if the sample had been prepared by SLC immediately after semen collection.

7.1.5. Selecting spermatozoa that survive cryopreservation

One way of avoiding the perishable nature of fresh semen would be to cryopreserve semen. However, not all stallions produce semen that can be frozen successfully. If the most robust spermatozoa are selected prior to freezing, sperm cryosurvival can be improved [29] and the thawed spermatozoa survive longer than control spermatozoa [32]. Since freezing protocols for stallion spermatozoa involve a centrifugation step to remove most of the seminal plasma, SLC can be used instead without creating extra effort and has the advantage of removing some of the sources of reactive oxygen species that will harmful to spermatozoa during cryopreservation. The longer survival time of the spermatozoa after thawing may allow more flexibility in the timing of insemination relation to ovulation [32].

7.1.6. Improving the sperm quality of poor quality frozen semen batches

SLC can be used to select robust spermatozoa after thawing [33–35]. Instead of discarding batches where post-thaw sperm motility is less than 30%, it may be possible to extract sufficient motile spermatozoa by SLC for insemination [36]. More straws may be needed than would normally be necessary to recover sufficient spermatozoa for an insemination dose but at least the poor quality batch has been "rescued." This is particularly relevant if the stallion is no longer available for semen collection for freezing.

7.1.7. Conservation of rare breeds or rare bloodlines

When the decision is reached to use conservation breeding to increase population numbers or to gene bank semen, there may be few males to choose from [37]. In such cases, it is important to preserve as many genes as possible, which in practice means using all available stallions regardless of sperm quality. Since no selection has been made for sperm quality, or those stallions that are available are elderly and with deteriorating sperm quality, SLC can be used to advantage to select motile spermatozoa with normal morphology, good membrane integrity and good chromatin integrity from immotile spermatozoa, abnormal spermatozoa and those with damaged chromatin. Since these parameters of sperm quality are linked to pregnancy rate after insemination, SLC can increase the chances of a mare becoming pregnant after insemination. In addition, improved sperm cryosurvival could lead to better success if frozen spermatozoa are used for insemination, thus increasing the likelihood that gene banking can actually help conservation breeding efforts.

7.2. Applications in pig breeding

Similarly to equine breeding, the pig breeding industry relies heavily on the insemination of fresh, cooled semen. Although cryopreservation protocols exist for boar semen, there is a

perception that pregnancy rates are lower and litter sizes smaller if cryopreserved semen is used for AI instead of fresh semen [30].

7.2.1. Improvement in sperm quality

Better linear motility, normal morphology and viability were observed in SLC-selected boar samples compared to unselected controls [38, 39]. Since these parameters of sperm quality have been linked to increased pregnancy rates in artificially inseminated sows, SLC-selected sperm doses would be expected to produce higher pregnancy rates when used for AI. However, pregnancy rates are normally high for boar semen doses, so the main benefit of SLC would be to enhance sperm quality for genetically valuable boars.

7.2.2. Reducing the number of spermatozoa required for an insemination dose

By selecting the most robust spermatozoa, it might be possible to reduce the sperm numbers used for insemination to 1×10^9 or even lower. Thus, even with the loss of approximately 33% of sperm numbers, more doses would be produced from each ejaculate.

7.2.3. Increasing the shelf life of stored semen

In SLC-selected preparations, sperm quality declines more slowly during storage than in unselected semen samples [38].

7.2.4. Removal of bacteria and viruses

Spermatozoa can be separated from bacteria [40], or viruses [41] in semen by SLC (see section on alternatives to antimicrobials).

7.2.5. Cryosurvival

Cryosurvival can be enhanced by SLC [42, 43]. In the first of these studies [42], boar sperm samples processed by SLC before freezing showed less membrane fluidity and less generation of reactive oxygen species after thawing than controls. In the second study, SLC in 500-mL tubes improved the quality and cryosurvival of boar semen [42]. However, the authors did point out that a high recovery rate would be required in order for SLC to be included routinely in cryopreservation protocols.

7.2.6. For IVF

SLC can be used to select spermatozoa for IVF, particularly where low numbers of spermatozoa are available. The rate of polyspermy was elevated when SLC-selected spermatozoa were used, indicating that SLC-selected spermatozoa are highly fertile [44]. When the sperm dose was drastically reduced to avoid polyspermy, the fertilization rate and blastocyst development rate were superior to unselected spermatozoa from the same boar.

7.2.7. Conservation breeding

Semen quality may be poor in rare breeds since they have not undergone selection for these traits [37]. Therefore, SLC may help to improve fertility.

7.3. Applications in cattle breeding

The cattle AI industry depends almost exclusively on cryopreserved semen in many parts of the world, except in New Zealand [1]. Cryopreservation prolongs the life of the semen sample and facilitates transport to other countries for insemination. Semen can be placed in quarantine, typically for one month after semen collection, to allow time for any disease in the donor animals to manifest itself. However, cryopreservation damages spermatozoa, although bull spermatozoa seem to be reasonably resistant to cryoinjury. The following uses of SLC have been identified for bull semen.

7.3.1. Improving sperm quality in poor semen samples prior to cryopreservation

Occasionally semen quality is poor, for example, if the proportion of spermatozoa with normal morphology is low [45], or if there is blood in the semen [46]. Even though SLC had no direct effect on total and progressive motility, it appeared to have a positive influence on several other kinematic parameters that may be important for fertilization after artificial insemination. In a previous study, the kinematics straightness (STR), Linearity (LIN) and beat cross frequency (BCF) of uncentrifuged bull sperm samples were positively correlated with the pregnancy rate at day 56 [47]. Thus, SLC samples might be expected to have better fertility than uncentrifuged ones.

7.3.2. Selecting good quality spermatozoa for IVF

Good quality spermatozoa can be selected from thawed semen samples for IVF, for example [48, 24]. Chromatin integrity particularly is improved in the SLC-selected samples: sperm chromatin damage (%DFI) was lower in SLC-selected samples than in unselected control samples (1.84 and 2.99% respectively, *p* = 0.036).

7.3.3. To select spermatozoa with high metabolic activity

Mitochondrial membrane potential ($\Delta\Psi$m) is one of the assays used to evaluate sperm metabolic activity and fertility [49]. Since SLC-selected spermatozoa were found to have a higher $\Delta\Psi$m than controls [50], it is possible that their fertility may be higher too.

8. Removal of pathogens

8.1. Removal of bacteria from semen samples

Most ejaculates are contaminated with bacteria despite being collected from healthy animals, and further contamination can occur during semen processing [51]. Bacteria can adversely

affect sperm quality, by competing for nutrients in the semen extender or from the production of metabolic by-products and endotoxins. In addition, they may cause uterine disease in inseminated females. To control the growth of bacteria, antibiotics are added to semen extenders according to national and international guidelines. The choice of antimicrobials is limited since they may be toxic to spermatozoa, and thus a cocktail of agents may be chosen to try to reduce the adverse effects on sperm survival. However, antibiotic use in general can lead to the development of microbial resistance, which can be passed on to other animals and also between humans and animals. Recent reports indicate that some microorganisms may be resistant to the antibiotics used in semen extenders for livestock [52–54]. Therefore, it might be prudent to develop alternatives to antimicrobials in semen extenders [55], although a change in the legislation would be required in the event that antibiotics are not added.

Human semen samples processed by DGC with a silane-coated silica colloid showed a 10-fold reduction in bacterial content [56]. Strict aseptic technique could reduce bacterial contamination almost completely. Increasing the g force used for centrifugation may have been associated with an increase in the number of positive samples for some bacteria. For 10 boar semen samples processed by SLC in a laminar air flow (LAF) bench, no bacteria were cultured from six of the SLC-selected sperm samples, and the number of bacteria in the remaining samples was reduced compared to the uncentrifuged controls [40]. Less than 1% of bacteria remained after the SLC processing. A possible bacteriostatic effect of the colloid was observed, despite the fact that no antibiotics were included in the colloid formulation. Removal of bacteria appeared to be affected by the bacterial species and the length of time between semen collection and SLC.

In a similar experiment with stallion semen, using a tube insert to facilitate harvesting of the sperm pellet [57], the removal of >90% of the total bacterial load was possible if the latter was >5 × 10^4 cfu/mL although for smaller bacterial loads removal was less efficient. Bacteria that tend to clump together, for example, forming palisades, chains and pairs, were more difficult to remove, possibly due to an alteration in the density of the bacterial unit by clumping [57]. Flagellated bacteria were also more difficult to remove.

Using SLC at a higher g force (600 g for 10 min) than in the previous studies, approximately 50% of the contaminating bacterial load was removed from stallion semen. Post-thaw sperm motility was increased if bacterial load was reduced compared to controls, confirming the beneficial effects of bacterial removal on sperm survival [53].

8.2. Reduction in viral load

Although AI was developed first as a means of reducing disease transmission between individuals, it is still possible to spread viruses in semen [58], for example, foot and mouth disease virus, porcine respiratory and reproductive syndrome virus, equine arteritis virus, human immunodeficiency virus (HIV) and, more recently, zika virus in humans. Breeding sires used for semen collection are tested routinely for serological evidence of infection, but virus may be shed in semen for several weeks before the antibody response is detected. In other cases, individuals may be permanent virus "shedders" without showing sero-conversion. Thus, a negative serological test indicates only that the male has not mounted an antibody

response at that point in time rather than being an absolute indicator of non-infection. Semen from shedding males represents a source of disease transmission via artificial insemination.

For several years, human fertility clinics have used a double semen processing technique of density gradient centrifugation followed by a swim-up to process semen from patients known to be shedding HIV or hepatitis C virus, where the female partner is not infected with the virus (so-called discordant couples) [59, 60]. These attempts to remove virus were apparently successfully in that there are no reports of infection of the female after the assisted pregnancy or of the birth of infected offspring. However, attempts to remove viruses from animal semen by a similar technique does not lead to the removal of all virus, possibly because unlike the human patients, the males are not treated with anti-viral drugs prior to semen collection.

In a preliminary experiment, aliquots of boar ejaculates were spiked with porcine circovirus 2 (PCV2) and processed by single layer centrifugation using Androcoll-P followed by a "swim-up" procedure. Samples before and after processing were tested for the presence of PCV2 in a tissue culture assay. The difference in virus titre corresponded to more than 99% reduction, and sperm quality was not affected by the selection procedure [41]. Other authors tested the removal of bovine viral diarrhoea virus (BVDV) from experimentally infected semen samples, comparing Percoll gradient centrifugation, swim-up, and a combination of swim-up and Percoll gradient centrifugation. They reported that no virus was detected from the combination of techniques, whereas a low level of virus was found in the swim-up preparation and a higher level in the Percoll preparation [61].

Using the "inner-tube" method described previously for some of the bacterial studies, it was possible to substantially reduce virus titre in semen samples from stallions known to be shedding equine arteritis virus [62]. However, some virus infectivity was still present, especially in stallions shedding large amounts of virus. Since it is not known how much virus in semen will cause infection in an inseminated mare, it would be unwise to assume that the use of SLC-selected samples is risk-free, unless a further modification of the procedure is developed.

9. SLC as an indicator of male fertility

The breeding industry wants to be able to identify sub-fertile individuals either to remove them from the breeding pool (bulls and boars) or to develop strategies to deal with their sub-fertility (stallions). Breeding companies would like to distinguish between highly fertile males and extremely highly fertile males. Attempts have been made to find laboratory tests that are predictive of sperm fertility, but whereas fertility is a complex multifaceted phenomenon, most assays evaluate only one parameter. However, the sperm yield after SLC is related to the quality and hence the potential fertility of the original ejaculates [63]; it is more highly correlated to fertility than the results of individual evaluations. Therefore, the suggestion was made that yield could be used as an indicator of stallion fertility in the absence of breeding data. The SLC is fast (30 min) and only requires a centrifuge, whereas assays of sperm quality require a fluorescent microscope or a flow cytometer and/or specialist skills. If the stud personnel do

not wish to carry out the SLC themselves, semen doses could be transported to a laboratory for this procedure. It is possible that SLC could be used predictively in other species as well.

10. Cost-benefit of SLC

Silane-coated silica colloids are expensive, but usually the improved sperm quality and enhanced fertilizing ability of the sperm sample is worth more than the cost of the procedure. The added value of SLC-selected sample depends on many factors, such as the perceived value of offspring from a particular male. The cost of SLC is less than DGC since it uses only one layer of colloid and needs less preparation time. Other factors increase the value of the technique: the availability of sperm samples, increased cryosurvival, biosecurity, ease of transport, etc. Thus, any processing steps that improve sperm survival and fertility will have an economic benefit. The price of the colloid will depend on the volume sold, because of economies of scale. The involvement of technical help in the laboratory affects preparation costs; the final price of the semen dose may include a premium for additional quality, which will, in turn, affect the demand for SLC-selected sperm samples.

11. Conclusion

In conclusion, colloid centrifugation, especially SLC, improves sperm quality in a variety of circumstances and for different species and a range of purposes within animal breeding. The procedure is straightforward and quick; it can be used by the personnel on any stud farm or semen collection centre where there is a swing-out centrifuge.

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References

[1] Morrell JM (2011) Artificial insemination: current and future trends. INTECH Open Access Publisher, Croatia. ISBN: 978-953-307-312-5, InTech, Available from: http://www.intechopen.com/articles/show/title/artificial-insemination-current-and-future-trends

[2] Morrell JM, Rodriguez-Martinez H: Biomimetic techniques for improving sperm quality in animal breeding: a review. The Open Andrology Journal. 2009;1:1-9.

[3] Suarez SS: Interactions of spermatozoa with the female reproductive tract: inspiration for assisted reproduction. Reproduction, Fertility and Development. 2007;19:103-110.

[4] Morrell JM: Stallion sperm selection: past, present and future trends. Journal of Equine Veterinary Science. 2012;32:436-440.

[5] Morrell JM, Johannisson A, Dalin A-M, Rodriguez-Martinez,H: Morphology and chromatin integrity of stallion spermatozoa prepared by density gradient and single layer centrifugation through silica colloids. Reproduction Domestic Animals. 2009;44:512-517.

[6] Morrell JM, Dalin A-M, Rodriguez-Martinez, H: Comparison of density gradient and single layer centrifugation of stallion spermatozoa: yield, motility and survival. Equine Veterinary Journal. 2009b;41:53-58.

[7] Morrell JM, Rodriguez-Martinez H, Johannisson A: Single layer centrifugation of stallion spermatozoa consistently selects the most robust spermatozoa from the rest of the ejaculate in a large sample size: data from 3 breeding seasons. Equine Veterinary Journal. 2010;42:579-585.

[8] Lindahl J, Dalin A-M, Morrell, JM: Pregnancies in mares inseminated with spermatozoa selected by single layer centrifugation and stored for 48h or 72h. Reproduction in Domestic Animals. 2011;46:P158.

[9] Morrell JM, Johannisson A, Rodriguez-Martinez H: Effect of osmolarity and density of colloid formulations on the outcome of SLC-selection of stallion spermatozoa. ISRN Veterinary Science. 2011. doi:10.5402/2011/128984.

[10] Pertoft H: Fractionation of cells and subcellular particles with Percoll. J Biochem Biophysical Methods 2000;44:1-30.

[11] Hunter RHF, Rodriguez-Martinez H: Analysing mammalian fertilisation. Reservations and potential pitfalls with an in vitro approach. Zygote. 2002;10:11-15.

[12] Bolton VN, Braude P: Preparation of human spermatozoa for in vitro fertilization by isopycnic centrifugation on self-generating density gradients. Archives Andrology. 1984;13:167-176.

[13] de Vries AC, Colenbrander B: Isolation and characterization of boar spermatozoa with and without a cytoplasmic droplet. International Journal Biochemistry. 1990;22:519-524

[14] Avery B, Greve T: Impact of Percoll on bovine spermatozoa used for in vitro insemination. Theriogenology. 1995;44:871-878.

[15] Motoishi M, Goto K, Tomita K, Ookutsu S, Nakanushi Y: Examination of the safety of intracytoplasmic injection procedures by using bovine oocytes. Human Reproduction. 1996;11:618-620.

[16] Mizuno K, Hoshi K, Huang T: Fertilization and embryo development in a mouse ICSI model using human and mouse sperm after immobilisation in polyvinlypyrrolidone. Human Reproduction. 2002;17:2350-2355

[17] Macpherson M, Blanchard TL, Love CC, Brinsko SP, Thompson JA, Varner DD: Use of a silane-coated silica particle solution to enhance the quality of ejaculated semen in stallions. Theriogenology. 2002;58:317-320

[18] Sharma RK, Seifarth K, Agarwal A: Comparison of single- and two-layer Percoll separation for selection of motile spermatozoa. International Journal Fertility. 1997;42:412-417.

[19] Sieme H, Martinsson G, Rauterberg H, Walter K, Aurich C, Patzoldt R, Klug E: Application of techniques for sperm selection in fresh and frozen-thawed stallion semen. Reproduction in Domestic Animals. 2003;38:134-140

[20] Varner DD, Love CC, Brinsko SP, Blanchard TL, Hartman DL, Bliss SB, Carroll BS, Eslick MC: Semen processing for the sub-fertile stallion. Journal Equine Veterinary Science. 2008;28: 677-685.

[21] Morrell JM, Dalin A-M, Rodriguez-Martinez H: Prolongation of stallion sperm survival by centrifugation through coated silica colloids: a preliminary study. Animal Reproduction. 2008;5: 121-126.

[22] Morrell JM, Johannisson A, Dalin A-M, Rodriguez-Martinez H: Single layer centrifugation with Androcoll™-E can be scaled-up to allow large volumes of stallion ejaculate to be processed easily. Theriogenology. 2009;72:879-884.

[23] Morrell JM, van Wienen M, Wallgren M: Single layer centrifugation can be scaled-up further to process up to 150 mL semen. ISRN Veterinary Science. 2011; doi: 10.5402/2011/183412.

[24] Abraham MC, Johannisson A, Morrell JM: Effect of sperm preparation on development of bovine blastocyst in vitro. Zygote. 2016; In press

[25] Stuhtmann G, Oldenhof H, Peters P, Klewitz J, Martinsson G, Sieme H: Iodixanol density gradient centrifugation for selecting stallion sperm for cold storage and cryopreservation. Animal Reproduction Science. 2012;133:184-190.

[26] Heutelbeck A, Oldenhof H, Rohn K, Martinsson G, Morrell JM, Sieme H: Use of density centrifugation for delayed cryopreservation of stallion sperm: perform sperm selection

directly after collection or after storage? Reproduction in Domestic Animals. 2015;50:76-83.

[27] Morrell JM, Mari G, Kutvölgyi G, Meurling S, Iacono E, Mislei B, Rodriguez-Martinez H: Spermatozoa from stallion ejaculates processed by single layer centrifugation with Androcoll™-E are capable of fertilization after artificial insemination. Reproduction Domestic Animals. 2011;46:642-645.

[28] Morrell JM, Richter J, Martinsson G, Stuhtmann G, Hoogewijs M, Roels K, Dalin A-M: Pregnancy rates are higher after artificial insemination with cooled stallion spermatozoa selected by single layer centrifugation than with control semen doses. Theriogenology. 2014;82:1102-1105

[29] Hoogewijs M, Morrell JM, Van Soom A, Govaere J, Johannisson A, Piepers P, De Schauwer C, de Kruif A, De Vliegher S: Sperm selection using single layer centrifugation prior to cryopreservation can increase post thaw sperm quality in stallions. Equine Veterinary Journal. 2011;43(Suppl 40):35-41.

[30] Morrell JM: Biomimetics in action: practical applications of single layer centrifugation for equine breeding. Veterinary Science and Technology. 2011;2:107. Doi: 10.4172/2157-7579.1000107

[31] Morrell JM, Macias Garcia B, Pena FJ, Johannisson A: Processing stored stallion semen doses by single layer centrifugation. Theriogenology. 2011;76:1424-1432.

[32] Hoogewijs M, Piepers S, Govaere J, De Schauwer C, de Kruif A, Morrell JM: Sperm longevity following pre-freeze sperm selection. Journal Equine Veterinary Science, 2012;32:489.

[33] Macias GB, Fernandez-Gonzalez L., Morrell J., Ortega Ferrusola C., Tapia JA, Rodriguez-Martinez H, Pena FJ: Single layer centrifugation through colloid positively modifies the sperm sub-population structure of frozen-thawed stallion spermatozoa. Animal Reproduction Science. 2009;114: 193-202.

[34] Macias GB, Morrell JM, Ortega Ferrusola C, Fernandez-Gonzalez L, Tapia JA, Rodriguez-Martinez H, Pena FJ: Centrifugation on a single layer of colloid selects improved quality spermatozoa from frozen-thawed stallion semen. Reproduction Domestic Animals. 2009;44:523-526.

[35] Mancill SS, Love CC, Brinsko SP, Edmond AJ. Foster ML, Teague SR, Waite JA, Varner DD: Effect of density gradient centrifugation on cryopreservation of equine spermatozoa. Animal Reproduction Science. 2010;121S:208-209.

[36] Stuhtmann G: Single layer centrifugation can improve poor quality frozen stallion ejaculates for AI. Reproduction in Domestic Animals. 2011;43:P257.

[37] Woelders H, Windig J, Hiemstra SJ: How developments in cryobiology, reproductive technologies and conservation genomics could shape gene banking strategies for (farm) animals. Reproduction in Domestic Animals. 2012;47 (Suppl. 4):264-273

[38] Morrell, JM, Saravia, F, Wallgren M, van Wienen, M & Rodriguez-Martinez, H: Selection of boar spermatozoa using centrifugation on a glycidoxypropyltrimethoxy-silane-coated silica colloid. Journal of Reproduction and Development 2009;55:547-552.

[39] van Wienen MVP, Wallgren M, Parvlevliet JM, Morrell JM: Boar spermatozoa survive freezing after single layer centrifugation with Androcoll-P. Reproduction in Domestic Animals. 2011; 46(Suppl. 2):Abst. P2.

[40] Morrell JM, Wallgren M: Removal of bacteria from boar ejaculates by single layer centrifugation can reduce the use of antibiotics in semen extenders. Animal Reproduction Science. 2011;123:64-69.

[41] Blomqvist G, Persson M, Wallgren M, Wallgren P, Morrell JM: Removal of virus from boar semen spiked with porcine circovirus type 2. Animal Reproduction Science. 2011;126:108-114.

[42] Martinez-Alborcia MJ, Morrell JM, Parrilla I, Barranco I, Vázquez JM, Martinez EA, Roca J: Improvement of boar sperm cryosurvival by using single-layer colloid centrifugation prior freezing. Theriogenology. 2012;78:1117-1125.

[43] Martinez-Alborcia MJ, Morrell JM, Barranco I, Maside C, Gil MA, Parrilla I, Vazquez JM, Martinez EA, Roca J: Suitability and effectiveness of single layer centrifugation using Androcoll-P in the cryopreservation protocol for boar spermatozoa. Animal Reproduction Science. 2013;140:173-179.

[44] Sjunnesson YC, Morrell JM, González R: Single layer centrifugation-selected boar spermatozoa are capable of fertilization in vitro. Acta Veterinaria Scandinavica. 2013;55:20-26.

[45] Morrell JM, Rodriguez-Martinez H, Andersson M: Colloid centrifugation selects normal spermatozoa from polymorphic bull ejaculates: a case study. Reproduction in Domestic Animals. 2014;49:281-284.

[46] Yusnizar Y, Abraham MC, Laskowski D, Johannisson A, Morrell JM: Changes in Bull Sperm Kinematics after single layer centrifugation. Reproduction in Domestic Animals. 2014;49:954-956.

[47] Al-Essawe E, Morrell JM: Relationship between bull sperm kinematics and fertility. Reproduciton in Domestic Animals. 2014;49:Suppl abst.3

[48] Thys M, Vandaele L, Morrell JM, Mestach J, Van Soom A, Hoogewijs M, Rodriguez-Martinez H: *In vitro* fertilising capacity of frozen-thawed bull spermatozoa selected by single-layer glycidoxypropyltrimethoxysilane-coated silica colloidal centrifugation. Reproduction in Domestic Animals. 2009;44:390-394.

[49] Oliveira BM, Arruda RP, Thomé HE, Filho MM, Oliveira G, Guimarães, Nichi M, Silva LA, Celeghini ECCC: Fertility and uterine hemodynamic in cows after artificial insemination with semen assessed by fluorescent probes Theriogenology. 2014;82:767-772.

[50] Johannisson A, Nongbua T, Edman A, Morrell JM: Effects of single layer centrifugation (SLC) on bull spermatozoa prior to freezing on post-thaw semen characteristics. Theriogenology. 2016;86:140

[51] Althouse, G.C.; Pierdon, M.S.; Lu, K.G. Thermotemporal dynamics of contaminant bacteria and antimicrobials in extended porcine semen. Theriogenology. 2008;70:1317-1323.

[52] Zampieri D, Santos VG, Braga PA, Ferreira CR, Ballottin D, Tasic L, Basso AC, Sanches BV, Pontes JH, da Silva BP, Garboggini FF, Eberlin MN, Tata A: Microorganisms in cryopreserved semen and culture media used in the in vitro production (IVP) of bovine embryos identified by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). Theriogenology. 2013;80:337-345.

[53] Guimaraes T, Lopes G, Pinto M, Silva E, Miranda C, Correia MJ, Damásio L, Thompson G, Rocha A: Colloid centrifugation of fresh stallion semen before cryopreservation decreased microorganism load of frozen-thawed semen without affecting seminal kinetics. Theriogenology. 2015;83:186-191.

[54] Gloria A, Contri A, Wegher L, Vignola G, Dellamaria D, Carluccio A: The effects of antibiotic additions to extenders on fresh and frozen-thawed bull semen. Animal Reproduction Science 2014;150:15-23 doi:10.1016/j.anireprosci.2014.08.012.

[55] Morrell JM, Wallgren M: Alternatives to antibiotics in semen extenders: a review. Pathogens. 2014;3:934-946.

[56] Nicholson CM, Abramsson L, Holm SE, Bjurulf EX: Bacterial contamination and sperm recovery after semen preparation by density gradient centrifugation using silane-coated silica particles at different g forces. Human Reproduction. 2014;15:662-666.

[57] Morrell JM, Klein C, Lundeheim N, Erol E, Troedsson MHT: Removal of bacteria from stallion semen by colloid centrifugation. Animal Reproduction Science. 2014;145:47-53.

[58] Foote RH: The history of artificial insemination: selected notes and notables. Journal Animal Science. 2002;80:1-10.

[59] Bujan L, Daudin M, Alvarez M, Massip P, Puel J, Pasquier C: Intermittent human immunodeficiency rtype 1 virus (HIV-1) shedding in semen and efficiency of sperm processing despite high seminal HIV-1 RNA levels. Fertility Sterility. 2002;78:1321-1323.

[60] Levy R, Bourlet T, Maertens A, Salle B, Lomage J, Laurent JL, Pozzetto B, Guerin JF: Pregnancy after safe IVF with hepatitis C virus RNA-positive sperm. Human Reproduction. 2002;17:2650-2653.

[61] Galuppo AG, Junior NB, Arruda NS, Corbellini AO, Chiappetta CM, Pavão DL, D'Angelo M, Canal CW, Rodrigues JL: Evaluation of the effectiveness of semen processing techniques to remove bovine viral diarrhea virus from experimentally contaminated semen samples. Journal of Virological Methods. 2013;187:443-448.

[62] Morrell JM, Timoney P, Klein C, Shuck K, Campos J, Troedsson M: Single layer centrifugation reduces equine arteritis virus titer in the semen of shedding stallions. Reproduction in Domestic Animals. 2013;48:604-612.

[63] Morrell JM, Stuhtmann G, Meurling S, Lundgren A, Winblad C, Macias Garcia B, Johannisson A: Sperm yield after single layer centrifugation with Androcoll-E is related to the potential fertility of the original ejaculate Theriogenology. 2014;81:1005-1011.

[64] Anel-López L, Martínez-Rodríguez C, Soler AJ, Fernández-Santos MR, Garde JJ, Morrell JM: The use of Androcoll-S after thawing improves the quality of electroeja-culated and epididymal sperm samples from red deer. Animal Reproduction Science. 2015;158:68-74.

[65] Morrell JM, Saravia F, Wallgren M, van Wienen M, Rodriguez-Martinez H: Selection of boar spermatozoa using centrifugation on a glycidoxypropyltrimethoxy-silane-coated silica colloid. Journal of Reproduction and Development. 2009;55:547-552.