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Colloid Centrifugation of Semen: Applications in Assisted Reproduction

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Abstract

Colloid centrifugation can be used to separate heterogeneous populations of cells particularly semen samples, which typically contain mixtures of spermatozoa at different stage of maturity together with dead and dying spermatozoa and also non-sperm cells. Colloids can be used to enrich the population of spermatozoa that are viable and functional; these are the spermatozoa that are needed for fertilization in Assisted Reproduction. Sperm samples obtained after colloid centrifugation may be enriched for motile, morphologically normal spermatozoa with intact chromatin. Insemination of mares with stallion sperm samples prepared by colloid centrifugation resulted in a higher pregnancy rate per cycle than controls, confirming that the good sperm attributes observed in the laboratory were indicative of functional spermatozoa. The method does not simply enrich for viable spermatozoa by inhibiting dead or dying spermatozoa from passing through the colloid since emerging evidence shows that colloid centrifugation can select for spermatozoa with certain properties such as metabolic activity. Other reproductive cells, such as spermatids or progenitor cells, may also be purified by colloid centrifugation. This review describes the history of colloid centrifugation for selecting spermatozoa, compares colloid centrifugation with alternative techniques, and finally describes some of the applications of the technique within the animal breeding industry.

Keywords

Animal Spermatozoa, Purification, Enrichment, Selection, Spermatids

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1. Introduction

Colloid centrifugation is a technique for separating different types of cells from heterogeneous suspensions [1]. One specialised application of this technique is in selecting spermatozoa for various assisted reproduction techniques *i.e.* where a pregnancy is created by a means other than natural mating. The most commonly encountered technique of assisted reproduction in animals is artificial insemination (AI); in Europe the vast majority of dairy cattle and pigs are bred using this technique [2]. Specialist laboratories may use *in vitro* fertilization (IVF), particularly for cattle, where sperm samples are added to oocytes in the laboratory and incubated to allow the fertilized oocytes to develop; and intracytoplasmic sperm injection (ICSI) e.g. in horses, where an individual spermatozoon is injected into an oocyte. The embryos generated by these processes can be transferred to recipients to create a pregnancy, either immediately or after cryopreservation and storage. These techniques are also adopted occasionally for conservation management *i.e.* the breeding of rare or endangered species. In contrast, human fertility treatments usually involve IVF or ICSI rather than intrauterine insemination. Regardless of the assisted reproductive technology (ART) that is to be employed, colloid centrifugation can be used to obtain functional spermatozoa, thereby increasing the chances of generating a pregnancy [3] and for that pregnancy to continue [4]. In addition to practical applications in ART, colloid centrifugation is a useful research tool in the elucidation of the mechanisms occurring in spermatozoa during maturation, capacitation and the acrosome reaction. These events are all necessary before spermatozoa can bind to the zona pellucida prior to fertilization of the oocyte.

If cell suspensions are centrifuged through colloids of different densities, the cells move to a point that matches their own density—the isopycnic point [1]. The degree of separation that can be achieved depends on how many layers of colloid of different densities are used and the differences in densities of the various cells that are to be separated. When colloids are used to separate sperm samples, it is usual to choose a colloid that is less dense than mature sperm cells so that the spermatozoa will pellet in the bottom of the centrifuge tube for ease of retrieval.

Colloid centrifugation may be used to select and purify epididymal spermatozoa [5] or precursor cells such as round and elongated spermatids [6], or even spermatogonia—the progenitor cells that will form spermatocytes at the start of spermatogenesis. Occasionally it may be necessary to use epididymal spermatozoa, either post-castration or after the death of the animal [7]. This is particularly relevant for conservation biology, when recovery of epididymal and testicular spermatozoa may represent the last chance to recover genetic material from a particular individual. However, such tissue is usually contaminated with blood and cellular debris, both of which have a detrimental effect on sperm survival. Transplantation of spermatogonial stem cells to other individuals has been achieved in mice and bulls [8], with production of donor gametes from the recipient, but extraction of the spermatogonia requires enrichment of the specific cells from a mixture of testicular cells.

This review will describe sperm selection by colloid centrifugation and compare this method to other sperm selection methods that have been reported. Species-specific applications of colloid centrifugation will be discussed, particularly for the equine, porcine and bovine breeding industries, as well as in conservation breeding

2. Sperm Selection

Spermatozoa to be used for ART must be functional *i.e.* able to fertilize an oocyte and to direct the future development of the zygote. Semen samples contain a heterogeneous mixture of spermatozoa at different stages of maturity and functional ability. Colloid centrifugation enables a sub-population to be selected consisting mainly of motile spermatozoa with intact membranes, stable DNA and normal morphology, and to separate them from the rest of the ejaculate [9]. These attributes of sperm quality have been associated with an increased likelihood of pregnancy [10]; therefore, selected sperm samples are more likely to generate a pregnancy than unselected samples. This process mimics the selection that is believed to occur in the female reproductive tract, where spermatozoa swim away from the seminal plasma at the site of semen deposition and then are subjected to selection at various sites in the reproductive tract, for example by selective binding to oviductal epithelial cells [11]. Since colloid centrifugation mimics a biological process, it is known as a biomimetic technique [3].

3. Colloid Centrifugation of Spermatozoa

Before spermatozoa can fertilize an oocyte, they must undergo a series of membrane changes, known as capaci-

tation, followed by the acrosome reaction just before they bind to the oocyte. The timing of these events is crucial because spermatozoa do not survive long after undergoing the acrosome reaction. Thus, the provision of a mixture of spermatozoa of different ages in the ejaculate is to ensure that there will be a progression of spermatozoa capable of fertilization in the oviducts over a period of time under physiological conditions. However, if the sperm sample is to be stored prior to being inseminated into a female, the presence of cellular debris and dead or dying spermatozoa may adversely affect the viable spermatozoa. Separation of the latter from the rest of the ejaculate therefore enables them to remain functional over a longer period of time during *in vitro* storage. A further advantage is that spermatozoa can be removed from seminal plasma (which contains decapacitating factors) and semen extender before use [12], thus enabling them to be used for IVF. Colloid centrifugation may select for spermatozoa with longer telomeres than in control samples; telomeres are believed to play a key role in early embryonic survival [13].

In colloid centrifugation, a sub-population of motile spermatozoa with normal morphology, intact membranes and good chromatin integrity is separated from the rest of the ejaculate, including the seminal plasma [3]. These spermatozoa pass through the colloid during gentle centrifugation and form a pellet in the bottom of the centrifuge tube, whereas non-motile spermatozoa or those with damaged chromatin are retained at the interface between the semen and the colloid [14]. Seminal plasma, with its content of non-sperm cells and microorganisms, such as bacteria, and semen extender are retained above the colloid. Typically, the selected spermatozoa in the pellet will exhibit better sperm quality than in the original unselected sample [9] [15], and will retain this quality [16] and fertilizing ability [17] for longer than those in the original samples. If too many spermatozoa are loaded on to the colloid, or high numbers of abnormal spermatozoa are present at the interface, some normal spermatozoa will be unable to pass into the colloid and will be found in the interface population. The centrifugation force and time, as well as the physical properties of the colloid [14] are critical in allowing as many normal spermatozoa as possible to reach the pellet while preventing immotile or morphologically abnormal spermatozoa from entering the colloid.

The colloids typically used to prepare spermatozoa are coated silica colloids e.g. Percoll (polyvinylpyrrolidone-coated silica), prepared in a buffered salt solution. When Percoll is used, buffered salt solutions are used to dilute the colloid to achieve the desired density. The dilutions must be made up just prior to use since they are not stable. In contrast, silane-coated silica e.g. Redigrad, is stable in solution and can be made up well in advance of use. Silane-coated silica colloids are available as ready-to-use formulations specifically for sperm selection. The salt composition, pH, osmolarity and density of the colloid formulation affect the outcome of centrifugation in terms of number of spermatozoa in the pellet and their quality [14]. Commercial colloid products are sterilized during manufacture, and typically do not contain antibiotics. Other substances may also be used e.g. Iodixanol (OptiPrep™, Progen, Heidelberg, Germany) [18].

4. Background to Sperm Selection by Colloid Centrifugation

4.1. Density Gradient Centrifugation

The first application of colloid centrifugation to sperm selection was as a density gradient, in which two or more layers of colloids of different densities were used e.g. [19]. The degree of separation achieved depends on the difference in density of the colloids used in the gradient and the difference in densities of the cells to be separated. The technique gained popularity 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 approximately 1.5 mL. 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, this technique was also used for preparing frozen animal semen (mostly bovine) for IVF e.g. [20]. Although sperm quality in animal semen could be improved by DGC in the laboratory, the consensus of opinion was that it was too impractical for processing whole ejaculates for AI in the field.

In the1990s, a debate occurred concerning whether Percoll™ was harmful to spermatozoa, with some researchers reporting adverse effects from the use of PVP-coated silica (e.g. [21]. However, PVP has been used to facilitate the capture of bovine spermatozoa for ICSI without any detrimental effects on the spermatozoa [22]. Subsequently, there were reports of problems with mouse zygote development following exposure of spermatozoa to PVP [23]. These conflicting results may be due to species differences in susceptibility to PVP or may re-

flect differences in the PVP from various sources [24]. Subsequently, some batches of Percoll were found to have high endotoxin levels [25], necessitating the testing of each batch to identify those suitable for use with sperm samples. Other problems potentially associated with the use of PVP-coated silica include structural alterations in human spermatozoa [26], although these were observed after exposure to 10% PVP, a high concentration *per se*.

Commercially available colloid formulations for DGC of semen became available early in the 21st century. Stallion sperm quality was improved in the selected samples [27] and the technique could be used to prepare small numbers of spermatozoa from problem ejaculates for low dose insemination [28]. However, sperm yields were too low and the method too impractical for routine application when preparing spermatozoa for conventional insemination in the field.

4.2. Centrifugation Though a Single Layer of Colloid

Towards the end of the 1990s, a modification of DCG was developed utilizing one layer of Percoll™ for preparing human spermatozoa [29]. Good results were obtained with oligospermic ejaculates but the sperm quality of normospermic semen was not improved. Thus the technique was not considered to be useful at that time, since there was a preference to have a standard protocol for all sperm samples. Similar results were later reported when one layer of Percoll™ was tested with stallion semen [30], with sperm quality being improved only in poor quality ejaculates.

Single Later Centrifugation (SLC) using one layer of a species-specific colloid for stallion spermatozoa (Androcoll-E) was reported by Morrell *et al.* [31]. The advantage of using only one layer of colloid is that the protocol is less time-consuming and easier to use than DGC. The same authors described a series of studies in which "normal" stallion ejaculates were 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 [9] [14]. Results from 250 ejaculates processed by SLC showed that the technique was repeatable and reliable under field conditions for stallion ejaculates at commercial stud farms [16]. In addition, development of a different colloid formulation for use in 50 mL tubes (Androcoll-E Large) enabled SLC to be scaled-up to process whole stallion ejaculates in 4 - 6 of these 50 mL tubes [32]. It would be too laborious to attempt to use DGC in this size of tube. This scaled-up technique has been slowly gaining popularity around the world, particularly for processing problem stallion ejaculates.

It is also possible to use SLC to process 25 mL of extended semen in 100 mL tubes or even 150 mL extended semen in 500 mL tubes [33]. The latter is particularly useful for processing boar ejaculates, which are voluminous. Thus protocols and colloids have been developed for the following sizes of tubes: 15 mL, 50 mL, 100 mL, 500 mL [34]. For optimum results, the specific colloid formulation for a particular size of tube should be used. These modifications in the SLC methodology are shown in **Table 1**.

The SLC technique was also modified to process small volumes of thawed bull semen. Whereas some studies were reported in which 1 mL of colloid was used in a microcentrifuge tube with a small volume of semen e.g. [35], it was found that a higher yield of spermatozoa cold be obtained using 1 ml of colloid in a 15 mL tube [36].

Table 1. Size of tube, volumes of colloid and semen used in various modifications of SLC (after [75]).

Tube size	Volume colloid (mL)	Volume extended semen* (mL)	Reference
12 - 15 mL	1.0	0.25	[36]
12 - 15 mL	4.0	Up to 4.5	[32]
50 mL	15	Up to 20	[32] [33] [59]*
100 mL	20	25	[33]
200 mL	60	60	[32]
500 mL	150	150	[33]

*Optimal sperm concentration of up to 100×10^6 is appropriate for boar and stallion semen; for bull semen, 50×10^6/mL should be used.

The silane-coated silica colloid is expensive, and therefore being able to use smaller volumes is attractive on economic grounds.

5. Selection or Enrichment?

Several researchers have argued that in fact there is no selection of particular sub-populations, only an enrichment of viable, motile spermatozoa by separating them from dead or dying spermatozoa. There is some evidence that colloid centrifugation does result in selection of spermatozoa; in a recent publication, Morrell *et al.* [37] showed that sperm samples after SLC had a different pattern of reactive oxygen species (ROS) production than uncentrifuged samples. The SLC-samples showed a significantly reduced production of hydrogen peroxide compared to controls, whereas the production of superoxide was slightly increased. These results are intriguing, since it is commonly believed that superoxide radicals are rapidly converted to hydrogen peroxide. These results suggest that either superoxide is not converted to hydrogen peroxide, as previously, thought or that SLC may allow the passage of a sperm sub-population that metabolise differently to the main population. In the latter case, it would suggest that selection of spermatozoa with certain properties is being achieved. However, whether the mechanism of colloid centrifugation is selection or enrichment, the end result is that a sperm sample is produced that survives longer than controls, may have better chromatin integrity and morphology than controls and, at least in horses, results in a higher pregnancy rate after AI.

6. Alternative Methods for Selecting Spermatozoa

Alternative sperm selection techniques include migration (where spermatozoa move from the sample into medium), and filtration through Sephadex. Migration techniques select for motile spermatozoa, but these spermatozoa may be morphologically abnormal or have damaged chromatin. The sperm recovery rate is low, being approximately 10%, the method is time-consuming and requires an incubator or at least a water bath in which the tube containing the sample can be placed at an angle. Sephadex purportedly selects for motile, morphologically normal spermatozoa with intact acrosomes. However, neither of these techniques has been adapted for routine field use. In addition, a sperm washing technique is used to remove most of the seminal plasma from the sperm sample. This technique does not select a sperm sub-population but may prolong sperm life during storage [38], although other authors have observed an increase in sperm chromatin damage after this type of handling. A comparison of these different techniques, including sperm washing and colloid centrifugation, is described in detail in [3] and is summarized in **Table 2**, modified from [3]. The species in which these techniques have been tested are listed in **Table 3**. A brief description of the most commonly tested techniques follows.

6.1. Sperm Washing

This method separates the spermatozoa from seminal plasma [39] and extender such as cryopreservation medium. Semen extender is added to the semen which is then centrifuged gently; the supernatant is removed and the sperm pellet resuspended in medium or fresh semen extender. Most of the spermatozoa that were present in the original sample are found in the sperm pellet, together with dead and abnormal spermatozoa and cellular debris [40]. However, there are some reports that the most motile spermatozoa may swim-up again from the sperm pellet while the centrifuge is decelerating, and are thus discarded with the supernatant. Up to 20% of the most motile spermatozoa may be lost through this route [41]. Sources of reactive oxygen species are not removed; these may be detrimental to sperm viability or may cause chromatin damage. In an effort to reduce the number of spermatozoa lost, a modification of the sperm washing technique is to use cushion fluid in the bottom of the centrifuge tube. A dense material is layered under the sperm sample before centrifugation, through which the spermatozoa cannot pass; thus the cushion fluid acts as a buffer between the spermatozoa and the walls of the centrifuge tube allowing the sample to be centrifuged at a much higher g force than where there is no cushion fluid. However, such samples also contain more damaged chromatin than samples prepared by other methods [42], suggesting that increasing the centrifugal force is not necessarily problem-free.

In a modification of the washing technique, low-molecular weight components of seminal plasma have been removed from boar ejaculates by dialysis [43]. More recently, a sperm filter similar to a coffee filter was reported to separate spermatozoa from seminal plasma without the need for centrifugation [44]. The recovery rate after the sperm filter was greater than after sperm washing (89% versus 81%).

Table 2. Properties of different sperm separation and selection methods (*modified from* [3]).

Property	Washing; simple filters	Migration e.g. swim-up	Filtration	Colloid centrifugation
Ease of use	Simple			Requires some attention to detail
Equipment required	Centrifuge	Special tubes needed for swim-through		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 integrity, acrosome integrity.
Seminal plasma removed	Mostly	Yes	Some removed	Yes
Pathogens and debris removed	No	?	?	Yes
Yield of motile spermatozoa	?	10% - 20%	ca. 60% - 85%	>50%
Leukocytes	Present	Removed	Removed	Removed
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 problems with Percoll™ (endotoxin levels, PVP etc)
Animal	Buffalo, ram, boar, stallion	Bull, ram, boar, dog	Ram, boar, buffalo, bull	Bull, ram, boar, stallion, turkey, dog, leopard

Table 3. Examples of sperm preparation techniques used in various species.

Sperm washing	Migration e.g. swim-up	Filtration e.g. glass wool, sephadex	Colloid centrifugation
Buffalo [76] Ram [77] bull [78] Boar [79] Stallion [80] [81]	Bull [40] [44] [78] [82] [83] Ram [77] [84] Boar [85] Buffalo [76] [86] Stallion [30]	Ram [77] Boar [87] Buffalo [50] [75] Stallion [51] [88] [89] Bull [74]-[76] [90] Dog [48]	Bull [45] [78] [82] Ram [77] Boar [62] [91] Stallion [92] Turkey [93] Dog [94] Goat [69] Red deer [33] Donkey [70] Brown bear [71] Cat [53] [72] Gray wolf [74] Camel [95]

6.2. Sperm Migration

Migration techniques rely on the ability of spermatozoa to move from the extended ejaculate or washed sperm pellet into another medium [25], thereby separating themselves from seminal plasma. The sperm sample can be underneath, on top of, or to one side of the second medium into which the spermatozoa will swim [25], although the most common arrangement is to have the sperm sample underneath (hence the name “swim-up”). Selection is based on motility; there is no selection for other parameters of sperm quality such as head morphology, viability, acrosome status or chromatin integrity [45]. Sperm samples may exhibit better midpiece- and tail-morphology after swim-up than after sperm washing, e.g. [40] because tail abnormalities may hinder motility and the spermatozoa

are therefore retained in the original sample. Migration through, media containing hyaluronic acid may select for spermatozoa with intact membranes [46] [47]. The major disadvantage of migration methods is the low recovery rate, e.g. 10% - 20% [40] thus making it impractical for preparing AI doses in most animal species, although it can be a useful technique for separating spermatozoa from seminal plasma prior to IVF.

6.3. Sperm Filtration

Substances such as glass wool and Sephadex beads have been used to select motile, viable spermatozoa with intact acrosomes [48]. Non-viable spermatozoa tend to adhere to the matrix more than motile spermatozoa [49]. The mechanism of action is unclear but may involve adherence of abnormal spermatozoa to the matrix due to surface charges [50], or proteins present on the sperm surface after capacitation [51]. Bull spermatozoa are reported to show improved freezability after Sephadex filtration [52]. Such methods help to eliminate leukocytes (and thus some sources of ROS) and fewer spermatozoa are lost than with other methods, e.g. a recovery rate of approximately 63% was reported by [53]. However, the filtrate is not as clean as with other sperm separation methods [53], because not all of the seminal plasma and cellular debris is removed.

6.4. Additional Selection Techniques

Newer technologies, such as microfluidics, electrophoresis, motile sperm organelle morphology examination (MSOME), and birefringence have been advocated for processing human semen [54]. One example is that the microfluidic device was used to select spermatozoa with better chromatin fragmentation, as evaluated by the DNA dispersion test, from oligospermic human samples [55]. However, such methods are too expensive and impractical to be considered for processing animal semen. In addition, the authors claim that eliminating the centrifugation steps should improve the selection of sperm with higher motility, normal morphology and DNA integrity, whereas colloid centrifugation achieves all these outcomes if performed correctly.

7. How to Perform Single Layer Centrifugation

The following directions for use apply to species-specific colloids developed by the authors, previously known as Androcoll with a suffix denoting the species e.g. Androcoll-E was for the equine species. It should be noted that the protocol has been developed only for this colloid formulation [32]; other colloid formulations may not give such good results.

The ready-to-use colloid should be equilibrated to room temperature before use, to avoid cold-shocking the spermatozoa. The colloid is poured into the centrifuge tube and the semen sample, adjusted to the appropriate sperm concentration (e.g. 100×10^6/mL for boar and stallion; 50×10^6/mL for bull), is pipetted on top to form a distinct separate layer. The semen should be added slowly so that the interface between the colloid and semen is not disrupted. The tube is placed in the centrifuge at 300 ×*g* for 20 minutes. The centrifuge should preferably have a swing-out rotor and the brake should not be used *i.e.* the centrifuge should be allowed to slow down gently at the end of the centrifugation time. The supernatant is removed carefully: first the seminal plasma and extender, then the interface layer (containing spermatozoa that have not passed into the colloid), and lastly the colloid, down to the last 1 - 2 mm above the sperm pellet. Using a clean pipette, the sperm pellet is retrieved from beneath the colloid and placed in a clean tube for suspension in semen extender to the desired concentration.

8. Practical Applications of SLC

Apart from sperm washing, which does not select spermatozoa, SLC is the only sperm selection technique that is used in the field. The recovery rate is linked to the quality of the original sperm sample, typically ranging from 20% - 90% with a mean recovery of >50%. Practical applications of SLC for equine spermatozoa have been reported previously [56]; however, applications of SLC for other species have not been reviewed. The following section describes some of these applications in livestock species and for conservation breeding.

8.1. Preparing Spermatozoa for Artificial Insemination

Pregnancy rates after AI are known to be linked to sperm quality in the ejaculate [10]; therefore, selecting the

most motile, morphologically normal spermatozoa with intact chromatin should, theoretically, result in a higher pregnancy rate after AI. This was found to be the case in an AI trial in horses, in which insemination of SLC-selected sperm samples resulted in significantly more pregnancies than unselected control samples [57]. The SLC-selected stallion sperm samples also retain motility, viability and chromatin integrity in storage at 6˚C, which unselected controls do not [58]. The SLC samples were still fertile 96 h after semen collection and selection, whereas unselected semen usually cannot be stored for more than 36 h after semen collection. The pattern of ROS-production is changed in the selected samples, with significantly less production of hydrogen peroxide in the SLC-selected samples than in unselected controls. This observation may help to explain the apparently longer life of the SLC-selected spermatozoa than unselected samples.

Fresh bull semen has also been prepared by SLC, using the scaled-up variant of a bovine-specific colloid in 50 mL tubes. Sperm chromatin integrity was reported to be improved in SLC samples compared to controls [59], although sperm motility was only improved in a haemospermic sample [60]. In other studies, a higher proportion of spermatozoa with high mitochondrial membrane potential was observed in the SLC samples than in controls [61]. Superoxide production was also increased; these two parameters indicate that the metabolic activity of the spermatozoa in the SLC samples may be increased compared to controls [61].

8.2. Improving Cryosurvival

In some species, such as cattle, the AI industry depends on cryopreserved semen. Cryopreservation not only prolongs the life of the semen sample and enables it to be transported around the world for insemination into females in other countries; it also permits the semen to be "quarantined" until the donors have been shown to be free of disease at the time of semen collection. However, spermatozoa are easily damaged by the processes of freezing and thawing, despite the addition of cryoprotectants; thus the sperm quality of thawed semen may be poor and the resulting pregnancy rate low. At the present time, success with freezing stallion and boar semen is not as good as with bull semen, with the result that the equine and swine breeding industries rely mostly on fresh (usually cooled) semen.

Selection of stallion and boar spermatozoa by SLC prior to cryopreservation results in better sperm quality in the thawed samples than in unselected controls e.g. stallion [42]; boar [62] [63]. The SLC-selected sperm samples survived longer after thawing than control samples [64]. However, using a different colloid, Mancill *et al.* [65] were unable to show a difference in pregnancy rates compared to controls when colloid-centrifuged stallion semen was frozen and subsequently used for AI. Clearly further research is needed to optimize the protocols.

8.3. Preparing Spermatozoa for *in Vitro* Fertilization or ICSI

The most frequently reported application for SLC in animal assisted reproduction is in preparing thawed sperm samples for IVF e.g. [66]. In this application, the spermatozoa have to be separated from the cryoextender in which the semen was frozen, and from seminal plasma that contains decapacitation factors. Selection of spermatozoa with normal morphology and intact membranes is associated with increased blastocyst formation and quality. It is not only the ability of the spermatozoa to penetrate and activate the oocyte that is important but also the ability of the zygote to continue to develop; chromatin integrity is implicated in this process. The advantages of SLC over "swim-up" are that the selection is not just based on motility, but also on other attributes of sperm quality, the procedure is quicker than for "swim-up" and a higher yield of spermatozoa is recovered. Stallion spermatozoa have been prepared for ICSI using SLC [67].

8.4. Conservation Breeding

Sperm quality may be a problem in rare breeds [68], particularly since there are a limited number of males from which to choose, and they will not have been selected for sperm quality. Thus, SLC can be useful either in selecting good quality spermatozoa prior to cryopreservation or in processing the thawed sperm samples. There have been reports of the use of SLC to improve sperm quality in several rare species: e.g. goats [69]; donkey [70]; brown bear [71], and domestic cat as a model for wild species [72]. Use of SLC with thawed sperm samples is more appropriate for IVF than for AI in the field but is theoretically possible for the latter if there is access to a centrifuge.

As mentioned previously, epididymal or testicular spermatozoa can be purified by SLC e.g. cat [73]; stallion

[5]. A similar method has been used to prepare wolf spermatozoa free of somatic cells for DNA extraction [74].

8.5. Potential Applications for Non-Reproductive Cells

Since silane-coated silica is non-cytotoxic, colloid centrifugation could potentially be used for other types of cells, such as stem cells and immune cells, as well as female reproductive cells. The composition of the salt solution used to dilute the colloid should be altered to suit the types of cells to be selected, and the pH and osmolarity adjusted. The density of the colloid should also be optimized for the particular cell type, depending on the application.

9. Conclusion

Of the various techniques available for selecting spermatozoa for ART, colloid centrifugation has the greatest potential for selecting spermatozoa with the desired attributes for fertilization and embryo development. In addition, good recovery rates can be obtained by paying attention to the physical and chemical properties of the colloids to be used, and considering the physical properties of the semen to be processed. The recovery rate of good quality spermatozoa is higher than other techniques. Colloid centrifugation, particularly SLC, offers many advantages for improving sperm quality for ART, such as selecting motile spermatozoa with normal morphology, intact membranes and good chromatin integrity. These spermatozoa have improved fertilizing ability compared to unselected spermatozoa. Colloid centrifugation has a range of applications for different species, including prolonging the usable life of fresh or cooled semen samples, and improving cryosurvival. The procedure is straightforward and quick to perform, can be used by the personnel on most stud farms or semen collection stations where there is a swing-out centrifuge, and in IVF laboratories; a similar technique may be used for other types of reproductive cells, such as spermatids or spermatogonia for germplasm banking, or may be used to select non-reproductive cells.

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