Bull Sperm Selection for Assisted Reproduction

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Abstract

The sperm population within an ejaculate is heterogeneous, reflecting differing ability to fertilize an oocyte. Selecting sperm sub-populations with certain desired characteristics may have a positive effect on pregnancy rate in assisted reproduction. This review describes different methods of biomimetic selection for bull spermatozoa, focussing particularly on colloid centrifugation. Migration, microfluidics and magnetic activated cell sorting are also described. Several versions of the colloid centrifugation technique known as single layer centrifugation are available, differing in the volume of sperm sample to be processed. Samples can be processed in volumes ranging from 0.25 to 150 mL, in appropriately sized tubes. Processing small volumes of semen (0.25 mL–1.0 mL semen on 1 mL colloid) is best done in a 15 mL tube, since the interface between the semen and colloid is greater than in a 1.5 mL tube. In addition to current applications, future uses of colloid centrifugation are described, for example, as biomarkers of fertility, for improving the semen quality of young bulls, and for removal of pathogens.

Keywords

Biomimetic selection · Colloid centrifugation · Fertilizing ability · Reproductive efficiency · Livestock production

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5.1 Introduction

Selection of bull spermatozoa for assisted reproduction has been reported frequently over the last three decades. The reasons for wanting to select spermatozoa vary from selection of X- or Y-chromosome bearing spermatozoa and thus to choose the sex of the calf before conception, to selecting the spermatozoa that are judged most likely to be able to reach the oviducts and fertilize an oocyte. Extensive reviews have focussed on sperm sex selection, which will therefore not be covered in the present review. Instead, this review will focus on biomimetic selection of the spermatozoa that are most capable of reaching the oocyte. The highlights and changes that have occurred over the last 30 years will be described, and the directions of potential future development of various methods will be discussed. The first section will consider the assisted reproduction technologies (ART) for which bovine spermatozoa are used and why sperm selection is needed for them, followed by a description of the different sperm selection methods available. The remainder of the review will focus on developments in one of these methods: colloid centrifugation.

5.2 Assisted Reproduction in Cattle

Artificial insemination (AI) is by far the most widely used reproductive biotechnology for breeding cattle. Estimates of the number of AIs performed in cattle are difficult to pinpoint because of differences in the way such figures are reported but in 1999, 264 million bull semen doses were produced worldwide (Thibier and Wagner 2002). It is likely that this figure has more than doubled in the last 20 years since the annual production of cattle and buffalo semen doses in India alone is now 115 million. Embryo transfer is the second most widely used ART, but accounts for a much smaller proportion of the animals bred than AI. In 2019, almost 1.5 million transferrable embryos were produced (Viana 2019). The embryos used are either derived in vivo or may be produced in vitro in some countries.

Another reproductive biotechnology, intracytoplasmic sperm injection (ICSI) of bovine oocytes, is possible (Magata et al. 2019) but is not performed routinely. There are problems with the technique in cattle, partly because of the dark ooplasm, making visualization of the internal structures of the oocyte difficult (Wei and Fukui 2020), and the large head of bull spermatozoa (Galli et al. 2003), but also due to lack of oocyte activation. These technical difficulties result in low efficiency of the technique in cattle (Unnikrishnan et al. 2021).

Embryo production in vitro (IVP) and ICSI have their own specific requirements for sperm preparation.

5.2.1 Sperm Preparation for Artificial Insemination

Bull semen receives minimal preparation; if the ejaculate reaches certain thresholds, e.g. for sperm membrane integrity, or morphology or motility, a suitable extender is

added to give the desired sperm concentration, calculated to deliver a set number of spermatozoa in a 250 μL straw. Different breeding companies have their own thresholds of acceptance and their own values for the optimum sperm number per dose. The extender usually contains a cryoprotectant such as glycerol if the semen is to be frozen, and antibiotics.

5.2.2 Sperm Preparation for Embryo Transfer

In ET, in vivo derived embryos may be produced by performing AI in a superovulated female, followed a week or so later by flushing to recover the resulting embryos. Alternatively, the embryos are produced in vitro (IVP), either following in vitro maturation (IVM) of immature oocytes aspirated from ovaries obtained from the slaughterhouse, or using mature oocytes obtained from a donor cow using ovum pick-up. Whatever the source of the oocytes, some form of sperm selection is performed, both to separate the spermatozoa from seminal plasma (which contains de-capacitation factors) and from the cryomedium in which they were frozen, as well as to select the most robust spermatozoa. Cryopreservation can inflict considerable injury on sperm samples; therefore, sperm selection separates the least damaged spermatozoa from the rest of the sample.

5.2.3 Sperm Preparation for Intracytoplasmic Sperm Injection

The sperm sample for ICSI is first prepared in the same way as a sample for IVF, but then an individual spermatozoon is selected. The operator chooses spermatozoa that are motile and with no apparent morphological defect. The tail is broken, and the spermatozoa are aspirated into the injection pipette before being inserted into the cytoplasm of the oocyte.

5.3 Why Select Spermatozoa?

Ejaculated semen contains a heterogeneous population of spermatozoa at different stages of maturity. These spermatozoa differ in their ability to reach an oocyte after deposition in the female reproductive tract. Selection mechanisms exist within the female to ensure that only the spermatozoa with certain attributes reach the oocyte for potential fertilization (Suarez 2007). Spermatozoa must be motile (to swim against fluid flow), have normal morphology, intact membranes and an intact acrosome. They must also be able to capacitate and to undergo the acrosome reaction in the presence of an oocyte. Thus, of the millions of spermatozoa present in the ejaculate, only a few hundred actually reach the oocyte and only one is able to fertilize it. In assisted reproduction, these natural selection mechanisms may be circumvented, for example, by depositing the semen in a different part of the reproductive tract than would occur during natural mating, resulting in a higher

proportion of the sperm population potentially being able to achieve fertilization. If the oocyte is fertilized by a spermatozoon that is abnormal, development may be impaired or interrupted at some stage, even after implantation. Clearly, in IVP and especially in ICSI, these physiological interactions with the reproductive tract of the cow are missing. Therefore, there is a chance that spermatozoa with sub-optimal characteristics might fertilize the oocyte.

Another reason for utilizing sperm selection is to choose spermatozoa with intact chromatin. Sperm DNA is in a condensed form (chromatin) that becomes increasingly tightly packed as the spermatozoon matures. A spermatozoon with damaged chromatin may have good motility and normal morphology and can compete with other spermatozoa to fertilize the oocyte. The oocyte has the ability to repair some DNA damage but the repair mechanisms may be overwhelmed if too much damage is present (González-Marín et al. 2012). Fertilization by a spermatozoon with damaged DNA can lead to subsequent embryonic development being halted. Some research suggests that, at least in pigs, spermatozoa with damaged chromatin do not reach the oviducts (Ardon et al. 2008), but studies in human patients (Flamigni and Coticchio 2006) suggest that this is not the case since an association was found between DNA fragmentation and post-implantation embryo loss. In horses, an negative association was observed between DNA fragmentation and fertility (Love and Kenney 1998).

The main reason for using sperm selection in ART is to ensure that most of the "unsuitable" spermatozoa are removed, thus enabling high quality spermatozoa to be available to fertilize the oocyte. Since the sperm selection techniques used in the laboratory mimic those occurring in the female reproductive tract, they are known as biomimetic.

5.4 Biomimetic Sperm Selection Methods

Sperm selection methods are based on separation of spermatozoa with certain physical characteristics; they have been reviewed in detail previously (Morrell and Rodriguez-Martinez 2009, 2010, 2016). Recently new methods, such as Magnetic Activated Cell Sorting (MACS) and microfluidic devices have become available which deserve consideration. Therefore, the focus of this section will be on these newer techniques, as well as migration and colloid centrifugation.

5.4.1 Sperm Selection by Migration

The sperm selection methods that have been consistently used in practice are sperm migration (in the form of "swim-up") and colloid centrifugation. These techniques are used when preparing bovine spermatozoa for IVF and enable the spermatozoa to be separated from seminal plasma and extender. In "swim-up", selection is based only on motility; there is no selection for normal morphology or intact chromatin. In contrast, colloid centrifugation selects for morphologically normal, motile

spermatozoa with intact chromatin (Morrell et al. 2009), and also removes seminal plasma proteins that are coating the surface of the spermatozoa (Kruse et al. 2011). "Swim-up" takes approximately 45–60 min, recovering 10–20% of the spermatozoa in the sample. Colloid centrifugation, in contrast, requires only 25 min preparation time (including the 20-min centrifugation) with a recovery rate of >50% for sperm samples transported overnight from the bull stud (Goodla et al. 2014), depending on the sperm quality of the original sample. Although migration methods were the first selection methods to be used when preparing spermatozoa for IVF, only a small proportion of the spermatozoa are recovered, and the procedure takes too long to be of use when preparing whole ejaculates for AI.

Another migration technique is potentially able to select spermatozoa exhibiting a particular type of motility, the ability to move against fluid flow or rheotaxis. Spermatozoa must move against the direction of fluid flow in the female reproductive tract (which flows caudally) to enable them to reach the site of fertilization. Recently, the characteristics of sperm sub-populations separated by rheotaxis were examined (Rappa et al. 2018). Although the velocity of spermatozoa exhibiting rheotaxis flow was different to those that did not, sperm morphology and hyaluronic acid binding did not differ between the two sub-populations. This microfluidic device may be useful as a research tool for investigating which characteristics are relevant for spermatozoa to move within the female tract, but it would not be practical in its present form for routine selection of spermatozoa with high velocity for AI. An AI trial involving low doses of microfluidic-sorted sperm samples ($<10^6$ spermatozoa) was reported to give a pregnancy rate of 37% compared to almost 40% with a standard dose of unsorted controls (Nagata et al. 2018). Chromatin integrity was better, and high mitochondrial membrane potential was higher in the selected samples than in controls. However, a higher proportion of the spermatozoa were starting to capacitate in the microfluidic-selected samples than controls. These results are intriguing, raising the possibility that if the method could be scaled–up and speeded up to process more spermatozoa in a reasonable time, and capacitation could be avoided, it could be feasible to prepare sperm samples for AI in this manner.

5.4.2 Sperm Selection by Magnetic Activated Cell Sorting

Magnetic activated cell sorting (MACS) selects cells on the basis of binding of surface molecules to coated magnetic beads. Thus, spermatozoa that have phosphatidylserine exteriorized on their membranes because they are entering apoptosis will bind to Annexin V- coated magnetic particles and can then be removed from the sample in a magnetic field (Faezah et al. 2014). Although technically feasible for sperm samples, MACS does not appear to have been widely adopted. There are some reports of its use to prepare human spermatozoa for intracytoplasmic sperm injection (ICSI), although no improvements in fertilization, pregnancy, embryo quality, implantation, and live birth rates were observed between controls and selected sperm samples (Nadalini et al. 2014; Romany et al. 2010). Other studies used magnetic nanoparticles coated either with antibody to ubiquitin or with lectin

that binds to glycan exposed on the sperm surface. Similar conception rates were obtained for the nanoparticle-purified sperm samples and the controls, even though the sperm number was half the normal dose in the selected samples (Odhiambo et al. 2014). Similarly, cat or bull spermatozoa selected using nanoparticles were reported to have similar fertilization rates to controls in IVF (Durfey et al. 2019). The technique was considered to require further refinement (Nagata et al. 2018). These results are to be expected since the spermatozoa that are being removed are dead or damaged and would not succeed in binding to the oocyte in IVF. A more relevant aspect to investigate would be whether sperm quality deteriorated more slowly in the selected sperm samples than in controls, because of the removal of damaged spermatozoa that are potential sources of reactive oxygen species.

5.4.3 Sperm Selection by Colloid Centrifugation

This technique has received considerable interest from the equine semen industry, where problems with sperm quality can be encountered since stallions are selected as breeding sires based on their performance in competition rather than on seminal attributes. However, interest in colloid centrifugation from the bovine semen industry has been slight, mainly because bull sperm quality is generally considered to be good. In most countries, there is no market for bull sperm samples that do not survive freezing (possibly with the exception of New Zealand and Ireland where fresh semen is inseminated), resulting in continual selection for ejaculates that reach certain thresholds of post-thaw sperm quality. However, it is possible that the criteria commonly used for accepting bull semen for commercial distribution are not the most relevant in terms of fertility, since only approximately 40% of inseminated dairy cows produce a calf. Various combinations of sperm quality parameters have been suggested as biomarkers of fertility (Kumaresan et al. 2017) that may serve as a better indicator of in vivo fertility than the ones currently employed.

The principle of colloid centrifugation of spermatozoa is that mature motile spermatozoa with intact membranes and good chromatin integrity are separated from the rest of the ejaculate by passage through a colloid during gentle centrifugation (Morrell and Rodriguez-Martinez 2009). Poorly motile or immotile spermatozoa and those with damaged membranes are retained at the interface between the colloid and the semen, provided that the centrifugal force used is low and the centrifugation time is restricted (typically 300 g and 20 min). A detailed description of the protocol for stallion semen was provided previously (Morrell and Nunes 2018). The method for bull semen is similar except that the semen should first be extended to a sperm concentration of approximately 50×10^6 spermatozoa/mL (Nongbua et al. 2017). The selection is based on the ease with which the spermatozoa can pass between the colloid particles; motile spermatozoa will pass through the colloid more easily than immotile spermatozoa, as will spermatozoa with normal morphology and intact acrosomes. Selection also occurs according to density. In the nucleus, the chromatin becomes more and more tightly packed as spermatozoa mature, resulting in an increase in density. Thus, those spermatozoa

Table 5.1 Comparison of migration methods, magnetic activated cell sorting, microfluidics and colloid centrifugation for improving bovine sperm quality

Method	Advantage	Disadvantage
Swim-up	Cheap; readily available; improves fertility	Lose most of the spermatozoa; time consuming, need an incubator and possibly a centrifuge
Microfluidics	Good selection	Low yield; need specialist devices; takes time; only suitable for IVF or possibly low-dose AI; expensive? Effect on fertility unknown. Needs further development
Magnetic activated cell sorting	Rapid	Columns of magnetic particles are expensive; cannot process large volumes of ejaculate; need a magnet, i.e. not readily available in sperm labs. Does not improve fertility
Colloid centrifugation	Good selection; available for all; improves fertility	Colloid is expensive; need a centrifuge

that are less dense remain at the semen/colloid interface whilst the spermatozoa with mature chromatin pass through the colloid. Spermatozoa in which DNA strand breaks are present may be less dense than those with intact DNA although this speculation has not been investigated. Therefore, the sperm pellet will be enriched for motile spermatozoa with normal morphology and intact chromatin, compared to the original sperm sample.

A summary of the different methods of sperm selection discussed in this review is shown in Table 5.1.

Some of the uses of colloid centrifugation in processing bull sperm will now be considered.

5.5 Uses of Colloid Centrifugation in Bovine Assisted Reproduction Technologies

5.5.1 Early Uses of Colloid Centrifugation

Some of the first reported uses of colloid centrifugation in bovine ART were in preparing sperm samples for IVF using Percoll density gradients (de Vries and Colenbrander 1990). The outcome was mixed, with some reports mentioning an increase in the number of acrosome-reacted spermatozoa in the density gradient prepared samples. The problem may have arisen from the use of synthetic human tubal fluid (HTF) to prepare the different colloids needed for the density gradient, since the osmolarity of the resulting colloids may have been conducive for capacitation and acrosome reaction to occur. Using colloid formulations with optimized physical characteristics should avoid this issue (Morrell and Wallgren 2011). A further problem was that a toxic effect of some batches of Percoll was reported, necessitating testing of each batch before use (Avery and Greve 1995). Since then there has been a move to using silane-coated silica instead of polyvinylpyrrolidone

(PVP)-coated silica as the basis of the colloid formulations; the PVP-coated silica preparations at the time could not be autoclaved, which may have been a contributing factor to a detrimental effect perceived with some batches. No further reports of toxicity of Percoll to bull spermatozoa appeared although problems with murine zygote development were observed following exposure of the sperm to PVP (Mizuno et al. 2002).

The next development in colloid centrifugation was the modification of the density gradient technique to use only one layer of colloid, i.e. only one density of colloid and therefore not a density gradient. This modification was described for animal semen in 2006; its use has increased dramatically since then. The advantages of using only one layer of colloid is that there is no need to prepare several colloids of different densities (Morrell and Rodriguez-Martinez 2009) and the ready-to-use species-specific colloid formulations available allow consistency between batches (Morrell 2006). Crucially for its use with animal semen, the SLC technique can be scaled-up to process larger volumes of semen. Although the bull ejaculate is typically only a few millilitres in volume, the sperm concentration is very high, requiring considerable extension before colloid centrifugation. It is important not to overload the colloid if high recovery rates are to be achieved, to minimize sperm competition to enter the colloid (Morrell et al. 2010). Theoretically, a whole bull ejaculate could be processed in approximately eight 50-mL centrifuge tubes, depending on the sperm concentration in the original ejaculate.

Currently, the major use of colloid centrifugation for bovine sperm samples is still in sperm preparation for IVF. Freshly ejaculated bull semen is usually of very good quality, in terms of motility, membrane integrity and morphology. Therefore, semen producing centres do not perceive the need to select spermatozoa by colloid centrifugation, since this method inevitably results in the loss of some robust spermatozoa along with the poorly motile ones. However, an association between chromatin integrity and fertility was reported for Norwegian Red bulls (Narud et al. 2021), showing similar trends to the associations seen in the stallion (Love and Kenney 1998). If an oocyte is fertilized by a spermatozoon with damaged chromatin, embryonic development may be initiated but is halted at some stage (Gopalkrishnan et al. 2000; Lazaros et al. 2011), possibly even after implantation has occurred. In studies with human IVF and ET, implantation rates were lower where the sperm donor had higher rates of damaged chromatin than where the sperm chromatin was normal (Simon et al. 2014). Therefore, we can speculate that selecting for bull spermatozoa with intact chromatin for AI might be beneficial in avoiding some early embryonic loss, which is high in cattle. In a recent study in Swedish dairy cattle, approximately 45% of pregnancies were lost in the early stages (Ask-Gullstrand et al. 2020); the chromatin integrity status of the sperm samples was not reported. It would be interesting to establish whether there is an association between chromatin status and early embryonic loss.

5.5.2 Colloid Centrifugation to Improve Sperm Quality Pre-Freezing

Despite the high quality of most bull ejaculates, it is still possible to improve some aspects of sperm quality further by colloid centrifugation. Goodla et al. (2014) reported less DNA fragmentation and a higher mitochondrial membrane potential in bull sperm samples processed by SLC than controls. Normal morphology was increased in four of the 20 bulls in her study (Goodla et al. 2014). Membrane integrity and total and progressive motility were not different between SLC and control samples. Production of some reactive oxygen species was also increased: thus, there was a higher proportion of spermatozoa producing superoxide, corresponding to the higher mitochondrial membrane potential observed in the SLC samples, but also a higher production of hydrogen peroxide, although levels were very small in all samples. In summary, although there was a less obvious beneficial effect of SLC in bull semen than reported for stallion semen (Morrell et al. 2010), there was still an improvement in some parameters of sperm quality. This result may be due to the quality of bull semen being high to start with.

5.5.3 Colloid Centrifugation to Improve Sperm Quality Post-Freezing

Similar beneficial effects of preparing bull ejaculates by SLC prior to freezing on post-thaw sperm quality were reported (Nongbua et al. 2017). Thus, SLC samples had better chromatin integrity and a greater proportion of spermatozoa with high mitochondrial activity post thaw than controls. However, a study on bulls in Thailand showed that both sperm motility and normal morphology were greater in SLC samples than in controls. These results suggest that the degree of improvement seen depends on the initial quality of the samples, with a greater beneficial effect in the lower quality samples. This result is in keeping with studies on stallion semen (Morrell et al. 2010). In an AI study carried out in Thailand, the pregnancy rate following insemination with SLC-selected samples was approximately twice that of control samples (Thanapol Nongbua, personal communication).

Chromatin integrity can be evaluated in many ways. Apart from detecting single strand breaks, as in the Sperm Chromatin Structure Assay, evaluating free thiols indicates the degree of condensation of the chromatin, while the presence of retained histones has been associated with lower fertility and also indicates potential sites for epigenetic change. Preliminary results indicated that there were fewer free thiols in SLC samples than in controls (Morrell et al. 2017).

5.5.4 Colloid Centrifugation to Prepare Thawed Spermatozoa for IVF

The first stage in preparing spermatozoa for IVF is to separate the spermatozoa from medium and seminal plasma. Colloid centrifugation has several advantages over swim-up for this purpose since it allows selection of the most robust spermatozoa, which are assumed to be the most capable of fertilization. Whereas swim-up (or other migration techniques) enables motile spermatozoa to be separated from the rest of the sample, the spermatozoa may not have other desirable attributes such as normal morphology or intact chromatin (Samardzija et al. 2006). However, IVF cannot mimic fertilization in vivo, not the least because the spermatozoa do not need to traverse the whole of the reproductive tract in order to locate an oocyte. The spermatozoa compete with each other to fertilize the oocytes in the same drop of medium. Furthermore, protocols for bovine IVF are designed to produce the maximum number of fertilized oocytes, including using an excess of spermatozoa. Using either swim-up or colloid centrifugation selects motile or good quality spermatozoa, respectively, thus negating the effect of the quality of the original sample (Morrell et al. 2016). Thus, if the purpose of the IVF is to look for potential differences in fertilizing ability between different sperm treatments, it is important to reduce the number of spermatozoa used to a threshold level so that differences in fertilizing ability can be detected (Sabés-Alsina et al. 2020).

Another interesting development is the use of smaller and smaller centrifuge tubes in an effort to reduce costs. Thus, there are several reports of "mini-gradients" in the literature. A study comparing sperm yield from different sizes of colloid preparations, however, deduced that the recovery rate is greater if 1 mL colloid is used in a 15 mL tube instead of a 1.5 mL (Eppendorf-type) tube (Abraham et al. 2016). This could be an important finding if sperm numbers are limited, e.g. when using commercial sexed semen for IVF. Another point to note is that many protocols using mini-colloid preparations use very high g forces to try to maximize sperm yield. Studies in other species showed that high g forces are associated with considerable DNA damage. Since spermatozoa with damaged DNA can compete with "normal" spermatozoa to fertilize an oocyte, and embryo development can proceed for some time before being halted because of problems with male DNA, it would seem to be preferable to avoid causing DNA damage during sperm preparation. However, no studies have been done to date to show an association with early embryonic death in vivo after transfer of these embryos.

The different version of SLC are depicted in Fig. 5.1 and their uses are summarized in Table 5.2.

Table 5.2 Summary of different versions of single layer centrifugation processing whole ejaculates or sperm samples

Sample	Size of tube (mL)	Volume of sample (mL)	Volume of colloid (mL)	Potential purpose
Thawed straw	15	0.25–1.0	1.0	IVF, ICSI
Aliquot	15	1.0–4.5	4	IVF, AI
Aliquot	50	20	15	AI
Whole ejaculate	500	Up to 200	150	AI

Note: *AI* artificial insemination, *IVF* in vitro fertilization, *ICSI* intracytoplasmic sperm injection.

5.6 Future Uses of Colloid Centrifugation

5.6.1 Colloid Centrifugation as an Indicator of Fertility

The number of spermatozoa passing through the colloid reflects the sperm quality of the original ejaculate. A study with stallion spermatozoa showed that the recovery rate of motile spermatozoa is strongly associated with sperm quality and also with the fertility of the uncentrifuged ejaculate in artificial insemination (Morrell et al. 2014). In a small study with bull semen samples, in which extended semen was sent overnight to the laboratory at 6 °C and SLC was performed on arrival, the recovery rate (proportion of the loading dose appearing in the sperm pellet) was found to vary among ejaculates (mean ± SD 49 ± 18%). This figure is quite similar to the general non-return rates of 40–45% currently reported for dairy cattle in Sweden (Ask-Gullstrand et al. 2020), based on 12,219 inseminations. This preliminary result indicates that it might be possible to use the recovery rate after colloid centrifugation as an indicator of the potential fertility of bull ejaculates. This is an interesting possibility and requires following up.

5.6.2 Improving the Sperm Quality of Young Bulls

Genomic selection enables interesting candidates for future breeding sires to be identified at a young age (Meuwissen et al. 2001). There is considerable interest from breeding companies in being able to use these young bulls as semen donors for AI as soon as possible. However, the sperm concentration, and motility (Murphy et al. 2018) and also sperm morphology (Karabinus et al. 1990) of young bulls tend to be inferior to older bulls. Preliminary results with colloid centrifugation suggest that it could enable some ejaculates to be used earlier than currently possible.

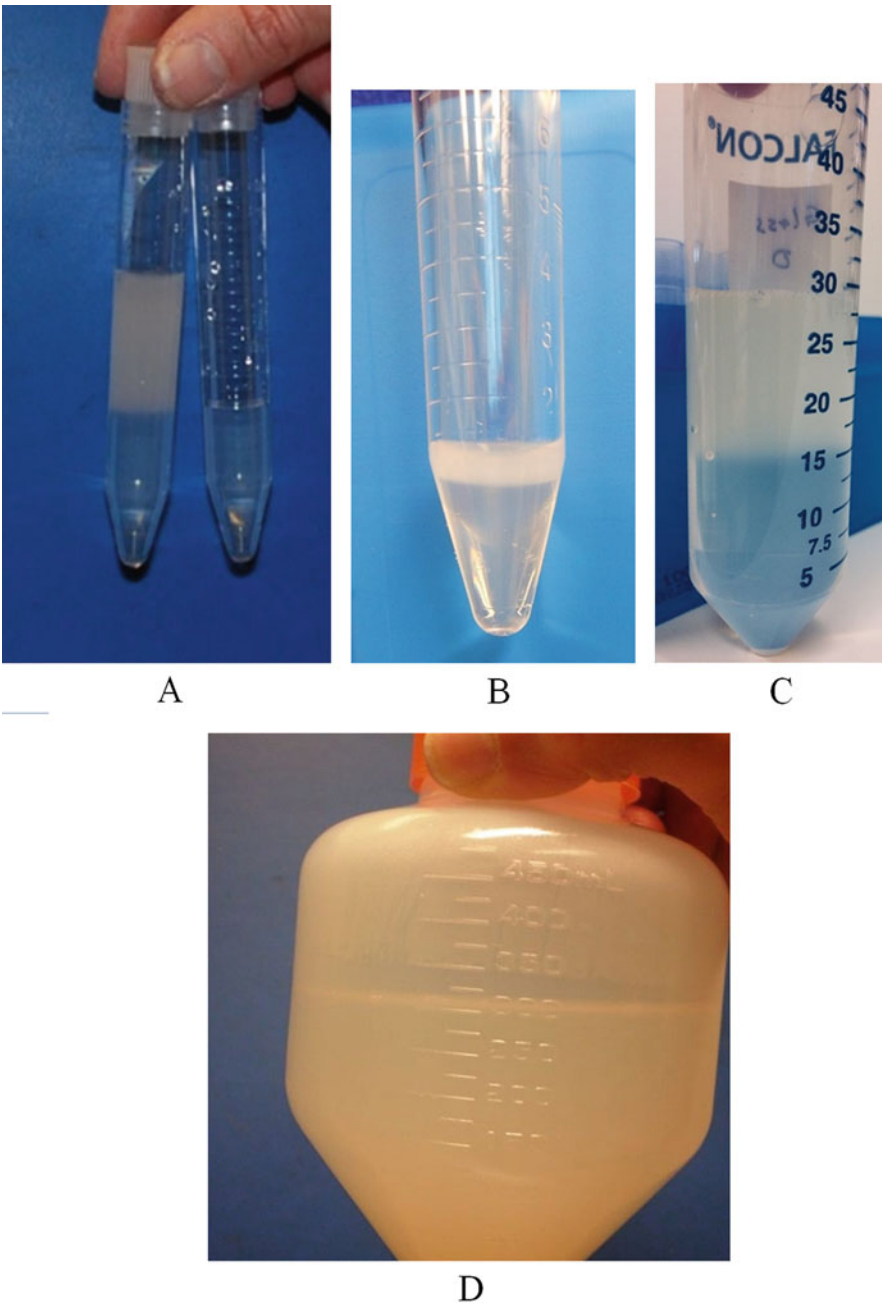

Fig. 5.1 Single Layer Centrifugation (SLC) in tubes of different sizes: (**a**) Small SLC, before and after centrifugation in 12 mL tubes; in the left-hand tube, 4.5 mL extended semen is layered over 4 mL colloid. In the right-hand tube, the sperm pellet is clearly visible. The white line at the interface between the colloid and the seminal plasma and extender contains spermatozoa that have not been able to pass into the colloid. (**b**) Mini-SLC, consisting of 1 mL colloid and 0.25 mL thawed semen before centrifugation. (**c**) Large SLC comprising 15 mL Bovicoll and up to 20 mL extended

5.6.3 Removal of Seminal Plasma

Although much effort has been devoted to determining links between sperm quality and fertility, the contribution of the fluid portion of semen, i.e. seminal plasma, has often been neglected. Recent studies showed that seminal plasma can affect the viability of bovine epithelial endometrial cells in culture and the release of pro-inflammatory cytokines in a fertility-dependent manner (Nongbua et al. 2018a, b, 2020). Seminal plasma from bulls of lower fertility had a detrimental effect on the cultured cells, whereas seminal plasma from bulls of high fertility had either no effect or a less deleterious effect. During natural mating in cattle, semen is deposited in the vagina (Alghamdi et al. 2009), and the spermatozoa move away from seminal plasma through the cervix. Thus, the seminal plasma itself does not enter the uterus. During artificial insemination, however, the semen dose is deposited at the entrance of the uterus, or just inside the cervical canal, and therefore uterine cells are exposed to seminal plasma (Bromfield 2016; Robertson 2005). We speculate, therefore, that the seminal plasma from lower fertility bulls may disrupt the normal signaling mechanisms that are thought to occur at mating to prepare the uterus to receive the embryo several days later. If this is the case, it should be possible to improve fertility of semen doses from bulls of low fertility by removing the seminal plasma using colloid centrifugation prior to freezing. If the non-return rates of the bulls are unknown, it might prove to be better to remove the seminal plasma from all samples anyway. The possibility of using the scaled-up versions of SLC, i.e. in 50 mL centrifuge tubes or larger, would expedite such preparation at the semen collection facility.

5.6.4 Separation of Spermatozoa from Pathogens in Semen

The development of AI occurred in an effort to control disease transmission among herds, in which it was highly successful. However, it is still possible to transmit viruses and bacteria in semen. Bulls for breeding are tested before entering the semen collection program and at regular intervals thereafter. In addition, frozen semen is usually quarantined for 28 days before use, to ensure that the donor was healthy at the time of semen collection. Despite rigorous serological testing of bulls, it is still possible for a male to be seronegative for a particular virus while shedding the virus in his semen, as occurs for bovine diarrhoea virus (Givens 2018). Alternatively, after a recent infection, the animal may shed high virus loads before mounting a serological response and can infect females via this route.

Studies based on colloid centrifugation followed by a "swim-up" procedure removed more than 99% of porcine circovirus particles from spiked semen samples

Fig. 5.1 (continued) semen before centrifugation. (**d**) Extra-large SLC, comprising 150 colloid and up to 200 mL extended semen in a 500-mL tube

(Blomqvist et al. 2011) and the majority of virus particles from semen of stallions with equine arteritis virus infection (Morrell et al. 2013). It is not known whether the remaining virus load would be sufficient to cause infection in inseminated animals since the threshold for infectivity for each virus is not known. Moreover, the establishment of infection may depend on intrinsic factors in the female, such as immune status, number of previous pregnancies, underlying health status. Therefore, further research is needed to see if the remaining virus can be inactivated to ensure that the selected sperm samples are not infective.

Apart from viruses, bacteria can also be present in semen. Bacteria from the skin and the animal's environment colonize the mucosa of the distal reproductive tract and contaminate the semen as it is ejaculated (Rota et al. 2011). The semen extender added to nourish and protect the spermatozoa during freezing acts as a nutrient medium for bacteria and could result in high numbers of bacteria being transferred to the uterus during artificial insemination. Therefore, antibiotics are added during semen processing, as stipulated in national and international legislation (Council of Europe, Directive 92/65/EEC. 1992) but this non-therapeutic application may be contrary to recommendations on the prudent use of antibiotics to avoid the development of antimicrobial resistance. Studies are currently underway to determine whether passing the spermatozoa through a low density colloid to separate all the spermatozoa from the seminal plasma would also remove the bacteria. This method produced promising results with boar semen (Morrell et al. 2019).

5.7 Conclusions

There are several biomimetic methods for bull sperm selection that are appropriate to prepare small volumes of semen but, at present, only colloid centrifugation in the form of single layer centrifugation is relevant for preparing whole bull ejaculates. This method is already used in the equine semen industry and could be used to advantage in the bull semen industry but its widespread use in processing bull semen has not been reported. In contrast, colloid centrifugation is frequently used to prepare bull sperm samples for IVF. Other methods of sperm selection, such as microfluidics, are promising but require further development and/or scaling-up if they are to be both economic and practical for widespread use.

References

Abraham MC, Johannisson A, Morrell JM (2016) Effect of sperm preparation on development of bovine blastocyst in vitro. Zygote 24:825–830

Alghamdi AS, Lovaas BJ, Bird SL, Lamb GC, Rendahl AK, Taube PC, Foster DN (2009) Species-specific interaction of seminal plasma on sperm-neutrophil binding. Anim Reprod Sci 114:331–344. https://doi.org/10.1016/j.anireprosci.2008.10.015

Ardon F, Helms D, Sahin E, Bollwein H, Töpfer-Petersen E, Waberski D (2008) Chromatin-unstable boar spermatozoa have little chance of reaching oocytes in vivo. Reproduction 135: 461–470

Ask-Gullstrand P, Strandberg E, Båge R, Christensen J, Berglund B (2020) Genetic parameters for reproductive losses estimated from the in-line milk progesterone profiles in Swedish dairy cows. J Dairy Sci 104:3231–3239

Avery B, Greve T (1995) Impact of Percoll on bovine spermatozoa used for in vitro insemination. Theriogenology 44:871–878

Blomqvist G, Persson M, Wallgren M, Wallgren P, Morrell JM (2011) Removal of virus from boar semen spiked with porcine circovirus type 2. Anim Reprod Sci 126:108–114

Bromfield JJ (2016) A role for seminal plasma in modulating pregnancy outcomes in domestic species. Reproduction 52:223–232. https://doi.org/10.1530/REP-16-0313

Council of Europe (1992) Directive 92/65/EEC. https://www.ecolex.org/details/legislation/council-directive-9265eec-laying-down-animal-health-requirements-governing-trade-in-and-imports-into-the-community-of-animals-semen-ova-and-embryos-not-subject-to-animal-health-requirements-laid-down-in-specific-community-rules-referred-to-in-annex-a-i-to-directive-90425eec-lex-faoc019208/. Accessed 18 Mar 2021

de Vries AC, Colenbrander B (1990) Isolation and characterization of boar spermatozoa with and without a cytoplasmic droplet. Int J Biochem 22:519–524

Durfey CL, Swistek SE, Liao SF, Crenshaw MA, Clemente HJ, Thirumalai RVKG, Steadman CS, Ryan PL, Willard ST, Feugang JM (2019) Nanotechnology-based approach for safer enrichment of semen with best spermatozoa. J Anim Sci Biotech 10:14–25

Faezah SS, Zuraina FM, Farah JH, Khairul O, Hilwani NI, Iswadi MI, Fang CN, Zawawi I, Abas OM, Fatimah SI (2014) The effects of magnetic separation on cryopreserved bovine spermatozoa motility, viability and cryo-capacitation status. Zygote 22:378–386

Flamigni C, Coticchio G (2006) Sperm DNA fragmentation: paternal effect on early post-implantation embryo development in ART. Hum Reprod 21:2876–2881

Galli C, Vassiliev I, Lagutina I, Galli A, Lazzari G (2003) Bovine embryo development following ICSI: effect of activation, sperm capacitation and pre-treatment with dithiothreitol. Theriogenology 60:1467–1480

Givens MD (2018) Review: risks of disease transmission through semen in cattle. Animal 12:165–171

González-Marín C, Gosálvez J, Roy R (2012) Types, causes, detection and repair of DNA fragmentation in animal and human sperm cells. Int J Mol Sci 13:14026–14052

Goodla L, Morrell JM, Yusnizar Y, Stålhammar H, Johannisson A (2014) Quality of bull spermatozoa after preparation by single layer centrifugation. J Dairy Sci 97:2204–2212

Gopalkrishnan K, Padwal V, Neherji PK, Gokral JS, Shah R, Juneja HS (2000) Poor quality of sperm as it affects repeated early pregnancy loss. Arch Androl 45:111–117

Karabinus DS, Evenson DP, Jost LK, Kaproth MT (1990) Comparison of semen quality in young and mature Holstein bulls measured by light microscopy and flow cytometry. J Dairy Sci 73: 2364–2371

Kruse R, Dutta PC, Morrell JM (2011) Colloid centrifugation removes seminal plasma and cholesterol from boar spermatozoa. Reprod Fertil Dev 23:858–865

Kumaresan A, Johannisson A, Al-Essawe E, Morrell JM (2017) Sperm viability, reactive oxygen species and DNA fragmentation index combined can discriminate between above- and below-average fertility bulls. J Dairy Sci 100:5824–5836

Lazaros LA, Vartholomatos GA, Hatzi EG, Kaponis AI, Makrydimas GV, Kalantaridou SN, Sofikitis NV, Stefo TI, Zikopoulos KA, Georgiou IA (2011) Assessment of sperm chromatin condensation and ploidy status using flow cytometry correlates to embryo quality and pregnancy following in vitro fertilization. J Assist Reprod Genet 28:885–891

Love CC, Kenney RM (1998) The relationship of increased susceptibility of sperm DNA to denaturation and fertility in the stallion. Theriogenology 50:955–972

Magata F, Tsuchiya K, Okubo H, Ideta A (2019) Application of intracytoplasmic sperm injection to the embryo production in aged cows. J Vet Med Sci 81:84–90

Meuwissen et al (2001) Prediction of total genetic value using genome-wide dense marker maps. Genetics 157:1819–1829

Mizuno K, Hoshi K, Huang T (2002) Fertilization and embryo development in a mouse ICSI model using human and mouse sperm after immobilisation in polyvinlypyrrolidone. Hum Reprod 17: 2350–2355

Morrell JM (2006) Update on semen technologies for animal breeding. Reprod Domest Anim 41: 63–67

Morrell JM, Johannisson A, Dalin A-M, Rodriguez-Martinez H (2009) Morphology and chromatin integrity of stallion spermatozoa prepared by density gradient and single layer centrifugation through silica colloids. Reprod Domest Anim 44:512–517

Morrell JM, Kumaresan A, Johannisson A (2017) Practical implications of sperm selection techniques for improving reproduction. Anim Reprod 14:572–580

Morrell JM, Nunes MM (2018) Practical guide to single layer centrifugation of stallion semen. Equine Veter Edu 30:392–398. https://doi.org/10.1111/eve.12658

Morrell JM, Núñez-González A, Crespo-Félez I, Martínez-Martínez S, Alborcia M-JM, Fernández-Alegre E, Dominguez JC, Gutiérrez-Martín CB, Martínez-Pastor F (2019) Removal of bacteria from boar semen using a low-density colloid. Theriogenology 126:272–278

Morrell JM, Rodriguez-Martinez H (2009) Biomimetic techniques for improving sperm quality in animal breeding: a review. Open Androl J 1:1–9

Morrell, Rodriguez-Martinez (2010) Practical applications of sperm selection techniques as a tool for improving reproductive efficiency. Veter Med Int. https://doi.org/10.4061/2011/2984767

Morrell JM, Rodriguez-Martinez H (2016) Colloid centrifugation of semen; applications in assisted reproduction. Am J Anal Chem 7:597–610

Morrell JM, Rodriguez-Martinez H, Johannisson A (2010) Single layer centrifugation of stallion spermatozoa consistently selects the most robust spermatozoa from the rest of the ejaculate in a large sample size. Equine Vet J 42:579–585

Morrell JM, Sabes-Alsina M, Abraham MC, Sjunnesson Y (2016) Practical applications of sperm selection techniques for improving reproductive efficiency. Anim Reprod 13:340–345

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

Morrell JM, Timoney P, Klein C, Shuck K, Campos J, Troedsson M (2013) Single layer centrifugation reduces equine arteritis virus titre in the semen of shedding stallions. Reprod Domest Anim 48:604–612

Morrell JM, Wallgren M (2011) Removal of bacteria from boar ejaculates by single layer centrifugation can reduce the use of antibiotics in semen extenders. Anim Reprod Sci 123:64–69

Murphy EM, Kelly AK, O'Meara C, Eivers B, Lonergan P, Fair S (2018) Influence of bull age, ejaculate number and season of collection on semen production and sperm motility patterns in Holstein Friesian bulls in a commercial artificial insemination center. J Anim Sci 96:2408–2418

Nadalini M, Tarozzi N, Di Santo M, Borini A (2014) Annexin V magnetic-activated cell sorting versus swim-up for the selection of human sperm in ART: is the new approach better than the traditional one? J Assist Reprod Genet 31:1045–1051

Nagata MPB, Endo K, Ogata K, Yamanaka K, Egashira J, Katafuchi N, Yamanouchi T, Matsuda H, Goto Y, Sakatani M, Hojo T, Nishizono H, Yotsushima K, Takenouchi N, Hashiyada Y, Yamashita K (2018) Live births from artificial insemination of microfluidic sorted bovine spermatozoa characterized by trajectories correlated with fertility. PNAS 115:3087–3096

Narud B, Khezri A, Tewoldebrhan Zeremichael T, Stenseth EB, Heringstad B, Johannisson A, Morrell JM, Collas P, Deinboll Myromslien F, Kommisrud E (2021) Sperm chromatin integrity and DNA methylation in Norwegian red bulls of contrasting fertility. Mol Reprod Dev. https://doi.org/10.1002/mrd.23461

Nongbua T, Al-Essawe E, Edman A, Johannisson A, Morrell JM (2018b) Effect of adding heterologous versus homologous bovine seminal plasma prior to cryopreservation on bull sperm quality after thawing. Zygote 26:388–394

Nongbua T, Guo Y, Edman A, Humblot P, Morrell JM (2018a) Effect of bovine seminal plasma on bovine endometrial epithelial cells in culture. Reprod Domest Anim 53:85–92. https://doi.org/10.1111/rda.13069

Nongbua T, Guo Y, Ntallaris T, Rubér M, Rodriguez-Martinez H, Humblot P, Morrell JM (2020) Bull seminal plasma stimulates in vitro production of TGF-β, IL-6 and IL-8 from bovine endometrial epithelial cells, depending on dose and bull fertility. J Reprod Immunol 142:103179

Nongbua T, Johannisson A, Morrell JM (2017) Effects of single layer centrifugation (SLC) on bull spermatozoa prior to freezing on post-thaw semen characteristics. Reprod Domest Anim 52: 596–602

Odhiambo JF, DeJarnette JM, Geary TW, Kennedy CE, Suarez SS, Sutovsky M, Sutovsky P (2014) Increased conception rates in beef cattle inseminated with nanopurified bull semen. Biol Reprod 91(97):1–10

Rappa K, Samargia J, Sher M, Pino JS, Rodriguez HF, Asghar W (2018) Quantitative analysis of sperm rheotaxis using a microfluidic device. Microfluid Nanofluid 22:100–110

Robertson SA (2005) Seminal plasma and male factor signalling in the female reproductive tract. Cell Tissue Res 322:43–52. https://doi.org/10.1007/s00441-005-1127-3

Romany L, Meseguer M, Gracia-Herrero S, Pellicer A, Garrido N (2010) Magnetic activated sorting selection (MACS) of non apoptotic sperm (NAS) improves pregnancy rates in homologous intrauterine insemination (IUI). Prelim Data Fertil Ster 94:S14

Rota A, Calicchio E, Nardoni S, Fratini F, Ebani V, Sgorbini M, Panzani D, Camillo F, Mancianti F (2011) Presence and distribution of fungi and bacteria in the reproductive tract of healthy stallions. Theriogenology 76:464–470

Sabés-Alsina M, Wallgren M, Sjunesson Y, Ntallaris T, Lundeheim N, López-Béjar M, Morrell JM (2020) Effect of season on the in vitro fertilizing ability of frozen-thawed Spanish bovine spermatozoa. J Dairy Sci 103:9525–9533

Samardzija M, Karadjole M, Getz I, Makek Z, Cergolj M, Dobranic T (2006) Effects of bovine spermatozoa preparation on embryonic development in vitro. Reprod Biol Endocrinol 4:58–64

Simon L, Murphy K, Shamsi MB, Liu L, Emery B, Aston KI, Hotaling J, Carrell DT (2014) Paternal influence of sperm DNA integrity on early embryonic development. Hum Reprod 29: 2402–2412

Suarez SS (2007) Interactions of spermatozoa with the female reproductive tract: inspiration for assisted reproduction. Reprod Fertil Dev 19:103–110

Thibier M, Wagner HG (2002) World statistics for artificial insemination in cattle. Livestock Prod Sci 74:203–212

Unnikrishnan V, Kastelic J, Thundathil J (2021) Intracytoplasmic sperm injection in cattle. Gen 12: 198–215

Viana J (2019) Statistics of embryo production and transfer in domestic farm animals. IETS data retrieval report. Embryo Technol Newsl 38:16

Wei H, Fukui Y (2020) Technical improvement in intracytoplasmic sperm injection (ICSI) in cattle. J Reprod Dev 46:403–407