

Review Article

Practical guide to single layer centrifugation of stallion semen

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Keywords: horse; sperm preparation; artificial insemination; colloid centrifugation; Androcoll-E; fertility

Summary

The technique of single layer centrifugation through species-specific colloids has been gaining acceptance over the last few years as a practical method of improving the sperm quality of stallion semen for artificial insemination. This article explains the background to the technique and the different colloid formulations used, suggests some applications of the technique with particular reference to the equine breeding industry, describes the method in detail, and finally provides some guidance for problem solving. It is intended as a practical guide for those considering using the technique to improve sperm quality and pregnancy rates in equine artificial insemination and embryo transfer.

Introduction

The equine breeding industry is extremely diverse, ranging from stud farms with a few mares for artificial insemination (AI) to enormous embryo transfer enterprises with several thousand. One feature that they have in common is the need for good quality semen doses for AI, by which one usually means good sperm motility, viability, morphology and chromatin integrity of the semen sample. Stallion semen varies considerably in quality, depending both on the individual donor and on the handling procedures to which it has been subjected. Sperm quality has been shown to be related to per cycle pregnancy rates (e.g. Parlevliet and Colenbrander 1999; Morrell *et al.* 2008; Love *et al.* 2015). Therefore, every effort must be made to prevent it from deteriorating before AI. The use of cooled semen rather than fresh semen has increased in recent decades and results in a mean per cycle pregnancy rate of approximately 65% (Rota *et al.* 2004). This means that approximately one-third of inseminated mares do not become pregnant in any one cycle, indicating that there is room for improvement.

Pregnancy is a multifactorial phenomenon with both mare and stallion factors being involved (Amann 2005). When AI is used, additional factors can have an adverse effect on the outcome, including semen handling at the time of collection, conditions during transport of the semen, handling of the dose at the receiving stud and also the skill of the inseminator. Selection methods for stallion semen, in particular the development of colloid centrifugation, have been reviewed previously (Morrell 2012). Colloid centrifugation using species-specific colloids (e.g. Androcoll-E, now known as Equicoll)¹ is one method that has been advocated to improve sperm quality in semen doses for AI (Morrell 2011). This method involves centrifuging extended semen through a colloid, which, broadly speaking, serves to separate spermatozoa with good motility, normal morphology

and intact chromatin from the rest of the sperm population and the seminal plasma. The separation is not perfect: for example, some immotile spermatozoa may be present in the sperm pellet but usually the proportion of motile spermatozoa with normal morphology and intact chromatin is better in single layer centrifugation (SLC)-selected samples than in uncentrifuged samples (Johannisson *et al.* 2009; Morrell *et al.* 2009a,b). These SLC-selected spermatozoa survive longer during storage than uncentrifuged spermatozoa (Morrell *et al.* 2010) and retain their fertilising capacity for up to 96 h after semen collection (Lindahl *et al.* 2012). Although the purpose of the latter study was to determine if SLC-selected stallion spermatozoa were capable of fertilisation after prolonged storage, and no comparison with untreated controls was possible, the study did show that fertilising capacity is retained in the SLC-selected spermatozoa. These results enabled a fertility trial to be conducted in which pregnancy rates SLC-selected and untreated control samples were compared (see later).

This method has been used to prepare spermatozoa from 'problem' stallions, i.e. those known to have a poor pregnancy rate after AI; pregnancy rates were higher when SLC-selected samples were used for AI than had been obtained previously using control samples (Morrell *et al.* 2011a). In a separate study using semen from 'normal' stallions, i.e. stallions not known to have a fertility problem, pregnancy rates after conventional AI using SLC-selected sperm samples at 24 h after processing were significantly higher than controls (Morrell *et al.* 2014). Thus the apparent improvement in sperm quality in the selected samples is reflected in increased pregnancy rates after AI.

Other authors using different protocols and other colloids have reported success only with problem stallions (e.g. Sieme *et al.* 2003; Varner *et al.* 2008), concluding that the method does not provide sufficient spermatozoa for conventional AI doses. However, the colloid formulation can influence the outcome of the procedure considerably (Morrell *et al.* 2011b). Thus it is important to use species-specific colloid formulations with a protocol that has been developed for the colloid in question and that is backed up by sound experimental results.

Hoogewijs *et al.* (2011) reported that SLC-selected sperm samples survived cryopreservation better than controls prepared by cushion centrifugation. Furthermore, the SLC-selected spermatozoa survived longer after thawing than controls (Hoogewijs *et al.* 2012). However, the authors did point out that the number of frozen doses that could be produced was reduced compared to cushion centrifugation (Hoogewijs *et al.* 2011). These observations suggest that the technique might be best applied to stallion semen that does

not freeze well by conventional means. Improvements in sperm motility and viability have also been observed if the thawed sample is processed by SLC (Table 1 and Fig 1). Studies by other researchers did not show a difference in pregnancy rate when the spermatozoa were prepared by SLC (Cerny *et al.* 2012); however, they had used a different colloid (EquiPure) and protocol compared to those used by Hoogewijs *et al.* (2012) (Table 2).

Proposed applications of SLC for stallion semen have been described previously (Morrell 2011, 2012) and are summarised in Table 2. The purpose of the present article is to provide a practical step-by-step guide to SLC of stallion spermatozoa using Equicoll (formerly known as Androcoll-E), describing the steps in the procedure in detail and indicating the pitfalls to be avoided.

The SLC method

Preparing stallion spermatozoa by SLC is straightforward (Fig 2). It takes only a few minutes to learn the technique but attention to detail will help to optimise the results obtained (Morrell *et al.* 2009c).

- Equilibrate both the semen sample and the colloid to the same temperature to avoid cold shock. The simplest way of

doing this is to take the colloid aliquot out of the refrigerator before semen collection. If more colloid is needed quickly, it can be placed in an incubator or water bath but it should not be made warmer than the semen sample. Sperm quality deteriorates if it is subjected to fluctuating temperatures such as pipetting cooling semen over hot colloid and then centrifuging it.

- Adjust the sperm concentration to approximately $100 \times 10^6/\text{mL}$ using the usual semen extender e.g. Kenny's extender. Do not use a freezing extender at this stage because these tend to have high osmolarity that will draw water out of the spermatozoa and thus change their density. It is important not to exceed the sperm concentration of $100 \times 10^6/\text{mL}$ to avoid the spermatozoa competing with each other to pass into the colloid and overloading the colloid's selection capacity (Morrell *et al.* 2010). If the original semen sample has a concentration of less than $100 \times 10^6/\text{mL}$, it can be used at this lower concentration or alternatively it can be concentrated first by cushion centrifugation and then the sperm pellet resuspended in semen extender to a sperm concentration of $100 \times 10^6/\text{mL}$. However, note that cushion centrifugation itself can damage the spermatozoa so

TABLE 1: Mean sperm motility and viability (%) before and after single layer centrifugation (SLC) of frozen thawed stallion semen (n = 18)

Batch	Motility before SLC	Motility after SLC	Viability before SLC	Viability after SLC
1	40 ± 6	60 ± 11	42 ± 22	56 ± 4
2	40 ± 8	65 ± 8	52 ± 1	70 ± 2
3	34 ± 8	65 ± 16	51 ± 2	69 ± 7
4	38 ± 10	46 ± 15	61 ± 1	59 ± 3
5	15 ± 3	31 ± 2	19 ± 4	24 ± 12
6	34 ± 9	35 ± 6	36 ± 3	39 ± 7
7	21 ± 8	30 ± 8	15 ± 9	21 ± 7

TABLE 2: Proposed applications of single layer centrifugation in the equine breeding industry (Morrell 2011, 2012)

1. To improve sperm quality in cooled AI doses, thus improving pregnancy rates (Morrell *et al.* 2010; 2014)
2. To increase the storage life of cooled AI doses (Morrell *et al.* 2010; Lindahl *et al.* 2012)
3. To improve sperm survival at temperatures above 5°C
4. To allow semen from 'poor coolers' to be used for cooled sperm doses (Morrell 2012)
5. To improve cryosurvival, particularly from stallions whose semen does not freeze well (Hoogewijs *et al.* 2012)
6. To separate viable spermatozoa from dead or dying spermatozoa after cryopreservation (Stuhtmann 2011).

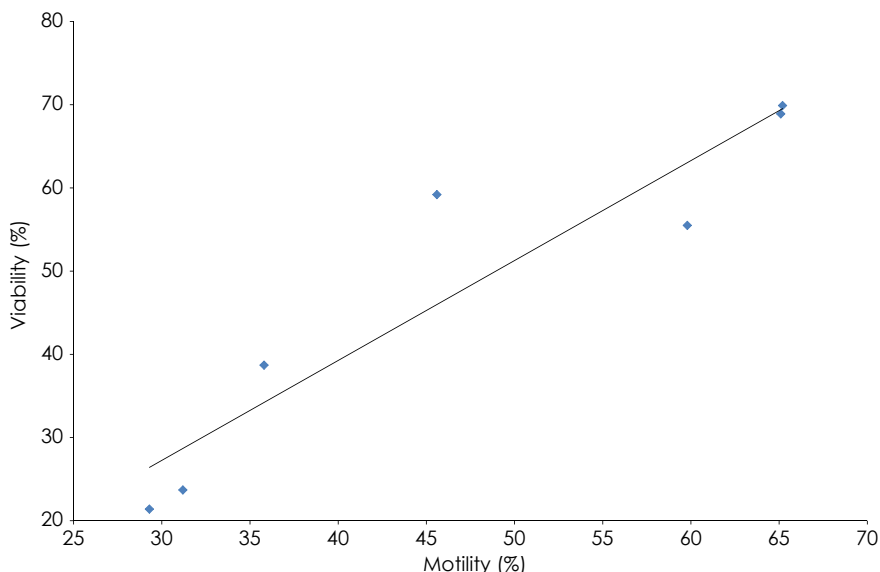


Fig 1: Relationship between sperm motility and viability after single layer centrifugation of frozen thawed semen (r = 0.93; P<0.01).

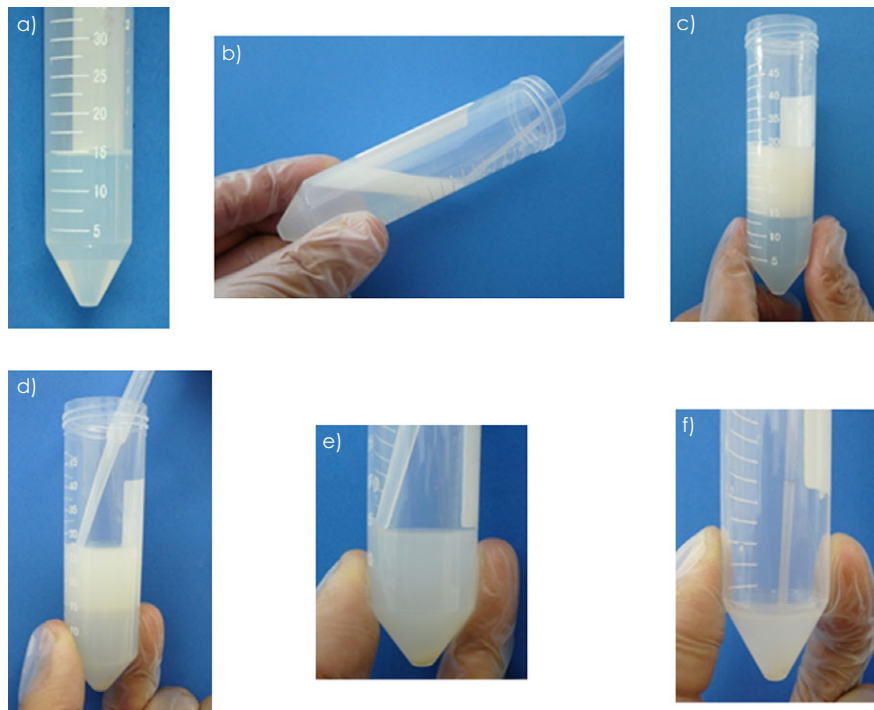


Fig 2: Step-by-step guide to single layer centrifugation. a) Pour 15 mL equilibrated colloid into a 50 mL tube; b) add 1–2 mL extended semen slowly from a Pasteur pipette; c) continue to add remainder of semen slowly, up to 20 mL; d) after centrifugation, start to remove semen extender; e) when all semen extender and spermatozoa at the interface have been removed, start to aspirate the colloid; f) remove colloid down to last 1–2 mL; pass a Pasteur pipette straight down through the remaining colloid into the sperm pellet; aspirate sperm pellet from beneath colloid.

that the sperm yield after SLC could subsequently be reduced (Hoogewijs *et al.* 2011).

- Pour 15 mL of Equicoll into a clean 50 mL conical centrifuge tube.
- Pipette up to 20 mL of extended semen slowly over the top of the colloid. The tube should be held at an angle of approximately 45°, which can be achieved by resting one side of the conical end against the bench. The first 1–2 mL of extended semen should be added very slowly, for example using a fine-bore Pasteur pipette, so that the semen forms a distinct layer above the surface of the colloid. There will be a clear line at the interface between the colloid and the semen, which is essential for good separation of the sperm subpopulations. The remaining semen can be added more quickly so long as it does not start to sink into the colloid. If the semen is seen to sink into the colloid and then return to the surface, the semen is being added too quickly and the integrity of the interface is reduced. The final volume of extended semen that can be added depends on the sperm quality but should not exceed 20 mL (a total of 200 million spermatozoa) in a 50 mL tube.
- Place the centrifuge tube in the centrifuge, which should have a swing-out rotor. Centrifuge at 300 *g* for 20 min without using the brake to slow down the centrifuge. If the centrifuge is calibrated in rpm, do not guess what setting to use since too high *g* force will damage the spermatozoa. Calculate the appropriate settings using the following formula: $\text{rpm} = \sqrt{[300/(1.118 \times r)]} \times 10^3$ where *r* = radius of rotor in mm. Alternatively, use a 'quick calculator' function from the

Internet e.g. <http://www.sciencegateway.org/tools/rotor.htm>. Provide the desired *g* force (300) and the radius of your rotor in mm and the rpm will be calculated for you. For example, if your centrifuge rotor has a radius of 150 mm, the rpm needed to provide 300 *g* will be 1300.

- Start the centrifugation as soon after layering the semen over the colloid as possible. The semen will start to mix with the colloid with time, reducing the effectiveness of the subsequent centrifugation.
- Once the centrifuge has stopped, remove the tubes as soon as possible and recover the sperm pellet. A delay at this stage will allow spermatozoa to swim out of the pellet into the colloid and thus be lost. The supernatant can be removed either using a pipette or with a water pump. Remove the extender and seminal plasma first, making sure that all of the interface between the semen and the colloid has been removed before starting to remove the colloid. The sperm subpopulation at the interface contains those spermatozoa that have not been able to pass into the colloid e.g. immotile or abnormal spermatozoa. If one starts to remove the colloid before this sperm subpopulation has been removed, it moves down the tube and can re-contaminate the sperm pellet.
- Remove the colloid down to the last 1 mL. Take a clean pipette and go right down into the sperm pellet through the remaining colloid; aspirate the sperm pellet from beneath the remaining 1 mL colloid. This is important to prevent re-contaminating the sperm pellet. Place the sperm pellet in 2–3 mL fresh extender, measure the sperm concentration and

adjust to the desired sperm concentration. Any residual colloid aspirated with the sperm pellet and subsequently inseminated will not harm the mare or impede conception. Do not resuspend the sperm pellet in the same tube that was used for the SLC since unselected spermatozoa and bacteria could be adhering to the sides of the tube, thus recontaminating the separated sperm population.

Notes

1. *Use the recommended volume of colloid:* some protocols have been suggested using smaller volumes of colloid. However, in a centrifuge with a swing-out rotor, the centrifuge tube is tilted to an almost-horizontal position during centrifugation. Thus, if a smaller volume of colloid is used, not only will the interface area available for selection be reduced, but also the interface population may actually come into contact with the bottom of the centrifuge tube where the sperm pellet will sit once the tube returns to the vertical position again, thus causing re-contamination of the selected sperm sample. The exception to this rule is if you are using colloid specifically designed for 15 mL tubes (Equicoll-Small)¹ when 4 mL colloid and up to 4.5 mL semen can be used (**Fig 3**).

2. *Use the recommended sperm concentration,* particularly if using 15 mL tubes (where up to 4.5 mL extended semen at a sperm concentration $100 \times 10^6/\text{mL}$ can be added). It has been shown previously that there is a strong negative correlation between recovery rate and the sperm concentration in the loading dose placed on top of the colloid in 15 mL tubes ($r = -0.40$, $P < 0.001$; Morrell *et al.* 2010). Thus there is a balance between volume of colloid used and recovering the maximum number of selected spermatozoa. However, when using 50 mL tubes, the relationship between recovery rate and sperm concentration in the loading dose is not as clear. In fact, for sperm concentrations $< 60 \times 10^6/\text{mL}$ (**Fig 4**), there is a positive relationship between recovery rate and sperm concentration in the loading dose ($r = 0.57$, $P < 0.01$). This relationship then

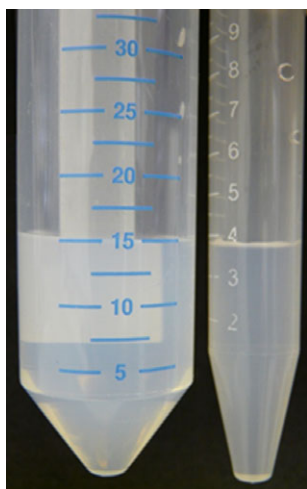


Fig 3: The colloid can also be used successfully in a 15 mL centrifuge tube. Note that the volume of colloid (4 mL) creates a column of approximately the same height as 15 mL in a 50 mL colloid in a centrifuge tube.

deteriorates as the sperm concentration increases, such that for sperm concentrations for $80\text{--}170 \times 10^6/\text{mL}$, there is a negative association between sperm concentration and recovery rate ($r = -0.18$; **Fig 5**).

3. *It is not advisable to combine cushion centrifugation with SLC in the same tube.* Cushion centrifugation purportedly allows sperm samples to be centrifuged at a high g force without damaging them, by protecting them from impacting on the bottom of the tube. However, it does cause some damage to spermatozoa and the higher g forces would result in more abnormal spermatozoa being collected in the pellet than would normally occur at a lower g force. Therefore, it is not recommended to combine the two techniques.

4. *Using SLC for stored or thawed semen.* If SLC is not possible immediately after semen collection it can be carried out on cooled semen up to 24 h after collection (Morrell *et al.* 2011c). Sperm survival is prolonged, although the sperm quality is not as good as if the SLC was carried out immediately after semen collection. With thawed semen, the most motile spermatozoa can be selected with SLC (Mancill *et al.* 2010; Stuhmann 2011). In our hands, good results were obtained when the contents of eight 0.5 mL straws of thawed semen were pooled and the sperm concentration adjusted to $100 \times 10^6/\text{mL}$ before layering on top of 15 mL of Equicoll-Large in a 50 mL tube (Stuhmann 2011).

Problem solving

The most common question that people have asked is why they do not recover as many spermatozoa as they put on the colloid. Since spermatozoa that are not motile or those that have abnormal morphology, poor membrane integrity or damaged chromatin do not readily pass into the colloid, these spermatozoa are largely absent from the sperm pellet. Therefore, the yield of spermatozoa recovered will depend on the proportion of spermatozoa that are motile, with normal morphology, intact membranes and intact chromatin in the original semen sample. Thus the sperm yield from very poor quality ejaculates will be low. However, these damaged spermatozoa that are 'lost' are not wanted for insemination anyway. Spermatozoa with damaged chromatin are still motile and able to fertilise but subsequent development of the embryo may be impaired (Aitken and De Iulius 2007). This means that these damaged spermatozoa are competing with normal spermatozoa to fertilise the oocyte; if one of these abnormal spermatozoa penetrates the oocyte, embryonic loss will occur at some stage and thus the chance of a pregnancy resulting from that insemination will have been lost.

If insufficient care is taken when layering the semen on top of the colloid, the semen will start to mix with the colloid. This is seen when semen passes below the surface of the colloid and then comes back to the surface. By the time that all the semen has been added, the interface is no longer sharp and well defined (**Fig 6**). The resulting sperm pellet is likely to contain more of the immotile or morphologically abnormal spermatozoa than would result from a well-layered preparation.

Note that not all of the abnormal spermatozoa will be removed, especially if using thawed spermatozoa. The longer the centrifugation time and the greater the centrifugal force, the more spermatozoa will be pelleted, irrespective of quality. Increasing centrifugation time and force will allow more

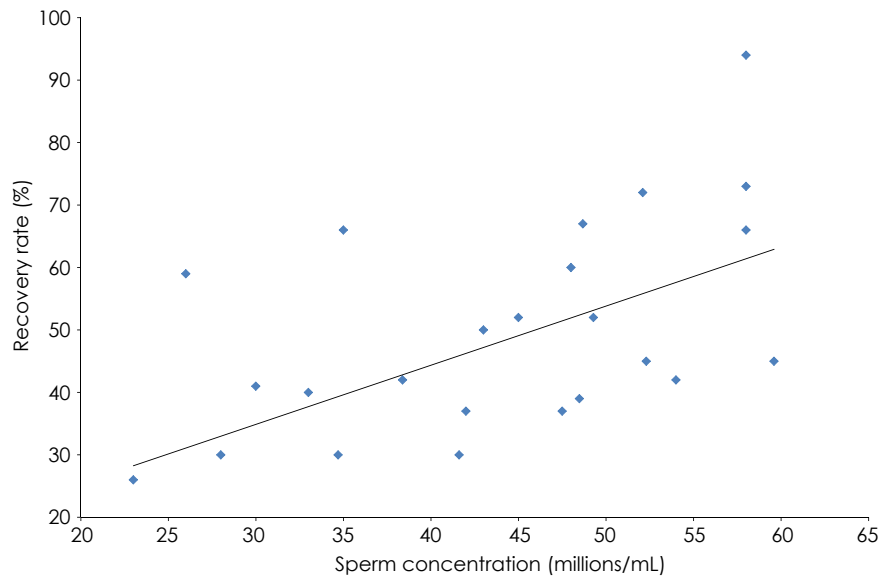


Fig 4: Association between sperm concentration in the loading dose ($<60 \times 10^6/\text{mL}$) and sperm recovery after single layer centrifugation (n = 27).

abnormal spermatozoa to be collected in the pellet. The present protocol is an optimisation of yield and quality, designed to produce the best result possible in as many cases as possible. It is recommended to always try this protocol first, only changing the protocol if good results are not obtained with a particular ejaculate.

Centrifuges need calibrating from time to time. Unless the centrifuge is relatively new, it could be a good investment to have it re-calibrated by the manufacturer before using it intensively for sperm preparation.

Another question that sometimes arises is that people have tried other colloids, such as Percoll² or Equipure³, or they have tried other protocols than the one recommended here and have not obtained a good result. It should be

noted that Percoll and Equipure were designed as colloids for density gradients, Percoll particularly being used to prepare frozen bull spermatozoa for IVF, rather than to prepare stallion spermatozoa for AI. Much smaller sperm numbers are required for bovine IVF than for conventional equine AI and the spermatozoa are needed to capacitate as soon as possible. Some success has been obtained using these protocols to prepare sufficient spermatozoa for deep uterine insemination in mares, but this method of insemination is only useful for centres that have access to specialist equipment and staff. To be generally applicable for the equine breeding industry, one must have a practical method for obtaining sufficient spermatozoa for conventional AI at any stud farm. Therefore, it is strongly recommended to use only a protocol

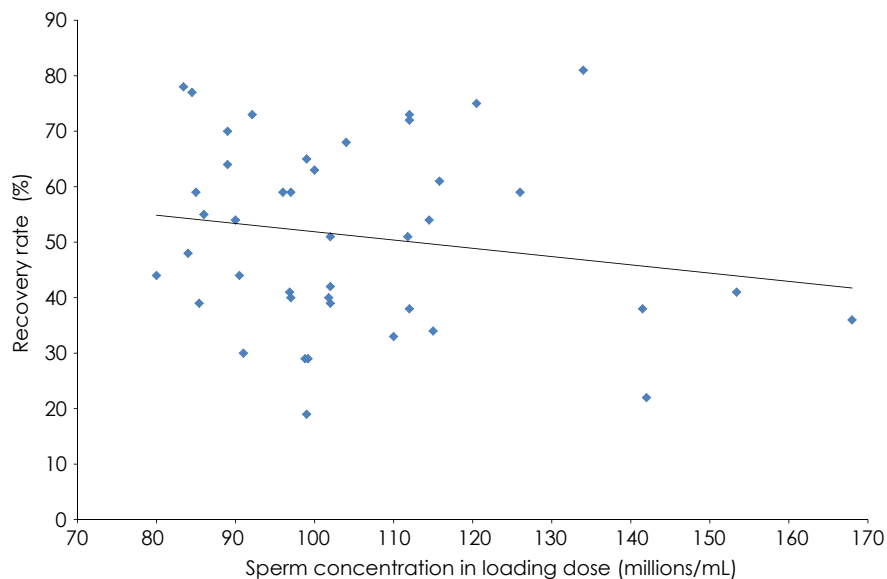


Fig 5: Association between sperm concentration in the loading dose ($80\text{--}170 \times 10^6/\text{mL}$) and sperm recovery after single layer centrifugation.

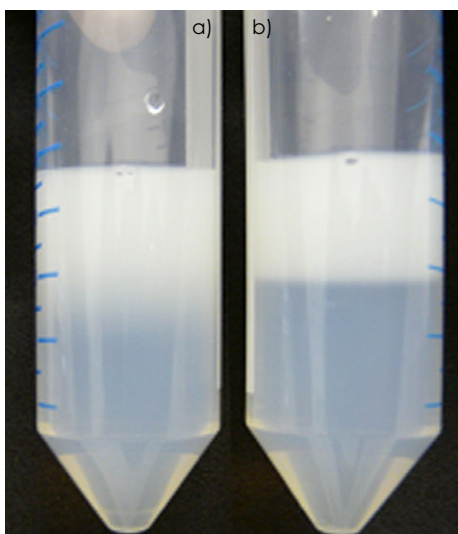


Fig 6: Semen layered on top of Androcoll-Equine for single layer centrifugation; a) incorrect (semen has mixed with colloid); b) correct (sharp interface). Note the sharp interface between the colloid and the semen appears to be important for good selection.

that has been developed for the specific colloid to be used and that is backed up by sound experimental results. The SLC method presented here is patented and has been shown to work when the protocol is followed properly.

In conclusion, SLC can be used to improve stallion sperm quality in a variety of circumstances and for a range of purposes within equine breeding. The procedure is straightforward and quick to perform, can be used by the personnel on most stud farms where there is a centrifuge, and has been shown consistently to improve sperm quality in stallion ejaculates.

Authors' declaration of interests

J.M. Morrell is the inventor of two of the colloids mentioned in this article (Androcoll/Equicoll and EquiPure) and is the patent holder for the colloid formulations and SLC technique. M. Nunes is employed by Minitube of Brazil.

Ethical animal research

Semen collection by artificial vagina is a routine husbandry procedure. Any semen used in this report was collected as part of a routine semen collection programme at a commercial stud farm. It does not compromise the health and welfare of the stallions. At this time, it is not a regulated procedure in Sweden.

Source of funding

Professor Morrell is funded by the Swedish Foundation for Equine Research.

Authorship

J.M. Morrell prepared the manuscript based on many years' experience with the technique. M. Nunes assisted with critical reading of the drafts and preparation of the figures. He has

also used this technique on stud farms in Brazil and provided the idea that a practical guide to the technique could be helpful.

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