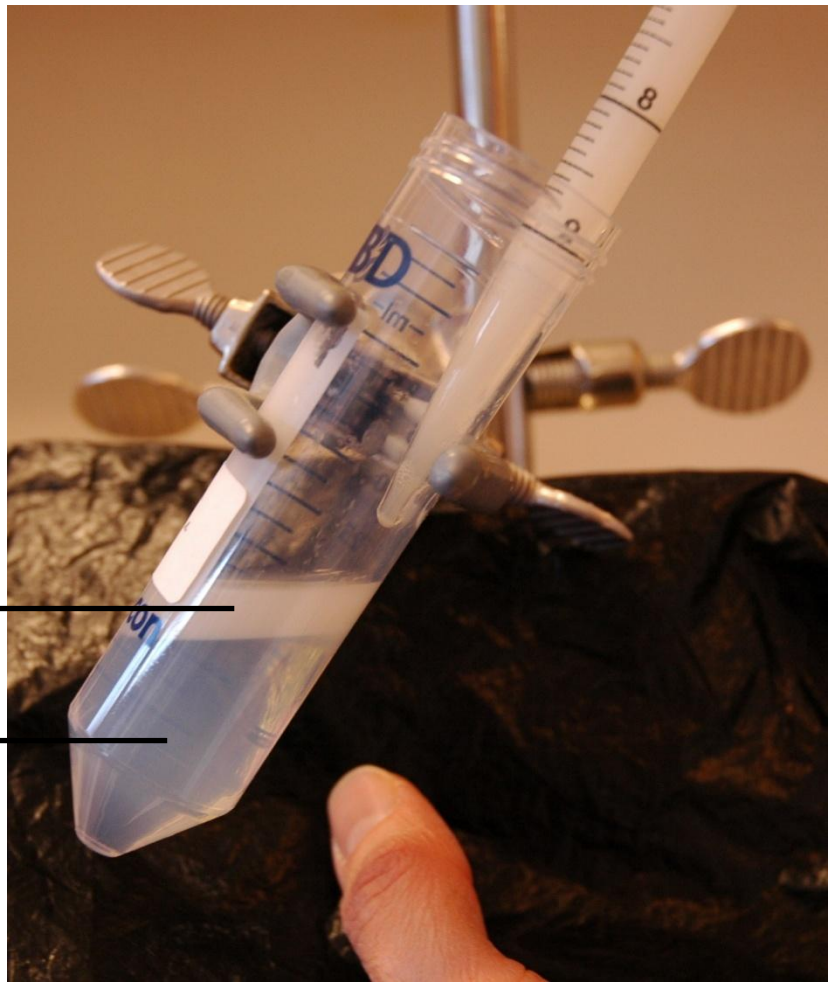
Effect of SLC on motility, morphology, viability and ROS production of boar spermatozoa



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Contents

Abstract:

The aim of this study was to investigate the effect of single layer centrifugation (SLC) on boar sperm samples. At the same time scaling up the SLC technique was tested with two volumes: 4.5 ml and 15 ml. In total, 32 ejaculates from 8 boars were used in this project. The boars were of different breeds: Swedish Landrace (n=1), Norwegian Landrace (n=2), Swedish Yorkshire (n=1) and Hampshire (n=4). Three treatments were used: control (untreated), SLC with 4.5 ml extended semen, and SLC with 15 ml extended semen. The evaluation of the samples was based on motility, linear motility, progressive motility, number of sperm, yield, viability and ROS production. With the SLC treatment it was possible to select spermatozoa with a more linear motility and higher progressive motility, and also spermatozoa with normal morphology. No difference in viability between the different treatments was detected. In contrast, ROS production was slightly increased in the SLC-selected samples. There was no significant difference in results between the 4.5 ml SLC volume and the 15 ml SLC volume, indicating that the technique can be scaled up to 15 ml without compromising sperm quality. For the future the technique must be scaled up more to be useful in practice.

Abbreviations :

AI	Artificial insemination
bSA	Bovine serum albumin
BTS	Beltsville thawing solution
CASA	Computer-assisted sperm analysis
DCFDA	Dichlorofluoresceïndiacetate
DGC	Density gradient centrifugation
FC	Flow cytometer
HE	Hydroethidine
HO	Hoechst 33258
IVF	In Vitro Fertilisation
PI	Propidium Iodide
PMI	Plasma membrane integrity
PVP	Polyvinylpyrrolidone
ROS	Reactive oxygen species
SLC	Single layer centrifugation
SLU	Sveriges lantbruksuniversitet
SRF	Sperm-rich fraction
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight linear velocity

Introduction:

Artificial Insemination (AI) is nowadays a common technique used in the agriculture industry. This biotechnology has improved the reproduction and genetics of farm animals. It is also of great economical importance, since the livestock producer can breed the herd with males having the desired selection criteria. Semen of good breeders can be extended and therefore can be used to fertilize more females than is possible by natural mating, and there are fewer geographical barriers to access the semen of good breeders. There are also other benefits, such as semen can be cryopreserved so there is a possibility of producing offspring from a specific male after its death; there is less chance of transfer of sexually transmitted diseases or other pathogens with AI than with natural mating (Foote, 2002).

In natural mating, semen is deposited in the female tract. At the deposition site, which is different between species, spermatozoa migrate away from the seminal plasma, which contains decapacitating factors, and can therefore start to decapacitate. The cervix of the female tract acts like a filtering system which filters poorly viable spermatozoa. Thus spermatozoa of poor quality will not progress further up the female reproductive tract and therefore will not reach the oöcyte. In some species (such as pigs, horses and dogs) the semen is naturally placed directly into the uterus and in these species the filtering function is achieved by the uterotubal-junction. In the oviduct active selection of viable spermatozoa is done by the oviductal epithelial cells. The result of these selections is that only highly motile, viable spermatozoa reach the oöcyte and can fertilize it (Morrell *et al.*, 2008c, Morrell & Rodriguez-Martinez, 2009).
With AI the spermatozoa are placed in the reproductive tract of the female by means of an insemination catheter, either in the cervix or the uterus. The uterotubal-junction functions as a barrier to filter poorly viable spermatozoa (Morrell & Rodriguez-Martinez, 2009).
Pregnancy is a very complicated and delicate event, requiring a balance of both sow and boar factors. Sow factors include time of insemination relative to ovulation, hygiene, illness of the sow, stress, environment, housing and so on (Colenbrander *et al.*, 2000). Boar factors include quality of spermatozoa, previous illness (spermatogenesis takes approximately 50 days), hygiene, temperature of the environment, nutrition, handling and preservation of the ejaculate, and so on (Colenbrander et al., 2000).

It is very hard to predict the outcome of an AI because there are so many factors that have an influence. Not only is the right quantity of spermatozoa needed, approximately 3×10^9 per insemination dose in the boar, but also the spermatozoa should be of good quality (Spinaci *et al.*, 2005). If the sperm dose is decreased, the quality of the spermatozoa becomes even more important (Waberski *et al.*, 2008).

Nowadays at boar stations only sperm concentration and motility are routinely assessed. Mostly motility is visually evaluated using a phase contrast microscope and the accuracy of the evaluation depends on the experience of the processor. Morphology is only checked when the boar enters the production line or when abnormalities are suspected (Tejerina *et al.*, 2008). Although there is interest in using more objective and accurate diagnostic methods to evaluate boar semen, they are not commonly used in the pig industry yet.

Since sperm quality is a combination of many factors, it is hard to quantify using only one or two factors. Several techniques can be used to assess sperm quality, both *in vitro* laboratory tests and *in vivo* tests (Turba *et al.*, 2007). Parameters such as morphology, motility and integrity of sperm plasma membrane play a very important role in boar sperm quality.

Motility assessment can easily be evaluated with a computer-assisted sperm analysis (CASA) instrument such as SM-CMA^{TM} (MTM Medical Technologies, Montreux, Switzerland). The CASA instrument records each individual spermatozoon over a certain time interval and digitalizes it through the attached camera. (Tejerina et al., 2008) Different parameters are reported, such as: immotile sperms, local motile sperms and motile sperms. The motile sperms may be subdivided into circular, non-linear motile and linear motile. The different findings are given in number, percentage, concentration (mil/ml). From the motile sperm the straight linear velocity (VSL), average path velocity (VAP) and the curvilinear velocity (VCL) are measured and given in μm/s.

Apart from the purchase price, the reason that CASA instruments are not in widespread commercial use is because the instrument needs proper programming with species-specific settings, and it requires validation. Furthermore, most instruments cannot handle a high concentration of spermatozoa.
The optimal concentration is approximately 100 million spermatozoa per milliliter. However, the use of CASA is increasing, especially because of its ability to profile the motility patterns of spermatozoa. These parameters are retrospectively correlated with individual fertility and are of high interest in the pig industry (Didion, 2008).

Viability assessment can be achieved using SYBR-14 and Propidium Iodide (PI) staining, and analyzing the stained spermatozoa either by fluorescence microscopy or by flow cytometry (FC). The advantages of the FC lie in the high number of spermatozoa analyzed, rapid sample analyzing, small sample volume and the analysis of individual spermatozoa. When using SYBR-14 and PI together, live and dead spermatozoa can be identified effectively. SYBR-14 can be used easily because it is not time dependent, with staining equilibrium being reached in less than 15 minutes and then remaining relatively stable. SYBR-14 stains the nuclei of living cells, whereas PI enters the nucleus only of damaged spermatozoa and it interacts with the DNA of the cell. Dying spermatozoa are stained with both SYBR-14 and PI. Boars usually have a high percentage of living spermatozoa in the sample (80-90%) (Garner & Johnson, 1995, Maxwell *et al.*, 2000). One reason for this high percentage is because of the long-term genetic selection of boars used at AI stations.

In the mitochondria from cells there is a high inner transmembrane potential. This potential is maintained by electron transport and oxidative phosphorylation. The potential is necessary to produce highly valuable mitochondrial ATP. Reactive oxygen species (ROS) can be produced in the mitochondria, which can induce a disturbance in electron transport in such a way that the coupling between the electron transport and the oxidative phosphorylation is disturbed. Consequently, the potential is not high enough to support mitochondrial ATP production, which is needed, for instance, for motility. Thus fertility of boar sperm can be affected by ROS (Guthrie *et al.*, 2008).
A FC can be used to measure the concentration of ROS in a semen sample. The samples can be run rapidly and accurately, because the flow cytometer measures individually a large number of spermatozoa per analysis (up to many thousands), (Maxwell *et al*., 2000). In the FC, laser lights are directed onto a stream of fluid sample. Each suspended particle present in the sample from 0.2 to 150 micrometers passing through the laser beam scatters the light in some way. The particles present in the sample which are stained with different fluorescent chemicals may be excited into emitting light at a longer wavelength than the light source. The detectors analyses the different light patterns and give information about the physical and chemical structure of each individual particle.
Hoechst 33258 (HO) can be used to stain dead or damaged spermatozoa because it does not penetrate the membranes of living cells (Maxwell *et al*., 2000). Hydroethidine (HE) staining can be used to measure the ROS concentration produced in living cells, because it is oxidized to ethidium by ROS (Guthrie *et al.*, 2008). Dichlorofluoresceindiacetate (DCFDA) is used as an indicator of ROS production.

To improve the fertilizing quality of sperm used in AI, methods have been developed to select spermatozoa. These methods are more frequently used for in vitro fertilization (IVF) than for AI when the natural selection processes occurring in the female reproductive tract are completely absent. Clearly for the IVF there is a greater emphasis to remove seminal plasma and select spermatozoa of good quality. The method of selection of spermatozoa and the removal of seminal plasma includes the use of density gradients (Thys *et al.*, 2008, Morrell & Rodriguez-Martinez, 2008). These methods have not been used for selecting spermatozoa for AI because of the large volumes of semen involved or large number of spermatozoa needed for an AI dose. With Density Gradient Centrifugation (DGC), two different layers of a colloid are placed in a centrifuge tube. The lowest layer will contain the highest density and a second layer of a lower density is placed on top. Usually Percoll^{TM} is used for the colloid, which consists of polyvinylpyrrolidone (PVP) - coated silica particles in a salt solution. Thereafter the aliquot of the semen will be pipetted on top of the two layers, the gradient will be centrifuged and the spermatozoa washed. At the Swedish University of Agricultural Sciences (SLU), a new method, Single Layer Centrifugation (SLC), has been developed. This method uses only one layer of a species-specific colloid formulation with a high density. The SCL method does not require time-consuming preparation of the colloid layers of different densities which are used in the DGC (Thys et al., 2008, Morrell & Rodriguez-Martinez, 2008, Morrell & Rodriguez-Martinez, 2009, Morrell *et al.*, 2009c). Morrell et al also showed that there is no difference in the motility of sperm preparations between the SLC method and the DGC (Morrell et al., 2009c).

The SLC-technique has already been used in several studies with bull and stallion semen (Morrell & Rodriguez-Martinez, 2009, (Morrell *et al.*, 2009a, Johannisson *et al.*, 2009) and also in boar semen (Morrell *et al.*, 2009b). The results suggest that the SLC-technique is easy to use, requires less time and provides gentler handling of the spermatozoa (Thys et al., 2008, Morrell *et al.*, 2008a). In addition, the use of the single layer facilitates scaling-up the volumes of colloid and ejaculate used. Therefore it may be possible to process the large number of spermatozoa which is required for AI-doses of boar and stallion semen (Thys et al., 2008, Morrell et al., 2008a, Wallgren *et al.*, 2008).

Aims of the study:

To investigate the effect of single layer centrifugation (SLC) through silica colloids on boar sperm samples, the samples being analyzed by computerized assisted sperm analysis (CASA) and by looking at other parameters of sperm quality, such as morphology and viability.

Hypothesis:

1) The quality of SLC selected samples of boars will be increased compared to the uncentrifuged samples.
2) Volumes of the SLC method can be scaled-up without decreaseing the quality of the sperm preparations.

Materials and methods:

Animals:

Semen from eight mature boars was used.

Group I:
Four boars from the Division of Reproduction, Faculty of Veterinary Medicine and Animal Sciences, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden. The boars were of different breeds: Swedish Yorkshire (n=1), Norwegian Landrace (n=2) and Swedish Landrace (n=1) and the average age was 3.1 year (SD 0.4)

Group II:
Four boars from Quality Genetics, Svinsemin Hållsta 106, Eskilstuna, Sweden. They were pure Hampshire and the average age was 1.4 year (SD 0.5).

Collection of semen

Group I:
Semen was collected once a week, for a period of 4 weeks. At least 4 ejaculates per boar were used. The sperm-rich fraction (SRF) of ejaculates from the boars was collected using the gloved hand technique into a plastic bag in a pre-warmed thermos flask. The semen was immediately extended 1:1 (v/v) in warm (38°C) Beltsville Thawing Solution (BTS).
The BTS was prepared on the day of collection. 200 ml of BTS contains 200 ml deïonised water, 7.4 g Glucose, 1.2 g Tri-Sodium citrate, 0.26 g Sodium bicarbonate, 0.26 g Sodium EDTA and 0.16 g Potassium chloride. After 20min. blending, the pH (±7.0) and Osmolarity (± 330) were measured using an ATC pH meter and ABEX Osmometer respectively.
The sperm concentration in the extended semen was measured using a Nucleocounter-SP100 (Chemometec, Denmark) and adjusted to $100*10^6$ sperm cells/milliliter.
SLC was carried out immediately after measuring the concentration.

Group II:
The semen was collected at the boar station around 8:00 that morning and dispatched to a collection point in Uppsala. The semen was extended with X-cell (IMV, L´Aigle, France) and adjusted to $100*10^6$ sperm cells/milliliter at the boar station, before transporting in a Styrofoam box at a temperature between 17-24°C. At SLU, the sperm concentration was measured using the Nucleocounter-SP100 (Picture 1A and 1B).

Nucleocounter-SP100:

Picture 1A: Nucleocounter-SP100 machine picture at: http://www.chemometec.com

Picture 1B: Nucleocounter-SP1 cassette picture at:http://www.chemometec.com

Specific settings for boar semen were installed in the Nucleocounter-SP100. An aliquot of 50 µl from each sample was placed in a sample cup and diluted with 5 ml reagent S100 solution. This solution was necessary to disrupt the plasma membrane in order to let the PI enter the spermatozoa and stain the DNA. The SP1 cassette was filled with this sample by pressing the piston. The SP1 cassette contains PI and after the fluid enters the cassette the PI was mixed with the fluid and stained the DNA of the spermatozoa. After filling, the cassette was placed in the Nucleocounter-SP100. The Total Cell Count was presented on the instrument display after 30 seconds.

Single Layer Centrifugation

Group I and group II:

Aliquots of the extended semen were layered on top of a single layer of a species-specific colloid (Androcoll™-P, SLU) (Picture 1). Different volumes were layered very gently on top of the colloid as follows: 4.5 ml of semen was layered on top of 4 ml colloid in a 10 ml centrifuge tube, 15 ml of semen was layered on 15 ml colloid in a 50 ml centrifuge tube. An aliquot of semen (uncentrifuged) was used as an unselected control sample. After centrifuging the SLC samples at 300 x *g* for 20 min, the resulting sperm pellets were resuspended in BTS containing bovine serum albumin (bSA) at 1.25 mg/ml (group I) or resuspended in X-cell (group II).

The three types of samples (Fig. 1),
(i) Unselected (only the extended semen, not centrifuged on colloid)
(ii) Selected 4.5 ml (4.5 ml extended semen centrifuged on 4 ml colloid), and
(iii) Selected 15 ml (15 ml extended semen centrifuged on 15 ml colloid), were stored in a Unitron Climate Box (Tørring, Denmark), regulated to a temperature of 17 to 18°C.

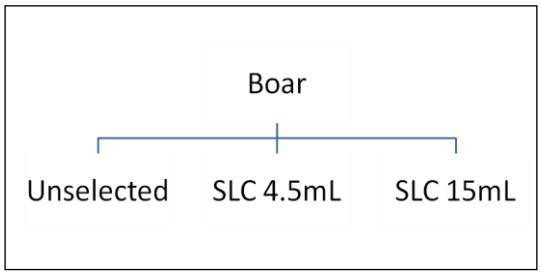


Figure 1: the three types of samples

Picture 2: preparation of tubes; Colloid layer on the bottom of the tube and semen aliquot is pipetted on top of it.

Sperm evaluation for both groups

Assessment of motility, linear motility, progressive linearity and VCL

The sperm motility was assessed with a CASA instrument. The settings from the CASA instrument were as follows: 32 frames with a spermatozoon present in at least 16, time resolution 20 ms (50 Hz), immotile when VAP ≤ 10 µm/s, motile when VAP ≥ µm/s, local motile when 10 µm/s < VAP > 25 µm/s as reported previously (Tejerina *et al*., 2008). If there was sufficient sample, an aliquot of at least 3 ml was taken out of the stock sample every day and used for the assessment. This was not possible for the 4.5 ml because the total volume available was not sufficient. On the day of collection (day 0) the sperm motility was assessed both before and after heating in a water bath at 38 °C for 30 minutes. On the following 4 days, motility assessment was made after incubating the samples for 30 minutes at 38°C.
An aliquot, (5 µl) of the sample was put on a Makler counting chamber 10 µl deep (Sefi Medical Instruments, Haifa, Israël) (pre warmed to 38°C) and placed on the warm (38°C) microscope stage of a phase-contrast microscope, at least 200 spermatozoa were assessed at 200 *x* magnification. Three parameters were measured: motile spermatozoa (%), linear motile spermatozoa (%), and the curvilinear velocity (VCL µm/s). Progressive motility was calculated subsequently by: motile spermatozoa*linear motile spermatozoa/100.

Assessment of number of sperm and Yield

Sperm concentration was measured using the Nucleocounter-SP100. For each measurement, a 50 µl aliquot of each sperm sample was mixed with 5 ml of reagent S100, and the total number of spermatozoa/ml was counted.
After that the percentage of sperm cells passing the colloid (=Yield or recovery rate), was calculated with the following formula:
Yield = (concentration of sperm in pellet*resuspended volume)/(concentration of sperm original on colloid*original volume)*100
Or using the definitions given in figure 3:
Yield = (concentration F * volume tube 3)/(concentration A * volume A)*100

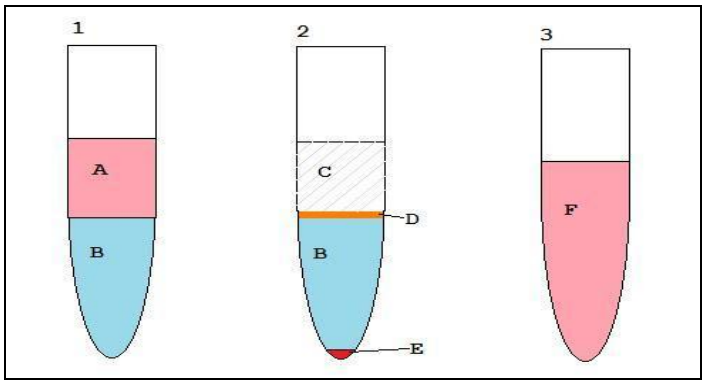


Figure 3: 1: tube with semen aliquot (A) and colloid (B); 2: Tube after centrifugation with extender (C), not selected sperm cells (D), colloid (B) and selected sperm pellet (E); 3: tube with resuspended sperm pellet in extender (F)

The data obtained from the CASA instrument is listed as in the model figure below (Fig.2)

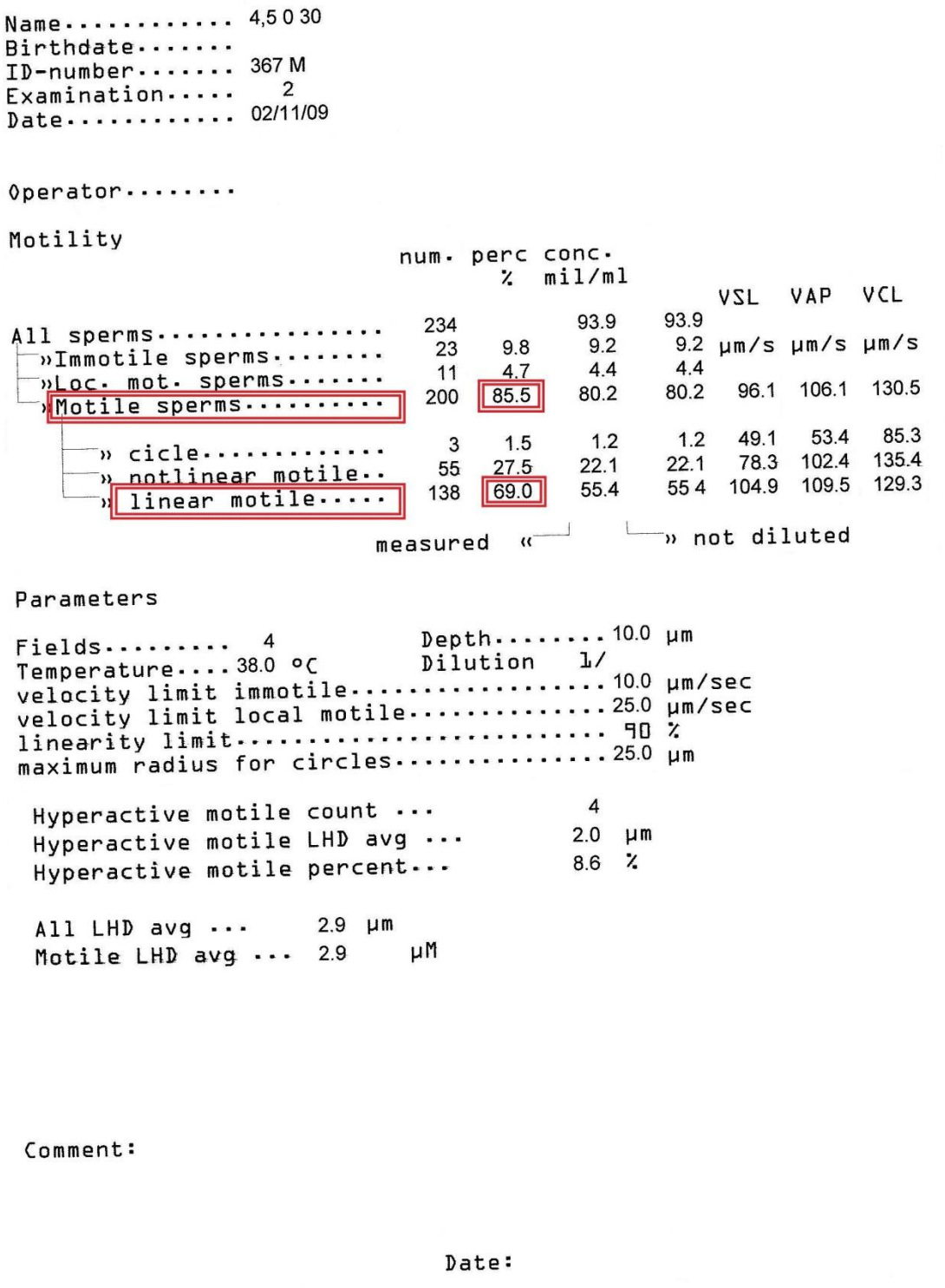


Figure 2: Data list from CASA instrument

Assessment of morphology

Aliquots from the unselected and the selected samples were fixed in a buffered formalin solution. A smear of another aliquot was made for subsequent staining with Williams' stain. After these treatments morphology was evaluated by skilled personnel according to standard protocol in the Swedish Sperm Reference Laboratory at SLU. The method for the standard protocol is described in Morrell at al. (2008b).

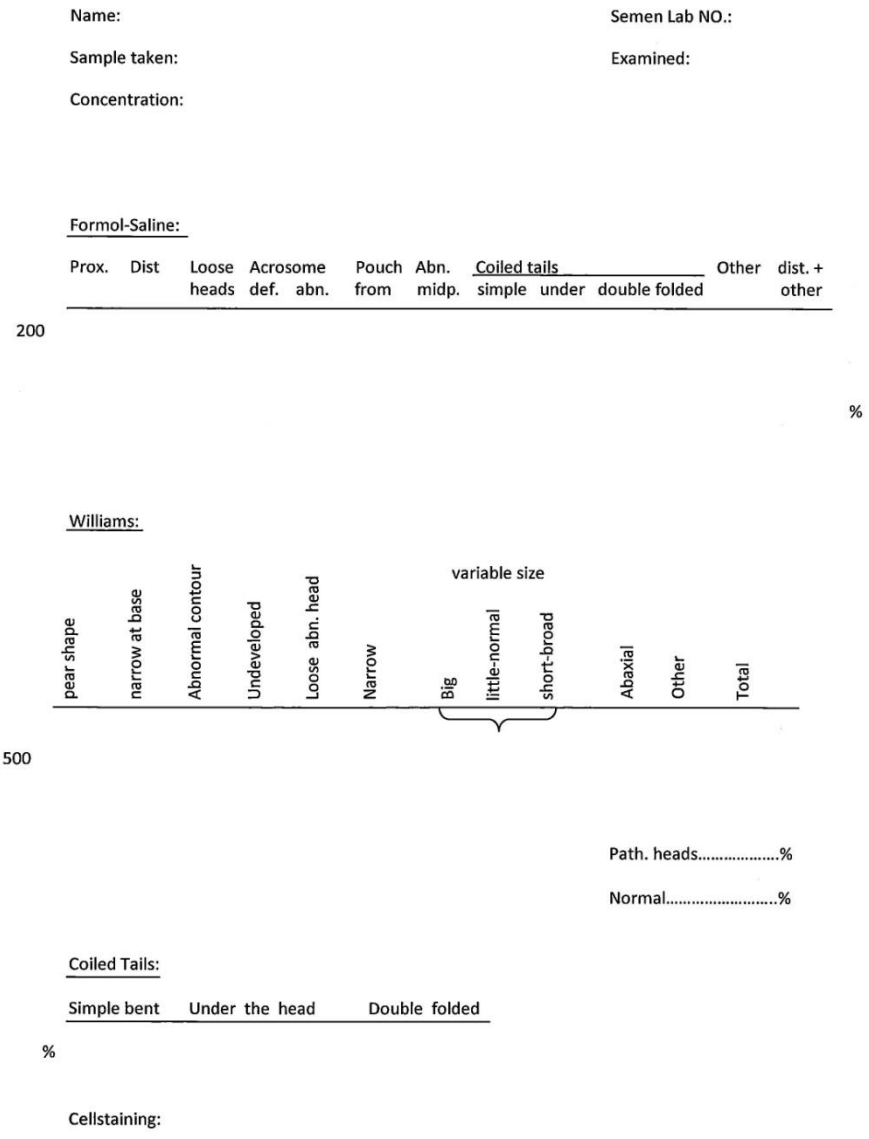


figure 4: Morphology result rapport

Assessment of viability and plasma membrane integrity

Aliquots from the unselected and selected samples were extended to a concentration of approximately $5*10^6$ sperm cells/ ml. For the extension, either BTS (group I) or X-cell (group II) was used.
Each sample was evaluated using a BD LSR flow cytometer (Beckon Dickinson, San José, CA, USA). Excitation was with an argon-ion laser (488 nm). Detection of green fluorescence was with a FL1 band pass filter (530/30 nm) while red fluorescence was measured using a FL3 long pass filter (>670 nm) A total of 50,000 sperm specific-events were evaluated and calculated as percentages.
For the assessment of the plasma membrane integrity (PMI) the samples were stained with SYBR14-PI. First 1000µl of the dilution was pipetted in a plastic tube and 1 µl of SYBR-14 (final stain concentration 0.02 µM) was added followed by 5 µl of PI (final stain concentration 12 µM). After incubating at 38°C for 10 minutes, the fluorescence was measured by using the FC. The cells were classified as: living (SYBR14-positive / PI-negative), dead (SYBR14-negative / PI-positive) or dying (SYBR14-positive / PI-positive).

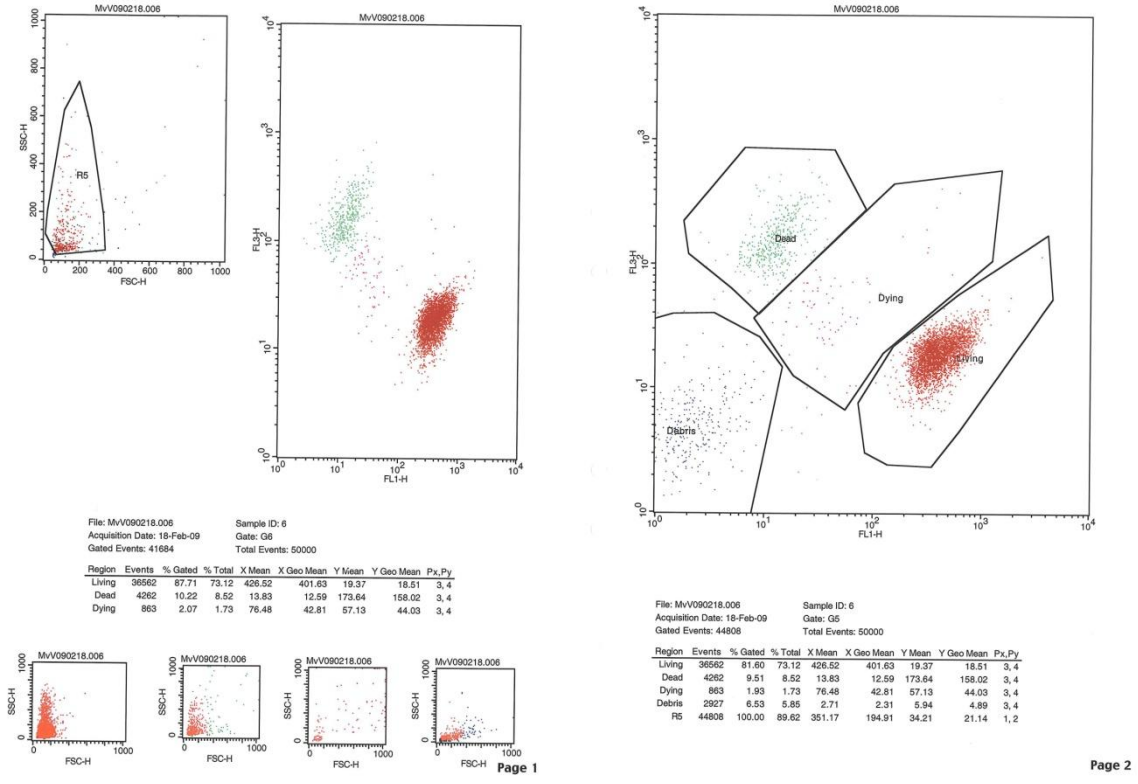


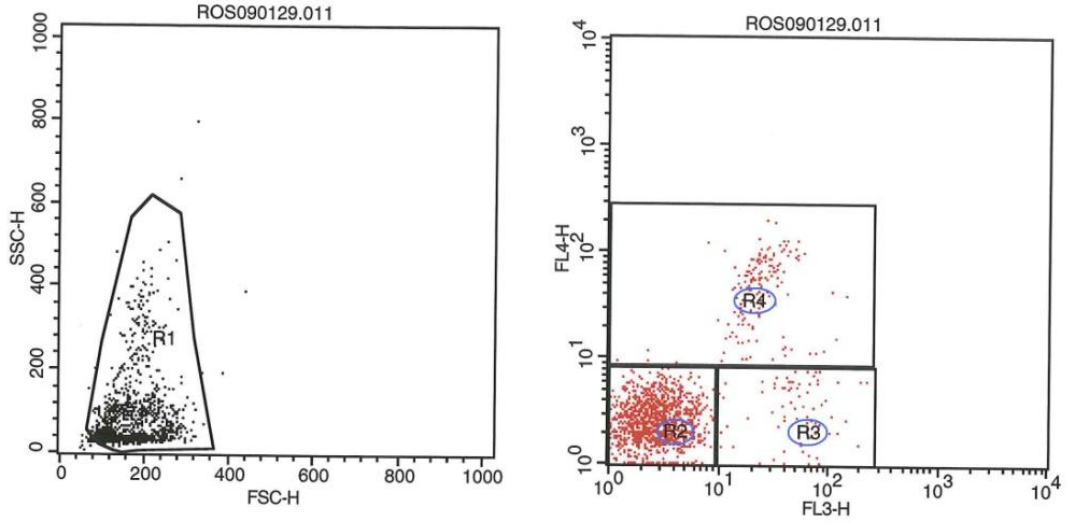
Figure 5: SYBR14-PI flow cytometer report

Assessment of Reactive Oxygen Species (ROS)

Aliquots from the unselected and selected samples were extended to a concentration of approximately $5*10^6$ sperm cells/ ml. For the extension either BTS (group I) or X-cell (group II) was used.
Each sample was evaluated using a BD LSR flow cytometer (Beckon Dickinson, San José, CA, USA). Excitation was with an argon-ion laser (488 nm) and a HeCd laser (325 nm). Detection of green fluorescence was with a FL1 band pass filter (530/30 nm), red fluorescence was measured using a FL3 long pass filter (>670 nm) and blue fluorescence was detected in FL4 with a band pass filter (510/20 nm). In total, 30,000 sperm specific-events were evaluated and calculated as percentages.
For the assessment of ROS production, the samples were stained as follows: a new aliquot of 300 µl from the diluted samples were put in a plastic tube and 3 µl of HO (final stain concentration 0.4 µM), 3 µl of HE (final stain concentration 0.4 µM) and 3 µl of DCFDA (final stain concentration 20 µM) were added. After gentle mixing the samples were incubated at 38°C for 30 minutes before running the FC. The cells were classified as: ROS-negative living, ROS-positive living or dead.

Statistical analysis

The statistical analyses were performed using the SAS software (Ver. 9, SAS Institute Inc., Cary, NC, USA). Variables were analyzed separately for each measuring occasion, using analysis of variance (PROC MIXED).
The statistical model included the fixed effects of group of boars (2; group I or group II), boar nested within group of boars (4+4), treatment (3; 4.5, 15, UN) and the interaction between treatment and group of boars. The statistical model also included the random effect of ejaculate nested within boar.
Least squares means were calculated for each level of the fixed effect, and levels of significance were estimated for differences between least squares means.



Region Statistics

File: ROS090129.011
Log Data Units: Linear Values
Sample ID: 87 50 Wed
Patient ID:
Tube:
Panel:
Acquisition Date: 29-Jan-09
Gate: G1
Gated Events: 28729
Total Events: 30000
X Parameter: FL3-H (Log)
Y Parameter: FL4-H (Log)

Region	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean	Px,Py
R1	28729	100.00	95.76	8.81	4.00	8.40	3.45	1, 2
R2	23877	83.11	79.59	3.00	2.67	2.69	2.45	7, 5
R3	1449	5.04	4.83	60.79	50.09	3.73	3.28	7, 5
R4	3340	11.63	11.13	27.46	23.36	51.22	40.00	7, 5

Figure 6: ROS flow cytometer report; Left top: R1=total gated; Right top: R2= ROS negative-living; R3= ROS positive living; R4=dead;

Results

Motility

There were no significances at d0t0 and d0t30 between groups and treatments.
After day 1, the motility was significantly better in the unselected samples than in the SLC-selected samples (P<0.001) (see Fig. 7).
Furthermore, there was a significant group*treatment interaction (P<0.001)

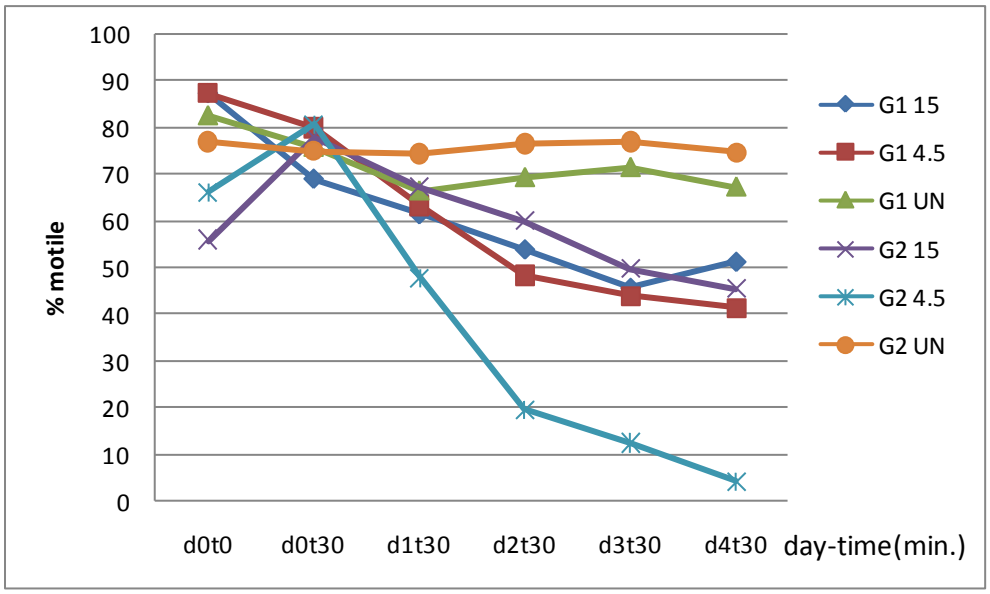


Figure 7: Mean % motility per group-treatment; G1 15 = group I treatment 15ml SLC; G1 4.5 = group I treatment 4.5ml SLC; G1 UN = group I untreated samples; G2 15 = group II treatment 15ml SLC; G2 4.5 = group II treatment 4.5ml SLC; G2 UN = group II untreated samples.

In contrast, both linearity and progressive motility (Figures 8 and 9) were better in the SLC-selected samples than in the unselected samples at all time points (P<0.001). In addition, there was a significant difference between treatments (P<0.001) and also a group*treatment interaction (P<0.001) from day 2 onwards.

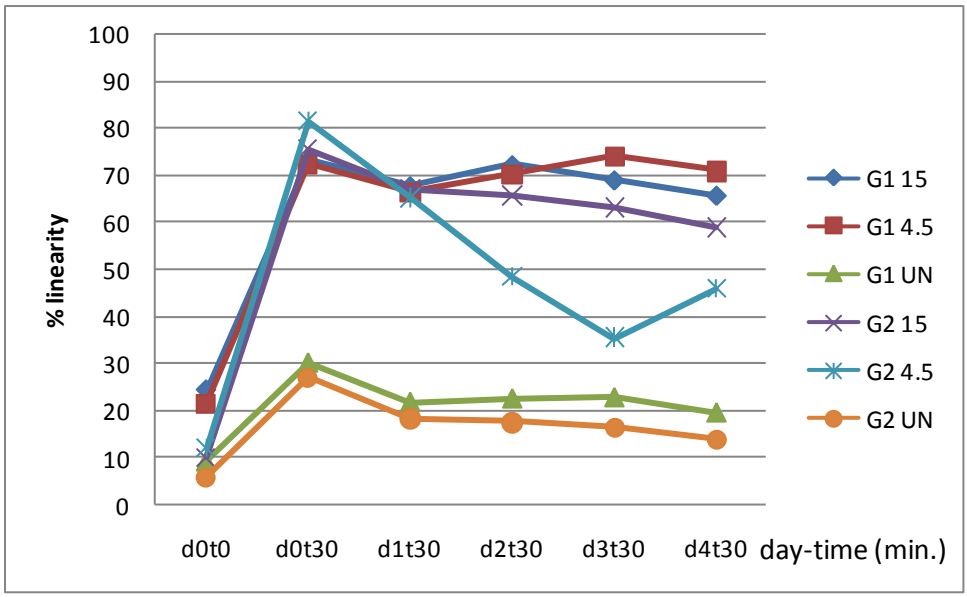


Figure 8: Mean % linearity per group- treatment; G1 15 = group I treatment 15ml SLC; G1 4.5 = group I treatment 4.5ml SLC; G1 UN = group I untreated samples; G2 15 = group II treatment 15ml SLC; G2 4.5ml = group II treatment 4.5ml SLC; G2 UN = group II untreated samples.

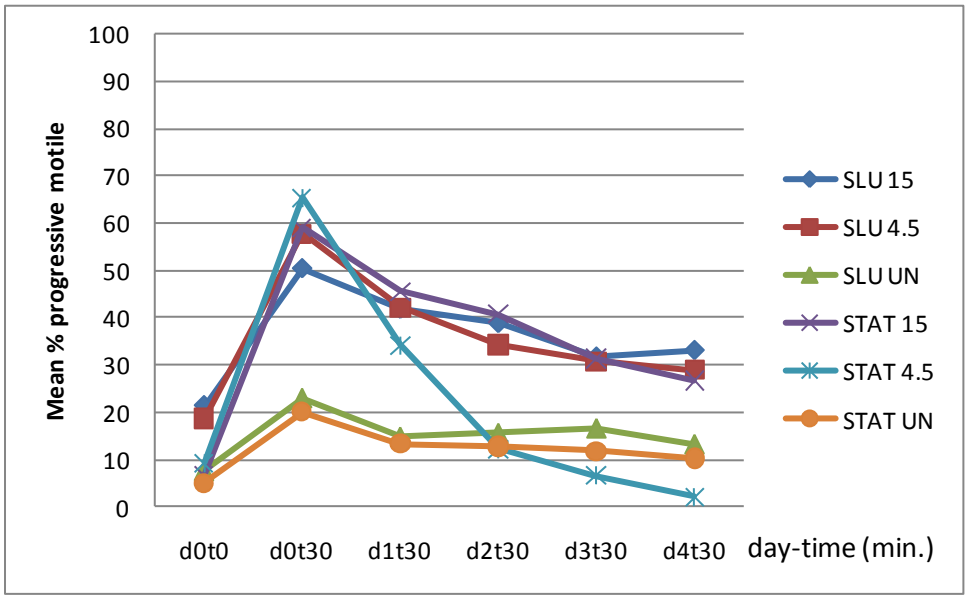


Figure 9: Mean % progressive motile spermatozoa per group-treatment; G1 15 = group I treatment 15ml SLC; G1 4.5 = group I treatment 4.5ml SLC; G1 UN = group I untreated samples; G2 15 = group II treatment 15ml SLC; G2 4.5ml = group II treatment 4.5ml SLC; G2 UN = group II untreated samples.

Sperm concentration and yield

The yields from SLC are shown in Figure 10. There was no difference in the mean yields from SLC 4.5 and SLC 15 overall (P>0.05), although there were differences between boars. Boar 4 in group II did have a significantly larger yield from SLC 15 than from SLC 4.5 (P<0.001). Although the yield from group I boars was higher than group II for the SLC 4.5 (P<0.001), there was no difference between the two groups for the yield from the SLC 15 (P>0.2).

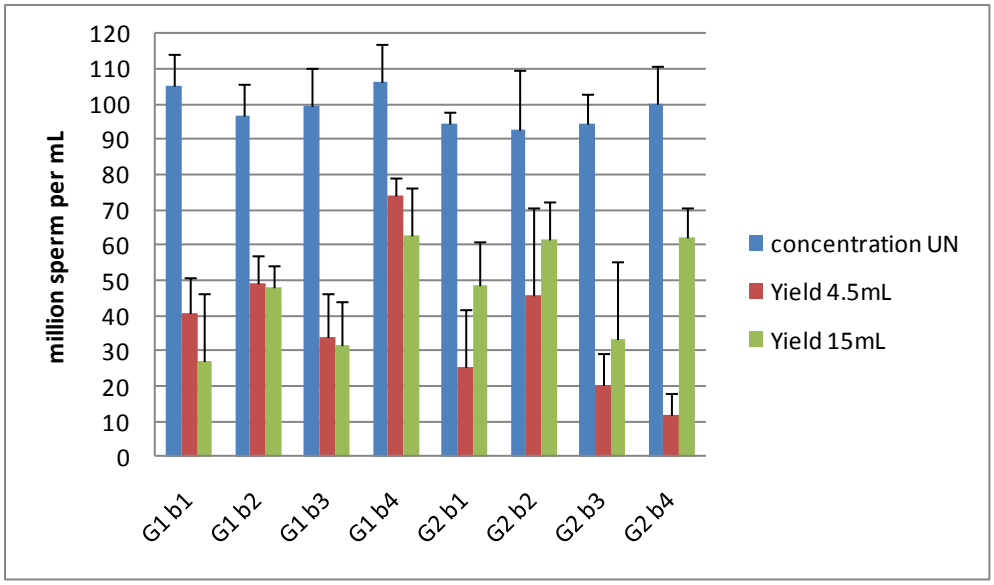


Figure 10: Mean concentrations and Yields per boar in million spermatozoa per ml;
G1 b1 = Group I boar 1; G1 b2 = Group I boar 2; G1 b3= Group I boar 3;
G1 b4= Group I boar 4; G2 b1= Group II boar 1; G2 b2= Group II boar 2;
G2 b3= Group II boar ; G2 b4= Group II boar 4.

Sperm morphology

The proportion of morphologically normal spermatozoa was significantly higher in the SLC samples than in the untreated samples (mean ±SD: 93.9 ± 7.0% and 87.9 ± 15.3% for 4.5 ml and 15 ml respectively and 81.1 ± 14.9% for the unselected samples; P<0.001). There was also a significant difference between the groups (P<0.001), with group I boars having a significantly higher percentage of morphologically normal spermatozoa than group II boars (mean ± SD: see Table1 and Figure 11)

	unselected	4,5mL	15mL
Group I	83.6 ± 13.6	96.5 ± 3.6	94.0 ± 5.1
Group II	80.0 ± 16.6	91.3±8.7	81.8 ± 19.6
total group	81.1 ± 14.9	93.9 ± 7.0	87.9 ± 15.3

Table 1: percentage of normal spermatozoa with formal saline (mean ± SD)

The difference in the proportions of some morphological abnormalities between the unselected and selected samples was not significant (see Figure 11):

- proximal cytoplasmic droplets: less in the treated samples in both groups
- tail defects: less in the treated samples in both groups

There were also some numerical differences between groups in the proportions of some morphological abnormalities (not significant) (see Figure 11)

- midpiece defects: less in group I than in group II
- head defects: less in group I than in group II
- tail defects: less in the unselected samples from group II than in the unselected samples from group I

All other morphological abnormalities were present at less than 1% and thus not mentioned.

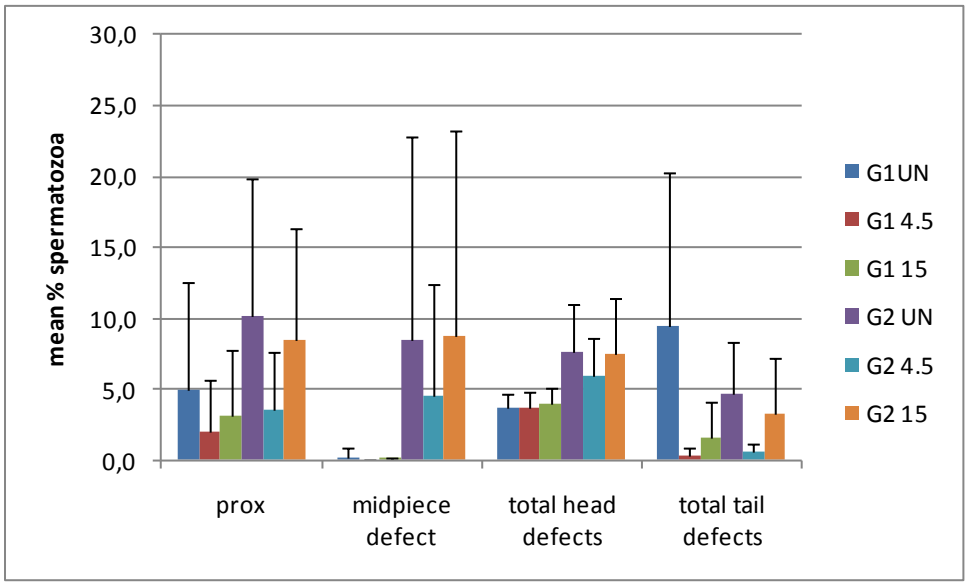


Figure 11: mean differences in morphological abnormal spermatozoa per group-treatment in percentage; G1 UN = Group I untreated samples; G1 4.5 = Group I 4,5ml samples; G1 15 = Group I 15ml samples; G2 UN = Group II untreated samples; G2 4.5= 4.5ml samples; G2 15 = Group II 15ml samples; Prox= proximal cytoplasmic droplets; All other morphological abnormalities were <1% and thus not mentioned in this figure.

Sperm viability

In group II there were significantly fewer living spermatozoa than in group I (mean ± SD: 93.7 ± 2.8% and 91.1± 4.3% for group I and II respectively; P<0.01)(see Table 2). In group I there were significantly fewer dying spermatozoa than in group II (mean ± SD: 4.8 ± 2.2% and 7.4 ± 3.7% for group I and group II respectively; P <0.,001).
There was no significant difference between the untreated and SLC-selected samples and no significant difference between the two different SLC treatments.

	viability		
	living	dying	dead
Group I	70.3 ± 2.8	3.6 ± 2.2	1.0 ± 0.7
Group II	91.1 ± 4.3	7.4 ± 3.7	1.5 ± 1.1

Table 2: Percentage of spermatozoa per group: living, dying and dead (mean ±SD) measured 24h after semen collection and SLC.

ROS production

In the unselected samples there was less ROS production than in the selected samples (mean ± SD: 6.6 ± 3.3% for unselected, 8.4 ± 3.2 % and 9.4 ± 5.3% for 4.5 ml and 15 ml SLC-selected samples respectively, p<0.01) (see Table 3) There was also a slightly lower concentration of dead spermatozoa in the untreated samples but these results were not significant from the treated samples.
Table 3 gives the means and SD for the parameters measured with the FC. There was a difference in % living spermatozoa in all treatments between the ROS staining method and the SYBR14-PI staining method.

		unselected	4,5mL	15mL
ROS	living	83.6 ± 4.8	75.3 ± 11.5	75.1± 12.4
	ROS prod.	6.6 ± 3.3	8.4 ± 3.2	9.4 ± 5.3
	dead	9.3 ± 2.7	15.7 ± 9.2	15 ± 8.6
SYBR14 PI	living	92.6 ± 3.6	92.6 ± 2.8	91.6 ± 5.1
	dying	5.9 ± 2.8	6.0 ± 2.4	7.0 ± 4.5
	dead	1.6 ± 1.1	1.2 ± 0.8	1.4 ± 1.0

Table 3: Percentage of sperm cells: living, ROS producing, dead and dying (mean ±SD)measured 24h after semen collection and SLC.

Discussion and conclusions

The objectives were to investigate the effect of single layer centrifugation (SLC) through silica colloids on boar sperm samples, the samples being analyzed by using a CASA instrument and a FC and by looking at other parameters of sperm quality, such as morphology and viability.
The hypotheses were that the quality of boar sperm could be increased when using SLC and that scaling-up the SLC method could be possible.

Motility

The results showed that sperm motility at d0t0 was significantly better in group I than in group II. A possible reason for this observation is that there was less delay between collecting the semen and measuring the motility with the CASA instrument for group I boars than for group II boars. With group II samples, there was an interval of at least 5hours between collecting the ejaculates and measuring motility. The samples also had to be transported from the boar station to the collection point and from there to SLU. Although the samples were packed in a Styrofoam box it was possible that the temperatures in the box were fluctuating too much and thereby affecting the spermatozoa negatively. Another explanation could be the fact that the boars from group I were of a different breed than the boars from group II. It is possible that genetic differences influenced the motility outcome.
The higher motility after day one in the untreated samples than in the SLC samples could be due to the higher sperm concentration in the untreated samples, or to a sub-optimal level of protein in the extender used for the SLC-samples. For the latter, BSA was used as protein to prevent spermatozoa from clumping together or sticking to the glass slide.
However, with the SLC treatment it was possible to select spermatozoa with a more linear motility and higher progressive motility. These two parameters are some of the essential parameters for fertility (Tejerina *et al*., 2008) and they can quantify the quality of the spermatozoa (Morrell & Rodriguez-Martinez, 2009). The sperm kinematics are important for sperm transport and fertilization in the female tract and so can be used to predict the function of the spermatozoa (Maxwell *et al*., 2000). Motility is also an important fertility indicator because of its importance for migration through the female tract and gamete interaction at fertilization. Robayo et al. found a positive correlation between VCL and VAP and migration in cervical mucus of sheep (Robayo *et al*., 2008). Holt et al. found that litter size in pigs was positive correlated with VCL and VAP (Holt *et al*., 1997) but positive correlations with fertility were also found in other species (Cox *et al*., 2006, Robayo et al., 2008).
The motility parameters measured by CASA can be affected by sperm concentration, and type of extender. Therefore, extending a sperm sample from a concentrated stock can introduce artefacts in the motility parameters. The supposed advantage of a new CASA instrument, the Qualisperm^{tm}, is its ability to handle high sperm concentrations, and therefore in a future study it might be interesting to use this instrument, to avoid the problems of extending the stock sperm suspension.

Sperm concentration and yield

Because there was no significant difference between the mean yields from the SLC 4.5 and the SLC 15, it can be concluded that there is no difference in treatments for this parameter, that is, the SLC 15 treatment is not influencing the yield negatively. There were significant boar differences but those differences were related to animal influences alone and not to the treatment influence.

Sperm morphology

It was possible to select for spermatozoa with normal morphology using SLC. Morphology is one of the factors influencing the quality of sperm. (Morrell & Rodriguez-Martinez, 2009) When a higher concentration of morphological normal stallion spermatozoa is used, the higher the chance of pregnancy (Morrell & Rodriguez-Martinez, 2009).
The difference between the groups can be explained by the fact that there was a difference in breed between group I and group II. That also explains the high variation (SD) between the samples. The boars in group I are checked more often for morphological abnormalities, because they are used for research purposes. The boars in group II, on the other hand, would not be checked for morphological abnormalities unless there were problems with fertility results, so there are no data on how long these abnormalities had existed.

Sperm viability

The SLC treatment does not improve boar sperm viability, according to the SYBR-14/PI results. This observation is in contrast to results with stallion spermatozoa where viability was improved by SLC-selection (Johannisson *et al.*, 2009). One explanation for this finding could be the fact that the viability in the samples was already high and therefore hard to improve significantly. Also the concentration of protein concentration used in the extender was lower than stated in the manufacturer´s product information sheet, because their recommended concentration (10% protein) was almost impossible to mix and caused the spermatozoa to aggregate. Another explanation could be that the staining concentrations were not optimal. Although Garner and Johnson found that staining with SYBR14-PI gave similar flow cytometric patterns in bulls, boars, rams, mice, rabbits and men, they also showed that staining with SYBR14-PI could give differences among individual boars due to true animal differences. With SYBR14-PI staining, the percentage living was 80-90% for boar spermatozoa (Garner & Johnson, 1995) (Maxwell et al., 2000). In the study reported here, a higher percentage living spermatozoa (around 85-95%) was found, possibly because the concentration of the stain and/or the concentration of the protein were not optimal. In a future study it can be useful to look at different dye combinations or using other assays e.g. the Nucleocounter-SP100 measures sperm viability in addition to sperm concentration.

ROS production

The findings that there was less ROS production in the untreated samples than in the SLC-selected samples is surprising, since density gradient centrifugation has been shown to reduce ROS production in boar sperm samples. Previously, a concentration of less than 4% of ROS production was seen, while in the study reported here, it was around 6% (Guthrie & Welch, 2006).
However, the SLC-samples used here contained a higher proportion of dead spermatozoa than in the uncentrifuged samples, which may have been a source of ROS.
Furthermore, there was a big difference in the concentration of living spermatozoa between the ROS staining method and the SYBR14-PI staining method, despite being measured for both parameters on the same day. It is possible that either the ROS staining procedure or the SYBR14-PI staining was not optimal (for boar semen).

Scaling up

For nearly all parameters, there were no significant differences between the 4.5ml and the 15ml SLC-treatments, indicating that the SLC can be scaled-up to 15ml without reducing sperm quality in the resulting samples. These results confirm earlier observations with stallion spermatozoa (Morrell et al, 2009e). Scaling-up has been tried with up to 50ml extended boar semen (unpublished data) but the sperm quality was not as good in the selected samples as for the 4.5 and 15 ml volumes. The technique and the formulation of the colloid require further adjustment to scale-up to a higher volume.
The 15ml volume is feasible to produce AI doses from very valuable boars. The total number of sperm produced in one tube was approximately $600*10^6$ spermatozoa per ml. So for one insemination dose of $3*10^9$ spermatozoa, 4 tubes would be needed.
For a future study it can be interesting to investigate the pregnancy rate using SLC-treated sperm samples.

In conclusion, SLC can be used to enhance the quality of boar sperm samples, particularly for normal morphology, linear motility and progressive motility. Furthermore, the technique could be scaled-up to process 15 ml extended ejaculate per tube, without compromising sperm quality in the selected samples. For the future the technique must be scaled up more to be useful in practice.

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