Removal of bacteria from boar semen using a low-density colloid

JM Morrell[1*], A Núñez-González[2], I. Crespo-Félez[2], S Martínez-Martínez[3], M-J Martínez Alborcia[4], E. Fernández-Alegre[2], J C Dominguez[2,5], CB Gutiérrez-Martín[3], F. Martínez-Pastor[2,6]

[1]Clinical Sciences, Swedish University of Agricultural Sciences, 75007 Uppsala, Sweden

[2]Institute of Animal Health and Cattle Development (INDEGSAL), Universidad de León, 24071 León, Spain

[3]Department of Animal Health, Universidad de León, 24071 León, Spain

[4]AIM Ibérica, Av. Playa de las Américas 2-7, 28290 Las Rozas (Madrid)

[5]Department of Animal Medicine, Surgery and Anatomy (Animal Medicine and Surgery), Universidad de León, 24071 León, Spain

[6]Department of Molecular Biology (Cell Biology), Universidad de León, 24071 León, Spain

Abstract

Antibiotics are added to semen extenders when preparing commercial semen doses for artificial insemination according to national and international guidelines. However, this addition of antibiotics represents non-therapeutic usage and could be contributing to the development of antibiotic resistance. Colloid centrifugation was shown to reduce the load of bacteria present in boar semen and was capable of removing all bacteria if performed directly after semen collection, albeit with some loss of spermatozoa. The present experiment was conducted with a low density colloid to investigate whether it was possible to separate all of the spermatozoa from seminal plasma i.e. without selection for robust spermatozoa, or whether this would have a detrimental effect on sperm quality. Ejaculates from nine boars were extended in Beltsville Thawing Solution without antibiotics and were transported to the laboratory for Single Layer Centrifugation (SLC) on modified Porcicoll i.e. at a low density (S). A further modification was that a sterile inner tube was included inside some of the 50 mL centrifuge tube to facilitate harvesting of the sperm pellet (M). Aliquots of all samples (control, S and M) were cultured for bacterial quantification and identification using standard microbiological methods. Sperm quality was evaluated daily. Three of the C and M samples and five of the S samples did not contain any bacteria. Mean bacterial counts for the remaining samples (colony forming units/mL) were as follows: C 259±216; S 30±22; M 33 ±15 ($P<0.05$). *Citrobacter* spp., *Staphylococcus simulans, Klebsiella variicola, Escherichia coli, Myroides odoratimimus, Proteus* spp. and *Enterococcus faecalis* were identified in the control samples. There were marginal differences in sperm quality among treatments, with sperm velocity and linearity being higher in S and M samples than in C at all time points. However, sperm viability, capacitation and acrosome status were marginally better in controls than in S or M on day 0, but these differences disappeared during storage. Conclusions: centrifugation through a low density colloid can remove or reduce bacterial contamination in

boar ejaculates without using antibiotics. Furthermore, it is possible to collect boar ejaculates without bacterial contamination by paying strict attention to hygiene.

Introduction

The emergence of antibiotic-resistant bacteria, particularly methicillin-resistant strains [1], is leading to a crisis in health care, with people contracting bacterial infections that are difficult, or in some cases, impossible to treat with antibiotics. Antibiotic resistance has been described by the World Health Organisation (WHO) as the biggest threat for people in the modern world [2].

Even a small amount of antibiotic usage can lead to considerable levels of antibiotic resistance developing within an animal species [3], and genes for antimicrobial resistance are readily exchanged between reservoirs in human beings and animals [4]. Therefore, there is now a concerted effort to restrict usage of antibiotics to therapeutic purposes only, choosing the appropriate antibiotics for their pharmacokinetic properties and the sensitivity of the specific bacteria involved. However, in spite of this effort, antibiotic resistance is still a major threat.

One non-therapeutic use of antibiotics is the addition of antibiotics to semen extenders when preparing semen doses for artificial insemination, as stipulated by various governmental Directives e.g. Council Directive 90/429/EEC, Annex C, in the European Union. The ejaculate becomes contaminated during semen collection by the microbiota colonising the lower sections of the reproductive tract and from the environment. Since the semen is usually stored for several days before artificial insemination (AI) in a nutrient-rich medium to maintain sperm survival, bacteria can multiply, even at the normal storage temperature for boar semen (16-18°C). Antibiotics are added to prevent bacterial-induced deterioration in sperm quality and the possibility of causing disease in inseminated females. However, the

efficacy of the antibiotics against the microorganisms commonly contaminating semen is now being questioned since bacteria have been detected in boar semen despite the presence of antibiotics [5].

An alternative to adding antibiotics to semen extenders would be to remove the contaminating bacteria by physical means immediately after semen collection. A new sperm preparation technique, known as Single Layer Centrifugation (SLC) through species-specific colloid formulations [6] selects the sperm sub-population that are highly motile, with normal morphology and good chromatin integrity, from the rest of the ejaculate [7, 8]. The technique can be scaled-up to process whole ejaculates in 500 mL tubes [9, 10]. The SLC technique was shown to reduce bacterial contamination in boar and stallion sperm samples in the laboratory [11, 12]. However, there is concern among pig breeders that some spermatozoa are lost during processing. This loss could be reduced by using a low density colloid formulation to simply separate spermatozoa from seminal plasma, but the effect on bacterial numbers and sperm quality has not been ascertained.

The purpose of this study, therefore, was to determine whether SLC with a low density colloid is effective in separating spermatozoa from bacteria and whether the sperm quality of the resulting samples is adversely affected by the procedure of centrifugation without selection. A further aim was to determine whether the SLC method could be modified to facilitate pellet retrieval without re-contamination.

Materials and Methods

Semen extender

Beltsville Thawing Solution (BTS), modified from Pursel & Johnson [13] was used to extend the semen. The BTS consisted of glucose (205.4 mM), tri-sodium citrate (20.4 mM), sodium hydrogen carbonate (14.9 mM), sodium EDTA (3.4 mM) and potassium chloride (10.1 mM). The modification was that no antibiotics were added to the BTS. After SLC, BTS with added

bovine serum albumin (5%) was used, to prevent the spermatozoa from aggregating or sticking to the tube or slide [14].

Animals and semen collection

The boars (4 x Large White, 2 x Landrace, 1 x Pietrain, 1 x Duroc, 1 x Synthetic) were kept at a commercial pig station (Technological Centre of Artificial Insemination; Topigs-Norsvin Spain, Campo de Villavidel, León, Spain) under standard husbandry conditions. Semen was collected using the semi-automatic collection device Collectis® according to the technique described by Perez-Patiño et al. [15]. The sperm concentration was measured photometrically and ejaculates were immediately extended in warm BTS at 34-35 ºC to a sperm concentration of approximately $100x10^6$/mL and were transported to the semen processing laboratory at the Institute of Animal Health and Cattle Development (INDEGSAL), University of León, Spain, in an insulated container.

Sample preparation

The colloid used was a silane-coated silica formulation for boar semen (Porcicoll, patent applied for; PI8163758), prepared at a density of 1.052 g/mL [14]. Each extended semen sample was split into three 15-mL portions: uncentrifuged control (C), SLC with a low density Porcicoll (S), and modified SLC with a low density Porcicoll (M), in which an inner tube was used to facilitate pellet retrieval (modified from [12]). Treatments S and M were centrifuged at $300 \times g$ for 20 min before resuspending the sperm pellet in sterile BTS containing 5% BSA. The sperm suspensions were transferred to 15 mL tubes for storage at 17 °C. An aliquot (0.5 mL) of each sample was immediately placed on ice and transferred to the microbiology department for culture.

Microbiological and MALDI-TOF analyses

Aliquots of the samples were used for bacterial count, culture and identification using standard protocols. Briefly, the samples (0.1 mL) were cultured for 24 h at 37 °C on a

116 microaerophilic atmosphere in different medium: Blood Columbia agar, Cled (Cystine-
117 Lactose-Electrolyte-Deficient) agar, McConkey agar and TSA (tryptone soy agar) agar
118 (OXOID, Hampshire, UK).
119 For the count, we performed dilutions from -1 to -6, following sowing 100 microliters in Agar
120 TSA. The number of colonies was expressed as colony forming units/mL. Plates were
121 incubated for a further 24 h and read again.
122 The bacteria were characterized using different methods depending on type: Gram stain,
123 oxidase and catalase activity and different biochemical test (API 20E, API 20NE, API Staph,
124 API Strep; Bio Merieux Inc., Durham, NC), according to the manufacturer's instructions.
125 Samples were analysed using a Bruker Daltonics UltrafleXtreme MALDI-TOF/TOF
126 equipment and the FlexControl software v. 3.0 (Brucker Daltonics, Bremen, Germany) for the
127 acquisition of mass spectra. The Biotyper Real Time Classification software v3.1 (Brucker
128 Daltonics) was used for microbial identification by comparison of the spectra with the
129 corresponding database provided by the manufacturer (MALDI Biotyper database, 5989
130 entries, Bruker Daltonics). This software generates a score, ranging from 0 to 3, showing the
131 similarity between a given sample and a reference spectra, and displays the top 10 matching
132 results with the highest scores. The reliability of the identification was evaluated according to
133 the standard manufacture interpretative criteria: 2.300 - 3.000, high species identification
134 probability; 2.000 - 2.290, high genus identification probability; 1700-1.999, presumable
135 species identification; 1.700 - 1.999 presumable genus identification; 0.000 - 1.699 unreliable
136 identification.
137 ***Motility analysis by CASA***
138 A 5 µl drop was prepared in a Makler counting chamber (10 µm depth; Haifa Instruments,
139 Israel) and examined with a phase contrast microscope (Nikon E400 with warmed stage at
140 37 °C; 10x negative contrast optics) provided with a Basler A312f camera (Basler AG,

Ahrensburg, Germany). Images from at least three fields were acquired at 53 frames/s and analyzed with ISAS 1.0.18 software (Proiser SL, Valencia, Spain). The kinematics reported were Total motility (MOT; %), Progressive motility (PROG; %), straight-line velocity (VSL; μm/s), linearity (LIN; %), and amplitude of the lateral movement of the head (ALH; μm).

Flow cytometry

The following fluorescent probes in PBS (0.5% BSA) were used in combination for evaluating sperm properties: Propidium iodide (PI, 3 μM) for membrane integrity, Mitotracker deep red (MTdr, 100 nM) for mitochondrial activity, PNA-FITC (1 μg/ml) for acrosomal status and Merocyanine 540 (M540, 2 μM) for capacitation status [16]. After adding the sperm samples (10^6 mL^{-1}), the mixture was incubated in the dark for 15 min at 37 °C; Hoechst 33342 (H342, 5 μM) was added to all tubes to enable debris to be excluded from the fluorescence profiles.

Analyses were performed using a MACSQuant Analyzer 10 cytometer (Miltenyi Biotech, Bergisch Gladbac, Germany), equipped with three diode lasers (violet at 405 nM, blue at 488 nM and red at 635 nM). The fluorescence was detected using filters 450/50 (violet line, blue fluorescence: H342), 530/40 (blue line, green fluorescence: PNA-FITC), 585/40 (blue line, orange fluorescence: M540), 655-730 (blue line, red fluorescence: PI) and 655-730 (red line, red fluorescence: Mitotracker deep red). All parameters were visualized in a ºlogarithmic scale. Spermatozoa were gated as $H342^+$ events, collecting at least 5000 spermatozoa. The gated data were analyzed using Weasel v3.4 (http://www.frankbattye.com.au/Weasel/), obtaining the proportions of viable spermatozoa (PI^+), of damaged-acrosome cells (PNA^+), of damaged-acrosome within viable (PNA^+ within PI^-), of capacitated within viable ($M540^+$ within PI^-), and of spermatozoa with active mitochondria ($MTdr^+/PI^-$).

Statistical Analysis

Data were analyzed using linear mixed-effects models (R statistical package) with treatments

and storage time as fixed effects. Pairwise comparisons were adjusted by Tukey's method. Results are presented as means ± SEM; the threshold for significance was set at $P < 0.05$.

Results

Bacteriology

No bacteria were cultured from three of nine C, five of nine S and three of nine M samples (Table 1). There were fewer bacteria in the samples prepared by either S or M than in C ($P<0.01$), with no difference in mean bacterial counts between S and M. Excluding the samples that did not contain bacteria in any treatments, mean±SEM colony forming units/mL (CFU/mL) observed for the different treatments were 325±47 for C, 28±28 for S and 37±20 for M (C vs. M and C vs. S, $p<0.01$). According to MALDI-TOF, the bacteria in the control samples were *Citrobacter spp., Staphylococcus simulans, Klebsiella variicola, Escherichia coli, Myroides odoratimimus, Proteus spp.* and *Enterococcus faecalis*. In both S and M, *Staphylococcus simulans, Klebsiella variicola* and *Escherichia coli* were identified. In addition, the biochemical tests identified *Pantoea agglomerans* in C, *Proteus vulgaris* in S and *Flavobacterium indologenes* in M that were not identified by MALDI-TOF.

Sperm quality

Sperm motility, viability, acrosomal status, capacitation status and mitochondrial activity for the three treatments immediately after preparation (day 0) and after 17 °C storage for up to seven days are shown in Tables 2 and 3. Overall, C showed a significantly higher quality on day 0, but differences were small and sperm quality tended to equalize with storage. Sperm quality decreased with storage time ($P<0.001$) except for ALH, where there was no significant change.

Although total motility (Table 2) was higher in the control samples than in selected samples after the centrifugation on day 0 (C>M>S, $P<0.01$), these differences decreased with storage

time, and by day 7 this pattern was reversed (C<M~S, P<0.05). Moreover, progressive motility was, in general, higher on days 1-2 and on day 7 for S and M than for C; velocity (VCL) and linearity (LIN) werehigher for the selected samples on any day of analysis (P<0.05) after day 0. However, ALH did not differ between treatments.

Sperm viability (Table 3) showed a higher average value for C than S and M on days 0 and 1 (P<0.05), although there was no difference between S and M. By day 2, viability in C and M were not different and only S was significantly lower (P<0.05). For the remaining storage times, there were no significant differences in viability between treatments. Acrosomal damage (both total and within the viable population) and the proportion of capacitated spermatozoa within the viable population were overall slightly higher for selected spermatozoa only at day 0 (P<0.05). The proportion of spermatozoa with active mitochondria tended to be higher for C up to day 3, although without a consistent pattern, but was not significant after day 3.

Discussion

The purpose of the present study was to investigate whether colloid centrifugation with a low density colloid could separate spermatozoa from bacteria in boar semen samples without having a detrimental effect on sperm quality. The centrifuged samples contained fewer bacteria than controls; in fact, in some cases there were no bacteria at all, and there was no noticeable effect on sperm quality that would be expected to impact on pregnancy rates or litter size in inseminated females. These results on reduction in bacterial numbers correspond to previous studies with boar semen [11] and stallion semen [12, 17], performed with species-specific colloids of higher density than the one used here.

These results are interesting since they suggest that SLC with a low density colloid could be used as a practical alternative to adding antibiotics to semen extenders to control bacterial

contamination in semen, without losing too many spermatozoa and without a detrimental effect on sperm quality. Although there were slight differences between the colloid-selected samples and controls on some days (marginal increases in capacitated or acrosome reacted spermatozoa immediately after sperm preparation), the changes disappeared with time. In any case, it is unlikely that such small differences would have an effect on pregnancy rate and litter size following artificial insemination. There were no differences in the bacterial counts from S and M, indicating that the presence of the inner tube did not provide an opportunity for bacteria to track down from the semen into the sperm pellet, at least not with the bacterial loads found in this study.

Although the previous results from our group showed that bacteria-free sperm samples could be produced if the SLC was done immediately after semen collection, leaving the spermatozoa in contact with seminal plasma for several hours before SLC during transport to the laboratory resulted in some bacteria appearing in the pellets [11]. In the present study, the first five samples were collected 2-3 hours before processing by SLC, whereas the remaining samples were collected 1-2 hours before processing. It would be interesting to see whether reducing the time between collection and processing could further reduce the bacterial count in the processed samples, although this may not be practical for field use.

The bacteria found in the present study were similar to the bacteria found in commercial semen samples in the previous study [11], and in other studies on boar semen. Thus, *P. aeruginosa*, *E. coli*, *Citrobacter* spp. and *Enterococcus* spp. were reported in a study on boars in Sweden [18]. In a study on 250 boar semen samples in the United States of America, *Enterococcus* spp., *Stenotrophomonas maltophilia*, *Alcaligenes xylosoxidans*, *Serratia marcescens*, *Acinetobacter wolffi*, *E. coli* and *Pseudomonas* spp. were observed, among others [19]. In boar semen in Cuba, *Klebsiella*, *Staphylococcus* and *Streptococcus* were identified [20]. Therefore, the bacteria found in the present study can be considered to be representative

241 of the species be found in boar semen, although no *Pseudomonas* spp. was seen. The bacteria
242 *Citrobacter* spp., *E.coli*, *Proteus* spp., *Enterococcus faecalis* and *Pantoea agglomerans* are
243 normally found in the intestine and are ubiquitous in soil and wastewater; *St. simulans* is
244 considered to be part of the normal skin microbiota, although it was identified as the
245 etiological agent of osteomyelitis and septicaemia in a human patient [21]; *K variicola,*
246 *Myroides odoratimimus* and *Flavobacterium indologenes* are environmental organisms,
247 although *K. variicola* has been associated with some cases of bovine mastitis [22], and
248 *Myroides* spp [23] and *Flavobacterium indologenes* [24] have been seen in infections in
249 immunocompromised human patients. Thus, these bacteria are not commonly associated with
250 infections of the reproductive tract in pigs, and an effect on sperm quality at the bacterial
251 loads observed here has not been reported previously.
252 Stressing the viable spermatozoa by centrifuging them without removing dead and dying
253 spermatozoa did not have an overall detrimental effect on sperm quality, although there were
254 small differences for individual parameters among the first days. These small differences
255 could be caused by technical errors, such as selected spermatozoa sticking to the Makler glass.
256 This result is in contrast to a previous study, in which centrifugation through a low density
257 colloid had a detrimental effect on sperm motility [25]. However, in the latter study, the
258 sperm suspensions after SLC were stored in partially filled tubes, whereas in the present study
259 they were stored in 15 ml tubes filled to the top, thus effectively producing anaerobic
260 conditions and helping to prevent a rise in pH due to loss of carbon dioxide from the medium
261 [26]. There were no differences in sperm quality between treatments S and M, suggesting that
262 the presence of the inner tube disrupting the integrity of the interface between the colloid and
263 the semen did not necessarily allow spermatozoa to bypass selection at the interface. However,
264 whereas six of the M samples contained bacteria only four of the S samples did. This result
265 requires confirmation with a larger number of samples, since it could indicate that the

disruption of the interface by the presence of the inner tube does permit bacteria to track through the colloid. However, more experiments are planned to elucidate this point. In addition, because of the promising results obtained in the present study, it is intended to compare sperm quality in further sperm samples prepared by low density colloid with conventional semen doses containing antibiotics.

The lack of a detrimental effect on sperm quality in the present study, as well as a reduction in the bacterial load in S and M samples, is very encouraging as a possible alternative to the use of antibiotics to control bacteria in semen samples. However, more extensive studies are needed before the method can be recommended to the swine industry. A larger sample size is needed, with ejaculates collected from boars kept under different husbandry conditions that might affect both bacterial numbers and the types of bacteria able to colonise the lower reproductive tract. Sepúlveda *et al.* [27] showed that increasing bacterial loads in boar semen were associated with deterioration in sperm motility and membrane integrity. The bacterial loads were not high in the present study, although previous studies showed that SLC could remove even bacterial loads as high as 10800 cfu/mL [11]. Additional studies will determine whether the proportion of silane-coated silica colloid in the formulation can be reduced even further, thus reducing the production cost and making the method more attractive for the pig industry. These additional studies will also determine whether bacteria that fasten on to spermatozoa by means of hooks, such as *E.coli*, are removed by passage through the low density colloid. Our previous study with boar semen showed that 95% of *E. coli* were removed by the high-density colloid. In a different study, we showed that seminal plasma proteins coating the sperm surface are removed by passage through the colloid [28]; it would be interesting to use electron microscopy to see whether bacteria are similarly removed by the low density colloid. Finally, a fertility trial is needed to ensure that there is no detrimental effect on reproductive efficiency after using colloid centrifuged samples. Studies with equine

AI showed that colloid-selected sperm samples resulted in the identification of more embryonic vesicles [10]. Although it was not possible to perform AI trials in the present pig study, previous studies showed that SLC-selected boar spermatozoa are highly fertile when used for *in vitro* fertilization [10], and that it is necessary to reduce the ratio of spermatozoa to oocytes substantially to avoid polyspermy [29].

With a breeding sow population of approximately 12.5 million within the European Union, each sow being expected to produce 2.3 litters of piglets per year following artificial insemination, the scale of usage of antibiotics added to semen doses is a cause for concern. However, to date no alternative to antibiotics has been proposed to safeguard sperm quality and sow health. Antimicrobial peptides have been identified e.g. cationic peptides [30], protegrine 1 [31], and defensins [32], but sperm toxicity (at least of the cationic peptides and defensins) renders them inappropriate for use in semen extenders. The advantage of colloid centrifugation to separate spermatozoa from bacteria is that no spermatotoxic effect has been identified. In addition, when the colloid is used, there is no subsequent detrimental effect from the presence of dead and dying bacteria, and production of bacterial metabolic byproducts is reduced. Intuitively, it would seem to be better to remove the bacteria at source (or prevent contamination in the first place) rather than adding antibiotics to kill the bacteria or prevent them from multiplying.

Paying strict attention to hygiene in the semen collection area enables some ejaculates to be collected without bacterial contamination, as shown here since three ejaculates did not contain any bacteria at all. An artificial vagina was used for semen collection. In our previous experiment with boar semen, one of the six ejaculates collected by the gloved hand method at the university did not produce any bacterial growth on culture [11]. Processing all semen doses in a laminar air flow hood has also been suggested to reduce bacterial contamination [34]. However, it should be noted that sale of insemination doses without antibiotics would

316 require a change in the current regulations covering biosecurity of semen, particularly as some
317 of the S and M samples contained low numbers of bacteria, although the numbers were much
318 lower than would be deposited in the female reproductive tract during natural mating. The
319 sow´s reproductive tract has well-developed mechanisms to deal with the bacteria introduced
320 during mating [11].

321 Council Directive 90/429/EEC, Annex C, in the European Union, mentions in particular
322 *Leptospira* and *Mycoplasma* spp. Neither of these bacterial genera were identified in the
323 control samples in this study and therefore the ability of the low density colloid to remove
324 these bacteria was not tested. Future experiments should investigate this aspect, which is
325 important for biosecurity. However, it should be noted that *Mycoplasma* spp. are difficult to
326 culture, requiring special culture conditions; therefore, they may have been missed in the
327 present study. This was also a problem with previous methods of microbiological
328 identification by colony morphology and biochemical analyses, since it is not possible to
329 detect bacteria that do not grow or are overgrown by other colonies. In addition, the presence
330 of extender in the present study might have enhanced the growth of particular bacteria at the
331 expense of others. To be certain that no additional bacteria were present, it would be
332 necessary to use a metagenomic technique such as 16S sequencing [33] in which all the
333 bacterial DNA present is identified, but this method would have been beyond the scope of the
334 present study. An issue with using the metagenomic technique instead of identifying actual
335 bacterial colonies is that one does not know whether the DNA comes from live or dead
336 bacteria and hence whether it could potentially be a problem to inseminated sows or to
337 spermatozoa, or not.

338 In conclusion, the results presented here suggest that colloid centrifugation could provide an
339 alternative to antimicrobial usage for controlling bacterial contamination in boar semen for
340 artificial insemination. There was no difference in the number of bacteria in S and M

treatments. Physically separating bacteria from spermatozoa is less likely to have a detrimental effect on sperm quality than killing the bacteria *in situ* and would be preferable in the fight against development of antibacterial resistance. However, changes in legislation would be necessary before such measures could be adopted by the pig semen production industry.

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Table 1: Total bacterial counts (cfu/mL, mean ± SD) in sperm samples prepared by low density colloid centrifugation compared to control samples immediately after collection/preparation (n=9 ejaculates; 3 replicates per sample).

Treatment	Boar								
	1	2	3	4	5	6	7	8	9
C	0	0	283±205	400±318	69±27	257±110	0	243±188	58±32

S	0	0	30±7	0	18±18	54±11	0	0	64±46
M	0	0	42±31	33±12	46±37	28±26	0	32±10	41±45

Note: C = control, S = SLC, M = modified SLC with inner tube;

Overall, the number of bacteria was lower ($P<0.01$) in S or M treatments comparing to C, excluding samples with 0 cfu/ml in all treatments.

459 Table 2. CASA results for the colloid experiment (mean±SEM) The effect of storage was
460 significant in all cases (P<0.001), except for ALH. Different superscripts indicate P<0.05
461 among treatments each day.

Day	Type	MOT	PROG	VCL	LIN	ALH
0	C	82.9±3.4^{a}	34.9±4.5^{a}	94±10.9	19.6±1.7^{a}	2.3±0.3
	S	62.2±1.9^{b}	43.4±2.1^{b}	101.6±2.8	39.9±1.5^{b}	2.1±0.1
	M	69.6±1.9^{c}	42.3±2.5^{b}	109.7±2.6	42.5±1.4^{b}	2.0±0.0
1	C	64.5±6.5	32.4±4.0^{a}	81.1±4.2^{a}	23.2±1.8^{a}	2.0±0.1
	S	58.9±2.0	43.0±2.0^{b}	100.8±3.1^{b}	40.6±1.3^{b}	2.1±0.1
	M	62.1±2.8	41.1±2.1^{b}	107.4±2.7^{b}	43.7±1.1^{b}	2.0±0.0
2	C	68.3±4.3^{a}	31.3±3.7^{a}	90.1±9.7^{a}	19.7±1.8^{a}	2.3±0.2
	S	59.6±1.5^{b}	42.9±1.9^{b}	101.4±3^{ab}	39.9±1.6^{b}	2.1±0.1
	M	63.7±1.5^{ab}	40.5±1.8^{b}	109±2.9^{b}	42.5±1.5^{b}	2.0±0.0
3	C	66.5±2.5	47.5±4.0	129.1±8.2	38.1±3.9	2.5±0.2
	S	69.3±2.9	49.9±3	121.2±5.4	44.0±4.4	2.2±0.2
	M	63.6±5.2	50.4±3.5	119.5±6.7	49.5±2.1	2.1±0.1
4	C	45.4±8.2	34.4±6.2	103.7±13.6^{ab}	32.1±4.1^{a}	2.3±0.2
	S	46.4±6.8	39.6±4.9	76.8±8.6^{a}	45.6±2.9^{b}	1.8±0.1
	M	60.4±3.9	43.1±1.9	112.0±7.5^{b}	44.8±2.4^{b}	2.1±0.1
7	C	27±7.8^{a}	18.8±5.7^{a}	77.1±11.6^{a}	27.8±4.7^{a}	2.0±0.2
	S	51.4±3.1^{b}	40.1±3.7^{b}	113.3±5.1^{b}	41.0±3.8^{b}	2.3±0.1
	M	40.4±5.6^{b}	31.4±5.0^{b}	107.7±6.1^{b}	44.4±3.3^{b}	2.1±0.1

462 C: Control; S: Single layer centrifugation; M: Modified SLC.

463 MOT: Total motility, %; PROG: Progressive motility, %; VCL: Curvilinear velocity, μm/s;
464 LIN: Linearity, %; ALH: Amplitude of the lateral movement of the head, μm.

465

466 Table 3. Flow cytometry results for the colloid experiment (%; mean±SEM). The effect of
467 storage was significant in all cases with P<0.001. Different superscripts indicate P<0.05
468 among treatments each day.

Day	Type	Viability (PI^-)	Damaged acrosomes (PNA^+)	Damaged acrosomes (PNA^+, PI^- ratio)	Capacitated ($M540^+$, PI^- ratio)	Active mitochondria (MT^+/PI^-)
0	C	85.3±1.5^a	5.9±1.9^a	2.5±0.3^a	2.3±0.2^a	86.2±1.8^a
	S	74.3±2.4^b	9.4±2.5^b	5.2±0.6^b	4.9±0.7^b	77.6±1.9^b
	M	71.8±1.6^b	8.8±2.5^b	5.5±0.5^b	5.8±0.5^b	75.5±1.6^b
1	C	85±1.3^a	10.4±0.4	3.3±0.3^a	2.8±0.2^a	87.8±1.9^a
	S	81.8±1.7^b	11.1±0.6	4.4±0.4^b	3.5±0.3^b	85.1±1.6^{ab}
	M	82.1±1.3^b	10.8±0.6	3.8±0.2^{ab}	3.2±0.3^{ab}	80.3±3.2^b
2	C	86.8±1.7^a	11.6±0.6^a	4.1±0.3^a	3.3±0.4^a	88.2±2^a
	S	78.7±1.1^b	14.9±0.9^b	5.9±0.4^b	4.7±0.5^b	80.9±1.4^b
	M	83.5±1.6^a	12.8±0.8^a	5.3±0.2^{ab}	3.8±0.3^{ab}	85±1.6^a
3	C	80.5±1.9	11.1±0.5^a	3.2±0.3^a	3.3±0.2^a	88±2.1^a
	S	79.1±1.2	14.6±1.1^b	5.9±0.4^b	4.7±0.3^b	79.9±3.3^{ab}
	M	78.1±1.4	13.8±0.6^b	5.5±0.4^b	4.2±0.6^{ab}	69.9±7.1^b
4	C	82.3±2.9	14.0±2.0	4.7±0.6	3.7±0.3^a	83.5±3.1
	S	78.2±0.9	15.9±1.2	6.7±0.4	5.2±0.4^b	77.2±2.8
	M	76.9±1.4	14.7±1.0	6.2±1.0	4±0.3^{ab}	70.9±4.8
7	C	71.5±4.6	16.3±1.6	3.2±0.4^a	4.5±0.5^a	73.3±4.5
	S	73.6±1.5	18.0±1.3	6.5±0.6^b	9.2±1.5^b	73.8±1.8
	M	74.6±3.3	17.4±1.2	6±0.7^b	9.1±0.7^b	75.4±3.9

469 C: Control; S: Single layer centrifugation; M: Modified SLC. PI^- = propidium iodine negative;
470 PNA^+ = peanut agglutinin positive; $M540^+$ = merocyanine 540; MT^+ = mitotracker positive.
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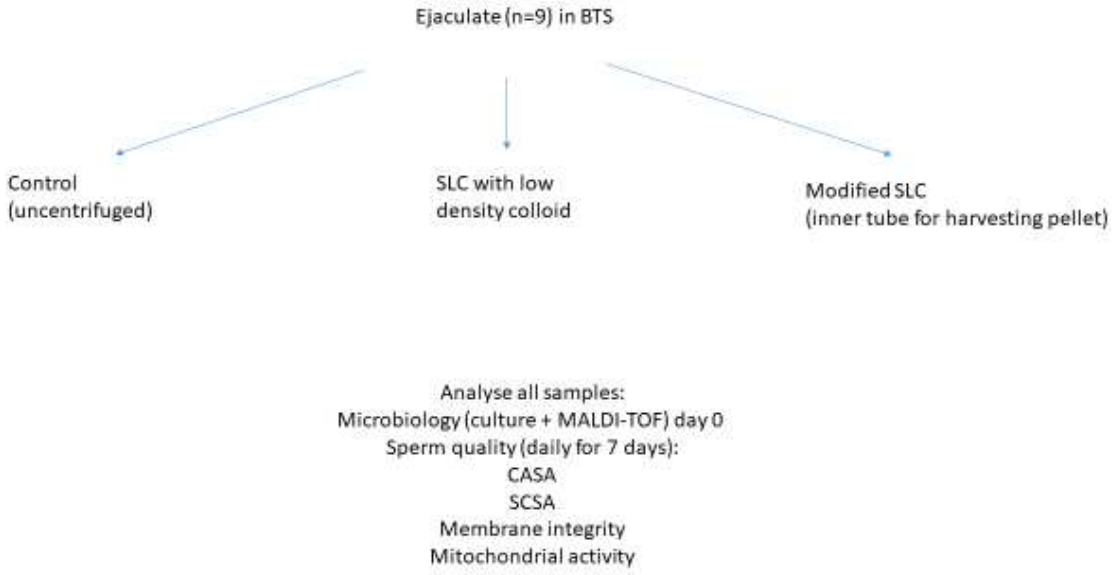
472
473

474 **Figure 1: experimental design**



475
476 Note: BTS = Beltsville Thawing Solution; SLC = Single Layer Centrifugation; MALDI-TOF
477 = matrix assisted laser desorption/ionisation; CASA = Computer assisted sperm analysis;
478 SCSA = Sperm Chromatin Structure Assay.

Highlights

Boar semen was centrifuged on a low density colloid to separate the spermatozoa from seminal plasma

Controls and resuspended sperm pellets were evaluated for sperm quality

Aliquots were taken for microbiology and identification by MaldiTof.

Colloid centrifugation removed all or most of the bacteria from the sample

Sperm quality was not different among treatments