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Effects of filtration through Sephadex columns improve overall quality parameters and "*in vivo*" fertility of subfertile refrigerated boar-semen

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ABSTRACT

This study was performed to test the effects of filtration through several chromatographic resins on the semen quality parameters (percentages of viability, altered acrosomes and morphological abnormalities, motion characteristicis and the response to the Osmotic Resistance Test) of boar ejaculates of poor quality. Our results indicate that filtration through a non-ionic Sephadex resin bed (Sephadex G-15), combined with a glasswool subjection bed, induced an overall improvement of semen quality parameters, especially seen in a significant ($P<0.05$) decrease in the percentages of morphological abnormalities and an increase of several motility parameters related to velocity and linearity. Similar results, although less intense, were observed when the filtration through G-15 resin was accompanied by an ionically neutral polypropylene disk bed instead of glasswool. On the other hand, filtration through two separate ion-exchange Sephadex resins, cationic C-50 and anionic A-50, have less beneficial, and even detrimental, effects on boar-semen quality. In all cases, filtration was accompanied by a significant ($P<0.01$) decrease in

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the final concentration of the samples. Ultrastructural and lectin studies showed that the interaction between sperm and chromatographic resins depends on the resin type utilized, and in the case of G-15 it seems that it works by trapping that sperm with not enough strength to overcome the physical resistance associated with chromatographic particles. When semen of poor quality was filtered through G-15 resin and then was utilized for "*in vivo*" fertility trials, a significant ($P < 0.05$) increase in the percentage of fertility was observed, when compared with the same, but unfiltered, samples. In summary, our results strongly indicate that filtration through ionically inert, Sephadex chromatographic resins could be a very useful and practical method to improve both boar-semen quality and fertilizing ability, especially from mediocre and/or subfertile samples.

1. Introduction

Sperm selection by filtration through chromatographic resins has been tested as a putative practical system to increase semen quality in ejaculates of several species. The rationale behind this system is based on the fact that dead and abnormal sperm cells of an ejaculate have a detrimental effect on the quality of live and normal sperm cells (Graham and Graham, 1990). In this way, the physical separation of dead/abnormal sperm cells from those that are viable/normal could render an increase in the quality of the ejaculate. One of the most commonly utilized resins in published experiments regarding sperm chromatographical separation is Sephadex beads. There are different Sephadex resin types according to their porous diameter and ionic charge. Thus, Hammit and Martin (1989), Loseth et al. (1992) and Busalleu et al. (2008) have studied boar-sperm separation through neutral Sephadex resins of a different porous diameter (from Sephadex G-15 to G-50). In all cases, the authors have observed an increase in the percentage of viable and normal sperm cells after filtration. Similar results have been observed in other species, such as bull (Chinnaiya et al., 1989; Graham and Graham, 1990; Kanakaraj and Easwaran, 1991; Vyas et al., 1991, 1992a, b), horse (Hellander, 1992; Samper et al., 1991, 1995; Samper and Crabo, 1993), dog (Mogas et al., 1998) and ram (Landa et al., 1980; Valcárcel et al., 1996). On the other hand, several authors have also tested sperm separation through several ion-exchange Sephadex resins, observing a significant improvement in motility, viability and normal spermatozoa in bull (Anzar and Graham, 1993) and dog ejaculates (Mogas et al., 1998), although in this last case the number of viable sperm yielded after filtration was much smaller (for instance, from 53.3×10^6 sperm/mL in unfiltered samples to 10.5×10^6 sperm/mL after filtration through Sephadex G-15 resin, see Mogas et al., 1998) than that observed after filtration through neutral Sephadex resins.

One of the most important advantages of Sephadex filtration, especially through ionically inert beds, when compared with other sperm separation methods, is that this system yields a high percentage of viable, recovered spermatozoa. This is important when designing a practical application of the system, which is needed for the obtainment of the maximal number of spermatozoa for their application at the artificial insemination center. However, a great deal of complementary investigation is needed in order to optimize both the choice of the appropriate Sephadex bed and its specific utilization in field conditions.

The main aim of this work is to test the effect of semen filtration through different Sephadex columns on the most evaluated semen quality analysis parameters of refrigerated boar-semen. For this, we initially studied the effect that the containment web placed under the Sephadex beds could itself have on boar-sperm quality. After this, the effect on boar-semen quality of the filtration through several Sephadex resins was observed. The Sephadex resins tested were the non-ionic resin Sephadex G-15 and the ion-exchange resins Sephadex A-50 (anionic exchange resin) and C-50 (cationic exchange resin). Finally, "*in vivo*" fertility and prolificacy of filtered sperm through the G-15 resin column were evaluated in order to determine the putative application of this technique in field conditions.

2. Materials and methods

2.1. Preparation of Sephadex columns filtration

Sephadex resins were hydrated for 24 h at 16 °C with the commercial extender routinely utilized for pig artifical insemination at the associated pig farm (Acromax®, Gestión Veterinaria Porcina S.L., Madrid, Spain). During this time, the filtration columns were prepared. These filtration columns were made from 5-mL disposable plastic syringes. The columns had two parts: the first part was the subjection filter needed to prevent the wastage of chromatographic resins. This subjection filter was either a small amount of loosely packed glasswool with a thickness of 1–1.5 mm (Merck, Damstadt, Germany) or a polypropylene disk of 45 ± 9 μm of porous diameter (Millipore Europe Corporation, Yvelines, France), in all cases placed at the bottom of the column. The second part was the corresponding Sephadex resin (Sigma, St. Louis, MO, USA), which was added to a final volume of 1 mL in each column, which corresponds to a dry weight of 50 mg resin/column. In the case of two resins being utilized together, the added volume of each of these resins was also of 1 mL. From this general scheme, we initially carried out two separate, consecutive experimental designs. In the first one, the effect of both subjection filters by themselves was tested. For this first experimental design, semen was eluted through either the packed glasswool or the polypropylene disk described above without the presence of any other filtration element. The number of replicates that was performed in this design was 8, by utilizing 8 separate ejaculates. The ejaculates in each replicate were divided into 3 aliquots. The first was analyzed unfiltered, the second was analyzed after filtration through the glasswool bed and the third was analyzed after filtration through the polypropylene disk. This allowed for a direct comparison between the results obtained after filtration through both the glasswool and the polypropylene disk. After this, the second experiment consisted of the determination of the filtration effects of the evaluated chromatographic resins, separately. For this purpose, the columns described above were filled with 1 mL of the specific Sephadex resin. The experiment was performed with 3 separate types of Sephadex resins: Sephadex A-50 (anionic exchange resin), Sephadex C-50 (cationic exchange resin) and Sephadex G-15 (non-ionic resin). Finally, the columns were filled up to the top with the same semen extender utilized to hydrate the chromatographic resins in order to maintain hydration constant, and were thus kept and stored for a maximal period of 8 h at 4 °C until the semen filtration procedure. This second experimental procedure was divided into two separate procedures. The first, the evaluation of the effects of filtration through the separate resins in the presence of a glasswool bed. The second, a similar evaluation but substituting glasswool by polypropylene disks. Each procedure was replicated 8 times, by utilizing 8 separate ejaculates. In each replicate, the ejaculate was split into 5 aliquots, which were processed as follows: the first aliquot was analyzed unfiltered, and the other 4 aliquots were analyzed after filtration through the separate Sephadex resins as explained above.

2.2. Semen processing and filtration

Eight healthy boars of 2–3 years of age from a commercial farm were used in this study. The boars were from 3 separate lines (Landrace, Large White and Pietrain). The sperm-rich fraction of each ejaculate utilized in this study was manually collected twice weekly using the gloved-hand method and analyzed to ensure the quality and the homogeneity of the ejaculates. Immediately after collection, the ejaculated semen was suspended (1:2, v/v) in the appropriate commercial extender (Acromax®). The extended semen samples were cooled and maintained at 17 °C for shipment to the laboratory of the Autonomous University of Barcelona within 24 h post-collection, for further processing and analyses.

Before starting the filtration process, the previously prepared chromatography columns were firstly warmed at room temperature for 2 h. Afterwards, the columns were checked in order to ensure that they were not leaking Sephadex beads, and then the excess of liquid that fills the columns was eliminated by elution through the columns. At this moment, 5-mL aliquots of semen samples previously warmed at 37 °C for 5 min were immediately applied to the top of the corresponding chromatography column. The eluate yielded during the phase in which semen samples were entering into the chromatography resin was discarded, since it corresponds to the liquid contained in the column before the semen application. We collected the eluate only after all of the semen sample was completely entered

into the chromatographic bed. From this moment onwards, 3 mL of eluate was collected, which corresponded to the filtered semen, that were immediately processed for analysis as detailed below. At the same time, several samples of the resin beads were taken after the filtration procedure, in order to perform the ultrastructural studies of the sperm that had adhered to the resin particles. Finally, 3-mL aliquots of semen that were not filtered were utilized as controls in all of the described experimental designs.

2.3. Semen analyses

Percentages of viability, altered acrosomes and morphological abnormalities were determined by using the Eosin–Nigrosin stain (Bamba, 1988). The determination of the above-mentioned percentages was performed after analyzing a minimum of 200 spermatozoa/sample through optical microscopy (magnification: 1000×). Sperm concentration and total sperm number was determined after counting in a haemocytometer chamber. On the other hand, the Osmotic Resistance Test (ORT Test) was carried out as described in Rodríguez-Gil and Rigau (1995).

Motility was evaluated by computerized system analysis (Sperm Class Analyser V5.0, Microptic, Barcelona). To do this, semen samples were incubated in a water-bath at 37 °C for 1 min. Then, 5-μL aliquots of semen were placed onto a warmed slide and covered with a 24-mm^2 coverslip. Afterwards, images were taken under positive contrast at 200× magnification. A minimum of two different optical fields were analyzed for each sample. This CASA system analyzes 16 consecutive, digitalized photographs in a time lapse of 0.64 s.

Motility data obtained after CASA analyses were statistically processed by using the SAS statistical package (SAS, 1996). First of all, normality of data distributions was assessed by the Shapiro–Wilkis Test, which is included in the UNIVARIATE procedure. Following this, the next step was to determine how many of the rendered motion parameters obtained after CASA analysis were really important in order to perform an optimal analysis of the results. The selection of the appropriate parameters was performed by using the clustering procedure VARCLUS of the SAS statistical package, as in Quintero-Moreno et al. (2004). VARCLUS analyses rendered 13 final parameters, which are described in Table 1. After the VARCLUS procedure, a FASTCLUS procedure, included in the SAS package, was utilized to perform the motile sperm subpopulations study, as in Quintero-Moreno et al. (2004). The FASTCLUS procedure performs a disjointed cluster analysis based on Euclidean distances computed from one

Table 1
Description of the chosen motility descriptors.

Name	Units	Description
Curvilinear velocity (VCL)	μm/s	Measures the sequential progression along the true trajectory
Linear velocity (VSL)	μm/s	Measures the straight trajectory of the spermatozoa per unit time
Mean velocity (VAP)	μm/s	Measures the mean trajectory of the spermatozoa per unit time
Linearity coefficient (LIN)	%	VSL/VCL × 100
Straightness coefficient (STR)	%	VSL/VAP × 100
Wobble coefficient (WOB)	%	VAP/VCL × 100
Mean lateral head displacement (ALH med)	μm	Measures the mean head displacement along the curvilinear trajectory
Maximal lateral head displacement (ALH max)	μm	Measures the maximal head displacement along the curvilinear trajectory
Dance (DNC)	μm^2/s	VCL × ALH med
Algebraic angular mean displacement (MADalg)	°	Measures the algebraic value of the advancing angle of sperm trajectory. Negative values indicate a clockwise displacement.
Frequency of the head displacement (BCF)	Hz	Measures the number of lateral oscillatory movements of the sperm head around the mean trajectory
Minor harmonic oscillation of the head (HLO)	μm	Measures the minimum value of the distance between the curvilinear trajectory, with respect to the mean trajectory
Maximal amplitude of the oscillation of the head (HMX)	μm	Measures the maximal distance between two successive crosses around the mean trajectory

or more quantitative parameters. Spermatozoa were divided into clusters such that each observation belonged to a single cluster. Sperm cells that shared similar motility characteristics were assigned to the same cluster, whereas spermatozoa that differed in motility characteristics were assigned to different clusters. A PROC GLM procedure was applied to evaluate significant differences ($P < 0.05$) and the LSMEANS procedure was applied to list these differences. Finally, a Chi-square procedure was applied to determine the subpopulational distribution percentage in every single experiment. Once the percentage distribution per experiment was determined, new PROC GLM and LSMEANS procedures were applied to determine and list, respectively, the differences among the different treatments. The total number of spermatozoa analyzed following this protocol was 2509. Total motility was defined as the percentage of spermatozoa which showed a mean velocity (VAP; see Table 1 for definition) above 10 μm/s.

2.4. Processing of samples for scanning electron microscopy

As mentioned above, a small amount of both resin beads and subjection filters were taken after the filtration procedure to perform the structural studies. These samples were immediately fixed in a 3% (v:v) glutaraldehyde and 3% (v:v) paraformaldehyde solution in Sörensen's buffer (2.4 mM $NaHPO_4$ and 1.8 mM KH_2PO_4, pH 7.2) for 30 min at 4 °C. Afterwards, samples were washed with Sörensen's buffer at 4 °C and they were then dehydrated through an increased-alcohol series, transferred to isoamyl acetate solution placed upon stubs and subjected to critical-point drying. Following this, dried samples were shadowed in a sputtering Polaron-300 with a 50-nm coating and were then observed under a Zeiss 940A scanning microscope (Karl Zeiss Gmbh, Jena, Germany) in order to determine the structural relationship between chromatography beads and filtered sperm.

2.5. In vivo fertility trials

A total of 34 sows were utilized in the artificial insemination trials. Whole ejaculates from 8 Large White boars with a previous low-fertility recorded history were obtained and diluted 1:1 (v:v) in the utilized commercial extender, as described above, in the commercial farms owned by the collaborating firm. These ejaculates were divided into two aliquots of the same volume, and one of these aliquots was filtered at room temperature through a chromatographic column of 100 mL with the polypropylene neutral disk at the bottom of the barrel. The column was filled with 90 mL of Sephadex G-15 hydrated under the same conditions as described above and connected to a hydraulic vacuum pump. Semen samples were maintained at 35 °C until filtration. Just before filtration, columns were warmed in a thermostatic bath at 37–38 °C and then the semen was deposited at the top of the column as explained before. The other aliquots of the whole ejaculate were not filtered, and were thus utilized as a control sample. Once filtration was finished, both filtered and control samples were further diluted to a final sperm concentration of 30×10^6 sperm/mL with commercial extender at 35 °C, and they were then packaged in 90-mL aliquots in the same way as were the commercial seminal doses produced in the farm. Afterwards, sample doses were maintained at 16 °C until the first insemination, which was conducted less than 24 h after filtration. Sows were inseminated 3 times in the 60 h subsequent to oestrus detection. Each sow was inseminated only with the same semen. Inseminated sows were maintained in a commercial farm owned by the collaborating firm, and they were managed following the routine of this farm. Gestations were recorded by transabdominal ecography after 23–24 days of the last insemination and pregnant sows were allowed to give birth in normal farm conditions, recording the values of prolificacy and neonatal survival.

2.6. Statistical analysis

All data were imported into a database (Excel, 2000) and analyzed by the SAS statistical package (SAS, 1996). A General Lineal Model (PROC GLM) with the LSMEANS procedure was applied to evaluate significant differences ($P < 0.05$) among semen samples in all of the performed semen analytical determinations. A Chi-square test was applied to determine the differences in fertility and prolificacy rates between filtered, and control semen insemination.

2.7. Suppliers

All reactives were of analytical grade and came from Sigma, and Merck (Darmstadt, Germany).

3. Results

3.1. Effects on boar-semen quality analysis of filtration through both glasswool and polypropylene disk subjection filters

Samples utilized in this assay were of mediocre quality, as indicated by their values of viability. This was done in order to make a better determination of the putative improving effects of semen filtration. Taking this into account, filtration through both glasswool and polypropylene subjection filters alone significantly ($P < 0.001$) decreased sperm concentration after elution, when compared to control samples, although the decrease was significantly ($P < 0.001$) more intense after filtration through glasswool (10.4 ± 0.3 sperm/mL after filtration through glasswool vs. 15.4 ± 0.2 sperm/mL in unfiltered samples and 13.0 ± 0.3 sperm/mL after filtration through polypropylene disks; see Table 2). Moreover, filtration through the containment filters had other effects on sperm quality. Thus, as shown in Table 2, filtration through glasswool alone induced a significant ($P < 0.001$) decrease in the percentages of viability, altered acrosomes and total morphological abnormalities, as well as on the results of the ORT test. Filtration through the polypropylene disks had a weaker effect, observing only a slight, but significant ($P < 0.001$), decrease in the percentages of viability and altered acrosomes, as well as in the ORT test values. Remarkably, filtration through polypropylene disks significantly ($P < 0.001$) increased the percentage of viability (from $58.1 \pm 1.0\%$ in unfiltered samples to $64.8 \pm 1.1\%$ after filtration through polypropylene; see Table 2). Finally, filtration through both containment filters significantly ($P < 0.001$) decreased the percentage of total motility in both cases (from $66.2 \pm 1.1\%$ in unfiltered samples to $53.8 \pm 1.3\%$ after filtration through glasswool, and $57.9 \pm 1.2\%$ after filtration through polypropylene disks; see Table 2). Nevertheless, there was no other effect on any of the CASA-determined motility parameters (data not shown).

3.2. Effects of filtration through separate Sephadex resins with a containment filter of glasswool on the overall boar-semen quality analysis

Filtration induced a significant decrease in the sperm concentration of the obtained eluates in all of the tested systems. In this respect, it is noteworthy that filtration through the anionic resin A-50 combined with a glasswool containment filter induced the greatest decrease in sperm concentration ($2.0 \pm 0.8 \times 10^6$ sperm/mL vs. $18.3 \pm 0.2 \times 10^6$ sperm/mL in unfiltered samples; see Table 3) when using single resins. This decrease was further accentuated when filtration was carried out through a combination of both A-50 and C-50 resins, in which sperm concentration dropped to values of $0.9 \pm 0.1 \times 10^6$ sperm/mL (Table 3). This indicates that ionic resins present stronger mechanisms to trap boar-sperm than those of the G-15 non-ionic resins. On the other hand, the effects of each resin were also different on percentages of viability, altered acrosomes and total abnormalities, as well as

Table 2
Effects of glasswool and propylene disc subjection filters upon mean quality parameters.

Parameter	Unfiltered	Glasswool	Polypropylene
Concentration (10^6 sperm/mL)	15.4 ± 0.2^a	10.4 ± 0.3^b	13.8 ± 0.3^c
Viability (%)	58.06 ± 1.0^a	52.7 ± 1.2^b	64.8 ± 1.1^c
Altered acrosomes (%)	15.5 ± 0.4^a	12.6 ± 0.5^b	13.3 ± 0.4^b
Total motility (%)	66.2 ± 1.1^a	53.8 ± 1.3^b	57.9 ± 1.2^b
Total abnormalities (%)	11.1 ± 0.2^a	6.2 ± 0.3^b	9.8 ± 0.3^c
ORT Test (%)	80.7 ± 0.6^a	64.8 ± 0.7^b	74.2 ± 0.7^c

Results are expressed as means ± S.E.M. of 8 separate experiments. Different superscript letters in a column indicate statistically significant differences ($P < 0.05$). Unfiltered: semen doses before filtration. Glasswool, polypropylene: semen doses after elution with the indicated subjection beds, as described in Section 2.

Table 3
Effects of filtration through glasswool-resins columns upon mean quality parameters.

	Unfiltered	A-50	C-50	G-15	A-50/C-50
Concentration (10^6 sperm/mL)	18.3 ± 0.2[a]	2.0 ± 0.8[b]	6.9 ± 0.3[c]	4.8 ± 0.3[b]	0.4 ± 0.9[b]
Viability (%)	87.4 ± 0.8[a]	49.9 ± 3.1[b]	77.5 ± 1.2[c]	83.7 ± 1.8[a]	48.1 ± 3.6[b]
Altered acrosomes (%)	6.49 ± 0.3[a]	17.1 ± 1.3[b]	7.1 ± 0.5[a]	4.8 ± 0.5[a]	3.5 ± 1.5[a]
Total abnormalities (%)	7.9 ± 0.2[a]	5.0 ± 0.8[b]	5.5 ± 0.3[b]	4.9 ± 0.3[b]	0.8 ± 0.9[c]
ORT Test (%)	89.4 ± 0.5[a]	77.9 ± 1.9[b]	88.5 ± 0.7[a]	81.2 ± 0.8[b]	49.0 ± 2.3[c]

Results are expressed as means ± S.E.M. of 8 separate experiments. Different superscript letters in a column indicate statistically significant differences ($P < 0.05$). Unfiltered: semen doses before filtration. A-50, C-50, G-15, A-50/C-50: semen doses after filtration with the indicated Sephadex resins, as described in Section 2.

on values of the ORT test. Thus, as shown in Table 3, resin A-50 had an overall impairing effect on the filtered semen, which showed a great decrease in viability (49.9 ± 3.1% vs. 87.4 ± 0.8% in unfiltered samples), a moderate decrease in ORT values (77.9 ± 1.9% vs. 89.4 ± 0.5% in unfiltered samples) and a significant ($P < 0.001$) and remarkable increase in the percentage of altered acrosomes (17.1 ± 1.3% vs. 6.5 ± 0.3% in unfiltered samples). These impairing effects were accompanied by a slight decrease in the percentage of total morphological abnormalities (5.0 ± 0.8% vs. 7.9 ± 0.2% in unfiltered samples). The effect of resin C-50 plus glasswool was less aggressive, showing only a slight, although significant ($P < 0.001$), decrease in viability (77.5 ± 1.2% vs. 87.4 ± 1.2% in unfiltered samples; see Table 3) combined with a slight decrease in the percentage of abnormalities. Resin G-15 showed the best action on semen quality, since filtration through this system induced a significant ($P < 0.001$) decrease in total abnormalities viability (4.9 ± 0.3% vs. 7.9 ± 0.2% in unfiltered samples; see Table 3) that was accompanied by no modification in either viability or ORT test values (Table 3). Finally, filtration through a combination of A-50 and C-50 resins plus glasswool induced a very great decrease in the percentage of morphological abnormalities viability (0.8 ± 0.1% vs. 7.9 ± 0.2% in unfiltered samples; see Table 3). Unfortunately, this decrease was accompanied by a concomitant, very great decrease in both viability and ORT values, as well as the above-mentioned great decrease in sperm concentration (Table 3).

Regarding the effect of filtration through resins plus glasswool on motion parameters, it is noteworthy that filtration through resin A-50, alone or in combination with C-50, but not filtration through C-50 alone, induced a significant ($P < 0.001$) decrease in the percentage of total motility (Table 3). On the other hand, filtration through both ionic resins had very few effects on motion parameters. On the contrary, filtration through resin G-15 plus glasswool induced an improving effect on semen motility characteristics. In this way, as shown in Table 4, this filtration induced a significant

Table 4
Effects of filtration through glasswool-resins columns upon mean motility parameters.

	Unfiltered	A-50	C-50	G-15	A-50/C-50
Total motility (%)	66.6 ± 0.8[ab]	29.1 ± 3.3[c]	64.4 ± 1.3[a]	71.6 ± 1.3[b]	41.3 ± 3.8[c]
VCL (μm/s)	70.4 ± 1.8[ab]	74.4 ± 7.0[ab]	80.4 ± 2.7[a]	69.7 ± 2.7[ab]	52.1 ± 8.0[b]
VSL (μm/s)	22.6 ± 0.9[a]	24.5 ± 3.6[ab]	27.6 ± 1.4[ab]	30.3 ± 1.4[b]	21.0 ± 4.1[ab]
VAP (μm/s)	35.3 ± 1.1[a]	35.1 ± 4.5[ab]	42.5 ± 1.7[b]	44.3 ± 1.7[b]	28.1 ± 5.1[ab]
LIN (%)	33.1 ± 1.0[a]	34.3 ± 4.1[ab]	34.7 ± 1.5[a]	42.6 ± 1.6[b]	30.8 ± 4.7[ab]
STR (%)	63.9 ± 1.1[a]	57.9 ± 4.3[a]	60.9 ± 1.6[a]	65.5 ± 1.7[a]	50.9 ± 4.9[a]
WOB (%)	49.2 ± 0.9[a]	45.3 ± 3.7[a]	52.2 ± 1.4[a]	61.5 ± 1.4[b]	41.6 ± 4.2[a]
ALHmed (μm)	3.0 ± 0.1[a]	3.4 ± 0.3[a]	3.4 ± 0.1[a]	2.9 ± 0.1[a]	2.3 ± 0.4[a]
ALHmax (μm)	7.2 ± 0.2[a]	8.9 ± 0.9[a]	8.2 ± 0.3[a]	7.1 ± 0.3[a]	5.3 ± 1.0[a]
DNC (μm)	257.7 ± 21.6[a]	378.3 ± 84.5[a]	333.2 ± 32.3[a]	250.7 ± 32.9[a]	204.4 ± 96.1[a]
MADalg (°)	−24.2 ± 1.4[a]	−15.6 ± 5.4[ab]	−16.4 ± 2.1[ab]	−14.2 ± 2.1[b]	−11.1 ± 6.2[ab]
BCF (Hz)	16.5 ± 0.4[a]	16.5 ± 1.5[ab]	17.1 ± 0.6[a]	13.9. ± 0.6[b]	11.2 ± 1.7[ab]
HLO (μm)	0.14 ± 0.02[a]	0.35 ± 0.19[a]	0.18 ± 0.04[a]	0.15 ± 0.04[a]	0.12 ± 0.05[a]
HMX (μm)	2.3 ± 0.1[a]	2.1 ± 0.3[a]	2.6 ± 0.1[a]	2.6 ± 0.1[a]	1.7 ± 0.3[a]

Results are expressed as means ± S.E.M. of 8 separate experiments. Different superscript letters in a column indicate statistically significant differences ($P < 0.05$). Unfiltered: semen doses before filtration. A-50, C-50, G-15, A-50/C-50: semen doses after filtration with the indicated Sephadex resins, as described in Section 2.

Table 5
Effects of filtration through polypropylene-resins columns upon mean quality parameters.

	Unfiltered	A-50	C-50	G-15	A-50/C-50
Concentration (10^6 sperm/mL)	18.9 ± 0.2^{a}	5.6 ± 0.4^{b}	8.0 ± 0.4^{c}	7.5 ± 0.4^{c}	1.9 ± 0.6^{d}
Viability (%)	82.1 ± 0.9^{a}	62.4 ± 1.6^{c}	81.0 ± 1.5^{ab}	75.9 ± 1.5^{b}	56.2 ± 2.6^{c}
Altered acrosomes (%)	11.0 ± 0.3^{a}	12.4 ± 0.7^{a}	7.8 ± 0.6^{b}	10.1 ± 0.6^{ab}	0.9 ± 1.1^{c}
Total abnormalities (%)	3.9 ± 0.2^{ab}	5.0 ± 0.4^{ab}	5.3 ± 0.4^{a}	4.5 ± 0.4^{ab}	2.7 ± 0.6^{b}
ORT Test (%)	83.5 ± 0.6^{a}	78.8 ± 1.0^{b}	80.4 ± 0.9^{ab}	78.8 ± 0.9^{b}	47.2 ± 1.6^{c}

Results are expressed as means ± S.E.M. of 8 separate experiments. Different superscript letters in a column indicate statistically significant differences ($P < 0.05$). Unfiltered: semen doses before filtration. A-50, C-50, G-15, A-50/C-50: semen doses after filtration with the indicated Sephadex resins, as described in Section 2.

($P < 0.001$) increase in the percentage of total motility ($71.6 \pm 1.3\%$ vs. $66.6 \pm 0.8\%$ in unfiltered samples), accompanied by a concomitant increase in VSL, VAP, LIN and WOB and decreases in MADalg and BCF (Table 3).

3.3. Effects of filtration through separate Sephadex resins with a containment filter of polypropylene on the overall boar-semen quality analysis

Results obtained through filtration of the separate resins plus polypropylene disks as containment filters were not very different from those observed after filtration in the presence of glasswool. Thus, as shown in Table 4, all of the tested filtration mechanisms induced a significant decrease ($P < 0.001$) in sperm concentration, which was much more pronounced after filtration using a combination of A-50 and C-50 resins ($1.9 \pm 0.6 \times 10^6$ sperm/mL vs. $18.9 \pm 0.2 \times 10^6$ sperm/mL in unfiltered samples). Furthermore, the resin that showed the most potent improving effect was, in this case, again G-15, seeing a significant ($P < 0.05$) decrease in the percentage of altered acrosomes (Table 5). On the other hand, both A-50 and C-50 resins induced a slight decrease of both viability and the ORT test, whereas the filtration with more impairing effects on semen quality was that through a combination of A-50 and C-50 plus polypropylene, in which both viability and ORT were greatly reduced, despite a very great decrease in the percentage of altered acrosomes ($0.9 \pm 0.1\%$ vs. $11.0 \pm 0.3\%$ in unfiltered samples) with a reduction of total abnormalities ($2.7 \pm 0.6\%$ vs. $3.9 \pm 0.2\%$ in unfiltered samples; see Table 5). Finally, the effects of the separate filtrations in the presence of polypropylene disks on sperm motility characteristics were not very intense, showing only an overall decrease in the percentage of total motility, which was less intense in the filtration through resin G-15 (Table 5). Motion parameters were not greatly modified either, and only in the case of the combined filtration through both A-50 and C-50 resins was a massive action observed, with a decrease of VSL, VAP, STR and WOB values, accompanied by an increase in BCF (Table 6).

3.4. Ultrastructural study of the interaction between spermatozoa and the filtration supports

The scanning electron-microscope images showed that spermatozoa trapped in the propylene disk after filtration in the absence of resins were not tightly bound to the filter, but rather it seems that they were only situated loosely on the polypropylene fibers (Fig. 1a). Regarding the sperm/resins interaction, tightly, diffuse junctions were observed after filtration through the A-50 resin. However, tightly bound sites between sperm and Sephadex A-50 were found (Fig. 1b). Sperm bound to C-50 resin was also linked to the beads by an apparently tight junction. Nevertheless, these junctions were located at specific points of the sperm's surface (Fig. 1c). Finally, sperm/G-15 beads interaction was evident, although no defined pattern of this interaction was found (Fig. 1d).

3.5. In vivo fertility and prolificacy trials

Insemination with semen doses filtered through Sephadex G-15 resin with polypropylene as the containment filter showed a clear and significant ($P < 0.05$) increase in *in vivo* fertility when compared

Table 6
Effects of filtration through polypropylene-resins columns upon mean motility parameters.

	Unfiltered	A-50	C-50	G-15	A-50/C-50
Total motility (%)	67.1 ± 1.0^{a}	30.7 ± 1.7^{bc}	34.8 ± 1.6^{b}	51.2 ± 1.6^{d}	23.2 ± 2.7^{c}
VCL (μm/s)	69.6 ± 2.1^{ab}	61.7 ± 3.6^{a}	78.5 ± 3.3^{b}	73.8 ± 3.4^{ab}	61.6 ± 5.7^{ab}
VSL (μm/s)	22.9 ± 1.1^{ab}	19.9 ± 1.8^{ab}	25.0 ± 1.7^{a}	26.9 ± 1.7^{a}	13.0 ± 2.9^{b}
VAP (μm/s)	34.0 ± 1.3^{ab}	30.3 ± 2.3^{ab}	38.7 ± 2.1^{a}	38.2 ± 2.1^{a}	23.5 ± 3.6^{b}
LIN (%)	32.3 ± 1.2^{ab}	36.4 ± 2.1^{ab}	31.4 ± 1.9^{ab}	36.5 ± 2.0^{a}	23.4 ± 3.3^{b}
STR (%)	64.5 ± 1.2^{a}	64.9 ± 2.2^{a}	59.0 ± 2.0^{ab}	65.9 ± 2.1^{a}	49.0 ± 3.5^{b}
WOB (%)	47.3 ± 1.1^{ab}	52.2 ± 1.9^{a}	47.9 ± 1.7^{ab}	51.3 ± 1.8^{ab}	39.9 ± 3.0^{b}
ALHmed (μm)	3.0 ± 0.1^{a}	3.2 ± 0.1^{a}	3.5 ± 0.1^{a}	3.4 ± 0.1^{a}	2.9 ± 0.3^{a}
ALHmax (μm)	7.2 ± 02^{a}	8.1 ± 0.4^{a}	8.2 ± 0.4^{a}	8.3 ± 0.4^{a}	7.0 ± 0.7^{a}
DNC (μm)	249.0 ± 24.9^{a}	270.1 ± 43.1^{a}	340.4 ± 39.9^{b}	311.9 ± 40.6^{ab}	222.8 ± 68.6^{a}
MADalg (°)	−25.1 ± 1.6^{a}	−20.0 ± 2.8^{a}	−21.6 ± 2.5^{a}	−20.3 ± 2.6^{a}	−35.2 ± 4.4^{a}
BCF (Hz)	16.6 ± 0.4^{a}	14.9 ± 0.8^{a}	18.3 ± 0.7^{ab}	15.8 ± 0.7^{a}	22.5 ± 1.2^{b}
HLO (μm)	0.17 ± 0.03^{a}	0.16 ± 0.05^{a}	0.21 ± 0.05^{a}	0.20 ± 0.05^{a}	0.16 ± 0.09^{a}
HMX (μm)	2.4 ± 0.1^{a}	2.5 ± 0.1^{a}	2.8 ± 0.1^{a}	2.6 ± 0.1^{a}	2.2 ± 0.2^{a}

Results are expressed as means ± S.E.M. of 8 separate experiments. Different superscript letters in a column indicate statistically significant differences ($P < 0.05$). Unfiltered: semen doses before filtration. A-50, C-50, G-15, A-50/C-50: semen doses after filtration with the indicated Sephadex resins, as described in Section 2.

with insemination with the corresponding, unfiltered control doses (53.3% vs. 78.9% with unfiltered doses; see Table 7). On the other hand, prolificacy obtained after insemination with filtered doses was not significantly different from that obtained after insemination with the corresponding control doses, thus indicating that the observed improving effects of *in vivo* filtration was centered on overall fertility.

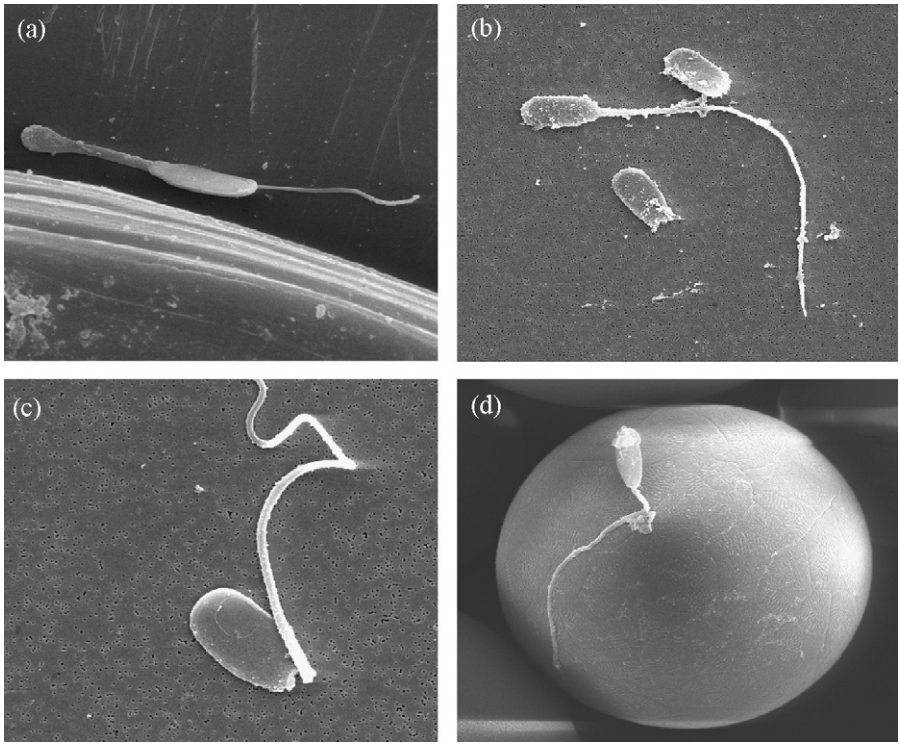

Fig. 1. Scanning electron microscopy of boar-sperm trapped in the separate chromatograpic resins. Ultrastructural techniques have been defined in Section 2. Spermatozoa attached at: (a) polypropylene disk bed, (b) A-50 Sephadex resin, (c) C-50 Sephadex resin and (d) G-15 Sephadex resin. Augmentations are: (a) 3776×; (b) 2681×; (c) 4055×; (d) 1543×.

Table 7
Effects of filtration through polypropylene/Sephadex G-15 resins columns upon *in vivo* fertility of subfertile boar-semen samples.

	n	Pregnant sows	Fertility (%)	Prolificacy (live piglets/farrowing)
Unfiltered samples	15	8	53.3[a]	11.9[a]
Filtered samples	19	15	78.9[b]	10.0[a]

Results are expressed as means ± S.E.M. Different superscript letters in a column indicate significant differences ($P < 0.05$). The experimental design has been described in Section 2.

4. Discussion

Our results state that filtration through resins columns, such as Sephadex G-15, could be a useful and practical system to improve boar-semen quality, as well as *in vivo* fertility under farming conditions. This could be of use, especially in farms geared towards genetic selection, in which semen quality is a much more important factor than the exact number of semen doses for artificial insemination that can be yielded by a single ejaculate. However, we must stress that the practical application of filtration must follow an accurate protocol and design, which involves good knowledge that each element of the filtration system can have on boar-semen quality. In this sense, to obtain the optimal filtration method it is very important not only to choose the correct resin, but also the most optimal containment filter under farming conditions. Thus, our results indicate that glasswool containment filters retain more sperm cells than the polypropylene disk, perhaps due to the fact that the glasswool device has a greater thickness than the disk. This can be an important factor in choosing polypropylene instead of glasswool as an effective subjection filter. Nevertheless, we must remember that filtration in systems which include glasswool commonly yields a smaller number of filtered sperm than those coming from polypropylene disks. Nonetheless, this is not the only factor to take into account when choosing the subjection filter, since the effects of boar-semen overall quality of these subjection filters can be very important. In this sense, our results also indicate that polypropylene disks have an overall improving effect slightly stronger than glasswool, thus indicating that polypropylene could be chosen as a first option as a subjection disk. These results do not greatly differ from others previously published. Thus, Loseth et al. (1992) obtained an improvement in viability and morphological and acrosomal abnormalities using glasswool alone, although these authors did not compare the effect of polypropylene, thus avoiding a direct comparison between both methods in their experimental conditions. Hence, glasswool could also be utilized, but in conditions in which this use does not affect viability.

Regarding the filtration effect of the separate resins, we have found that both the C-50 and the G-15 resins yielded the best viability percentages. On the contrary, filtration through the A-50 resin induced an overall important fall in seminal quality. These results can be observed regardless of the specific subjection filter utilized to contain the filtration resins, thus indicating that they are specific for the utilized resin. Furthermore, our results also suggest the anionic exchange between resin and sperm plasmatic membranes has an overall harmful effect on boar-sperm function. In this sense, it is noteworthy that sperm membranes have an overall negative charge, although there are local zones with a clear positive ionic charge that are able to interact with the anionic exchange resin (Holt, 1980; Anzar and Graham, 1993). The anionic exchange induced in these local, specific points of the sperm membrane during filtration would induce, in turn, local focuses of sperm-membrane stability, which would finally induce the observed decrease in sperm quality. In fact, the presence of local, specific points of junction and/or ionic exchange between sperm and the anionic resin is evident in the ultrastructural study of the sperm/resin interaction, in which we observed the presence of specific points of sperm/A-50 resin attachment that would correspond to sperm positive zones in the process of ionic exchange with the resin. The ionic-induced membrane de-stabilization would primarily affect membranes that were specially prone to being affected by these changes, like the acrosomal membrane, in a similar manner to that described by Cross and Meizel (1989). This would explain the great increase in altered acrosomes observed in sperm subjected to filtration through the anionic exchange resin. On the other hand, from a practical point of view, our results also suggest that the use of the A-50 resin or any other anionic exchange resin are not adequate to obtain improved semen for farm use.

It is worth noting that the results obtained with the C-50 cationic exchanger are completely different from those observed with the A-50 resin. Thus, filtration through the C-50 resin does not have the clearly impairing effects observed in A-50. In fact, several parameters indicate that filtration through the C-50 resin can have an overall improving effect on boar-semen quality. This result is similar to that previously reported in dog (Mogas et al., 1998). We have no clear explanation for this. Notwithstanding, it has been described that processes like capacitation followed by acrosome reaction are linked to the specific appearance of positive-charged components in the plasmatic membrane that would strongly interact with this resin (Anzar and Graham, 1993). In this way, sperm with some functional alteration that induces the appearance of these charged elements in their membranes would be more sensitive to being linked to the C-50 resin. The same phenomenon could be applied to dead sperm, which also would show these positive-charged points in their membranes (Anzar and Graham, 1993). Following this point, it would be possible that some sperm morphological alterations, such as cytoplasmic proximal droplets, would be related to changes in the membrane potential of the zones in and around these abnormalities, which would result in the appearance of additional sperm/resin linking zones. This would explain our results with scanning micrography, in which we observed the appearance of specific membrane points like cytoplasmic proximal droplets attached at the resin surface, which would be positive-charged zones. In any case, and under a practical point of view, our results show that filtration through a cationic resin exchange such as the C-50 one would be useful, especially in ejaculates in which poor quality would be linked to changes in the overall ionic charge of sperm membranes.

The improving effect of semen filtration through a neutral molecular-exclusion resin like G-15 has already been postulated before (Hammit and Martin, 1989; Correa and Zavos, 1996; Mogas et al., 1998). The filtration method consists of separating spermatozoa via gravity flow, following exclusion chromatography principles (Zavos and Centola, 1991). According to Samper et al. (1995), sperm with altered acrosomes is more prone to binding to neutral Sephadex, resulting in a decrease in acrosome alterations in filtered semen. Similar results have been reported in this study supporting this hypothesis. Nevertheless, our results regarding motility do not coincide with other published results on boar-semen. In this sense, other authors have detected a lack of effect (Januskauskas et al., 2005, and the results shown in this manuscript in the presence of polypropylene) or even a decrease in motility (Busalleu et al., 2008) after filtration. These discrepancies could be related to the specific characteristics of boar-sperm motility. It is noteworthy that several studies indicate that boar-sperm motility is highly dependent on environmental factors, such as temperature, extender and the type of the slides and coverslips used (Quintero-Moreno et al., 2004; Vyt et al., 2004). This implies that many factors that are very difficult to kept totally under control, like small temperature variations during the manipulation of both semen and resins during the filtration procedure, could affect boar-sperm motility. Moreover, it is evident that the election of a specific type of subjection filter can affect final sperm motility, thus indicating that the boar-sperm/subjection filter interaction is also important to understand the discrepancies in this point. In any case, and from a practical point of view, our results confirm that the neutral molecular-exclusion resins like Sephadex G-15 can be utilized in the field as a useful tool to improve boar-semen quality in mediocre samples.

Regarding the *in vivo* fertility study, we have only worked with polypropylene filters and G-15 resin due to two reasons. The first, we had a very low number of sows that could be utilized, and, in this way, we had to focus on the aspect that the previous experiments signaled as the most interesting under a practical point of view. The second was the good results obtained in the *in vitro* experiment, as this system showed a promising, great improvement in semen quality. We designed a large system for the whole ejaculate filtration, and we observed the described significant improvement in fertility from 53.3% in non-filtered to 78.9% in filtered semen. This improvement in fertility is related to good prolificacy results in farming conditions. Thus, although the low number of inseminated sows obliged us to be cautious, our results suggest that filtration could be of practical use in improving results of animals with very good genetic characteristics related to productive aspects (conversion indexes, etc.) but with concomitant, bad semen characteristics. In this way, genetic selection firms would optimize the utilization of these animals, which would be of the utmost importance in genetic selection for productive purposes other than reproduction.

In conclusion, boar-semen filtration through Sephadex columns could be a suitable system to improve semen quality of mediocre ejaculates, especially in field conditions. Furthermore, this filtration system only requires a minimal and very attainable infrastructure in relation to the high performance obtained using this filtration system.

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References

Anzar, M., Graham, E.F., 1993. Filtration of bovine semen. I. Development of a Sephadex ion-exchange filter. Anim. Reprod. Sci. 31, 187–195.
Bamba, K., 1988. Evaluation of acrosomal integrity of boar spermatozoa by bright field microscopy using an eosin–nigrosin stain. Theriogenology 29, 1245–1251.
Busalleu, E., Pinart, E., Rivera, M.M., Arias, X., Briz, M., Sancho, S., García-Gil, N., Bassols, J., Pruneda, A., Yeste, M., Casas, I., Rigau, T., Rodriguez-Gil, J.E., Bonet, S., 2008. Effects of filtration of semen dose from subfertile boars through neuter Sephadex columns. Reprod. Domest. Anim. 43, 48–52.
Chinnaiya, G.P., Sarma, P.V., Reddy, O., 1989. Standardisation of semen filtration technique through different media to improve its quality. Ind. J. Anim. Reprod. 10, 56–60.
Correa, J.R., Zavos, P.M., 1996. Preparation and recovery of frozen-thawed bovine spermatozoa via various sperm selection techniques employed in assisted reproductive technologies. Theriogenology 46, 1225–1232.
Cross, N.L., Meizel, S., 1989. Methods for evaluating the acrosomal status of mammalian sperm. Biol. Reprod. 41, 635–641.
Graham, E.F., Graham, J.K., 1990. The effect of whole ejaculate filtration on the morphology and the fertility of bovine semen. J. Dairy Sci. 73, 91–97.
Hammit, D.G., Martin, P.A., 1989. Correlations among assays of porcine semen quality following cryopreservation. Theriogenology 32, 369–384.
Hellander, J.C., 1992. Current practical use of glasswool/Sephadex filtration technique of frozen stallion semen. Acta Vet. Scand. Suppl. 88, 67–70.
Holt, W.V., 1980. Surface-bound sialic acid on ram and bull spermatozoa: deposition during epididymal transit and stability during washing. Biol. Reprod. 23, 847–857.
Januskauskas, A., Lukoseviciute, K., Nagy, S., Johannisson, A., Rodriguez-Martinez, H., 2005. Assesment of the efficacy of Sephadex G-15 filtration of bovine spermatozoa for cryopreservation. Theriogenology 63, 160–178.
Kanakaraj, P., Easwaran, B.M., 1991. Comparative merits of filtration techniques in improving the quality of bull semen. Cheiron 20, 139–143.
Landa, C.A., Almquist, J.O., Amann, R.P., 1980. Factors influencing Sephadex separation of bovine and ovine spermatozoa. J. Dairy Sci. 63, 277–282.
Loseth, K.J., Wolff, L., Hamilton, D.W., Crabo, B.G., 1992. Trapping of in vitro capacitated boar spermatozoa in Sephadex and glasswool filters. In: Proceedings of the 12th ICAR, The Hague, The Netherlands, p. 3.
Mogas, T., Rigau, T., Piedrafita, J., Bonet, S., Rodríguez-Gil, J.E., 1998. Effect of column filtration upon the quality parameters of fresh dog semen. Theriogenology 50, 1171–1189.
Quintero-Moreno, A., Rigau, T., Rodríguez-Gil, J.E., 2004. Regression analyses and motile sperm subpopulation structure study as improving tools in boar semen quality analysis. Theriogenology 61, 673–690.
Rodríguez-Gil, J.E., Rigau, T., 1995. Effects of slight agitation on the quality of refrigerated boar sperm. Anim. Reprod. Sci. 39, 141–146.
Samper, J.C., Hellander, J.C., Crabo, B.G., 1991. Relationship between the fertility of fresh and frozen stallion semen and semen quality. J. Reprod. Fertil. Suppl. 44, 107–112.
Samper, J.C., Crabo, B.G., 1993. Assay of capacitated, free damaged and extended stallion spermatozoa by filtration. Theriogenology 39, 1209–1220.
Samper, J.C., Hamilton, D.W., Pryor, J.L., Loseth, K.J., Troedsson, M.H.T., Crabo, B.G., 1995. Mechanism of Sephadex trapping of capacitated stallion spermatozoa. Biol. Reprod. Monogr. 1, 729–737.
SAS, 1996. SAS/STAC Software. SAS Inst. Inc., Cary, NC.
Valcárcel, A., de las Heras, M.A., Moses, D.F., Pérez, L.J., Baldassarre, H., 1996. Comparison between Sephadex G-10 and Percoll for preparation of nonspermic asthenospermic and frozen/thawed ram semen. Anim. Reprod. Sci. 41, 215–224.
Vyas, S., Dhami, A.J., Mohan, G., Sahni, K.L., 1991. Effect of Sephadex and glasswool column filtration on the quality and storage (at 5 °C) of crossbred bull semen. Ind. J. Anim. Sci. 61, 702–704.
Vyas, S., Mohan, G., Dhami, A.J., Sahni, K.L., 1992a. Comparative evaluation of different techniques for improving the semen quality of crossbred bulls. Ind. J. Dairy Sci. 45, 237–240.
Vyas, S., Mohan, G., Dhami, A.J., Sahni, K.L., 1992b. Effect of filtration through Sephadex and glasswool on the quality and freezability of semen of crossbred bulls. Ind. J. Anim. Sci. 62, 341–343.
Vyt, P., Maes, D., Dejonckheere, E., Castryck, F., Van Soom, A., 2004. Comparative study on five commercial extenders for boar semen. Reprod. Domest. Anim. 39, 8–12.
Zavos, P.M., Centola, G.M., 1991. Selection of sperm from oligozoospermic men for ARTA: comparisons between swim-up vs. SpermPrepTM filtration. J. Assist. Reprod. Technol. Androl. 1, 338–345.