

Enrichment of thawed boar spermatozoa with an intact membrane using Magnetic Activated Cell Sorting

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ABSTRACT

Not all boar sperm samples survive cryopreservation well. A method of eliminating damaged sperm might enable more cryopreserved boar semen to be used for pig breeding. In this study we investigated the use of Magnetic Activated Cell sorting (MACS) to eliminate damaged sperm from thawed boar semen samples. The thawed samples were mixed with Dead cell removal particles and were applied to the column in a SuperMACS II. Different fractions were collected: Original sample (O), Flow-through (FT), and Eluate (E). Sperm membrane integrity, mitochondrial membrane potential and reactive oxygen species were evaluated by flow cytometry after staining with SYBR 14 and propidium iodide, or 5', 6', 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolylcarbocyanine iodide, or hydroethidine and dichlorodihydrofluorescein diacetate, respectively. The FT samples had increased membrane integrity, a greater proportion of sperm with high mitochondrial membrane potential and a greater proportion of sperm negative for hydrogen peroxide than O samples ($P < 0.0001$), which in turn had increased membrane integrity than E samples ($P < 0.0001$). However, differences were seen between boars. The FT samples had increased values of live, superoxide positive sperm than O samples ($P < 0.0001$) and O samples had greater values than E samples ($P < 0.0001$), while there was no effect of boar. Sperm quality was best in the FT fraction, comprising approximately 32% of the sperm sample. In conclusion, although there were differences between boars, MACS separation can improve sperm quality in thawed semen samples. It would be interesting to see if this improvement is reflected in fertility outcomes.

1. Introduction

Despite considerable efforts, the cryosurvival of boar spermatozoa is still low (Rodríguez-Martínez and Wallgren, 2010). Spermatozoa are damaged by freezing and/or thawing, with deterioration of membranes and axonemes from the imbalance in osmolarity created by the change in temperature. In addition, there is loss of cholesterol and peroxidation of unsaturated fatty acids in the sperm membranes (Casas and Flores, 2013) leading to membrane destabilization, and changes in the sperm DNA packaging leading to a subsequent increase in DNA fragmentation (Fraser et al., 2011). Other types of structural damage have also been reported, as reviewed by Yeste (2015), including poor motility in thawed samples. This cryo-induced damage, coupled with a short functional life of thawed sperm, results in a lower farrowing rate and smaller litter size following artificial insemination with thawed sperm doses than when

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liquid semen is used.

One alternative to circumvent the effects of this cryo-induced sperm damage is to use modified insemination techniques, such as post-cervical or deep intrauterine deposition of semen instead of conventional deposition in the cervix (Khalifa et al., 2014). However, these techniques require more expertise in their performance than conventional artificial insemination and it is not always possible to pass the catheter all the way through the cervix in some females (Knox, 2016). Therefore, although these techniques require fewer functional sperm, their use is mainly restricted to specialised facilities.

Another approach is to try to improve cryosurvival by supplementing the cryomedium with a range of substances, including adding back boar seminal plasma (Hernández et al., 2007), or supplementing with e.g. cyclodextrins (Blanch et al., 2012), antioxidants such as glutathione (Estrada et al., 2014), or rice bran oil (Chanapiwat and Kaeoket, 2015). A different approach was to test different rates of freezing, comparing fast freezing in liquid nitrogen vapour with slow freezing in a computerized freezer (Pezo et al., 2023). However, although potentially promising results have been obtained from laboratory studies, no practical advances for insemination in the field have arisen from this research. Most porcine inseminations are still carried out with liquid semen, with the use of frozen semen being confined to genetic upgrades in nucleus facilities and transfer of sperm between countries (Khalifa et al., 2014).

For cryopreservation of boar spermatozoa to be useful for pig breeders, it is important to increase the proportion of spermatozoa that are of good quality after thawing. One way of doing this would be to eliminate spermatozoa with damaged membranes. A useful marker for boar spermatozoa with damaged membranes is Annexin V (Peña et al., 2003), which binds to spermatozoa that have exposed phosphatidylserine on their membranes.

Magnetic cell sorting (MACS, Miltenyi et al., 1990) is a method for removing or enriching fractions of cells labelled with magnetic particles through passage of the cell suspension through a steel-wool column in a magnetic field. This method has been utilised for enrichment of human spermatozoa with intact membranes using Annexin V (Gil et al., 2013). The aim of this present study was to determine if the proportion of spermatozoa with intact membranes could be enriched by MACS-separation of thawed boar sperm samples. A further aim was to study other indicators of sperm quality of the MACS-separated spermatozoa.

2. Materials and methods

2.1. Animals

Semen was obtained from Hampshire boars ($n=3$) housed at Svenska Köttföretagens facility in Hållsta, Sweden. The boars were housed and kept under standard conditions for the species, according to national and international regulations. Ejaculates were collected by the gloved hand method and were transported to the Swedish University of Agricultural Sciences (SLU) in Uppsala on three occasions for each boar. During transport, the semen was kept at 15–20 °C. Semen collection from boars using this method is considered to be a routine husbandry procedure and therefore no ethical approval was required for this study.

2.2. Semen processing

On arrival at SLU, the concentration of spermatozoa was determined using a Nucleocounter SP-100 (Chemometec, Allerød, Denmark) according to the instructions. The sperm samples were analyzed for membrane integrity (MI), mitochondrial membrane potential (MMP) and reactive oxygen species (ROS) as described below. Aliquots containing approximately 6×10^8 spermatozoa were frozen in mini-flatpacks (Saravia et al., 2005, Ekwall et al., 2007) and kept in liquid nitrogen. The flatpacks were thawed at 35°C for 20 s and the sperm concentration was determined with the Nucleocounter. In total, 24 flatpacks were thawed and processed.

2.3. MACS-separation

For the separation, 25×10^6 spermatozoa were mixed with 1 ml Dead cell removal particles (Miltenyi Biotec, Bergisch Gladbach, Germany). From this suspension, 1 ml was applied to an LS column in a SuperMACS II instrument (Miltenyi) that had previously been equilibrated with 7 ml Annexin binding buffer (Miltenyi Biotec). Then 7.5 ml Annexin binding buffer was applied to the column, and the flow-through fraction (FT) was collected. After that, the column was removed from the SuperMACS II, another 5 ml was applied, and the eluate fraction (E) was collected. The concentration in the collected fractions was determined using the Nucleocounter. From these measurements, the numbers of spermatozoa in the different fractions, as well as the numbers of spermatozoa left in the column was calculated (C). Where necessary, the concentration in the fractions for analysis was adjusted to 2×10^6 cells/ml with Annexin binding buffer. The remainder of the original sample (O), mixed with dead cell removal particles, was also diluted to 2×10^6 cells /ml using Annexin binding buffer.

2.4. Membrane integrity

Aliquots of all samples in 300 µl were stained with 0.07 µM SYBR 14 and 24 µM propidium iodide (PI, Live-Dead® Sperm Viability Lit L-7011; Invitrogen, Eugene, OR, USA). After incubating in the dark at 37°C for 10 minutes, the samples were evaluated using a FACSVerse flow cytometer (BDBiosciences; Franklin Lakes, NJ, USA). Excitation was induced by a blue laser (488 nm). Green fluorescence (FL1) from SYBR 14 was detected with a band-pass filter (527/32 nm) and red fluorescence (FL3) from PI was measured using a band-pass filter (700/54 nm). In total, 30,000 events were evaluated for each sample. After gating to identify spermatozoa, the cells were classified as membrane intact (SYBR14-positive/PI-negative), or membrane damaged (SYBR14-negative/PI-positive or SYBR14-

positive/PI-positive). For the purposes of this study, only proportions of membrane intact spermatozoa are reported.

2.5. Mitochondrial membrane potential

Aliquots of all samples (300 μ l) were stained with 12 μ M of the lipophilic cationic probe 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Molecular Probes). After incubating the mixture at 37 °C for 30 minutes in the dark, analysis was carried out using a FACSVerse flow cytometer (BDBiosciences). Excitation of stained cells was obtained with a blue laser (488 nm); emitted fluorescence was detected using both FL1 (527/32 nm) and FL2 (586/42 nm) filters with compensation applied between channels. Evaluation of 30,000 cells was followed by gating to identify spermatozoa and classifying them into two groups: spermatozoa with high MMP (orange fluorescence) and those with low MMP (green fluorescence). Only high MMP results are reported here.

2.6. Reactive oxygen species

Aliquots (300 μ L) were stained with Hoechst 33258 at 0.4 μ M (HO; Sigma, Stockholm), 0.4 μ M hydroethidine (HE; Invitrogen Molecular Probes, Eugene, OR, USA) and 20 μ M dichlorodihydrofluorescein diacetate (DCFDA; Invitrogen Molecular Probes). The samples were incubated at 37°C for 30 minutes before analyzing using a FACSVerse (BDBiosciences) flow cytometer (FC). Excitation was with a blue laser (488 nm) and a Violet laser (405 nm). Detection of green fluorescence (FL1) was via a band-pass filter (527/32 nm), red fluorescence (FL3) was measured using a band-pass filter (700/54 nm), and blue fluorescence (FL5) was detected via a band-pass filter (528/45 nm). In total, 30,000 sperm-specific events were evaluated. After gating for spermatozoa in the FSC-SCC dotplot, they were classified as living SO- or H₂O₂ negative, living SO- or H₂O₂ positive, or dead. Only the ROS content of living spermatozoa is reported here.

2.7. Statistical analysis

The statistical evaluation was performed in JMP version 15 (SAS institute). After testing data for normality, a mixed effects model was used for the flow cytometry parameters, with boar and fraction as fixed factors, and the measured parameters as response factors. The results were considered significant if $P < 0.05$. For comparisons of numbers of cells between different fractions, the Wilcoxon test was used.

3. Results

The results for the different evaluated populations before and after freezing (O sample) are shown in Table 1. Proportions of MI+, MMP+, H₂O₂- and SO- decreased after freezing-thawing, compared with the pre-freeze samples, while the proportion of SO+ increased. No change was found for the H₂O₂+ population. The proportions of spermatozoa in the three fractions after MACS separation are depicted in Fig. 1a-f. Although the variances were high due to some of the samples showing poor membrane integrity after thawing, there was an effect of treatment ($P < 0.0001$), with the FT samples having greater membrane integrity than O samples ($P < 0.0001$) and O samples having greater membrane integrity than E samples ($P < 0.0001$). There was no significant effect of boar. The same significance levels and effects were also found for MMP-high as well as for H₂O₂-, although in this case samples from boar B contained a decreased proportion of H₂O₂- spermatozoa compared to boar C ($P = 0.04$). The E samples contained a decreased proportion of H₂O₂+ spermatozoa compare to O samples ($P = 0.01$). There was no effect of treatment on live SO- spermatozoa, but boar B exhibited a decreased proportion of spermatozoa in this category compared to boar C. However, treatment affected the proportion of SO+ spermatozoa; the FT samples had greater values than O samples ($P < 0.0001$) and O samples had greater values than E samples ($P < 0.0001$), while there was no effect of boar.

The number of spermatozoa in the different fractions is shown in Fig. 2. The number of spermatozoa in the C fraction was increased compared to the FT fraction ($P = 0.04$), and the E fraction ($P = 0.0002$). The number of spermatozoa in the FT fraction was greater than in the E fraction ($P = 0.04$).

Table 1

Proportions (mean \pm SD) of membrane intact, high mitochondrial membrane potential, live hydrogen peroxide negative, live hydrogen peroxide positive, live superoxide negative and live superoxide positive spermatozoa for different boars before and after freezing ($n=24$).

Boar	MI+ (%)		MMP+ (%)		H2O2- (%)		H2O2+ (%)		SO- (%)		SO+ (%)	
	Pre-freeze	Post-freeze	Pre-freeze	Post-freeze	Pre-freeze	Post-freeze	Pre-freeze	Post-freeze	Pre-freeze	Post-freeze	Pre-freeze	Post-freeze
A	87 \pm 15	32 \pm 20	81 \pm 9.7	30 \pm 14	80 \pm 14	53 \pm 15	2.9 \pm 2.6	1.3 \pm 2.6	69 \pm 20	33 \pm 19	14 \pm 7.2	22 \pm 10
B	85 \pm 14	18 \pm 18	82 \pm 14	29 \pm 14	79 \pm 12	42 \pm 19	1.6 \pm 1.3	1.3 \pm 2.8	72 \pm 11	14 \pm 13	8.3 \pm 2.0	29 \pm 8.4
C	92 \pm 5.3	42 \pm 13	84 \pm 8.4	42 \pm 9.3	84 \pm 1.7	60 \pm 5	2.7 \pm 3.6	3.0 \pm 4.1	75 \pm 1.1	27 \pm 20	11 \pm 3.1	36 \pm 18

Notes: MI = membrane integrity, MMP = high mitochondrial membrane potential, H₂O₂ = hydrogen peroxide, SO = superoxide.

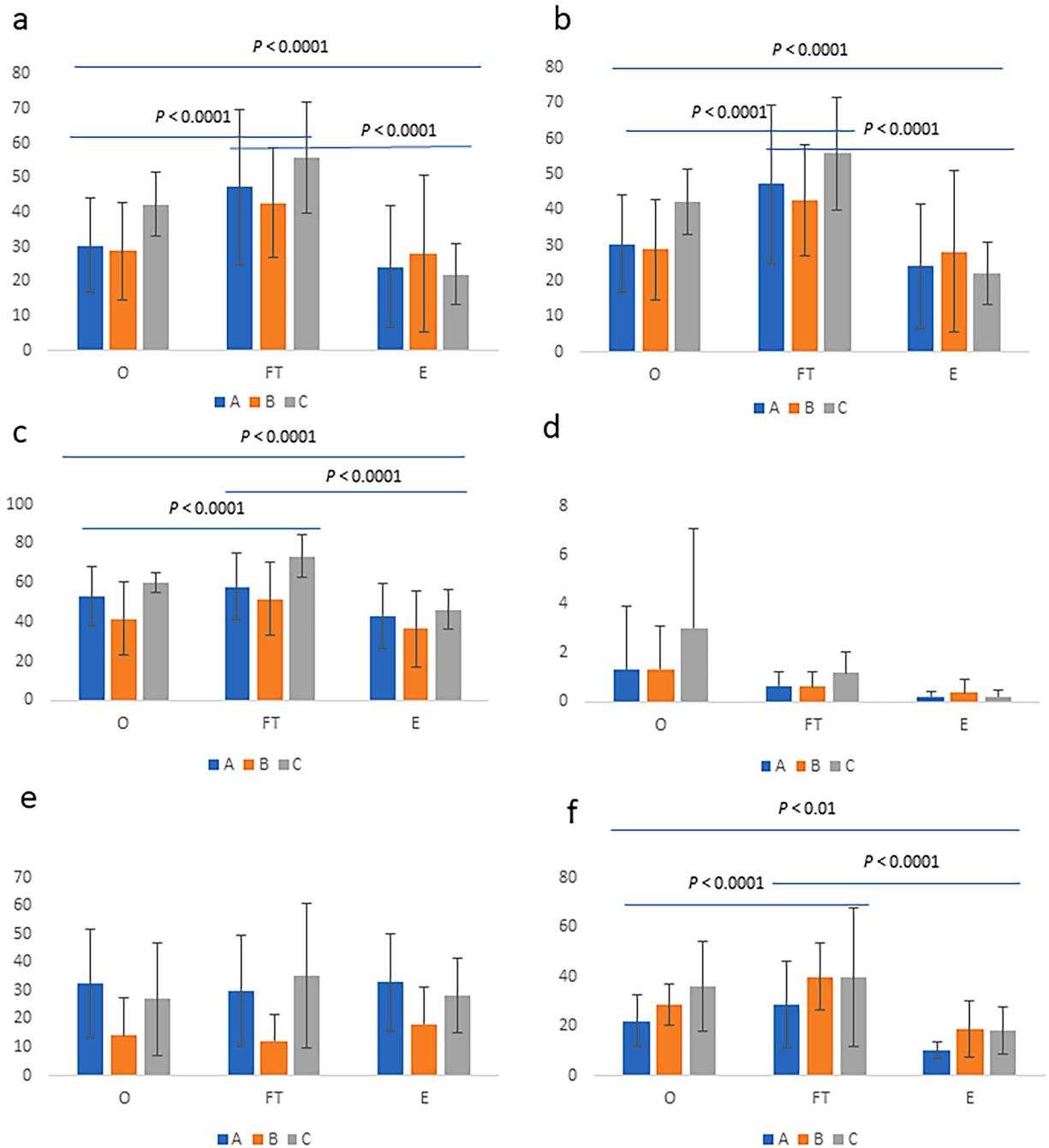


Fig. 1. mean (\pm SD) proportions of various boar sperm characteristics in the three different fractions from Magnetic Active Cell Sorting: FT = Flow-through, E = Eluate, C = Spermatozoa not in the other fractions i.e. left in the column, after applying 25 million sperm to the column ($n=24$). Note: a = membrane integrity, b = mitochondrial membrane potential, c = hydrogen peroxide negative, d =hydrogen peroxide positive, e = Superoxide negative, f =superoxide positive. The following treatments were different: a) FT>O>E, $P < 0.0001$; b) F c FT>O>E, $P < 0.0001$; c) FT>O>E, $P = 0.04$; c) FT>O>E, $P < 0.0001$; f) FT>O>E, $P < 0.0001$.

4. Discussion

The aim of this study was to determine if the proportion of spermatozoa with intact membranes and other indicators of sperm quality could be enriched by MACS-separation of thawed boar sperm samples and to study the quality of thawed boar sperm samples. The different fractions achieved by MACS separation did indeed show differences in sperm properties. Sperm quality was best in the FT fraction, which comprised approximately 32% of the spermatozoa in the sample. In contrast, no differences in membrane and

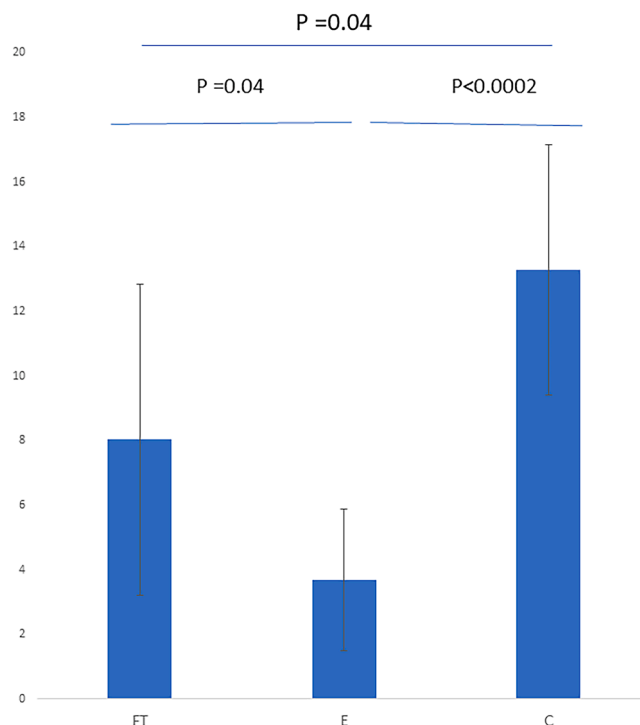


Fig. 2. The number of boar spermatozoa (in millions) in the different fractions: from Magnetic Activated Cell Sorting: FT = Flow-through, E = Eluate, C = Spermatozoa not in the other fractions i.e. left in the column ($n=24$). Note: 25 million sperm were applied to the column.

acrosome integrity, as well as production of reactive oxygen species and fertility, were observed in boar sperm samples selected by MACS (Durfey et al., 2019). However, the MACS-selected samples were reported to have better motility than non-selected sperm. Our results are more similar to those of Assumpção et al. (2021), who conducted a study with bull semen. Their MACS-selected samples were enriched for non-apoptotic sperm, although motility was decreased compared to fresh semen or from density gradient processing.

A different application for MACS, namely to sex bull sperm, was achieved using magnetic beads coated with antibody to a Y-sperm specific antibody (Paitoon et al., 2024). The bound sperm population showed reduced motility compared to non-bound population or controls. The sperm not bound to the beads (X-bearing sperm) did not differ in quality from unselected controls, and did not differ in pregnancy rate in an AI trial. However, they reported that significantly more female calves were produced than controls.

In contrast to the paucity of published work on MACS selection of animal sperm, some studies have been performed on human sperm involving samples from patients attending fertility clinics. In a review of the outcome of fertility treatments in 499 patients undergoing fertility treatment, pregnancy rate was reported to be increased where MACS-selected sperm were used compared to swim-up or density gradient selection methods (Gil et al., 2013). A similar result was reported by Pacheco et al., (2020), who observed a positive effect of MACS on the outcome of ICSI performed for human fertility treatment. The latter authors claimed that the MACS selected sperm samples had less DNA fragmentation than samples without MACS selection. A similar improvement in sperm DNA fragmentation was reported by Mantravadi and Gedela (2022), in their comparison of MACS and a microfluidic method for sperm selection. However, Gil et al. (2021), reviewing data from a much larger patient cohort, showed that there was little clinical impact of using MACS to select sperm for assisted reproduction. They pointed out that the way in which data are obtained from case reports has a profound effect on the apparent result (Gil et al., 2021), while Garrido and Gil Juliá (2024) were critical of the scientific quality of such studies.

5. Conclusion

From our results, albeit with semen from a small number of males, MACS selection of thawed boar semen produces an enriched population of membrane intact sperm with high mitochondrial membrane potential, which could be an advantage for use in IVF if fertility is not adversely affected by the procedure. However, unless the procedure could be scaled-up to deal with much larger numbers of sperm, the application of MACS technology in producing boar sperm samples for artificial insemination remains doubtful. The outlook for producing sufficient bull sperm for AI doses using this method would be more promising, since far fewer sperm are required than for a pig insemination dose. However, a method of selecting thawed boar sperm for in vitro fertilization (IVF) is also needed – an application that is currently increasing. The number of sperm obtained after MACS selection would be more than sufficient for IVF. Thus, based on the results reported here, a larger study is warranted, involving more boars and evaluation of fertilizing ability.

CRediT authorship contribution statement

Jane Morrell: Writing – review & editing, Supervision, Investigation, Funding acquisition. **Anders Johannisson:** Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Margareta Wallgren:** Writing – review & editing, Methodology, Data curation, Conceptualization.

Declaration of Competing Interest

none

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References

- Assumpção, T.I.D., Severo, N.C., Zandonaide, J.P.B., Macedo, G.G., 2021. Magnetic-activated cell sorting improves high-quality spermatozoa in bovine semen. *J. Anim. Reprod. Biotech.* 36, 91–98. <https://doi.org/10.12750/JARB.36.2.91>.
- Blanch, E., Tomás, C., Graham, J.K., Mocé, E., 2012. Response of boar sperm to the treatment with cholesterol-loaded cyclodextrins added prior to cryopreservation. *Reprod. Domest. Anim.* 47, 959–964. <https://doi.org/10.1111/j.1439-0531.2012.01999.x>.
- Casas, I., Flores, E., 2013. Gene banking: the freezing strategy. In: Bonet, S., Casas, I., Holt, W.V., Yeste, M. (Eds.), *Boar Reproduction*. Springer, Berlin, pp. 551–588.
- Chanapiwat, P., Kaeoket, K., 2015. Breed of boar influences the optimal concentration of gamma-oryzanol needed for semen cryopreservation. *Reprod. Domest. Anim.* 50, 221–226. <https://doi.org/10.1111/rda.12473>.
- Durfey, C.L., Swistek, S.E., Liao, S.F., Crenshaw, M.A., Clemente, H.J., Thirumalai, R.V.K.G., Steadman, C.S., Ryan, P.L., Willard, S.T., Feugang, J.M., 2019. Nanotechnology-based approach for safer enrichment of semen with best spermatozoa. *J. Anim. Sci. Biotechnol.* 10, 14–25. <https://doi.org/10.1186/s40104-018-0307-4>.
- Ekwall, H., Hernández, M., Saravia, F., Rodríguez-Martínez, H., 2007. Cryo-scanning electron microscopy (Cryo-SEM) of boar semen frozen in medium-straws and MiniFlatPacks. *Theriogenology* 67, 1463–1472. <https://doi.org/10.1016/j.theriogenology.2007.03.004>.
- Estrada, E., Rodríguez-Gil, J.E., Rocha, L.G., Balasch, S., Bonet, S., Yeste, M., 2014. Supplementing cryopreservation media with reduced glutathione increases fertility and prolificacy of sows inseminated with frozen-thawed boar semen. *Andrology* 2, 88–99. <https://doi.org/10.1111/j.2047-2927.2013.00144.x>.
- Fraser, L., Strzeżek, J., Kordan, W., 2011. Effect of freezing on sperm nuclear DNA. *Reprod. Domest. Anim.* 46 (Suppl 2), 14–17. <https://doi.org/10.1111/j.1439-0531.2011.01815.x>.
- Garrido, N., Gil Juliá, M., 2024. The use of non-apoptotic sperm selected by magnetic activated cell sorting (MACS) to enhance reproductive outcomes: what the evidence says. *Biology* 13, 30–53. <https://doi.org/10.3390/biology13010030>.
- Gil, M., Sar-Shalom, V., Melendez Sivira, Y., Carreras, R., Checa, M.A., 2013. Sperm selection using magnetic activated cell sorting (MACS) in assisted reproduction: a systematic review and meta-analysis. *J. Assist Reprod. Genet* 30, 479–485. <https://doi.org/10.1007/s10815-013-9962-8>.
- Gil Juliá, M., Hervás, I., Navarro-Gómez Lechón, A., Quintana, F., Amorós, D., Pacheco, A., González-Ravina, C., Rivera-Egea, R., Garrido, N., 2021. Sperm selection by magnetic-activated cell sorting before microinjection of autologous oocytes increases cumulative live birth rates with limited clinical impact: a retrospective study in unselected males. *Biology* 10, 430–440. <https://doi.org/10.3390/biology10050430>.
- Hernández, M., Roca, J., Calvete, J.J., Sanz, L., Muñio-Blanco, T., Cebrián-Pérez, J.A., Vázquez, J.M., Martínez, E.A., 2007. Cryosurvival and in vitro fertilizing capacity postthaw is improved when boar spermatozoa are frozen in the presence of seminal plasma from good freezer boars. *J. Androl.* 28, 689–697. <https://doi.org/10.2164/jandrol.107.002725>.
- Khalifa, T., Rekkas, C., Samartzi, F., Lymberopoulos, A., Kousenidis, K., Dovenski, T., 2014. Highlights on artificial insemination (AI) technology in pigs (i-xxx). *Maced. Vet. Rev.* 37 (1). <https://doi.org/10.14432/j.macvetrev.2013.09.001>.
- Knox, R., 2016. Artificial insemination in pigs today. *Theriogenology* 85, 83–93. <https://doi.org/10.1016/j.theriogenology.2015.07.009>.
- Mantravadi, K.C., Gedela, D.R., 2022. MACS vs microfluidics sperm sorting for raised sperm DNA fragmentation index. *deac104.108 Hum. Reprod.* 37 (Suppl 1). <https://doi.org/10.1093/humrep/deac104.108>.
- Miltenyi, S., Müller, W., Weichel, W., Radbruch, A., 1990. High gradient magnetic cell separation with MACS. *Cytometry* 11, 231–238. <https://doi.org/10.1002/cyto.990110203>.
- Pacheco, A., Blanco, A., Bronet, F., Cruz, M., García-Fernández, J., García-Velasco, J.A., 2020. Magnetic-activated cell sorting (MACS): a useful sperm-selection technique in cases of high levels of sperm DNA fragmentation. *J. Clin. Med* 9, 3976. <https://doi.org/10.3390/jcm9123976>.
- Paitoon, P., Sartsook, A., Thongkham, M., Sathanawongs, A., Lumsangkul, C., Pattanawong, W., Hongsibsong, S., Sringarm, K., 2024. Sperm quality variables of sex-sorted bull semen produced by magnetic-activated cell sorting coupled with recombinant antibodies targeting Y-chromosome-bearing sperm. *Theriogenology* 219, 11–21. <https://doi.org/10.1016/j.theriogenology.2024.02.016>.
- Peña, F.J., Johannisson, A., Wallgren, M., Rodríguez-Martínez, H., 2003. Assessment of fresh and frozen-thawed boar semen using an Annexin-V assay: a new method of evaluating sperm membrane integrity. *Theriogenology* 60, 677–689. [https://doi.org/10.1016/S0093-691X\(03\)00081-5](https://doi.org/10.1016/S0093-691X(03)00081-5).
- Pezo, F., Zambrano, F., Uribe, P., de Andrade, A.F.C., Sánchez, R., 2023. Slow freezing of preserved boar sperm: comparison of conventional and automated techniques on post-thaw functional quality by a new combination of sperm function tests, 10 pages *Animals* 13, 2826. <https://doi.org/10.3390/ani13182826>.
- Rodríguez-Martínez, H., Wallgren, M., 2010. Advances in boar semen cryopreservation. *Vet. Med Int* 2010, 396181. <https://doi.org/10.4061/2011/396181>.
- Saravia, F., Wallgren, M., Nagy, S., Johannisson, A., Rodríguez-Martínez, H., 2005. Deep freezing of concentrated boar semen for intra-uterine insemination: effects on sperm viability. *Theriogenology* 15, 1320–1333. <https://doi.org/10.1016/j.theriogenology.2004.06.012>.
- Yeste, M., 2015. Recent advances in boar sperm cryopreservation: state of the art and current perspectives. *Reprod. Domest. Anim.* 5, 71–79. <https://doi.org/10.1111/rda.12569>.