

DEPLETION OF DEAD SPERMATOZOA DID NOT SUFFICIENTLY IMPROVE THE QUALITY OF RAM SEMEN: SHORT COMMUNICATION

Jaromír VAŠÍČEK^{1,2*}, Andrea SVORADOVÁ¹, Andrej BALÁŽI¹, Rastislav JURČÍK¹, Jakub VOZAF², Peter CHRENEK^{1,2}

¹NPPC – Research Institute for Animal Production Nitra, Hlohovecká 2, 951 41 Lužianky, Slovak Republic

²Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Nitra, Slovak Republic

ABSTRACT

The main objective of this study was to improve the motility and overall quality of spermatozoa from the fresh ram semen samples using more sensitive depletion programmes of AutoMACS Pro Separator to remove dead sperm cells. Briefly, ram spermatozoa, given either at high (10^8) or low (10^7) concentrations, were incubated with the Dead Cell Removal kit reagent and magnetically sorted using two very sensitive depletion programmes (Depl05 and Depl025) with different sample loading rates. Fresh unsorted semen samples (control) as well as both sorted fractions (negative and positive) were analysed using computer-assisted sperm assay (CASA) to assess the motility parameters and using flow cytometry to evaluate the proportion of dead cells and overall magnetic-activated cell sorting (MACS) efficiency. We obtained significantly ($P < 0.01$) lower percentage of dead spermatozoa only after sorting the high concentrated spermatozoa using a Depl025 programme compared to control samples. However, the negative fractions still contained more than 20 % of dead cells irrespective of the sorting programme used. In addition, the motility parameters were significantly improved neither by the used sorting strategy nor by the adjustment of spermatozoa concentration. In conclusion, further optimization of this method is required in order to sufficiently remove dead spermatozoa and to improve the spermatozoa motility.

Key words: ram semen; CASA; MACS; depletion; dead spermatozoa; flow cytometry

INTRODUCTION

In general, assisted reproductive technology (ART) is commonly used to solve the infertility in humans (Tournaye, 2012) or on commercial animal breeding farms (Niemann *et al.*, 2011). One of the ART techniques is also a cryopreservation of spermatozoa. However, it has been reported recently that increased presence of dead spermatozoa in raw semen is associated with poor cryopreservation and insufficient outcomes of *in vitro* fertilization (IVF) (Roca *et al.*, 2013) compared with the spermatozoa from a standard semen sample (Oehninger *et al.*, 2000;

Di Santo *et al.*, 2011). On the other hand, sub-standard semen samples are usually characterized by the presence of a large population of morphologically altered and/or dead spermatozoa (Esteves *et al.*, 2012).

Magnetic-activated cell sorting (MACS) also belongs to ART techniques that has been used in human assisted reproduction (Said *et al.*, 2006; Oseguera-López *et al.*, 2019) to eliminate spermatozoa with deteriorated membranes and apoptotic-like features (Grunewald *et al.*, 2001) mainly in humans (Glander *et al.*, 2002; Agarwal *et al.*, 2009; Vendrell *et al.*, 2014; Bucar *et al.*, 2015) but also in animals (Vasicek *et al.*, 2014a; b; Mrkun

*Correspondence: E-mail: jaromir.vasicek@nppc.sk
Jaromír Vašíček, NPPC – Research Institute for Animal Production Nitra,
Hlohovecká 2, 951 41 Lužianky, Slovak Republic

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et al., 2014) using manual MASC instruments. We have already applied this technique for the first time for ram semen samples that were sorted using fully automated magnetic sorter (Vašíček *et al.*, 2020). However, the used depletion programmes (Deplete and Deletes) were not sensitive enough to efficiently remove dead spermatozoa from the fresh semen samples. Moreover, the spermatozoa motility, which is one of the primary indicators of semen quality (Baláži *et al.*, 2020), was not improved after sorting. Therefore, in this study in order to improve sperm motility we used more sensitive depletion programmes for the elimination of dead spermatozoa.

MATERIAL AND METHODS

Clinically health and sexually mature rams of Native Wallachian ($n = 2$) and Improved Wallachian ($n = 1$) sheep breeds aged 2–4 years were used in this study. Rams were kept under external conditions in individual stalls at the breeding facility (NPPC, RIAP Nitra, Lužianky, Slovak Republic). They were fed with hay bale and oats; water and mineral salt were supplied *ad libitum*. Prior to the experiment, semen samples were collected by an electro-ejaculation once a week for the duration of several weeks, as described previously (Baláži *et al.*, 2020). All semen collections were realized in autumn. Ram semen samples immediately after collection were transferred to the laboratory in a water bath for the subsequent processing. The experiments were carried out in accordance with the Code of Ethics of the EU Directive 2010/63/EU for animal experiments.

Freshly collected semen samples were diluted and analysed by CASA (Sperm Vision™, MiniTübe, Germany) for concentration (10^9 per mL), total motility (motility $> 5 \mu\text{m}\cdot\text{s}^{-1}$) and progressive motility (motility $> 20 \mu\text{m}\cdot\text{s}^{-1}$) of spermatozoa, as described previously (Baláži *et al.*, 2020). The CASA analyses were performed again after MACS sorting only in negative fractions.

In this study, we performed four types of experiments in order to determine the most appropriate selection strategy and the number of sorted spermatozoa. At first, ram spermatozoa at high concentration (10^8) were sorted using two

different sensitive depletion programmes (Depl05 and Depl025; first and second type of experiment, respectively). Next, ram spermatozoa at low concentration (10^7) were sorted using aforementioned programmes (third and fourth type of experiment, respectively). Briefly, aliquots of each semen samples (high and low concentrated) were diluted in 1 or 0.5 ml of a reagent from the Dead Cell Removal kit (Miltenyi Biotec, Germany), respectively and incubated for 15 min at room temperature according to the producer's manual. After incubation, dead spermatozoa were removed from the ram semen samples by AutoMACS Pro Separator (Miltenyi Biotec, Germany). A HEPES buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 1.8 mM CaCl_2 , at pH 7.2) was used as a sheath fluid instead of the standard MACS running buffer, since the commercial kit required a buffer with calcium for a proper binding of nanoparticles to the cells. The both depletion programmes (Depl05 and Depl025) differ mainly in the loading rate. The Depl05 programme loads 0.5 mL of sample per minute, while the Depl025 provides slower loading rate ($0.25 \text{ mL}\cdot\text{min}^{-1}$).

The negative and positive fractions obtained from MACS sorting were stained with allophycocyanin (APC)-conjugated labelling check reagent (LCR; Miltenyi Biotec, Germany) in order to determine the proportion of spermatozoa with bound nanoparticles. Moreover, all sorted samples, including fresh unsorted (control) samples, were mixed with a propidium iodide (PI at $50 \mu\text{g}\cdot\text{mL}^{-1}$; Molecular Probes, USA) prior to the analysis in order to reveal the dead spermatozoa. Stained samples (at least 10,000 cells) were immediately analysed using a FACSCalibur flow cytometer (BD, San Jose, CA, USA).

The experiments were replicated for three times. Semen samples, which contained more than 50 % of dead spermatozoa, were used for the immunomagnetic sorting. Motility parameters and the percentage of dead spermatozoa were statistically evaluated using a GraphPad Prism version 9.0.2 for Windows (GraphPad Software, San Diego, CA, USA) with a one-way ANOVA (Dunnnett's and Sidak's method, resp.) and expressed as the mean \pm SEM. P-values at $P < 0.05$ were considered as statistically significant.

RESULTS AND DISCUSSION

We tested the possibility of the potential use of immunomagnetic dead sperm removal to improve the semen quality. Two different depletion strategies (Depl05 and Depl025) for very sensitive fully automated cell sorting were examined. Ram spermatozoa were sorted at two different concentrations (10^8 and 10^7) in order to define a proper concentration according to the column capacity. High concentrated samples (10^8) showed significantly reduced dead cell number in negative fractions in comparison to control, when separated using Depl025 programme ($P < 0.01$). On the other hand, the proportion of dead spermatozoa also decreased slightly but insignificantly after sorting

using a Depl05 programme. However, negative fractions of those samples still contained more than 20 % of dead spermatozoa. On the other hand, the proportion of dead spermatozoa in positive fractions using both programmes, Depl05 and Depl025, increased significantly (over 80%; $P < 0.05$ and $P < 0.001$, respectively) in comparison to the control samples (Figure 1A). On the contrary, no significant changes in the proportion of dead cells were observed either in negative or positive fractions of low concentrated semen samples (10^7 of cells) irrespective of a used sorting programme (Figure 1B). Therefore, decreased concentration of spermatozoa did not facilitate their sorting, and ram spermatozoa could be effectively sorted at the higher concentration (10^8 of cells).

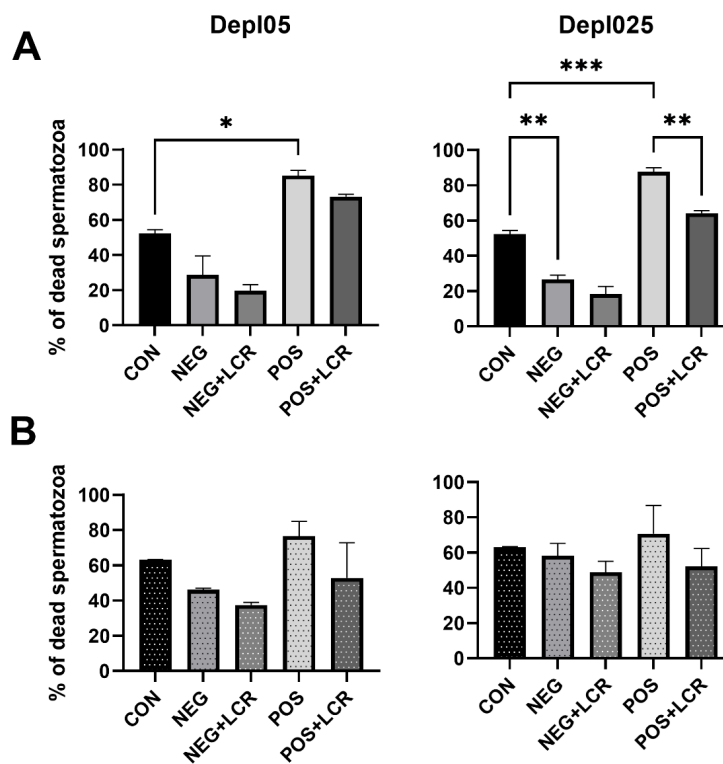


Figure 1. Changes in the proportion of dead spermatozoa after MACS sorting of high concentrated (A) and low concentrated (B) ram semen samples using two depletion programmes

Depl05 – sorting programme with loading rate at $0.5 \text{ mL} \cdot \text{min}^{-1}$; Depl025 – sorting programme with loading rate at $0.25 \text{ mL} \cdot \text{min}^{-1}$. Percentage of dead cells was determined as a proportion of the spermatozoa positively stained with a propidium iodide; CON – fresh semen samples before sorting; NEG – negatively sorted spermatozoa; NEG+LCR – negatively sorted spermatozoa co-stained with a labelling check reagent (LCR); POS – positively sorted spermatozoa; POS+LCR – positively sorted spermatozoa co-stained with a labelling check reagent (LCR); * – statistical significance at $P < 0.05$; ** – statistical significance at $P < 0.01$; *** – statistical significance at $P < 0.001$.

Interestingly, a slightly lower proportion of spermatozoa positive for both, PI and LCR, was observed in all samples (NEG+LCR and POS+LCR), when compared to the total percentage of dead (PI positive) cells in the NEG and POS fractions (Figure 1). Similar difference, though significant ($P < 0.01$), was noticed between positive fractions in high concentrated samples sorted using Depl025 (Figure 1A). This might indicate, that some portion of dead cells was not detected by the nanoparticles, and/or that those dead cells were appeared as a result of the sorting procedure itself, since ram spermatozoa are very sensitive to any physiological changes.

The aim of this study was to remove the dead cells from the semen samples in order to improve their quality in terms of better motility parameters. However, the total and progressive motility of high concentrated spermatozoa slightly decreased after sorting by a Depl05 programme. On the other hand, the progressive motility of spermatozoa slightly

but insignificantly increased after sorting using a Depl025 programme (Figure 2A). Furthermore, both motility parameters in low concentrated semen samples had decreasing tendency after sorting using both programmes (Figure 2B). The same observation was reported in our previous study (Vašíček *et al.*, 2020), where the motility of ram spermatozoa after depleting the dead cells using fewer sensitive programmes (Deplete and Deletes) decreased insignificantly, and the proportion of dead cells in negative fractions was not changed, when compared to the control samples. Similarly, another our study (Vasicek *et al.*, 2014b), focused on the removal of dead cells from rabbit semen samples using the manual MACS instruments, did not result in the improving the motility in negative fractions, as well as did not significantly decrease the number of dead spermatozoa.

On the contrary, in this study we used fully automated sorter in order to facilitate the sorting

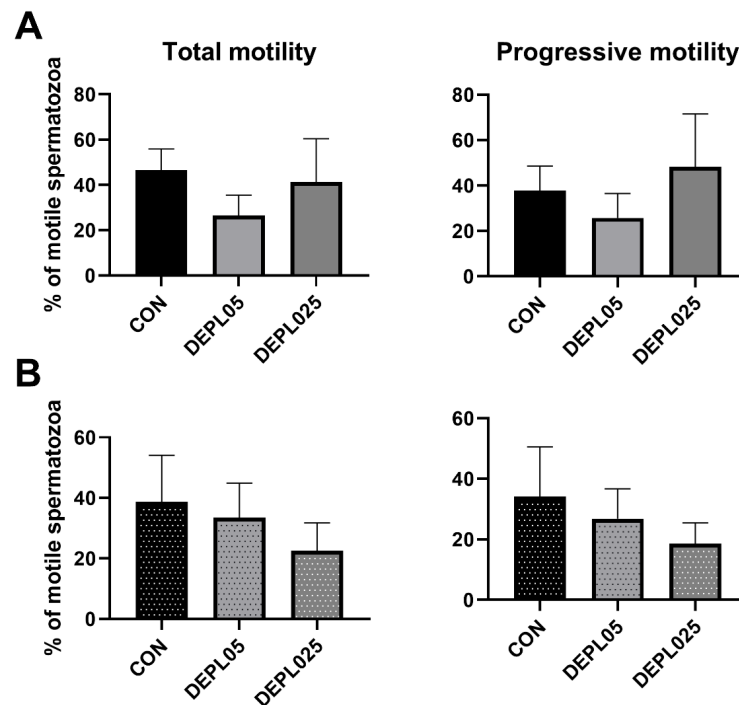


Figure 2. Changes in the motility parameters of spermatozoa after MACS sorting of high concentrated (A) and low concentrated (B) ram semen samples using two depletion programmes

CON – fresh semen samples before sorting; Depl05 – negative fractions of spermatozoa sorted using programme with loading rate at $0.5 \text{ mL} \cdot \text{min}^{-1}$; Depl025 – negative fractions of spermatozoa sorted using sorting programme with loading rate at $0.25 \text{ mL} \cdot \text{min}^{-1}$.

process and make it more sensitive to ram spermatozoa. Nevertheless, manual MACS instruments are commonly used to remove dead or apoptotic spermatozoa from human semen samples by this technique with variable results (Paasch *et al.*, 2003; Delbès *et al.*, 2013; Grunewald and Paasch, 2013; Merino-Ruiz *et al.*, 2019). Paasch *et al.* (2003) demonstrated an insignificant loss of progressive motility in negative fractions after MACS. On the other hand, a significant improvement in the quality (including motility) of negatively sorted spermatozoa in combination with density gradient centrifugation was observed by Delbès *et al.* (2013) and Merino-Ruiz *et al.* (2019).

Although we obtained a significant reduction in the number of dead spermatozoa in high concentrated ram semen samples after MACS (Depl025), nevertheless their presence in samples was still more than 20 %. It has been reported that high proportion of dead spermatozoa in fresh semen samples significantly increased ROS generation and nuclear DNA fragmentation in frozen-thawed boar spermatozoa and, thus, negatively affected the IVF outcomes (Roca *et al.*, 2013). Therefore, it is important to find an optimal method to decrease dead spermatozoa number in fresh ram semen of valuable breeding males to the minimum.

CONCLUSION

Present study indicates that fresh ram spermatozoa with high proportion of dead cells (over 50 %) can be free of these cells using a very sensitive depletion programme (Depl025), when MACS sorted at high concentrations. However, a considerable proportion of dead cells (over 20 %) still remains in semen samples after sorting. Therefore, further studies are needed in order to optimize this method and significantly improve the quality of ram semen samples, what could increase the cryosurvival rates and IVF outcomes of frozen-thawed spermatozoa.

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REFERENCES

- Agarwal, A., Varghese, A. C. & Sharma, R. K. (2009). Markers of oxidative stress and sperm chromatin integrity. In *Molecular Endocrinology* (pp. 377–402). Humana Press, Totowa, NJ. https://doi.org/10.1007/978-1-60327-378-7_24
- Baláži, A., Vašíček, J., Svoradová, A., Macháč, M., Jurčík, R., Huba, J., Pavlík, I. & Chrenek, P. (2020). Comparison of three different methods for the analysis of ram sperm concentration. *Slovak Journal of Animal Science*, 53(2), 53–58.
- Bucar, S., Gonçalves, A., Rocha, E., Barros, A., Sousa, M. & Sá, R. (2015). DNA fragmentation in human sperm after magnetic-activated cell sorting. *Journal of Assisted Reproduction and Genetics*, 32(1), 147–154. <https://doi.org/10.1007/s10815-014-0370-5>
- Delbès, G., Herrero, M. B., Troeung, E. T. & Chan, P. T. K. (2013). The use of complimentary assays to evaluate the enrichment of human sperm quality in asthenoteratozoospermic and teratozoospermic samples processed with Annexin-V magnetic activated cell sorting. *Andrology*, 1(5), 698–706. <https://doi.org/10.1111/j.2047-2927.2013.00106.x>
- Di Santo, M., Tarozzi, N., Naldini, M., & Borini, A. (2011). Human sperm cryopreservation: update on techniques, effect on DNA integrity, and implications for ART. *Advances in Urology*, 2012. <https://doi.org/10.1155/2012/854837>
- Esteves, S. C., Zini, A., Aziz, N., Alvarez, J. G., Sabanegh Jr, E. S., & Agarwal, A. (2012). Critical appraisal of World Health Organization's new reference values for human semen characteristics and effect on diagnosis and treatment of subfertile men. *Urology*, 79(1), 16–22. <https://doi.org/10.1016/j.urology.2011.08.003>
- Glander, H. J., Schiller, J., Süß, R., Paasch, U., Grunewald, S. & Arnhold, J. (2002). Deterioration of spermatozoal plasma membrane is associated with an increase of sperm lyso-phosphatidylcholines. *Andrologia*, 34(6), 360–366. <https://doi.org/10.1046/j.1439-0272.2002.00508.x>
- Grunewald, S. & Paasch, U. (2013). Sperm selection for ICSI using annexin V. In *Spermatogenesis* (pp. 257–262). Humana Press, Totowa, NJ. https://doi.org/10.1007/978-1-62703-038-0_23
- Grunewald, S., Paasch, U. & Glander, H. J. (2001). Enrichment of non-apoptotic human spermatozoa after cryopreservation by immunomagnetic cell sorting. *Cell and Tissue Banking*, 2(3), 127–133. <https://doi.org/10.1023/A:1020188913551>
- Merino-Ruiz, M., Morales-Martínez, F. A., Navar-Vizcarra, E., Valdés-Martínez, O. H., Sordia-Hernández, L. H., Saldívar-Rodríguez, D. & Vidal-Gutiérrez, O. (2019).

- The elimination of apoptotic sperm in IVF procedures and its effect on pregnancy rate. *JBRA Assisted Reproduction*, 23(2), 112. <https://doi.org/10.5935/1518-0557.20190007>
- Mrkun, J., Dolenšek, T., Knific, T., Pišlar, A., Kosec, M., Kos, J. & Zrimšek, P. (2014). Elimination of apoptotic boar spermatozoa using magnetic activated cell sorting. *Acta Veterinaria Brno*, 83(1), 13–18. <https://doi.org/10.2754/avb201483010013>
- Niemann, H., Kuhla, B., & Flachowsky, G. (2011). Perspectives for feed-efficient animal production. *Journal of Animal Science*, 89(12), 4344–4363. <https://doi.org/10.2527/jas.2011-4235>
- Oehninger, S., Duru, N. K., Srisombut, C., & Morshedi, M. (2000). Assessment of sperm cryodamage and strategies to improve outcome. *Molecular and Cellular Endocrinology*, 169(1–2), 3–10. [https://doi.org/10.1016/s0303-7207\(00\)00343-9](https://doi.org/10.1016/s0303-7207(00)00343-9)
- Oseguera-López, I., Ruiz-Díaz, S., Ramos-Ibeas, P. & Pérez-Cereales, S. (2019). Novel techniques of sperm selection for improving IVF and ICSI outcomes. *Frontiers in Cell and Developmental Biology*, 7. <https://doi.org/10.3389/fcell.2019.00298>
- Paasch, U., Grunewald, S., Fitzl, G. & Glander, H. J. (2003). Deterioration of plasma membrane is associated with activated caspases in human spermatozoa. *Journal of Andrology*, 24(2), 246–252. <https://doi.org/10.1002/j.1939-4640.2003.tb02669.x>
- Roca, J., Martínez-Alborcia, M. J., Gil, M. A., Parrilla, I., & Martínez, E. A. (2013). Dead spermatozoa in raw semen samples impair *in vitro* fertilization outcomes of frozen-thawed spermatozoa. *Fertility and Sterility*, 100(3), 875–881. <https://doi.org/10.1016/j.fertnstert.2013.05.020>
- Said, T., Agarwal, A., Grunewald, S., Rasch, M., Baumann, T., Kriegel, C. & Paasch, U. (2006). Selection of nonapoptotic spermatozoa as a new tool for enhancing assisted reproduction outcomes: an *in vitro* model. *Biology of Reproduction*, 74(3), 530–537. <https://doi.org/10.1095/biolreprod.105.046607>
- Tournaye, H. (2012). Male factor infertility and ART. *Asian Journal of Andrology*, 14(1), 103. <https://doi.org/10.1038/aja.2011.65>
- Vašíček J., Baláži A., Kuželová L. & Chrenek P. (2014b). Comparison of two commercial kits for the elimination of rabbit spermatozoa with damaged membrane via MACS technique. In *Animal Physiology 2014* (pp. 277–283), Prušánky, 2014. Brno: Mendel University in Brno. ISBN 978-80-7375-971-1
- Vasicek, J., Pivko, J. & Chrenek, P. (2014a). Reproductive performance of New Zealand White rabbits after depletion of apoptotic spermatozoa. *Folia Biologica*, 62(2), 109–117. https://doi.org/10.3409/fb62_2.109
- Vašíček, J., Svoradová, A., Baláži, A., Jurčík, R., Macháč, M., & Chrenek, P. (2020). Use of dead cell removal kit for the improvement of ram semen quality. *Slovak Journal of Animal Science*, 53(04), 205–208.
- Vendrell, X., Ferrer, M., García-Mengual, E., Munoz, P., Trivino, J. C., Calatayud, C. & Ruiz-Jorro, M. (2014). Correlation between aneuploidy, apoptotic markers and DNA fragmentation in spermatozoa from normozoospermic patients. *Reproductive Biomedicine Online*, 28(4), 492–502. <https://doi.org/10.1016/j.rbmo.2013.12.001>