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USE OF DEAD CELL REMOVAL KIT FOR THE IMPROVEMENT OF RAM SEMEN QUALITY: SHORT COMMUNICATION

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ABSTRACT

The main objective of this preliminary study was to improve the ram semen quality by the removal of dead and apoptotic cells from the ejaculates. For this purpose, ram spermatozoa were incubated with the Dead Cell Removal kit and magnetically sorted using two procedures with different sample loading rate (Deplete and Depletes) by a fully automated cell sorter. Fresh semen samples (control) as well as both sorted fractions (negative and positive) were analysed for motility parameters using CASA and for the proportion of dead cells using flow cytometry. As expected, a significant increase ($P < 0.05$) in the number of dead cells and decrease ($P < 0.05$) in the spermatozoa motility were observed in the positive fractions when sorted by both procedures. However, the viability of negatively sorted spermatozoa was not improved and their motility was insignificantly decreased. In conclusion, although the presented study demonstrates the possible use of a MACS technique for the elimination of ram spermatozoa with compromised membrane, the chosen sorting procedures seem to be insufficient to obtain high purity of spermatozoa with intact membranes. More sensitive depletion programmes should be tested in further studies.

Key words: ram semen; CASA; MACS; dead cell removal; flow cytometry

INTRODUCTION

Generally, the proportion of healthy spermatozoa within the semen affects its fertilizing ability, as the increased number of spermatozoa with damaged membrane or with poor motility obviously is a reason for the decrease in fertility (Januskauskas *et al*. 2003; Dogan *et al*. 2013). Magnetic-activated cell sorting (MACS) has been well established in human assisted reproduction in recent years (Said *et al*., 2006; Oseguera-López *et al*., 2019). This technique is predominantly used to eliminate spermatozoa with externalized phosphatidylserine that is translocated from the inner to the outer plasma membrane during apoptosis (Grunewald *et al*., 2001). Annexin V MicroBead Kit (Miltenyi Biotec, Germany) was used mainly in human (Glander *et al*., 2002; Agarwal *et al*., 2009; Vendrell *et al*., 2014; Bucar *et al*., 2015) or animal (Vasicek *et al*., 2014a; Mrkun *et al*., 2014) studies with the MACS selection of apoptotic spermatozoa.

Additionally, there is another product at the market, named Dead Cell Removal Kit (Miltenyi Biotec, Germany), which according to the producer recognizes a moiety in the plasma membrane of apoptotic or dead cells and can be, thus, used for their removal from the cell suspension using MACS. We have already used this kit together with Annexin V MicroBead Kit in our previous comparable study on rabbit spermatozoa (Vasicek *et al*., 2014b).

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However, in this study, like in the above-mentioned studies, the manual MACS instruments were used for the sorting of low concentrated semen samples. Moreover, any of published papers was focused on the MACS sorting of ram semen.

Thus, in the present preliminary study an automatic magnetic cell sorter was used in order to eliminate spermatozoa with the compromised membrane from the ram semen samples thereby improving their quality.

MATERIAL AND METHODS

Five sexually mature and clinically healthy rams of Native Wallachian (n = 3) and Slovak Dairy (n = 2) sheep breeds aged 2-4 years were used in this preliminary study. They were kept in external conditions in individual stalls at the breeding facility (NPPC, RIAP Nitra, Lužianky, Slovak Republic), fed with hay bale and oats; water and mineral salt were supplied *ad libitum*. Prior to the experiment, semen samples were collected once a week by an electro-ejaculation for the duration of several weeks, as described previously (Baláži *et al*., 2020). The experimental ram sperm samples were immediately after collection transferred to the laboratory in the water bath for the subsequent processing.

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

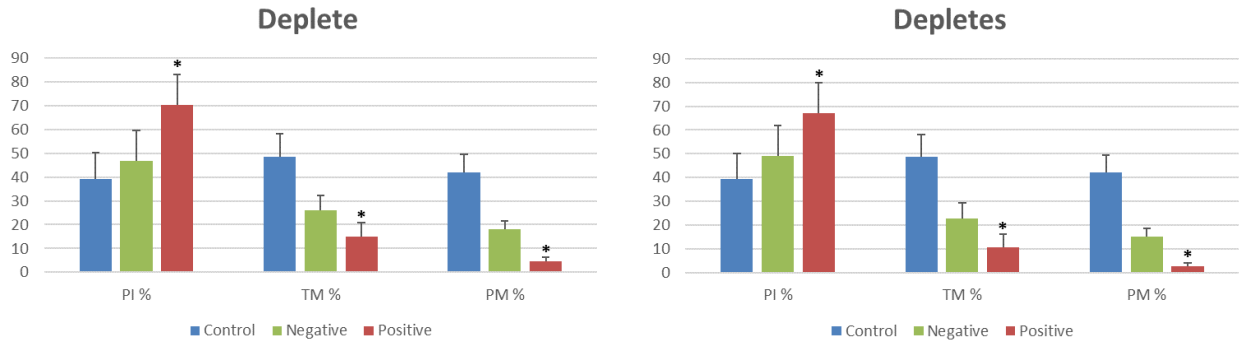
Aliquots of each semen samples (10^8 cells) were diluted in 1 ml of Dead Cell Removal kit (Miltenyi Biotec, Germany) and incubated for 15 min at room temperature according to the producer's manual. After incubation, AutoMACS Pro Separator (Miltenyi Biotec, Germany) was used to remove the dead spermatozoa from the ram semen sample. Since the commercial kit required a buffer with calcium for a proper binding of nanoparticles to the cells, 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. This instrument provides several sorting procedures. Two basic depletion programmes (Deplete and Depletes) were used in this preliminary study to sort semen samples from each ram. According to the producer's manual, first depletion procedure with loading rate at 4 mL.min^{-1} should be used in case if recovery of sample is of the highest priority. On the contrary, the second one should be used when a purity of sample is of the highest priority. This programme operates in a sensitive mode with a loading rate at 1 mL.min^{-1}.

Each fresh ram semen sample (control) as well as the negative and positive fractions obtained from MACS sorting were stained with propidium iodide (PI at 50 µg.mL^{-1}; Molecular Probes, USA) in order to determine the proportion of dead cells within the control and MACS-sorted semen samples. Stained samples (at least 10,000 cells) were immediately analysed using a FACS Calibur flow cytometer (BD, San Jose, CA, USA)

Motility parameters and the percentage of dead cells were evaluated using the SigmaPlot software (Systat Software Inc., Germany) with one-way ANOVA (Dunnett's method) and expressed as the mean ± SEM. *P*-values at $P < 0.05$ were considered as statistically significant.

RESULTS AND DISCUSSION

As far as we know, this is the first study aimed at the MACS removal of apoptotic and dead ram spermatozoa. Ram spermatozoa were sorted by fully automated cell sorter using two depletion procedures that differ in the speed of sample loading. Independently of the used sorting programme, the proportion of dead cells analysed by PI staining increased significantly ($P < 0.05$) in the positive fractions compared to control samples (Figure 1). On the other hand, the number of PI positive cells in the negative fractions did not differ from the control samples. A significant decrease ($P < 0.05$) in the total and progressive motility of spermatozoa was observed in positive fractions. However, the spermatozoa motility in negative fractions was not improved after both MACS sorting procedures and a slight decrease in both parameters, although insignificant in comparison to control samples, was noticed. No differences in the percentage of dead cells between



Deplete – sorting programme with loading rate at 4 mL.min^{-1}; Depletes – sorting programme with loading rate at 1 mL.min^{-1}; PI – percentage of dead cells stained with propidium iodide; TM – percentage of totally motile spermatozoa; PM – percentage of progressively motile spermatozoa; Control – fresh semen samples before sorting; Negative – negatively sorted spermatozoa; Positive – positively sorted spermatozoa; asterisk (*) – statistically significant at $P < 0.05$ in comparison to control sample.

Figure 1. Changes in the motility parameters and proportion of dead spermatozoa after MACS sorting of ram semen samples using two depletion programmes

negative fractions and control samples were noticed also in our previous study (Vasicek *et al*., 2014b), where the effect of Annexin V Microbeads kit and Dead Cell Removal kit on rabbit sperm quality was compared. Moreover, similarly to present study, the motility parameters of negatively sorted spermatozoa also decreased significantly ($P < 0.05$), when compared to controls.

Dead cell removal kit requires a special binding buffer that contains calcium. Thus, the principle of the staining procedure is similar to those for Annexin V Microbeads. In addition, several previous studies (Paasch *et al*., 2003; Delbès *et al*., 2013; Grunewald and Paasch, 2013; Merino-Ruiz *et al*., 2019) referred that this kit contains annexin V microbeads. We can therefore conclude that both kits are very similar in the terms of their function. Moreover, an insignificant loss of progressive motility was also noticed in the negatively sorted human sperm after MACS procedure (Paasch *et al*., 2003), whereas the positively sorted spermatozoa were almost immotile.

CONCLUSION

Present preliminary study indicates the possible use of MACS technique to remove the spermatozoa with compromised membrane from the ram semen samples. However, any of the used automatic cell sorting procedures did not significantly improve the quality of tested semen samples in the terms of their viability and motility parameters. Moreover, the chosen AutoMACS programmes seem not to be effective enough in case of the obtained purity of negatively sorted spermatozoa that could be a reason for these unsatisfying results. Anyway, more sensitive sorting procedures that are provided by the used automatic cell sorter should be tested in further studies.

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