

*Short communication***THE POTENTIAL USE OF MAGNETIC ACTIVATED CELL SORTING FOR ELIMINATION OF RABBIT APOPTOTIC SPERMATOZOA**J. VAŠÍČEK^{1,2}, V. PARKÁNYI¹, Ľ. ONDRUŠKA¹, A. MAKAREVICH¹, P. CHRENEK^{1,2*}¹Animal Production Research Centre Nitra; Slovak Republic²Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Slovak Republic**ABSTRACT**

The aim of our study was to verify the potential use of the MACS technique for elimination of rabbit apoptotic spermatozoa from insemination dose in order to improve fertilization and kindling rate in rabbit farming. MACS, using annexin V–conjugated superparamagnetic nanoparticles, proved to be an effective method for separating non-apoptotic spermatozoa from those with deteriorated plasma membranes based on the externalization of phosphatidylserine. Ejaculates from 11 New Zealand White rabbit males, chosen basing on motility parameters, were collected using an artificial vagina, mixed to make heterospermic pool and routinely diluted in a commercial insemination diluent (MiniTüb, Tiefenbach, Germany) at the ratio of 1:6. The incubation with annexin V and the binding to nanoparticles in magnetic field did not essentially affect the fertilizing ability of rabbit spermatozoa. No significant differences between the control and MACS – group were found both in the average number of liveborn kits (8.57 ± 0.59 vs. 7.50 ± 0.65) or the kindling rate (62.9% vs. 75.8%). In conclusion, these preliminary results may indicate the possible harmless and efficient use of MACS technique for improving the quality of rabbit insemination dose. However, further experiments are required in order to consider about applying of this technique into breeding practice.

Key words: rabbit; spermatozoa; magnetic-activated cell sorting (MACS); kindling rate**INTRODUCTION**

Apoptosis (genetically programmed cell death) is a physiological process running continuously, which maintains optimal number of generative cells in testes with the assistance of Sertoli cells (Sinha Hikim and Swerdloff, 1999). The phenotypic expression of apoptosis has been in relation to the presence of abnormal spermatozoa in semen (Sakkas et al., 1999; Barroso et al., 2000; Sakkas et al., 2002). The failure to eliminate these abnormal spermatozoa during the spermatogenesis can lead to their presence in semen (Sakkas et al., 1999;

Barroso et al., 2000; Sakkas et al., 2002).

One of the features of apoptosis in mammalian spermatozoa is an externalization of phospholipid – phosphatidylserine (PS), which is normally present on the inner leaflet of the sperm plasma membrane (Oosterhuis et al., 2000). Annexin V is a phospholipid-binding protein that has a high affinity for PS, but lacks the ability to pass through an intact sperm membrane (van Heerde et al., 1995). Therefore, any binding between annexin V and PS indicates that the membrane integrity has been compromised (Glander and Schaller, 1999). MACS, using annexin V–conjugated superparamagnetic nanoparticles,

***Correspondence:** E-mail: chrenekp@yahoo.comPeter Chrenek, Animal Production Research Centre Nitra, Hlohovecká 2,
951 41 Lužianky, Slovak Republic

Tel.: +421 37 6546285

Fax: +421 37 6546189

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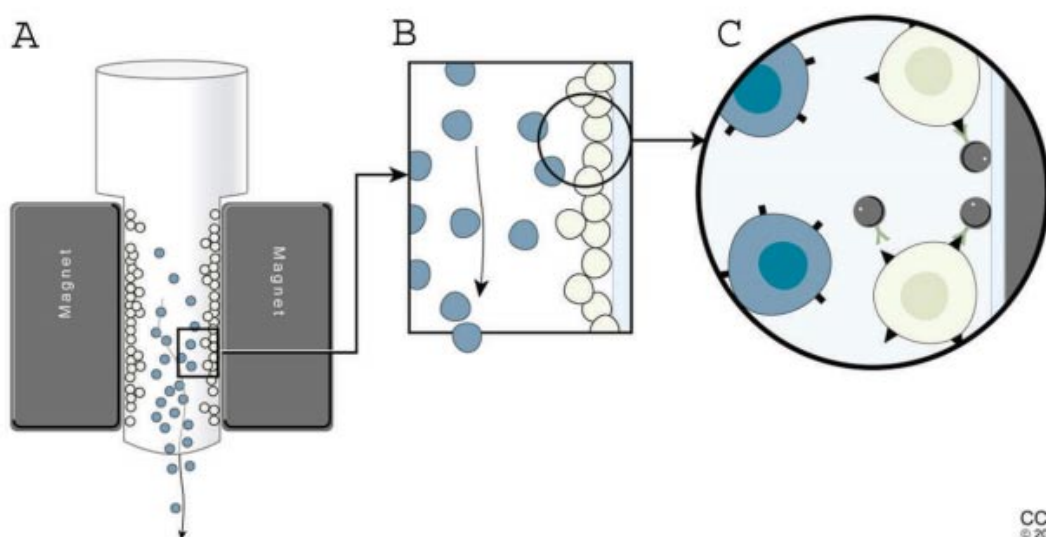
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proved to be an effective method for separating non-apoptotic spermatozoa from those with deteriorated plasma membranes based on the externalization of PS.

As the colloidal magnetic particles are extremely small (<100nm), the use of a high-gradient magnetic field is required to retain the labeled cells, approaching 1 tesla (1 tesla = 10,000 gauss) and the local gradients of up to 1000 tesla per meter (Williams et al., 1999; Zhang et al., 2005). The MidiMACS column (Miltenyi Biotec, Bergisch Gladbach, Germany) is specifically designed to generate this strong magnetic field while maintaining optimal cell viability and function. By using a MACS column with a biocompatible coating of ferromagnetic solid support placed in a powerful magnet,

the magnetic force is sufficient to retain the target cells labeled with minimum microbeads (Manz et al., 1995). MACS microbeads are superparamagnetic particles that are coupled to highly specific monoclonal antibodies. They are used to magnetically label the target cell population. They are not visible with light microscopy, are biodegradable, and are gentle on cells (Miltenyi et al., 1990). The column schematic is shown in Figure 1. By rinsing the column with buffer, the unlabeled cells are washed out without affecting the labeled cell fraction, thus ensuring optimal recovery. Once the column is removed from the magnet, the labeled fraction can be obtained (Abts et al., 1989).

Figure 1: Schematic diagram of MiniMACS magnetic cell separation column (Said et al., 2008)



MACS separation of spermatozoa using annexin V-conjugated superparamagnetic nanoparticles yields two fractions: annexin V negative (intact membranes, non-apoptotic) and annexin V positive (externalized PS, apoptotic; Grunewald et al., 2001; Glander et al., 2002). Said et al. (2005) and de Vantéry Arrighi et al. (2009) prepared sperm separation protocol that combines MACS with double density gradient centrifugation. This new combination yields spermatozoa with superior quality in terms of motility, viability and apoptotic indexes compared with others conventional methods.

Our goal was to verify a potential use of the MACS technique for elimination of rabbit apoptotic

spermatozoa from insemination dose in order to improve fertilization and kindling rate in rabbit farming.

MATERIAL AND METHODS

Semen collection and analysis

Semen samples from 25 New Zealand White (NZW) males were collected using an artificial vagina. Each sample of fresh ejaculate was evaluated using CASA (Computer Assisted Semen Analysis; MiniTüb, Tiefenbach, Germany) system for concentration and motility. For artificial insemination (A.I.) the best 11

bucks were chosen basing on motility parameters. Their ejaculates were mixed to make heterospermic pool and routinely diluted in a commercial insemination diluent (MiniTüb, Tiefenbach, Germany) at the ratio of 1:6. From the diluted rabbit semen 7 ml were took off and placed into prepared tubes for both groups, the control group and the experimental group intended for the magnetic separation.

Magnetic separation

The diluted rabbit spermatozoa (7 ml) were incubated for 15 min at 37 °C in 200 µl of annexin V-conjugated nanoparticles (Annexin V Microbead Kit, Germany) according to the producer' manual. The MidiMACS Magnetic Cell Sorting system (Miltenyi Biotec, Germany) was used for the MACS assay of rabbit spermatozoa in insemination dose (I.D.) at 37 °C in order to avoid heat shock.

Insemination

Sexually mature and clinically health rabbits, involved in the rearing programme, were used for artificial insemination (2 repeats, in September and April). For the experiments 57 does and 11 bucks of New Zealand White breed, reared in partially air-conditioned hall of a local rabbit farm, were used.

Females of NZW rabbits were inseminated either with fresh doses of diluted heterospermic semen (control; n = 28; 0.5 ml I.D. per female) or with magnetically separated semen (n = 29; 0.5 ml I.D. per female). PMSG (25 I.U.; Sargon, Bioveta, Czech Republic) was administrated to each doe 48 hours before A.I.

Immediately following A.I. synthetic GnRH (2.5 µg; Supergestran, Ferring-Pharmaceuticals, Czech Republic) was intramuscularly injected into each doe.

The ratio of kindled does to the number of inseminated does (kindling rate) and also the average number of liveborn kits per 1 inseminated doe were evaluated. Chi-square (χ^2) test was used for the evaluation of kindling rate. Average number of liveborn kits per doe was statistically evaluated using t-test in SigmaPlot (Systat Software Inc., Germany). Results are expressed as means \pm SEM.

RESULTS AND DISCUSSION

The control group of does, which was inseminated with diluted but untreated spermatozoa, resulted in an average of 8.57 ± 0.59 liveborn kits. In comparison, in the experimental group of does inseminated with MACS separated spermatozoa, there was a slight decrease in average number of liveborn kits per 1 inseminated doe (7.50 ± 0.65 ; Table 1), but this difference was not statistically significant. On the other hand, the kindling rate in the experimental group was about 12.9% higher than the kindling rate in the control group (75.8% vs. 62.9%; Table 1), although this difference was not statistically significant. Similarly, higher kindling rate in the experimental group of NZW rabbit does, inseminated with annexin V-negative (non-apoptotic) spermatozoa, was observed in our previous experiments (Vasicek et al., 2010).

Table 1: The fertility traits of MACS separated and control (untreated) rabbit spermatozoa

Trait	Control (untreated) sperm	MACS separated sperm
Number of inseminated does (n)	28	29
Average number of liveborn kits per doe	8.57 ± 0.59	7.50 ± 0.65
Kindling rate (%)	62.9	75.8

The incubation with annexin V and the reaction with nanoparticles in magnetic field did not essentially affect the fertilizing ability of rabbit spermatozoa that is proved by in vivo insemination test (average number of liveborn kits per 1 inseminated doe; Table 1). Paasch et al. (2003) reported that the separation columns and their magnetic field did not exert detectable adverse effect on the motility of spermatozoa in their experiments, which satisfies the prerequisite for harmless application in the clinical laboratory. Magnetic tagging and separation has

been shown not to affect cell viability or proliferation of various cell types and is already used to extract active germ cells from animal testis (von Schönfeldt et al., 1999) as well as from human testicular cancer (Meng et al., 1996).

MACS method has been developed as a flexible, easy and rapid cell separation technique that has ability to separate spermatozoa based on molecular characteristics of the sperm (Makker et al., 2008). The ability of annexin V to detect PS in spermatozoa suspensions depends on

the primary spermatozoa membrane structure, cytological stress treatments (Glander and Schaller, 2001), or the quality of the membrane bilayer (Schiller et al., 2000). Apoptosis has a detrimental effect on the male fertility. The specificity of annexin V binding to PS allows a magnetic depletion of dead and apoptotic cells (Martin et al., 1995) and thus potentially improves the quality of semen used for artificial insemination in rabbit farming.

CONCLUSION

Our preliminary results may indicate the possible harmless and efficient use of MACS technique to improve the quality of the rabbit insemination dose. However, further experiments are required in order to consider about applying of this technique into breeding practice.

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