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IMPACT OF THE MACS ON ELIMINATION OF APOPTOTIC SPERMATOZOA FROM RABBIT EJACULATES

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ABSTRACT

The objective of this study was to assess the effectiveness of the MACS technique used for the elimination of apoptotic rabbit spermatozoa from heterospermic pool (Experiment 1) as well as from the ejaculates of individual bucks (Experiment 2). The semen samples from control (untreated) and magnetically separated spermatozoa (in both E1 and E2) were evaluated by fluorescence analysis using Annexin-V-FLUOS Staining Kit (Roche Slovakia, Slovak Republic). Superparamagnetic microbeads conjugated with annexin V eliminated spermatozoa with externalized phosphatidylserine via MACS. MACS separation of spermatozoa yields two fractions: the annexin V-negative (AnV^-) and the annexin V-positive (AnV^+). The number of AnV^+ sperm was significantly lower ($P<0.001$) in the AnV^- fractions than in the AnV^+ fractions (in both E1 and E2). Our observations indicate that MACS technique could be an adequate method for the elimination of apoptotic spermatozoa with externalized phosphatidylserine from the rabbit ejaculates. However, further experiments are required in order to prove this suggestion.

Key words: rabbit; spermatozoa; MACS; annexin V

INTRODUCTION

When artificial insemination (AI) is applied in a rabbitry, it is estimated that one single buck may affect the fertility and prolificacy of about one hundred does (Seleem, 2005). Thus, the bucks employed in AI must have good genetic characteristics and provide a good semen yield both in terms of quality and quantity (Panella and Castellini, 1990; Battaglini, 1992; Castellini and Dal Bosco, 1998). There are many factors influencing the quality and quantity of rabbit semen such as breed (Amin *et al*., 1987), male (Castellini, 1996), age (Gogol *et al*., 2002), season (Bodnar *et al*., 2000), photoperiod (Theau-Clement *et al*., 1995), nutrition (Fodor *et al*., 2003), collection rhythms (Nizza *et al*., 2003) and transgenesis (Chrenek *et al*., 2007).

Henkel *et al.* (2004) and Seli *et al*. (2004) observed that the presence of apoptotic spermatozoa during in vitro fertilization (IVF) can be one of the reasons for obtaining suboptimal fertility results. The phenotypic expression of apoptosis has been in relation to the presence of abnormal spermatozoa in semen. 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). Therefore, selection and elimination of apoptotic spermatozoa is one of the necessary requirements for achieving optimal assisted reproduction outcomes. For this purpose the MACS (magnetic-activated cell sorting) technique is used in human medicine (Said *et al*., 2006a).

Principle of the magnetic separation is difference in membrane characteristics of separated cells (Said *et al*., 2006b). The translocation of phospholipid phosphatidylserine (PS) is one of the earliest detectable features of cells undergoing the initial steps of apoptosis. PS has a high and selective affinity for annexin V (Van Heerde *et al.*, 1995). Superparamagnetic microbeads

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conjugated with annexin V eliminated spermatozoa with externalized phosphatidylserine via MACS (Meng *et al.*, 1996). MACS separation of spermatozoa yields two fractions: the annexin V-negative (intact membranes, non-apoptotic) and the annexin V-positive (externalized PS, apoptotic) (Glander *et al*., 2002). The selection of non-apoptotic spermatozoa may improve sperm quality complementary to other separation techniques and assure optimal conception rates in human and animal assisted reproduction (Said *et al.*, 2005; Vasicek *et al*., 2010, 2011a).

The objective of this study was to assess the effectiveness of the MACS technique used for the elimination of apoptotic rabbit spermatozoa from heterospermic pool as well as from the ejaculates of individual bucks.

MATERIAL AND METHODS

Animals

Sexually mature (2 - 3 years old) and clinically health rabbit bucks (n = 11) of broiler New Zealand White (NZW) line reared in a partially air-conditioned hall of a local rabbit farm at APRC Nitra (Animal Production Research Centre, Lužianky, Slovak Republic) were used in the experiments. The animals were housed in individual cages, under a constant photoperiod of 14 h of light day. Temperature and humidity in the building were recorded continuously by means of a thermograph positioned at the same level as the cages (average relative humidity and temperature during the year was maintained at 60 ± 5 % and 17 ± 3°C). The rabbits were fed *ad libitum* with a commercial diet (KV; TEKRO Nitra Ltd., Slovak Republic) and water was provided *ad libitum* with nipple drinkers.

The treatment of the animals was approved by the Ministry of Agriculture and Rural Development of the Slovak Republic, no. SK P 28004 and Ro 1488/06-221/3a.

In this study the control (untreated) and magnetically separated spermatozoa from heterospermic pool (Experiment 1) as well as from the ejaculates of individual bucks (Experiment 2) were used for fluorescence analysis.

Semen collection and handling

Semen samples from 25 NZW bucks were collected using an artificial vagina. Each sample of fresh ejaculate was evaluated for the concentration and motility using Sperm Vision™ (Minitübe, Tiefenbach, Germany), a computer assisted sperm motion analyser (CASA). For magnetic separation, the best 11 bucks (Experiment 1) or the best four bucks (Experiment 2) were chosen basing on motility parameters. Ejaculates from chosen bucks were collected using an artificial vagina once a week during each experiment. In the Experiment 1 (E1) the ejaculates from 11 bucks were mixed to make heterospermic pool and routinely diluted in a commercial insemination diluent (MiniTüb) at the ratio of 1:6, whereas in the Experiment 2 (E2) the ejaculates from four bucks were handled separately and diluted at the same ratio.

Before magnetic sperm separation, the sperm cells were washed out of seminal plasma to facilitate better annexin V binding to PS. For this purpose the diluted semen was carefully filtered through a Sartorius filter (2 ml per filter) with a pore size of 1.2 µm, so that seminal plasma with a diluent passed through a membrane, which was then discarded. The rabbit spermatozoa retained by filter membrane were carefully flushed out from the filter to the collection tube with 2 ml of a binding buffer (Annexin V Microbead Kit, Miltenyi Biotec, Germany). The filtered spermatozoa were diluted in a binding buffer at the ratio of 1:3.66 (E1) or 1:8 (E2). Filtered and diluted rabbit semen was divided into the experimental group, intended for the magnetic separation, and the control group (untreated semen).

MACS separation of rabbit spermatozoa

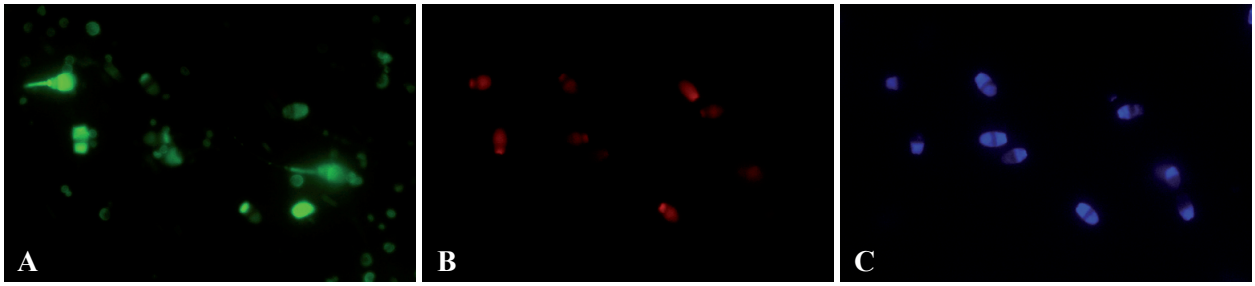
In the Experiment 1, the filtered rabbit spermatozoa were incubated with 200 µl of annexin V-conjugated nanoparticles (Annexin V Microbead Kit, Germany) for 15 min at room temperature according to the original protocol (Miltenyi Biotec). The MidiMACS Magnetic Cell Sorting system (Miltenyi Biotec, Germany) was used for MACS assay of rabbit spermatozoa at room temperature. The MACS LD column was placed into the magnetic field of a MACS Separator and prepared by washing with 1 ml of a binding buffer. The filtered rabbit spermatozoa (7 ml for LD column) incubated with annexin V-conjugated nanoparticles were applied onto the column. The annexin V-negative (AnV^-) spermatozoa passed through the column into the collection tube. Then the column was rinsed with 2 ml of a binding buffer, removed from the separator and placed onto a suitable collection tube. For the recovery of an annexin V-positive (AnV^+) fraction 1 ml of a binding buffer was pipetted onto the column and firmly flushed out using the plunger supplied with the column.

The filtered rabbit spermatozoa in the Experiment 2 were processed as described previously by Vasicek *et al.* (2011b).

Apoptosis assay *in situ* (annexin V/PI/DAPI)

For annexin V analysis semen samples obtained from control group (untreated), negative (AnV^-) and positive (AnV^+) fractions were in both experiments (E1 and E2) processed using Annexin-V-FLUOS Staining Kit (Roche Slovakia, Slovak Republic). Each sample

(10^6 cells) was centrifuged at 670 g for 8 min, resuspended in 500 µl of binding buffer (provided with the Kit) and centrifuged again as previously. Semen suspension (30 µl) was mixed with 50 µl of AnV/PI staining solution and incubated for 25-30 min at room temperature. Annexin V – FLUOS (4 µl), PI (4 µl) and buffer (192 µl; Annexin-V-Fluos staining kit) were mixed together in order to prepare 200 µl of the AnV/PI staining solution. After incubation samples were washed in 500 µl of binding buffer and centrifuged. Then aliquots of the semen suspension (4 µl) were placed between microslide and coverslip into 4µl of the Vectashield anti-fade medium containing DAPI fluorescent dye (Vector Laboratories, Burlingame, CA, USA). At least 200 spermatozoa were checked for staining and counted under a Leica fluorescent microscope (Leica Microsystem, Germany) at magnification 400x using 488 nm, 535 nm or 420 nm wave-length filters, respectively. The spermatozoa with the annexin V-positive membrane exhibited green fluorescence, dead spermatozoa exhibited red fluorescence, whilst total spermatozoa count was identified by blue signal due to DAPI staining (Fig. 1).



A – fluorescein - FITC (apoptotic spermatozoa), B – propidium iodide (dead spermatozoa), C – DAPI (total spermatozoa count)

Fig. 1: Fluorescent staining of the rabbit spermatozoa

Statistical analysis

Obtained results were evaluated statistically by one-way ANOVA (Holm-Sidak) using SigmaPlot software (Systat Software Inc., Germany) and expressed as the means ± SEM. P-values at $P<0.05$ were considered as statistically significant.

RESULTS AND DISCUSSION

In the Experiment 1, significantly higher ($P<0.001$) proportion of apoptotic spermatozoa was found in the AnV^+ fraction compared to the AnV^- fraction as well as to control samples. However, there were no significant differences in percentage of apoptotic cells between AnV^- spermatozoa and control samples as well as in proportion of dead cells among the all semen samples. Similarly, in the Experiment 2, we observed significantly higher ($P<0.001$) percentage of apoptotic as well as dead cells in AnV^+ fractions in comparison to AnV^- fractions and control samples, whereas there were no differences in percentage of apoptotic or dead cells between AnV^- spermatozoa and control samples (Table 1).

The use of the annexin V assay in livestock animals for the identification of different sperm subpopulations has already been documented (Chaveiro *et al*., 2007; Peña *et al.*, 2003). Staining of cells with a combination of Annexin V and PI allows simultaneous distinguishing among live, apoptotic or necrotic sperm populations. This method has been used by two authors to investigate sperm apoptosis, but conflicting results have been obtained (Glander and Schaller, 1999; Oosterhuis *et al*., 2000). In the first study the percentage of apoptotic sperm in the ejaculate positively correlated with motility, while in the second study a negative correlation was observed between apoptotic cells and sperm motility and concentration. This difference could be due to the different method used and/or to the different patient population studied, or more probably to the fact that in one study the analysis was carried out on a whole semen (Oosterhuis *et al.*, 2000) and in the other study it was carried out on sperm separated from seminal plasma by Percoll density gradient centrifugation (Glander and Schaller, 1999). In our experiments (E1 and E2) we noticed similar observation as in the second mentioned study (Glander and Schaller, 1999). Percentage of apoptotic sperm in AnV^+ fractions (Table 1) that were washed out from seminal plasma by filtration through Sartorius filter apparently negatively correlated with the total and progressive spermatozoa motility (data not published). Moreover, we compared PS externalization (annexin V assay) between the annexin V-negative and -positive fractions separated by MACS to assess the efficiency of MACS separation. The technique appears

Table 1: Proportion of apoptotic (AnV) or dead (PI) cells in MACS treated and control (untreated) rabbit spermatozoa

SEMEN SAMPLE		AnV/DAPI (%)	PI/DAPI (%)
Heterospermic pool	Control	4.69 ± 0.69a	2.74 ± 0.94
(Experiment 1)	AnV^-	3.48 ± 0.78a	3.48 ± 1.00
	AnV^+	16.83 ± 0.92b	3.00 ± 0.96
Individual buck	Control	5.97 ± 0.84a	7.12 ± 1.24a
(Experiment 2)	AnV^-	6.60 ± 0.78a	5.22 ± 1.06a
	AnV^+	59.53 ± 6.00b	34.95 ± 6.40b

Results are expressed as means ± SEM; a vs b were statistically significant at $P<0.001$

to be adequate because the number of PS-positive (annexin-positive) sperm was lower in the AnV^- fractions than in the AnV^+ fractions (Table 1) similarly as reported by Said *et al.* (2006a) (3.4 ± 1.7 % vs. 54.9 ± 18.1 %, $P<0.001$), although there were no statistical differences in proportion of apoptotic and dead cells between AnV^- fractions and control samples. Minimal PS externalization was noted in the AnV^- fractions, whereas a considerable number of spermatozoa that stained negative for PS were found in the AnV^+ fractions. The absence of PS externalization in some spermatozoa in the AnV^+ fractions may be because beads have already blocked the PS binding sites. In addition, annexin V can also bind to other enzymes such as protein kinases, and phospholipids such as PE (phosphatidylethanolamine), despite high affinity for PS (Said *et al*., 2006a).

We found some difference in the number of apoptotic spermatozoa within AnV^+ fractions between Experiment 1 and Experiment 2 (Table 1). This could be due to several factors. Since experiments were carried out during different seasons, there may be a seasonal influence on male fertility parameters. Other factors may be different batch of the kit or the type of column used for magnetic separation in Experiment 1 and Experiment 2. The use of flow cytometry for the annexin V assay could be more objective. Moreover, the small size of microbeads, about 50 nm in diameter, is advantageous in flow cytometry because bound microbeads are unable to change the scatter properties of spermatozoa (Miltenyi *et al*., 1990).

CONCLUSION

Our obtained results indicate that the MACS technique could be an adequate method for the elimination of apoptotic spermatozoa with externalized phosphatidylserine from the rabbit ejaculates. However, because of some discrepancies further experiments are required in order to prove this suggestion.

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REFERENCES

AMIN, S. O. – EL-FOYLY, M. A. – EL-SHOBHY, H. – EL-SHEEBINY, A. H. 1987. Effect of season, breed and sequence of ejaculation on some physical characteristics of rabbit semen. *Proceedings of the First Conference on Agriculture Development*, Cairo: Sham University, Anim. Prod., vol. 1, 1987, p. 54-67.

BARROSO, G. – MORSHEDI, M. – OEHNINGER, S. 2000. Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. *Hum. Reprod.*, vol. 15, 2000, p. 1338-1344.

BATTAGLINI, M. 1992. Fecondazione artificiale, attenti al maschio. *Riv. Coniglic.*, vol. 5, 1992, p. 47-51.

BODNAR, K. – SZENDRO, Z. – NEMETH, E. B. – EIBEN, C. – RADNAI, I. 2000. Comparative evaluation of abnormal spermatozoa in Pannon White, New Zealand White and Angora rabbit semen. *Arch. Anim. Breed.,* vol. 43, 2000, no. 5, p. 507-512.

CASTELLINI, C. – DAL BOSCO, A. 1998. Inseminazione artificiale nel coniglio: fisiologia del maschio, produzione e conservazione del seme. *Large Animals Review*, vol. 4, 1998, p. 87-94.

CASTELLINI, C. 1996. Confronto tra fecondazione natural e inseminazione artificiale con mestrui differenti nel coniglio. *Riv. Coniglic.*, vol. 2, 1996,

p. 57-59.
CHAVEIRO, A. – DA SILVA, F. – SANTOS, P. 2007. Assessment of Sperm Apoptosis in Cryopreserved Bull Semen After Swim-up Treatment: A Flow Cytometric Study. *Reprod. Domest. Anim*., vol. 42, 2007, p. 17-21.
CHRENEK, P. – TRANDZIK, J. – MASSANYI, P. – MAKAREVICH, A. – LUKAC, N. – PESKOVICOVA, D. – PALEYANDA, R. K. 2007. Effect of transgenesis on reproductive traits of rabbit males. *Anim. Reprod. Sci.*, vol. 99, 2007, p. 127-134.
FODOR, K. – ZOLDAG, L. – FEKETE, S. G. – BERSENYI, A. – GASPARD, A. – ANDRASOFSZKY, E. – KULCSAR, M. – ESZES, F. – SHANI, M. 2003. Influence of feconding intensity on the growth, body composition and sexual maturity of male New Zealand White rabbits. *Acta Vet. Hung*., vol. 51, 2003, no. 3, p. 305-319.
GLANDER, H. J. – SCHALLER, J. 1999. Binding of annexin V to plasma membranes of human spermatozoa: a rapid assay for detection of membrane changes after cryostorage. *Mol. Hum. Reprod.*, vol. 5, 1999, p. 109-115.
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*, vol. 34, 2002, p. 360-366.
GOGOL, P. – BOCHENEK, M. – SMORAG, Z. 2002. Effect of rabbit age on spermatozoa chromatin structure. *Reprod. Dom. Anim*., vol. 37, 2002, no. 2, p. 92-95.
HENKEL, R. – HAJIMOHAMMAD, M. – STALF, T. – HOOGENDIJK, C. – MEHNERT, C. – MENKVELD, R. – GIPS, H. – SCHILL, W. B. – KRUGER, T. F. 2004. Influence of deoxyribonucleic acid damage on fertilization and pregnancy. *Fertil. Steril.*, vol. 81, 2004, p. 965-972.
MENG, F. J. – ZHOU, Y. – SKAKKEBAEK, N. E. – MARKS, A. – GIWERCMAN, A. 1996. Detection and enrichment of carcinoma-in-situ cells in semen by an immunomagnetic method using monoclonal antibody M2A. *Int. J. Androl*., vol. 19, 1996, p. 365 -370.
MILTENYI, S. – MÜLLER, W. – WEICHEL, W. – RADBRUCH, A. 1990 High gradient magnetic cell separation with MACS. *Cytometry*, vol. 11, 1990, p. 231-238.
NIZZA, A. – DI MEO, C. – TARANTO, S. 2003. Effects of collection rhythm and season on rabbit semen production. *Reprod. Dom. Anim.*, vol. 38, 2003, p. 436-439.
OOSTERHUIS, G. J. – MULDER, A. B. – KALSBEEK-BATENBURG, E. – LAMBALK, C. B. – SCHOEMAKER, J. – VERMES, I. 2000. Measuring apoptosis in human spermatozoa: a biological assay for semen quality? *Fertil. Steril*., vol. 74, 2000, p. 245 -250.
PANELLA, F. – CASTELLINI, C. 1990. Fattori ambientali e genetici che influiscono sulle caratteristiche del seme di coniglio. *Riv. Coniglic.*, vol. 8, 1990, p. 39 -41.
PEÑA, F. – 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*, vol. 60, 2003, p. 677-689.
SAID, T. M. – AGARWAL, A. – GRUNEWALD, S. – RASCH, M. – BAUMANN, T. – KRIEGEL, C. – LI, L. – GLANDER, H. J. – THOMAS, A. J. JR. – PAASCH, U. 2006a. Selection of nonapoptotic spermatozoa as a new tool for enhancing assisted reproduction outcomes: an in-vitro model. *Biol. Reprod.*, vol. 74, 2006a, p. 530-537.
SAID, T. M. – AGARWAL, A. – GRUNEWALD, S. – RASCH, M. – GLANDER, H. J. – PAASCH, U. 2006b. Evaluation of sperm recovery following annexin V magnetic-activated cell sorting separation. *Reprod. Biomed. Online*, vol. 13, 2006b, p. 336-39.
SAID, T. M. – GRUNEWALD, S. – PAASCH, U. – GLANDER, H. J. – BAUMANN, T. – KRIEGEL, C. – LI, L. – AGARWAL, A. 2005. Advantage of combining magnetic cell separation with sperm preparation techniques. *Reprod. Biomed. Online*, vol. 10, 2005, p. 740-746.
SAKKAS, D. – MARIETHOZ, E. – ST JOHN, J. C. 1999. Abnormal sperm parameters in humans are indicative of an abortive apoptotic mechanism linked to the Fas-mediated pathway. *Exp. Cell Res*., vol. 251, 1999, p. 350-355.
SAKKAS, D. – MOFFATT, O. – MANICARDI, G. C. – MARIETHOZ, E. – TAROZZI, N. – BIZZARO, D. 2002. Nature of DNA damage in ejaculated human spermatozoa and the possible involvement of apoptosis. *Biol. Reprod.*, vol. 66, 2002, p. 1061-1067.
SELEEM, T. S. T. 2005. Some reproductive; productive and physiological aspects of purebred and crossbred Elander and New-Zealand White rabbits under Egyptian environmental conditions. *Proc. of the 4th Inter. Con. on Rabbit Prod. in Hot Clim.*, Sharm El-Sheikh (Egypt), 2005, p. 161-168.
SELI, E. – GARDNER, D. K. – SCHOOLCRAFT, W. B. – MOFFATT, O. – SAKKAS, D. 2004. Extent of nuclear DNA damage in ejaculated spermatozoa impacts on blastocyst development after in vitro fertilization. *Fertil. Steril*., vol. 82, 2004, p. 378 -383.
THEAU-CLEMENT, M. – ESPARBIE, M. N. – BOLET, G. 1995. Effects of artificial photoperiods on sexual

behaviour and sperm output in the rabbit. *Anim. Sci.*, vol. 60, 1995, p. 143-149.

VAN HEERDE, W. L. – DE GROOT, P. G. – REUTELINGSPERGER, C. P. 1995. The complexity of the phospholipid binding protein Annexin V. *J. Thromb. Haemost.*, vol. 73, 1995, p. 172-179.

VASICEK J. – MAKAREVICH A. V. – CHRENEK P. 2011b. Effect of the MACS technique on rabbit sperm motility. *Central Eur. J. Biol.*, vol. 6, 2011b, p. 958-962.

VASICEK, J. – PARKANYI, V. – ONDRUSKA, L. – MAKAREVICH, A. – CHRENEK, P. 2011a. Elimination of Apoptotic Spermatozoa from Rabbit Insemination Dose Using Annexin V Associated with the MACS Technique. A Preliminary Study. *Folia Biol. (Krakow)*, vol. 59, 2011a, p. 65-69.

VAŠÍČEK, J. – PARKÁNYI, V. – ONDRUŠKA, L. – MAKAREVICH, A. – CHRENEK, P. 2010. The potential use of magnetic activated cell sorting for elimination of rabbit apoptotic spermatozoa. *Slovak J. Anim. Sci.*, vol. 43, 2010, p. 205-209.