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Reproductive Performance of New Zealand White Rabbits after Depletion of Apoptotic Spermatozoa*

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The objective of this study was to assess the utility of the magnetic-activated cell sorting (MACS) technique used for improving characteristics and quality of insemination doses by the elimination of apoptotic rabbit spermatozoa from a heterospermic pool (Experiment 1) as well as from the ejaculates of individual bucks (Experiment 2). Superparamagnetic microbeads conjugated with annexin V eliminated spermatozoa with externalized phosphatidylserine via MACS. The control (untreated) and magnetically separated spermatozoa (in both E1 and E2) were used for artificial insemination of hormonally treated rabbit does. MACS separation of spermatozoa yielded two fractions: annexin V–negative (AnV^-) and annexin V-positive (AnV^+). The CASA analysis after MACS sperm sorting revealed that the proportion of apoptotic spermatozoa in the semen of New Zealand White bucks varied from 7 to 20%. Transmission electron microscopy revealed that MACS treatment might eliminate spermatozoa with membrane damages and released acrosomal matter. However, the MACS separation (in both E1 and E2) did not affect the reproductive parameters of rabbit does.

Key words: rabbit, spermatozoa, fertility, MACS, CASA, TEM

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One of the most immediate benefits of artificial insemination (AI) has been the reduction in the number of males and the use of genetically best males for breeding programmes. The reliable evaluation of both semen and the fertilizing ability of bucks are of vital importance to the success of the AI technique (SAFAA *et al.* 2008a). Many factors affect seminal traits (BOITI *et al.* 2005) and therefore proper protocols to improve spermatozoa characteristics are needed (BRUN *et al.* 2002).

Successful fertilization requires functional sperm with a normal membrane status (FLESCH & GADELLA 2000). Early phases of membrane disorders are associated with asymmetry of the membrane phospholipids and changes in lipid composition (SCHILLER *et al.* 2000). In vital cells with an intact plasma membrane, the phospholipid phosphatidylserine (PS) is located on the inner leaflet of the plasma membrane only (VERMES *et al.* 1995). The externalization of PS is reported to be a membrane marker for early apoptosis, since PS exposure precedes the nuclear changes that define apoptosis and also precedes the loss of membrane integrity by several hours (MARTIN *et al.* 1995).

In the mammalian testis, germ cells clonally expand through many rounds of mitosis before undergoing the differentiation and maturation steps that result in spermatozoa. This clonal expansion is excessive, requiring that a mechanism exists to match the number of germ cells with the supportive capacity of Sertoli cells (LEE *et al.* 1997). Overproliferation of early germ cells is tempered by selective apoptosis of their progeny (BARTKE 1995; ALLAN *et al.* 1992; BILLIG *et al.* 1995). Testicular germ cell apoptosis occurs normally and

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continuously throughout life (BARTKE 1995; BILLIG *et al.* 1995).

The phenotypic expression of apoptosis has been associated with the presence of abnormal spermatozoa in semen. The failure to eliminate these abnormal spermatozoa during spermatogenesis can lead to their presence in semen (SAKKAS *et al.* 1999, 2002; BARROSO *et al.* 2000). 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. 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.* 2006). MACS separation of spermatozoa yields two fractions: annexin V-negative (intact membranes, non-apoptotic) and 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.* 2005a; VASICEK *et al.* 2010, 2011a).

The objective of this study was to assess the MACS technique used for improving characteristics and quality of insemination doses by the elimination of apoptotic rabbit spermatozoa from a heterospermic pool as well as from the ejaculates of individual bucks.

Material and Methods

Animals

Sexually mature (6-36 month old) and clinically healthy rabbit bucks (n = 11) and does (n = 314) 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, Lu ianky, Slovak Republic) were used in the experiments. The animals were housed in individual cages, under a constant photoperiod of 14 h of daylight. 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.

Experimental design

In this study the control (untreated) and magnetically separated spermatozoa from a heterospermic pool (Experiment 1) as well as from the ejaculates of individual bucks (Experiment 2) were used for artificial insemination of hormonally treated rabbit does.

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 and artificial insemination (AI), the best 11 bucks (Experiment 1) were chosen based on motility parameters. In experiment 2, the best four bucks in terms of motility characteristics chosen from the 11 bucks (experiment 1) were used. Ejaculates from the chosen bucks were collected using an artificial vagina once a week for the duration of each experiment. In experiment 1 (E1) the ejaculates from 11 bucks were mixed to make a heterospermic pool and routinely diluted in a commercial insemination diluent (MiniTüb, Tiefenbach, Germany) at the ratio of 1:6, whereas in experiment 2 (E2) the ejaculates from four bucks were handled and diluted at the same ratio separately.

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 (rabbit sperm head width is 3.9-4.2 μm, sperm head length is 8-8.4 μm; SAFAA *et al.* 2008b), 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 a collection tube with 2 ml of 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 magnetic separation and the control group (untreated semen).

Depletion of apoptotic spermatozoa

In 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 Midi-MACS 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 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 binding buffer, removed from the separator and placed onto a suitable collection tube. For the recovery of annexin V-positive (AnV^+) fraction 1 ml of binding buffer was pipetted onto the column and firmly flushed out using the plunger supplied with the column.

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

The untreated (control group) and MACS separated (experimental group) rabbit semen in both experiments (E1 and E2) were then used for AI. The insemination doses (I.D.) for the control group were prepared by the same procedure as those for the experimental group, but the spermatozoa were not treated with the MACS technique. The commercial insemination diluent used for the dilution of fresh semen and the binding buffer used for the magnetic separation should protect spermatozoa from early capacitation, which is necessary if the spermatozoa are intended for artificial insemination.

CASA

In both experiments (E1 and E2) semen samples from the control group, AnV^- and AnV^+ fraction were placed into a Standard Count Analysis Chamber Leja 20 micron (MiniTüb, Tiefenbach, Germany) and evaluated using the CASA system (Sperm Vision™) under a Zeiss Axio Scope A1 microscope. In each sample the following parameters were evaluated: the concentration ($x10^9$ per ml), percentage of motile spermatozoa (motility>5 μm/s) and percentage of progressively motile spermatozoa (motility>20 μm/s), VCL – velocity curved line (μm/s), VSL – velocity straight line (μm/s), STR – straightness (VSL:VAP) and BCF – beat cross frequency (Hz).

Evaluation of sperm membrane status using TEM (transmission electron microscopy)

Morphology of sperm head membranes (membrane status) was determined using TEM. In both experiments (E1 and E2), the semen samples (spermatozoa suspensions) from the control group and AnV^- fraction were prepared according to the protocol described by PIVKO *et al.* (2009). The sperm cells were washed in 0.15 M cacodylate buffer (pH 7.1-7.3) and embedded into agar to form pellets for electron microscopy. Agar pellets of rabbit semen were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.15 M cacodylate buffer (pH 7.1-7.3) for 1 h and then washed in cacodylate buffer. Afterwards samples were post-fixed in 1% osmium tetroxide in cacodylate buffer, washed in distilled water, dehydrated in acetone and embedded into Durcupan ACM (Fluka Analytical – Sigma-Aldrich, Switzerland). The blocks of semen were cut into semithin sections (1-2 μm) using a UC 6 Leica ultramicrotome (Leica Microsystems, MIKRO Ltd., Bratislava, Slovakia). The sections were collected on copper grids, contrasted with uranyl acetate and lead citrate, and examined on a transmission electron microscope (JEM 100 CX-II, Jeol, Japan) operating at 80kV.

Membrane status was assessed by evaluating at least 400 spermatozoa heads which were classified according to the grade of acrosome morphology and divided into four groups: grade I – sperm with intact plasma membrane of the head and intact acrosome, grade II – sperm with waved plasma membrane, grade III – sperm with swollen or damaged acrosome and grade IV – sperm with pseudo-acrosomal reaction formed by vesicles and with loss of acrosomal content (PIVKO *et al.* 2009).

Artificial insemination of rabbit does

In Experiment 1 (six repeats, in December-June) NZW rabbit does (n = 142) were inseminated either with fresh doses of filtered heterospermic semen (control; n = 62; 0.5 ml I.D. per female) or with magnetically separated semen (n = 80; 0.5 ml I.D. per female). In the second experiment (E2; four repeats, in October-February) females of NZW rabbits (n = 172) were inseminated similarly either with fresh doses of filtered individual buck semen (control; n = 20 for each buck's semen; 0.5 ml I.D. per female) or with magnetically separated semen (n = 23 for each buck's semen; 0.5 ml I.D. per female). In both experiments the insemination doses with concentration of $0.03x10^9$/ml were used for insemination. PMSG at 25 I.U. (Sergon, Bioveta, Czech Republic) was administered to each doe 48 hours before AI. Immediately following AI 2.5 μg of synthetic GnRH (Supergestran, Ferring-Pharmaceuticals, Czech Republic) was intramuscularly injected into each doe.

The conception rate (the ratio of pregnant does to the number of inseminated does) was determined by abdominal palpation performed in each doe 17 days after AI. The kindling rate (the ratio of kin-

dled does to the number of inseminated does), number of liveborn and stillborn kits were determined at parturition. Total breeding effectiveness (kindling rate x number of liveborn kits) was also calculated.

Statistical analysis

Observed results were evaluated statistically using a χ^2-test (TEM, conception and kindling rate), *t*-test (number of liveborn and stillborn kits) and one-way ANOVA (Duncan's method) using SigmaPlot software (Systat Software Inc., Germany) and expressed as means ± SEM. P-values at $P<0.05$ were considered as statistically significant.

Results and Discussion

Since there are several external or internal factors that may affect the ejaculate quality of males used for AI, it is appropriate to use some sperm preparation technique that provides enhanced quality of insemination doses. For these reasons, in this study we used the unique MACS separation technique to retrieve spermatozoa free of apoptotic or dead cells with impaired plasma membrane.

CASA

In both experiments (heterospermic pool – E1 and individual buck's semen – E2) the MACS separation divided the basic spermatozoa population into two distinct subpopulations (fractions) with a lower spermatozoa concentration. The MACS separation revealed that the proportion of apoptotic spermatozoa in the semen of NZW bucks varied from 7% (Table 1) to about 20% (VASICEK *et al.* 2011b). Similar values of sperm concentration in the control and AnV^- group were recorded in a previous study (VASICEK *et al.* 2011a). The separation column and the magnetic field had no significant influence on sperm motility. There is apparently a tendency to increase the percentage of motile and progressively motile spermatozoa in the AnV^- fractions, whereas the AnV^+ sperm had lower motility values than the AnV^- fractions and the control samples ($P<0.05$ for heterospermic pool), similarly as in individual bucks II, III and IV (VASICEK *et al.* 2011b). The motility data of the heterospermic pool (E1) are similar to those obtained in previous experiments in which the total motility and progressive motility of spermatozoa were not statistically different between control and the MACS separated group (VASICEK *et al.* 2011a). Thus, the MACS technique evidently divided rabbit spermatozoa into two subpopulations: one (AnV^- sperm) with the same semen quality as in the fresh (control) ejaculate and the other (AnV^+ sperm) with significantly worse semen quality in terms of sperm motility.

In the present study (E1 and E2) the magnetic cell sorting apparently separated out AnV^+ spermatozoa (Table 1) with decreased spermatozoa vigour (VCL and BCF) and progressiveness (VSL and STR), thus eliminating spermatozoa with insufficient fertilizing ability. Although we found no significant differences in sperm motility between AnV^- and control samples, SAID *et al.* (2005b) re-

Table 1

Motility parameters of MACS treated and control (untreated) rabbit spermatozoa

Semen sample			Before treatment	After treatment	
		Parameter	Control	AnV^-	AnV^+
Experiment 1	Heterospermic pool	Concentration %	0.291 ± 0.079 –	0.287 ± 0.067 98.6 ± 8.5[a]	0.019 ± 0.004 6.5 ± 0.5[b]
		% Motile	64.6 ± 12.3[a]	68.5 ± 6.4[a]	10.2 ± 5.0[b]
		% Progressive	50.1 ± 16.3[a]	55.1 ± 11.9[a]	3.3 ± 3.3[b]
		VCL	143.5 ± 16.9[a]	146.6 ± 16.3[a]	39.5 ± 39.5[b]
		VSL	49.0 ± 8.1	54.6 ± 8.4	15.0 ± 15.0
		STR	0.8 ± 0.1	0.8 ± 0.0	0.2 ± 0.2
		BCF	28.9 ± 3.6[a]	31.4 ± 2.9[a]	3.9 ± 3.9[b]
Experiment 2	Average values for four bucks	VCL	159.9 ± 9.5[a]	144.4 ± 6.2[a]	39.6 ± 15.7[b]
		VSL	53.2 ± 1.2[a]	55.4 ± 4.2[a]	17.4 ± 6.7[b]
		STR	0.7 ± 0.0[a]	0.8 ± 0.0[a]	0.5 ± 0.1[b]
		BCF	31.0 ± 1.3[a]	32.7 ± 1.6[a]	12.9 ± 5.1[b]

VCL – velocity curved line, VSL – velocity straight line, STR – straightness, BCF – beat cross frequency. Results are expressed as means ± SEM; [a] vs [b] were statistically significant at $P<0.05$.

ported that annexin V- negative human spermatozoa had significantly higher motility values (76 ± 15.06, P=0.03) than the raw samples (64.5 ± 6.43). However, on the basis of the observed sperm traits, the rabbit sperm quality was sufficient for artificial insemination (THEAU-CLEMENT *et al.* 1996).

Evaluation of sperm membrane status using TEM

Undamaged cell membranes are necessary for sperm viability. Membrane destabilization leads to functional capacitation, enabling fertilization of the egg by sperm. However, if sperm is deposited in the female reproductive tract at a distance from the fertilization site, or maintained *in vitro* at standard temperatures, their longevity is shortened, resulting in pre-matured sperm death (MORTIMER & MAXWELL 2004) and reduced fertility. Fertile spermatozoa have an intact acrosome and low fertility spermatozoa have either damaged acrosomes or none at all. Therefore the acrosome plays an important role in sperm survival and fertility (PAASCH *et al.* 2003).

The ultrastructural changes on the plasma membrane, acrosomal membrane and acrosome of rabbit sperm were determined using TEM (Figure 1). We observed that the elimination of apoptotic spermatozoa resulted in obtaining semen with insignificantly decreased proportion of "bad" spermatozoa (grade III plus IV) and thus insignificantly increased proportion of "good" spermatozoa (grade I plus II) in both Experiments 1 and 2 (Table 2). Sperm classified into the first two grades are expected to have a higher fertilizing capacity com-

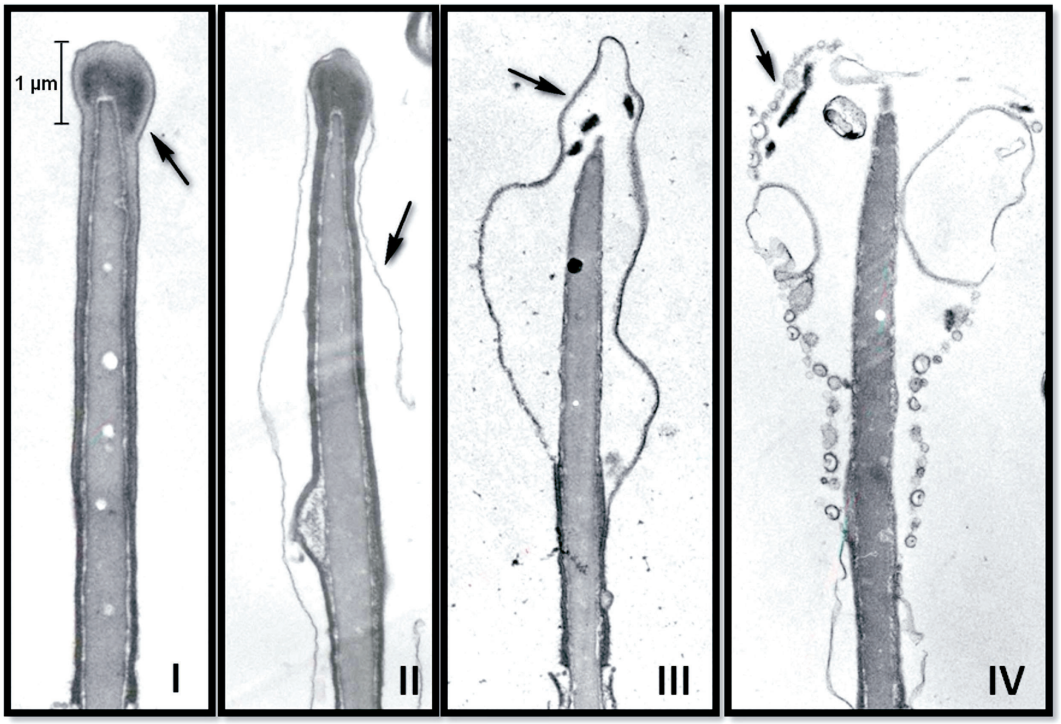

Fig. 1. Rabbit sperm heads classified to 4 grades according to state of membranes and acrosome. I – grade I – sperm with intact plasma membrane of the head and intact acrosome; II – grade II – sperm with waved plasma membrane; III – grade III – sperm with swollen or damaged acrosome; IV – grade IV – sperm with pseudo-acrosomal reaction formed by vesicles and with loss of the acrosomal content (I – and II – × 10 000; III – × 7 200; IV – × 10 000).

Table 2

Ultrastructural categorization of MACS treated and control (untreated) rabbit sperm heads

Sperm sample		Distribution to the grades of morphology n/%			
		I	II	III	IV
Heterospermic pool (Experiment 1)	Control	3/0.8	342/85.5	8/2.0	47/11.8
	AnV^-	8/2.0	350/87.5	4/1.0	38/9.5
Individual buck (Experiment 2)	Control	109/27.3	196/49.0	15/3.8	80/20.0
	AnV^-	136/34.0	191/47.8	25/6.3	48/12.0

n – number of spermatozoa classified into corresponding group according to the membrane status.

pared to sperm of grade III and IV in which damage to plasma and acrosomal membranes have already occurred (PIVKO *et al.* 2009). Electron microscopic study of the ultrastructure of frozen-thawed human spermatozoa was supported by clinical tests (Heath et al., 1985) and correlation was observed between the degree of damage, changes in the ultrastructure and the fertilizing ability of the spermatozoa (MAHADEVAN & TROUNSON 1984; HEATH *et al.* 1985).

In the bull, boar and ram, damage to the acrosome is the major cause of reduced fertility. Electron microscopy evaluation of the membrane and acrosomal integrity revealed that the sperm of different species was divided into several groups, from three to eight, according to the extent of morphological changes in the sperm head, membranes and the acrosome (MAHADEVAN & TROUNSON 1984; HEATH *et al.* 1985; ZIBRÍN *et al.* 1987; BWANGA *et al.* 1991; KROGENAES *et al.* 1994; LÓPEZ *et al.* 1999; OKADA *et al.* 2001; NISHIZONO *et al.* 2004; SA-ARDRIT *et al.* 2006). The authors mentioned above classified morphology of sperm based on the changes in the membrane and acrosome of the apical part of the sperm head. For the evaluation of the status of plasma membrane, outer and inner acrosomal membrane as well as acrosome, a classification system involving 4 grades of morphology is sufficient (PIVKO *et al.* 2009).

We assumed that the observed ultrastructural changes in spermatozoa membrane occur naturally as a result of capacitation and acrosome reaction since similar plasma and acrosomal membrane disruption and formation of vesicles were noticed by BEDFORD (2004) during sperm-egg interaction. According to this, the MACS technique might eliminate also spermatozoa with externalized PS that undergo capacitation (KOTWICKA *et al.* 2002) or acrosomal reaction (MARTIN *et al.* 2005). However, KURZ *et al.* (2005) observed that PS became accessible upon capacitation only in a subpopulation of PI-positive sperm cells. This confirms our assumption that the MACS technique using Annexin V-conjugated nanoparticles might eliminate dead and/or apoptotic spermatozoa.

Although the differences were not statistically significant, the CASA analysis (Table 1) apparently correlated with the membrane status of observed rabbit spermatozoa (Table 2). Furthermore, the specificity and sensitivity of the magnetic separation technique using annexin V-conjugated nanoparticles have already been shown through ultrastructural analysis by electron microscopy, i.e. about 73% of AnV^+ cells exhibited the presence of microbeads (PAASCH *et al.* 2003).

Artificial insemination of rabbit does

The MACS technique in both experiments (E1 and E2) did not affect the reproductive parameters of rabbit does (Table 3). Although we found significantly higher ($P<0.01$) conception rate in the AnV^- fraction compared to the control sample (for

Table 3

Reproductive performance outcomes observed in rabbit does after insemination with MACS treated and control (untreated) rabbit spermatozoa

Semen sample			Fertility parameters			
			Conception rate (%)	Kindling rate (%)	No. of liveborn kits	Total breeding effectiveness
Experiment 1	Heterospermic pool	Control	73.7	75.3	8.9 ± 0.4	670
		AnV^-	74.2	72.2	8.5 ± 0.4	614
Experiment 2	Buck I	Control	41.7[a]	41.7	8.7 ± 0.9	363
		AnV^-	59.2[b]	52.5	6.8 ± 1.1	357
	Buck II	Control	54.2	49.2[c]	7.2 ± 0.9	354
		AnV^-	48.3	25.0[d]	9.8 ± 1.1	245
	Buck III	Control	66.7	62.5	7.9 ± 1.0	494
		AnV^-	60.0	56.7	8.4 ± 1.1	476
	Buck IV	Control	66.7	66.7	8.2 ± 1.1	547
		AnV^-	82.5	81.2	8.6 ± 0.9	698
	Average values for four bucks	Control	57.3	55.0	8.0 ± 0.5	440
		AnV^-	62.5	53.8	8.3 ± 0.5	447

Results are expressed as means ± SEM; [a] vs [b] were statistically significant at $P<0.01$ and [c] vs [d] were statistically significant at $P<0.001$.

buck I), the difference in kindling rate was insignificant. This could be due to the subjective evaluation technique (abdominal palpation) or pseudopregnancy that is characterized by low sexual receptivity and low fertility of does (THEAU-CLEMENT 2007). On the other hand we noticed a significantly lower ($P<0.001$ for buck II) kindling rate using the AnV^- fraction compared to the control sample, whereas the number of liveborn kits was slightly higher (9.8 ± 1.1 vs. 7.2 ± 0.9, respectively). These contrary results may be due to factors affecting doe fertility such as parity, state of lactation and sexual receptivity at insemination, physiological state etc. (THEAU-CLEMENT 2007). Moreover, since experiments were carried out during different seasons, there may also be a seasonal influence on male and female fertility parameters (SAFAA *et al.* 2008a).

In previous studies (VASICEK *et al.* 2010, 2011a) we observed that after the application of a heterospermic pool for AI, the kindling rate of rabbit does varied from 62 to 74% in the control groups and from 75 to 81% in the AnV^- groups. Similarly in the present study, a kindling rate higher than 70% was found in the control as well as the AnV^- groups, whereas the kindling rate was on average only 55% for individual buck semen (Table 3). Thus it is better to use a heterospermic pool for AI to obtain higher reproductive outcomes of rabbit does, since one single buck may affect the fertility and prolificacy of about one hundred does (SELEEM 2005). We found no differences in the number of liveborn kits using AnV^- spermatozoa for AI between Experiment 1 and 2 (8.5 ± 0.4 vs. 8.3 ± 0.5, respectively; Table 3). Moreover, data in Table 3 clearly show that independently of the semen used for insemination (heterospermic or individual, untreated or MACS separated semen), each doe gave birth to about 8 kits. Similar number of liveborn kits (9.1) was observed in a previous study (VASICEK *et al.* 2011a). No differences were found in the number of stillborn kits using control or AnV^- spermatozoa for AI in both experiments (E1 and E2). The number of stillborn kits was about 1 kit per kindled doe. Total breeding effectiveness (kindling rate x number of liveborn kits) was much higher using untreated (control) or MACS separated semen for AI in Experiment 1 compared to Experiment 2, thus confirming that it is better to use a heterospermic pool for AI. On the other hand the total breeding effectiveness was not affected when MACS separated semen was used for AI in both E1 and E2 (Table 3).

Although the MACS elimination of apoptotic spermatozoa did not significantly increase the kindling rate of rabbit does or the total breeding effectiveness, this technique is widely and effectively used in treatment of human infertility (SAID *et al.* 2006). The effectiveness of this sperm preparation method in human medicine was proved using a sperm penetration assay. A significantly higher percentage of penetrated zona-free hamster oocytes were detected in the annexin V-negative fraction compared to the annexin V-positive sperm ($P<0.001$) as well as the controls ($P=0.001$) (SAID *et al.* 2006). What is more, the MACS technique was successfully tested as a fast and effective method of enriching viable spermatogonia from Djungarian hamster, mouse and marmoset monkey testes for the purposes of germ cell transplantation (VON SCHÖNFELDT *et al.* 1999). Moreover, recently the method for magnetic sorting of mammalian spermatozoa with damaged membranes using carboxyl-group functionalized magnetic particles conjugated to propidium iodide was patented by FOX *et al.* (2012).

Conclusion

The magnetic-activated cell sorting proved to be a quite harmless and potentially effective method for the elimination of rabbit apoptotic spermatozoa, although the insemination doses free of apoptotic sperm cells did not significantly improve the number of liveborn kits or kindling rate of rabbit does. Perhaps using higher concentrations of annexin V-conjugated nanoparticles for MACS technique could bring better kindling rates in rabbit husbandry, but then the economic costs should be taken into consideration. Moreover, in human medicine AnV^- cells display higher fertilization rates when a few sperm cells are used for animal model IVF and intracytoplasmic sperm injection (ICSI), whereas a much bigger spermatozoa concentration is used for artificial insemination in farm animal husbandry. Therefore, from our point of view the future of this sperm preparation technique for agricultural applications is not in the artificial insemination of farm animals, but in spermatozoa cryopreservation of different animal species for the purposes of farm animal gene banks.

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