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Effect of the MACS technique on rabbit sperm motility

Communication

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Abstract: Magnetic-activated cell sorting (MACS) separates apoptotic spermatozoa by the use of annexin V-conjugated nanoparticles which bind to phosphatidylserine that is externalized on the outer leaflet of the sperm plasma membrane. This technique yields two fractions: annexin V-negative (AnV^-) and annexin V-positive (AnV^+). The aim of the study was to evaluate the effect of MACS application on the motility parameters of rabbit spermatozoa. Rabbit semen samples collected separately from 4 bucks (I, II, III, and IV) were filtered and separated in a MACS system. The semen samples from a control (untreated) group, AnV^- and AnV^+ fraction were evaluated using CASA system. The experiment was replicated 4 times for each buck. The AnV^+ sperm had significantly lower concentration than the AnV^- fractions and the control samples ($P<0.05$ for bucks I, II, III, but not IV). We observed that the proportion of apoptotic spermatozoa in the semen of NZW bucks is about 20%. There was no significant difference in the percentage of motile and progressively motile spermatozoa between the AnV^- fractions and control samples. In conclusion, the MACS technique has no harmful effect on the rabbit sperm concentration and motility.

Keywords: *Rabbit • Sperm motility • CASA • Annexin V • Magnetic-activated cell sorting*

1. Introduction

Successful fertilization requires a sperm plasma membrane with normal integrity and function [1]. The numerous functions of the membrane are related to the cell metabolism for maintaining sperm motility, capacitation, acrosome reaction [2] and sperm–egg interaction [3]. The assessment of membrane integrity is based on the examination of sperm morphology and motility, hypo-osmotic swelling tests [4], supravital stains and flow cytometric techniques [5].

At the early phases of disturbed membrane function, asymmetry of membrane phospholipids occurs prior to a progressively disturbed integrity of the plasma membrane. The phospholipid phosphatidylserine (PS), which is normally present on the inner leaflet of the plasma membrane, becomes externalized to the outer leaflet [6]. The externalization of PS is a known early marker for apoptosis [7]. Annexin-V has a high affinity for PS [8], but it cannot pass through an intact sperm membrane. Therefore, when annexin-V binds to spermatozoa, it signifies that the integrity of the membrane has been disturbed [9]. Superparamagnetic microbeads (about 50 nm in diameter) conjugated with annexin V (called ANMBs) allow these low-quality spermatozoa to be eliminated *via* magnetic-activated cell sorting (MACS) [10]. MACS separation of spermatozoa yields two fractions: annexin V–negative (intact membranes, non-apoptotic) and annexin V-positive (externalized PS, apoptotic [9]).

Negative (non-apoptotic) sperm display higher fertilization rates when used for animal model IVF and intracytoplasmic sperm injection (ICSI) [11]. Furthermore, annexin-negative sperm separated by MACS had significantly higher motility following cryopreservation–thawing than sperm that were not separated. Similarly, annexin-negative spermatozoa also had higher cryosurvival rates than sperm cryopreserved without magnetic-activated cell sorting and sperm that were annexin-positive [12].

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In recent years, a number of techniques for objective assessment of movement characteristics of human and animal spermatozoa have been introduced using computer–assisted (automated) semen analysis (CASA) systems [13]. For the conventional analysis, a simple classification system, which provides the best possible assessment of sperm motility with no need for complex equipment, is recommended [14-17]. The use of computer-assisted (automated) semen analyzer – CASA is a promising alternative to the traditional approach of microscopic visualization of spermatozoa motility and heamocytometric evaluation of spermatozoa concentration [13].

Our goal was to evaluate the effect of the magnetic-activated cell sorting technique on the motility parameters of rabbit spermatozoa.

2. Experimental Procedures

2.1 Semen collection and handling

Semen samples from 25 New Zealand White (NZW) males were collected using an artificial vagina. Each sample of fresh ejaculate was evaluated using the CASA (Computer Assisted Semen Analysis; MiniTüb, Tiefenbach, Germany) system for concentration and motility. Basing on the motility parameters, the best four bucks (2 – 3 years old) were chosen for the experiment. Ejaculate from each buck was routinely diluted in a commercial insemination diluent (MiniTüb, Tiefenbach, Germany) at the ratio of 1:6 and 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 the filter membrane were carefully flushed out from the filter to the collection tube using 2 ml of binding buffer (Annexin V Microbead Kit, Miltenyi Biotec, Germany). The filtered spermatozoa were diluted in binding buffer at a ratio of 1:8. Filtered and diluted rabbit semen was divided into the experimental group (3.5 ml) intended for the magnetic separation and the control group (the rest of the sample).

Since the commercial insemination diluent was used for the dilution of fresh semen and the binding buffer was used for the magnetic separation, spermatozoa handling did not include capacitation. Both of those diluents protected spermatozoa from early capacitation which is necessary if the spermatozoa would be used for artificial insemination.

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.

2.2 Magnetic separation

The filtered rabbit spermatozoa were incubated for 15 min at room temperature in 120 µl of annexin V-conjugated nanoparticles (Annexin V Microbead Kit, Germany) according to the original protocol. The MiniMACS Magnetic Cell Sorting system (Miltenyi Biotec, Germany) was used for MACS assay of rabbit spermatozoa at room temperature.

The MACS MS column was placed in the magnetic field of a MACS Separator and prepared by washing with 0.5 ml of binding buffer. The filtered rabbit spermatozoa (3.5 ml) incubated with annexin V-conjugated nanoparticles were applied onto the column. The annexin V–negative spermatozoa passed through the column into the collection tube. Then the column was rinsed with 1.5 ml of binding buffer, removed from the separator and placed on a suitable collection tube. For the recovery of annexin V-positive fraction 1 ml of binding buffer was pipetted onto the column and firmly flushed out using the plunger supplied with the column.

2.3 CASA analysis

The semen samples from the control group, annexin V–negative (AnV^-) and annexin V-positive (AnV^+) fraction were placed into 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: concentration (10^9 per ml); percentage of motile spermatozoa (motility >5 µm/s) and percentage of progressively motile spermatozoa (motility >20 µm/s).

2.4 Statistics

The experiment was replicated 4 times for each buck (semen collection from October to November in autumn, and from December to February in winter). The results were evaluated statistically using a one-way ANOVA in SigmaPlot software (Systat Software Inc., Germany) and expressed as means ± SE. P-values at $P<0.05$ were considered as statistically significant.

3. Results and Discussion

Table 1 shows the basic CASA parameters of rabbit semen collected separately from 4 bucks, either before or after MACS treatment. The MACS separation divided the basic spermatozoa population into two distinct subpopulations (fractions) with a logically lower spermatozoa concentration. The semen concentration was insignificantly lower in the AnV^- fractions in comparison to the control samples. On

	Semen sample	Before treatment	After treatment	
	Parameter	Control	AnV^-	AnV^+
Buck I	Concentration	0.200 ± 0.034[a]	0.164 ± 0.026[b]	0.025 ± 0.011[c]
	% Motile	54.78 ± 16.39	51.61 ± 14.14	19.64 ± 6.89
	% Progressive	38.34 ± 14.12	37.92 ± 12.90	11.50 ± 5.93
Buck II	Concentration	0.188 ± 0.048[a]	0.155 ± 0.032[b]	0.028 ± 0.012[c]
	% Motile	63.39 ± 14.26[a]	64.54 ± 8.82[b]	8.80 ± 2.09[c]
	% Progressive	45.06 ± 14.83[a]	45.24 ± 10.4[b]	4.17 ± 3.15[c]
Buck III	Concentration	0.269 ± 0.051[a]	0.254 ± 0.036[b]	0.064 ± 0.050[c]
	% Motile	50.77 ± 14.61[a]	54.64 ± 14.25[b]	5.59 ± 1.60[c]
	% Progressive	40.84 ± 15.01[a]	46.52 ± 15.05[b]	1.09 ± 1.09[c]
Buck IV	Concentration	0.205 ± 0.072	0.168 ± 0.046	0.054 ± 0.037
	% Motile	50.80 ± 8.90[a]	53.01 ± 7.22[b]	7.46 ± 3.12[c]
	% Progressive	34.35 ± 10.69[a]	40.45 ± 8.48[b]	4.33 ± 1.72[c]
Average values for four bucks	Concentration (%)	0.216 ± 0.018[a]-	0.185 ± 0.023[b] 85.2 ± 3.1[b]	0.043 ± 0.010[c] 19.4 ± 3.4[c]
	% Motile	54.93 ± 2.97[a]	55.95 ± 2.93[b]	10.37 ± 3.16[c]
	% Progressive	39.65 ± 2.25[a]	42.53 ± 2.08[b]	5.27 ± 2.21[c]

Table 1. CASA parameters of MACS treated and control (untreated) rabbit spermatozoa.

Results are expressed as means ± SE; [a] vs. [c] and [b] vs. [c] were statistically significant at $P<0.05$.

the other hand, the AnV^+ sperm had significantly lower concentrations than the AnV^- fractions and the control samples (P<0.05 for bucks I, II, III, but not IV). We observed that the proportion of apoptotic spermatozoa in the semen of NZW bucks is about 20% (Table 1). The separation column and the magnetic field had no significant influence on sperm motility. Although there is 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 bucks II, III, IV, but not I), more repeats of the experiment are required in order to demonstrate the effectiveness of the MACS separation in terms of spermatozoa motility improvement. These data are similar to those obtained in our previous experiments, where the total motility and progressive motility of spermatozoa were not statistically different between control and MACS separated group [18].

Although 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 [19,20], on the basis of observed CASA parameters the MACS technique had no harmful effect on the rabbit sperm concentration and motility. Paasch *et al*. [21] also reported that the separation columns and their magnetic field did not exert detectable adverse effect on the motility of spermatozoa in their experiments. Moreover, MACS combined with the density gradient centrifugation (DGC) may significantly improve the motility of spermatozoa as reported by Said *et al*. [12] and de Vantéry Arrighi *et al*. [22].

In male reproduction, abnormal spermatozoa may be eliminated *via* apoptosis [23]. Those abnormal or immature spermatozoa and leukocytes are two main sources of reactive oxygen species (ROS) in semen that are one of the common forms of free radicals. Physiologically, free radicals control sperm maturation, capacitation and hyperactivation, the acrosome reaction (AR), and sperm-oocyte fusion. Pathologically, free radicals induce lipid peroxidation (LPO), DNA damage and apoptosis [24]. Aitken and Clarkson [25] showed that techniques of sperm preparation that involve a centrifugation step before the separation of motile cells are associated with a burst of ROS production from a subpopulation of cells, characterized by impaired motility and fertilizing ability. This burst of ROS has the effect of compromising the functional competence of normal spermatozoa in the same suspension [25]. The MACS technique does not require centrifugation before separation of the cells and therefore, together with elimination of apoptotic spermatozoa, eventually prevents releasing of ROS that could damage the membrane of the annexin V-negative spermatozoa.

MACS separation according to the externalization of PS results in the extraction of apoptotic spermatozoa and those with damaged membranes. Therefore, the selection of non-apoptotic spermatozoa may be used to enhance sperm quality following preparation techniques and subsequently achieve optimal conception rates in assisted reproduction [26] and potentially also in artificial insemination of the farm animals [18,27].

4. Conclusions

The MACS technique seems to be quite effective and harmless method for the elimination of apoptotic spermatozoa from the rabbit ejaculate, but further experiments are required in order to successful put this separation method into breeding practice.

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