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Short Communication

Elimination of Apoptotic Spermatozoa from Rabbit Insemination Dose Using Annexin V Associated with the MACS Technique. A Preliminary Study*

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The aim of this study was to verify whether the separation and elimination of the apoptotic fraction in rabbit semen using a MACS technique may improve sperm fertility potential and consequently rabbit kindling rate. Semen samples from 25 New Zealand White (NZW) rabbit males were collected using an artificial vagina and evaluated using the CASA system for concentration and motility. For artificial insemination the best 11 bucks were chosen based on motility parameters. Their ejaculates were mixed to make a heterospermic pool and routinely diluted in a commercial insemination diluent (MiniTüb, Tiefenbach, Germany) at a ratio of 1:6. Diluted heterospermic spermatozoa were filtered through a Sartorius filter to wash out seminal plasma, re-diluted in binding buffer (Annexin V Microbead Kit, Miltenyi Biotec, Germany) at a ratio of 1:3.66 and divided into two groups: an experimental group intended for MACS separation and control group without MACS separation. Then hormonally treated females of NZW rabbits were inseminated with fresh doses of filtered heterospermic semen (n=27; 0.5 ml I.D. per female) and MACS separated semen (n=28; 0.5 ml I.D. per female). Separation and subsequent elimination of apoptotic spermatozoa (positive selection) from the insemination dose (after negative MACS selection) was verified under *in vivo* conditions on the basis of increased kindling rate in the experimental group in comparison with kindling rate in the control group (81.3% vs. 73.8%). In conclusion, elimination of apoptotic spermatozoa by the use of the MACS technique results in a slight improvement in kindling rate of rabbit does.

Key words: Rabbit, spermatozoa, apoptosis, magnetic-activated cell sorting, annexin V, kindling rate.

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The mammalian reproduction and fertility potential of spermatozoa can be affected by many factors. In human medicine, assisted reproduction techniques (ARTs) are widely used for infertility treatment. 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.

Apoptosis (genetically programmed cell death) is a physiological process running continuously, maintaining an optimal number of generative cells in testes with the assistance of Sertoli cells (SINHA HIKIM & SWERDLOFF 1999). Unlike the somatic and germ cells of testes, the presence and localization of sperm apoptosis is more difficult to detect (OEHNINGER *et al.* 2003). Although spermatozoa exhibit several similar features characteristic for

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apoptosis in somatic cells, these related events do not necessary mean death as a result of apoptosis (TAYLOR *et al.* 2004). Despite this fact the phenotypic expression of apoptosis has been described in relation to the presence of abnormal spermatozoa in semen (SAKKAS *et al.* 1999, 2002; BARROSO *et al.* 2000). 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). 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, 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 phospholipid phosphatidylserine (PS). MACS separation of spermatozoa 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.* (2005b) and DE VANTÉRY ARRIGHI *et al.* (2009) prepared a 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 other conventional methods.

The use of the annexin V assay in livestock animals has already been documented. The annexin-V-binding assay allowed the identifcation of different sperm subpopulations in bull semen samples after freezing/thawing and swim-up treatment (CHAVEIRO *et al.* 2007). Similarly, PEÑA *et al.* (2003) and PEÑA *et al.* (2006) found significant differences between boar ejaculate fractions in fresh and frozen samples using an annexin-V assay to determine early changes in plasma membrane stability.

Our goal was to verify the hypothesis that elimination of apoptotic spermatozoa from rabbit insemination dose might improve fertility results (kindling rate) following insemination of does. This method was used on an animal model for the first time.

Material and Methods

Semen collection and analysis

Semen samples from males of 25 New Zealand White (NZW) lines 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. For artificial insemination (A.I.) the best 11 bucks were chosen based on motility parameters. Their ejaculates were mixed to make a heterospermic pool and routinely diluted in a commercial insemination diluent (MiniTüb, Tiefenbach, Germany) at a ratio of 1:6. The diluted rabbit semen was divided into control and experimental groups intended for magnetic separation. After each semen modification the sperm sample was placed into a Standard Count Analysis Chamber Leja 20 micron (MiniTüb, Tiefenbach, Germany) and evaluated using the CASA system (Sperm Vision™) under Zeiss Axio Scope A1 microscope.

Magnetic separation

Before magnetic sperm separation, the sperm cells were washed out from the 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.* 2008), so that seminal plasma with the diluent passed through a membrane and was then discarded. The rabbit spermatozoa retained by the filter membrane were carefully flushed out to a collection tube using 2 ml of binding buffer (Annexin V Microbead Kit, Miltenyi Biotec, Germany).

The filtered spermatozoa (1.5 ml) were diluted in 5.5 ml of binding buffer (1:3.66) and subsequently incubated in 200 μl of annexin V-conjugated nanoparticles (Annexin V Microbead Kit, Germany) for 15 min at room temperature. The MidiMACS Magnetic Cell Sorting system (Miltenyi Biotec, Germany) was used for MACS of rabbit spermatozoa in the insemination dose.

The insemination doses (I.D.) for the control group were prepared in the same way as those for the experimental group, but the spermatozoa were not subjected to the MACS technique.

Insemination

Sexually mature and clinically healthy rabbits that were included in the rearing programme were used for artificial insemination (2 repeats, in December-February). For the experiments 55 does and 11 bucks of New Zealand White lines were used, reared in a partially air-conditioned hall of a local rabbit farm.

Females of NZW rabbits were inseminated with fresh doses of filtered heterospermic semen (n=27; 0.5 ml I.D. per female) and the semen after annexin V separation (n=28; 0.5 ml I.D. per female). PMSG at 25 I.U. (Sergon, 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. The obtained results were analyzed using χ^2 - test.

Results and Discussion

Table 1 shows the basic CASA parameters of rabbit heterospermic semen collected from 11 rabbit males, either before or after MACS treatment. Following the treatment the semen concentration had decreased insignificantly (229.30x10^6/ml vs. 214.30x10^6/ml). The total motility and progressive motility of spermatozoa were not statistically different between the control and MACS separated groups (77.30 % vs. 79.39 % of motile spermatozoa and 66.87 % vs. 72.66 % of progressive motile spermatozoa). Moreover, other motility parameters were similar between the groups (VCL – velocity curved line – 167.07 μm/s vs. 174.54 μm/s, VSL – velocity straight line – 55.00 μm/s vs. 66.42 μm/s, STR – straightness – 0.73 vs. 0.79 and BCF – beat cross frequency – 28.57 Hz vs. 32.48 Hz). SAID *et al.* (2005b) reported that annexin V-negative human spermatozoa had statistically signifcant higher motility values (76 ± 15.06, P = 0.03) than the raw samples (64.5 ± 6.43). On the basis of the observed sperm traits, the rabbit sperm quality was sufficient for artificial insemination (THEAU-CLÉMENT *et al.* 1996; CASTELLINI *et al.* 2000).

Rabbit spermatozoa following MACS separation retained their fertilizing ability, confirmed by 81.3 % of does kindling an average of 9.05 liveborn kits in comparison to the control group, in which 73.8 % of does kindled an average of 9.45 liveborn kits (Table 2). SAID *et al.* (2006) used sperm a penetration assay as an *in vitro* model for evaluating human sperm fertilization potential af-

Table 1

CASA parameters of rabbit heterospermic semen collected from 11 bucks

Semen sample	Control (untreated) sperm	MACS separated sperm
Concentration (10^6 per ml)	229.30	214.30
% of motile spermatozoa (motility 5 μm/s)	77.30	79.39
% of progressive motile spermatozoa (motility 20μm/s)	66.87	72.66
VCL (μm/s)	167.07	174.54
VSL (μm/s)	55.00	66.42
STR (VSL:VAP)	0.73	0.79
BCF (Hz)	28.57	32.48

VCL – velocity curved line, VSL – velocity straight line, STR – straightness, BCF – beat cross frequency.

Table 2

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

Trait	Control (untreated) sperm	MACS separated sperm
Number of inseminated does (n)	27	28
Average number of liveborn kits per doe	9.45	9.05
Kindling rate (%)	73.8	81.3

ter MACS separation. Following the 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 ($P = 0.001$).

Annexin V has a high affinity for phosphatidylserine (PS) but lacks the ability to pass through an intact sperm membrane (VAN HEERDE *et al.* 1995). Therefore, the presence of annexin V binding on the sperm surface indicates that membrane integrity has been compromised (GLANDER *et al.* 2002).

Various sperm preparation techniques are currently used as the main components of ART procedures (HENKEL & SCHILL 2003). The main objective for applying these techniques is the selection of a sufficient number of viable motile spermatozoa capable of fertilizing the oocyte(s). Current standard spermatozoa preparation techniques which use a sedimentation or migration approach to separate spermatozoa are based on sperm motility or density (SAID *et al.* 2008). Among these, the double density gradient centrifugation (DGC) and the swim-up procedures are currently used as standard preparation techniques. In addition, the glass wool filtration (GWF) technique is known to provide spermatozoa samples with comparable recovery rates, motility, morphology and fertilizing capacity if appropriate types of glass wools are used (HENKEL & SCHILL 2003).

Separation and subsequent elimination of apoptotic spermatozoa (positive selection) from the insemination dose (after negative MACS selection) was verified under *in vivo* conditions on the basis of increased kindling rate in the experimental group in comparison to kindling rate in the control group (81.3% vs. 73.8%; Table 2). However, the differences in kindling rates as well as the differences in average number of liveborn kits per doe between experimental and control groups of NZW rabbit does (9.05 vs. 9.45; Table 2) were not statistically significant.

Our results may indicate that magnetic-activated cell sorting (MACS) with annexin V-conjugated nanoparticles can be an effective and vital separation technique useful for retrieving spermatozoa with a better fertility potential appropriate for insemination, which correlates with the observations of the other authors (GRUNEWALD *et al.* 2001; GLANDER *et al.* 2002; SAID *et al.* 2005a).

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