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Magnetic nanopurification of ram semen using Mase V particles for depletion of morbid and DNA fragmented spermatozoa

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Abstract

The aim of this study was to enhance the quality of fresh ram semen by purification of spermatozoa using combination of annexin V and lectin (PNA and LCA) coated nanoparticles. Semen samples from two rams were collected regularly during the breeding season using electro-ejaculation. Samples (n=5) with poor quality (decreased sperm motility and viability) were chosen for the nanopurification procedure. Fresh (control) samples and purified (depleted from damaged spermatozoa) samples were analysed by CASA for total and progressive motility. Flow cytometry was used to check presence of apoptotic-like changes (annexin V-FITC) and integrity of acrosome (PNA-AF488 and rhodamine LCA) as well as proportion of dead cells (propidium iodide) in semen samples before and after nanopurification. Transmission electron microscopy (TEM) was performed to assess changes in sperm ultrastructure and interaction with nanoparticles. Purified ram spermatozoa exhibited increased viability ($p<0.01$) and both motility parameters ($p<0.05$) and decreased proportion of spermatozoa with damaged acrosome ($p<0.05$). On the other hand, no significant differences were observed for annexin V and LCA positive spermatozoa between fresh and purified samples. TEM analysis revealed occurrence of spermatozoa with swollen acrosome and damaged plasma membrane as well as abundance of nanoparticles surrounding the cells or bound to the membrane in positively stained spermatozoa. In conclusion, nanopurification process can improve the quality of ram semen, mainly in terms of sperm viability, motility and presence of damaged spermatozoa, which might result in better fertilization ability of insemination doses or even higher cryosurvival rates of stored spermatozoa.

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Introduction

In general, animal semen may contain different proportions of intact viable, damaged or even dead spermatozoa, which can greatly affect the overall fertility. It has been reported that high number of dead spermatozoa in semen sample is associated with insufficient reproductive outcomes of *in vitro* fertilization as well as with poor cryopreservation (ROCA et al., 2013). The elimination of non-viable spermatozoa, which cause the main damage, from the semen using current technology has been previously studied with different results. The most recent, a nanotechnology-based technique (nanopurification) has been developed to non-invasive target and remove the moribund spermatozoa. Due to the interactions with specific magnetic nanoparticles, the process of nanopurification may have a potential to enrich insemination doses with viable spermatozoa resulted in the improvement of animal breeding efficiency (DURFEY et al., 2017).

In human assisted reproduction, magnetic-activated cell sorting (MACS) has been used to eliminate spermatozoa with deteriorated membranes and apoptotic-like features (AGARWAL et al., 2009; BUCAR et al., 2015; GLANDER et al., 2002; VENDRELL et al., 2014). In addition, the same technique using manual MACS instruments were used in rabbit (VASICEK et al., 2014) or boar (MRKUN et al., 2014). Recently we used for the first time a fully automated magnetic cell sorter to remove deteriorated spermatozoa from ram semen samples (VAŠÍČEK et al., 2020; VAŠÍČEK et al., 2021). Unfortunately, the used technique was not sensitive enough either to efficiently deplete the dead spermatozoa, or to improve the sperm motility itself. In fact, all above mentioned studies applied nanoparticles conjugated with annexin V, which bind to phosphatidylserine exposed on the surface of cells undergoing apoptosis. However, other ligands such as those of lectins, PNA (peanut agglutinin from *Arachis hypogaea*), PSA (*Pisum sativum* agglutinin), and LCA (*Lens culinaris* agglutinin) are present on the surface of defective spermatozoa with revealed acrosome (CROSS & WATSON, 1994; ODHIAMBO et al., 2011). Nanopurification of animal spermatozoa using these lectins in combination with or without of annexin V has already been reported in bull (ODHIAMBO et al., 2014), boar (DURFEY et al., 2017; DURFEY et al., 2019; FEUGANG et al., 2015) or camel (RATEB, 2021). On the other hand, such combination of glycoproteins has never been used to improve the quality of ram spermatozoa.

The main objective of this study was to magnetically eliminate the ram defective spermatozoa using the combination of nanoparticles conjugated with annexin V, PNA and LCA proteins.

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Material and methods

Sexually mature and clinically healthy rams (n=2) of the Native Wallachian and Slovak dairy sheep breed at the age of 2.5–5 years were used in this study. Animals were kept at a breeding facility of NPPC – RIAP Nitra (Lužianky, Slovak Republic) and fed as reported previously. Semen samples were collected twice a week by electro-ejaculation and immediately transported to the laboratory throughout the whole study as reported previously (VOZAF et al., 2021). The basic semen quality assessment consists of sperm motility and viability analysis. Based on this assessment, five semen samples with impaired quality were chosen for further nanopurification process to improve the ram sperm attributes.
The animals and sample collection were carefully handled in accordance with ethical guidelines as stated in the Slovak Animal Protection Regulation RD 377/12, which conforms to European Union Regulation 2010/63..

CASA analysis

The motility and sperm movement were analysed by CASA (SpermVision™ software, Minitube, Tiefenbach, Germany) with light microscope (at the 200× magnification; AxioScope A1, Carl Zeiss Slovakia, Bratislava, Slovakia) and Makler counting chamber (Microptic, Barcelona, Spain) as described previously (VOZAF et al., 2021). Briefly, samples were diluted by saline (0.9% NaCl; Braun, Nuaille, Germany) at ratio 1:20 (v/v). A drop of diluted semen (10 µL) was transferred to a counting chamber and analysed with manufacturer's pre-set parameters for rams. Mainly total (TM) and progressive motility (PM) were observed in this study.

Flow-cytometric analyses

Viability and apoptosis
The viability of spermatozoa in ram semen samples were evaluated as the proportion of dead spermatozoa stained by propidium iodide (PI) dye at the final concentration of 50 µg/mL. The apoptotic-like changes in ram spermatozoa were observed by staining with Annexin V-FITC (AnV) in combination with dead cell dye DRAQ7 as described previously (VASÍCEK et al., 2022).

Acrosomal status

The integrity of acrosome was determined using two different fluorescent probes: Alexa Fluor 488 conjugated PNA in combination with DRAQ7 dye and rhodamine conjugated LCA in combination with SYTOX Green dye as reported in previous study (VASÍCEK et al., 2022).

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Stained semen samples were immediately analyzed using flow cytometer FACS Calibur equipped with a Cell Quest Pro software (BD Biosciences, San Jose, CA, USA). A least 10,000 events (spermatozoa) were acquired and analysed in each sample.

Nanopurification of ram spermatozoa

To remove dead and morbid spermatozoa from the ram semen, Mase V iron nanoparticles (Clemente Associates Inc., USA) were used. The particles were coated with three glycoproteins, Annexin V, PNA and LCA, which bind either to the apoptotic-like spermatozoa, or spermatozoa with compromised acrosome. Briefly, an aliquot of ram semen (10^8 spermatozoa) diluted in 1.3 mL of HEPES buffer (VAŠÍČEK et al., 2020; VAŠÍČEK et al., 2021) was incubated at orbital shaker for 15 min. at room temperature with a proper volume of Mase V nanoparticles (at final concentration of 4 mg/mL) calculated according to the producer's manual and following formula:

Y (mL) = ax + b, a = - 0.22 b= 22.23 and x is the sperm motility

Then magnet was placed against the tube for 10 min. Afterwards, supernatant containing intact viable spermatozoa (negative fraction) was gently decanted to new tube, while the DNA fragmented and damaged spermatozoa (positive fraction) were still attached to the particles against the wall of the tube and magnet. The sorted (negative) spermatozoa were analysed by CASA system to check the sperm motility parameters and by flow cytometry to determine sperm viability (PI), apoptosis-like changes (AnV) and acrosomal status (PNA, LCA) as mentioned above.

Transmission electron microscopy of sorted ram spermatozoa

To analyse the interaction of nanoparticles with damaged ram spermatozoa, the sample of positive fraction after nanopurification was processed for transmission electron microscopy (TEM) as described previously (VOZAF et al., 2022). Briefly, positively sorted ram spermatozoa were fixed in a fixative solution (2% paraformaldehyde and 2.5% glutaraldehyde in 0.15 mol/L sodium cacodylate buffer, pH 7.1–7.3). Then, sample was washed three times in cacodylate buffer for 15min. and postfixed in 1% osmium tetroxide in cacodylate buffer during 1h and embedded into 2% agar. Subsequently, sample was dehydrated by passing them through an acetone series and embedded into PolyBed resin (Polysciences Inc., USA). Ultrathin sections (70 nm) were placed on nickel grids, contrasted and examined under the transmission electron microscope (JEM-2100, JEOL, Japan) operating at 200 kV.

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Statistical Analysis

Experiments with nanopurification of ram spermatozoa were performed five times. Due to the high variability of obtained data, an unpaired nonparametric Mann-Whitney t test was used for statistical analysis using GraphPad Prism version 9.5.1 for Windows (GraphPad Software, San Diego, CA, USA). The data are represented as means ± SD. Differences at $p<0.05$ were considered statistically significant.

Results and discussion

The removal of defective spermatozoa is one of the big challenges how to improve the assisted reproductive outcomes. Here, we used the magnetic nanoparticles to purified ram spermatozoa by depleting spermatozoa with apoptotic-like changes bound to annexin V and/or spermatozoa with compromised acrosome integrity bound to PNA or LCA. The flow cytometry revealed that apoptosis-like process was not significantly present either in fresh, or purified spermatozoa (Figure 1A). On the other hand, significant ($p<0.05$) enrichment of acrosome intact spermatozoa was achieved in purified spermatozoa compared to fresh samples (8.8±4.8% vs. 21.7±12.9% of PNA-positive cells, respectively; Figure 1B). The decrease of LCA-positive spermatozoa in purified samples was also observed, although not significant (Figure 1C). However, a significant ($p<0.01$) removal of dead spermatozoa was noticed after nanopurification of ram spermatozoa compared to fresh sperm samples (5.9±3.6% vs. 24.8±11.7%, respectively; Figure 1D). The nanopurification even significantly ($p<0.05$) improved the motility attributes of ram spermatozoa, both total and progressive motility in comparison to fresh sperm samples (82.9±9.4% vs. 71.0±6.2 and 80.0±8.6% vs. 65.2±8.7%, respectively; Figure 1E and 1F).

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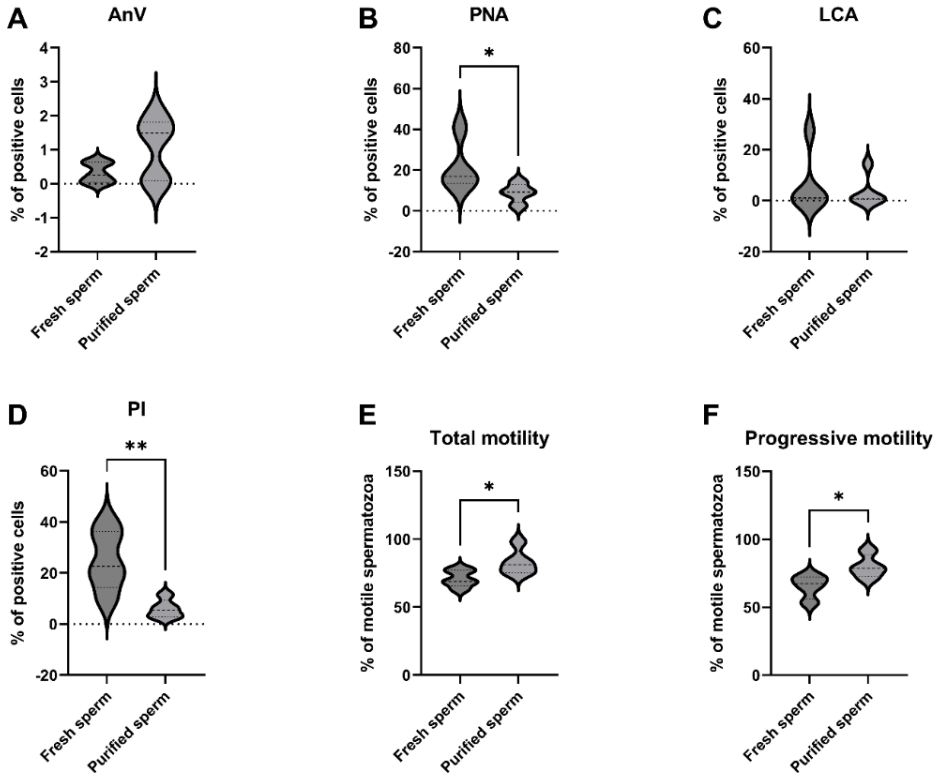

Figure 1: Sperm quality attributes in fresh and purified ram spermatozoa analysed by flow cytometry and CASA system. Fresh and magnetically purified ram spermatozoa were stained with annexin V to detect apoptotic-like changes (A), with PNA and LCA to detect disturbed acrosome (B and C, respectively) and with PI to target dead spermatozoa (D). CASA analysis was used to assess total and progressive motility (E and F, respectively) of fresh and purified ram spermatozoa. The data are expressed as the means ± SD; difference is statistically significant at * $p<0.05$ and ** $p<0.01$.

Similarly, increase in the viability of cryopreserved bull spermatozoa or in the total and progressive motility of fresh boar spermatozoa, both nanopurified with PNA conjugated nanoparticles, was observed in previous studies (FEUGANG et al., 2015; ODHIAMBO et al., 2014). DURFEY et al. (2017; 2019) also found significantly higher sperm progressive motility in fresh boar semen samples, in which defected spermatozoa were depleted with nanoparticles coated with lectins and annexin V. On the other hand, plasma integrity or acrosome reaction of nanopurified spermatozoa determined by PI or PNA staining respectively, was not significantly different compared to nonpurified spermatozoa (DURFEY et al., 2019). Contrarily, a dramatic improvement in post-thaw sperm motility and viability as well as in the

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proportion of spermatozoa with intact acrosome was reported in the camel cryopreserved semen after purification with lectins and annexin V conjugated nanoparticles (RATEB, 2021).

To confirm the effective interaction of nanoparticles with ram spermatozoa as well as to check the quality of sperm plasma membranes, we performed an ultrastructural analysis of purified sperm sample using TEM. Majority of the positively selected spermatozoa exhibited swollen acrosome and damaged plasma membrane with nanoparticles surrounding the cells or bound to the membrane (Figure 2). Similar observation was made in nanopurified boar spermatozoa (DURFEY et al., 2019).

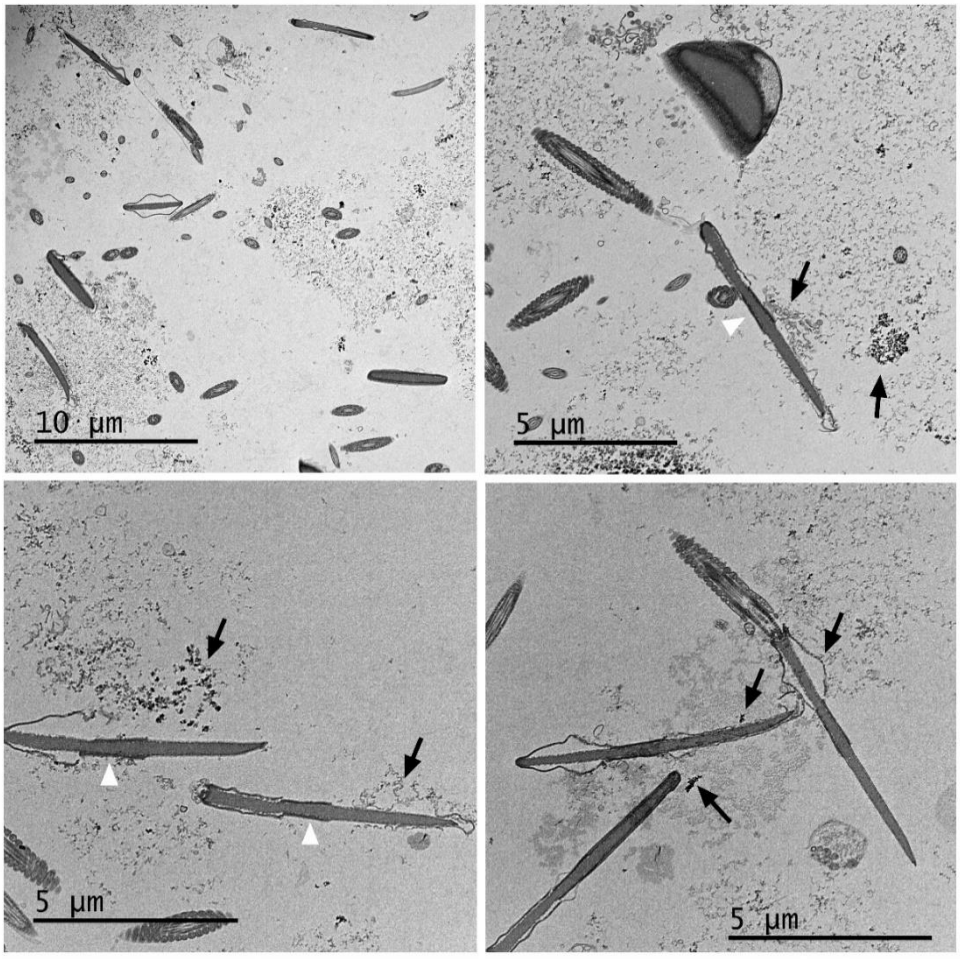

Figure 2: TEM analysis of ram spermatozoa retained using magnetic nanoparticles and magnetic field within the sorting tube. Nanoparticles are indicated with black arrows, while spermatozoa with damaged plasma membrane are marked by white arrowheads.

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Conclusion and recommendation

In conclusion, gentle manual magnetic nanopurification of ram semen samples may be a better alternative to automated MACS sorting regarding great sensitivity of ram spermatozoa. Moreover, purification of spermatozoa with the combination of annexin V and lectins such as PNA evidently much more improve the ram semen quality in terms of the sperm viability, motility and acrosome integrity than sperm sorting with nanoparticles coated only with annexin V itself. The presented method might be eventually used to enhance the semen quality of valuable ram intended for cryopreservation or artificial insemination. Anyway, further study is required to investigate if this method could also improve the post-thaw quality of already cryopreserved semen samples.

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