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Original Research

Sperm Sexing Mediated by Magnetic Nanoparticles in Donkeys, a Preliminary In Vitro Study

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ABSTRACT

Preselection of sex before conception has been one of the objectives pursued by scientists and breeders for many years. The donkey milk industry has shown a greater commercial interest, since jennies' demand is increasing nowadays. Therefore, to get a female animal, the oocyte must be fertilized with previously sexed semen. The current technique used for sperm sexing separates spermatozoa containing the X or Y chromosome based on each cell DNA content. However, this technique exposes spermatozoa to high-pressure speed flow, DNA fluorescent stain, and UV light, factors that may affect sperm quality. The aim of this study was to test a new technique to isolate spermatozoa carrying the X chromosome by means of magnetic nanoparticles (MNPs). Results show that the MNP technique is highly effective in select X spermatozoa, without affecting several physiological sperm parameters. In conclusion, the MNP technique provides an X sperm population with similar sperm physiological state than the control sample. The new sexing technology presented here can be mainly applied to improve the donkey's milk industry.

1. Introduction

Preselection of sex before conception has been one of the objectives pursued by scientists and breeders for many years. The financial benefit of selecting one sex has been already evaluated by dairy and meat cattle industries, where females or males are preferred, respectively [1], or mares for polo sports due to her better sport performance. In addition, since the use of donkey milk is preferred as replacement of cow's milk for allergic children, dietary complement, and cosmetic industry [2–4], the demand of jennies is increasing nowadays. Therefore, to get a female animal, the oocyte must be fertilized with previously sexed semen.

The current technique used for sperm sexing separates spermatozoa containing the X or Y chromosome based on DNA content, by cell sorting [5]. However, this technique exposes spermatozoa to high flow pressure, DNA fluorescent stain, and UV light, factors that may affect sperm quality [6–9]. Moreover, the number of selected spermatozoa obtained by this method is not enough to inseminate mares that require a large number of sperm [10]. In equines, the efficiency of this sexing technique is around 90% with a bias to the spermatozoa containing the X chromosome [11]. Furthermore, when mares are deep-horn inseminated with sexed semen, the pregnancy rate per cycle is highly variable and low (0%–60%) [12–15]. Thus, the application of the current sperm sexing technique is not only limited by the logistics of having the stallion, mare and flow cytometer in the same facility, but also by the poor success with frozen/thawed sorted sperm.

To obtain a good-quality sperm subpopulation, different sperm selection techniques have been developed [16]. For instance, nanoparticles combined to fluorescent dyes, antibodies, or

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magnetism have been used to separate out apoptotic spermatozoa from the semen sample [17]. Magnetic nanoparticles (MNPs) have been successfully used to select spermatozoa with better cryopreservation tolerance and fertilization potential for assisted reproduction in humans [18,19]. Considering that the Z electrical potential occurs between the sperm plasma membrane and the surrounding environment [20–22], this property may be used to discriminate the X and Y spermatozoa according to their differential capability to migrate along an electrophoretic field. Thus, most Y spermatozoa have a Z potential of −16 mV, whereas the X spermatozoa have Z potential of −20 mV [23]. Therefore, the use of MNPs combined to the sperm Z potential could be used as an alternative sperm sexing technique that might be useful for donkeys where sperm separation according to the sexual chromosome has not been performed yet.

The aim of this study was to test a new technique to isolate spermatozoa carrying the X chromosome by means of MNPs.

2. Materials and Methods

2.1. Semen Collection

Three Jack Donkeys of different breeds (aged 3–8 years) were used. To deplete spermatozoa from the sperm reservoir, semen were collected three to four times per week during 3 weeks, stabilizing daily sperm output. Afterward, donkey's ejaculates were collected three times in 1 week until a total of nine, by using a Missouri artificial vagina.

2.2. Sperm Sexing by Magnetic Nanoparticles

Gel-free semen samples were diluted to a concentration of 50 million sperm/mL with an extender (Equiplus, Mintube, Germany) and then centrifuged at 300 g for 15 minutes. The pellet was resuspended in HTF (modified Human Tubal Fluid; Irvine Scientific, CA) at 100 million sperm/mL. Then, the sperm sample was divided into two groups: one was incubated with three MNPs per spermatozoon (sample that was going to be sexed, hereafter called “sexed group”), and the other one was not incubated with MNPs (called “control group”). After that, each group was slowly mixed for 5 minutes by hand. The MNPs have a diameter of 50 nanometers, are composed of an iron magnetite nucleus covered with silica, and are negatively charged (provided by Clemente Associates Inc). The interaction between the negative charge of MNPs and the Z electrical potential of spermatozoa is different for those spermatozoa carrying an X chromosome (−20 mV) and those carrying a Y chromosome (−16 mV). Therefore, the Y chromosome bearing spermatozoa remained closer to MNPs, to which they bind forming a complex. Both sperm samples (sexed group and control group) were exposed to a magnet for 20 minutes. As a consequence, in the sexed group, the Y bearing sperm-MNP complexes remained attached to the tube inner wall due to the magnetic force, whereas the X chromosome spermatozoa did not bind to the MNP and remained suspended in the medium. Then, suspended spermatozoa carrying the X chromosome were slowly aspirated by a vacuum-controlled pump, transferred to a new tube, and cryopreserved. In the control group, the sperm suspension was also vacuum aspirated and cryopreserved. A summary of this process is presented in Fig. 1.

2.3. Cryopreservation and Thawing

Each sample has been frozen at 200 million spermatozoa per mL, in 0.5 mL straws using BotuCrio (Botupharma, Brazil) equine freezing extender, following the manufacturer's indications and

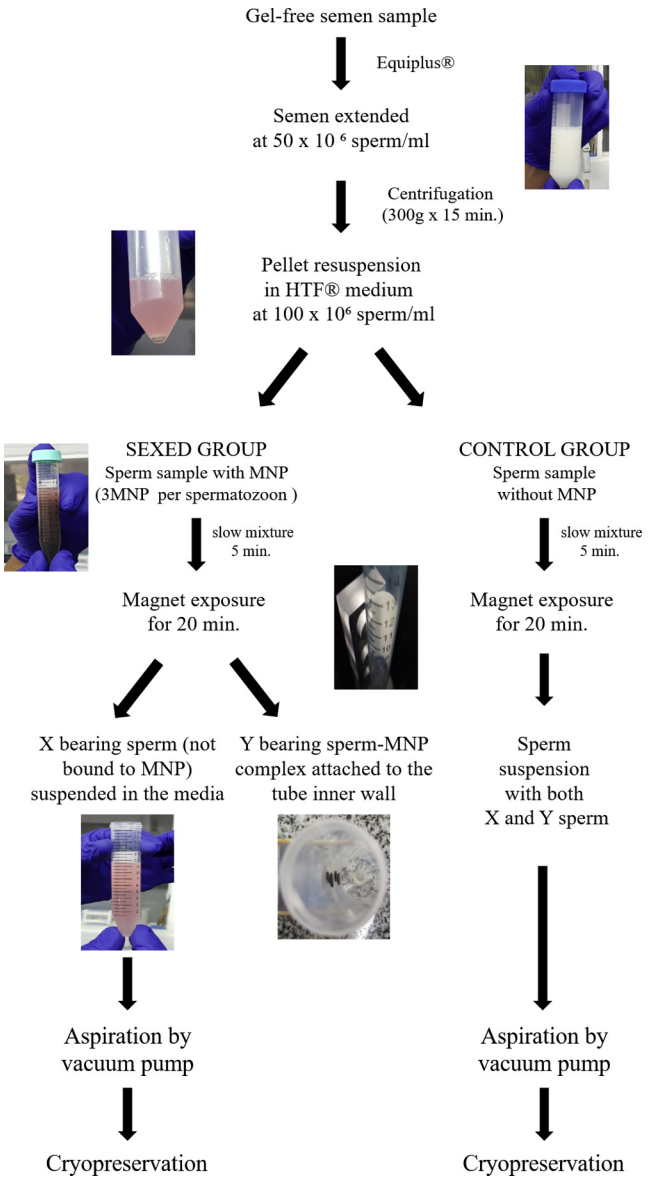

Fig. 1. The experimental procedure to obtain sexed spermatozoa by the MNP technique. MNP, magnetic nanoparticle.

standard cryopreservation protocols [24,25]. Briefly, samples were placed in a styrofoam box, using the following cooling and freezing curves: 1.3°C/minute (+25°C to +5°C), 6.3°C/minute (+5 to −120°C), and 76°C/minute (−120°C to −196°C). The straws were then thawed at 37°C for 30 seconds.

2.4. Sperm DNA Content and Viability

The efficiency of the sperm sex sorting and viability were evaluated by flow cytometry. For sperm DNA content determination in sexed and control samples, sperm number was adjusted at 6 million sperm/mL and stained with Hoechst 33342 (Sigma-Aldrich) at 0.5 μg/mL concentration for 30 minutes in a warm bath. For each experimental group (sexed and control), 100 μL of the cell suspension were placed in a cytometer tube, and 100 nM propidium iodide (PI) was used to determine sperm viability, which was added 1 minute before collecting data. Data were recorded as individual cellular events using an FACSCanto II cytometer (Becton Dickinson).

Forward scatter (FSC) and side scatter (SSC) fluorescence data were collected from 20,000 events per sample. Threshold levels for FSC and SSC were set to exclude signals from cellular debris. To better determine the DNA content, the fluid speed was adjusted to improve spermatozoa orientation. Thus, the sheath flow velocity setting was low (14 μL/min), allowing for a small core size (~10 μm). Appropriate cytometer settings were selected for Hoechst and PI. Hoechst 33342 was excited using a UV laser (350 nm) and their fluorescent signal detected as fluorescence of wavelength 461 nm, and PI was excited using a 488-nm argon excitation laser. Nonviable cells became PI positive, and their red fluorescent signal detected as fluorescence of wavelength >670 nm. Unstained control samples were used to verify that threshold settings were appropriate and to create the corresponding gates needed to discriminate debris from cells. Positive control for PI was performed with dead sperm cells. Data were analyzed using FACSDiva, FlowJo, and RStudio software. The rationale for flow cytometry analysis is explained in Fig. 2.

2.5. Physiological Sperm Parameters

2.5.1. Motility Parameters

Video camera and microscope settings were established according to Giaretta et al. [26] with few modifications. A light microscope (Nikon Instruments Inc, NY) with a 10x negative phase-contrast plan objective was used. The video camera, Nikon Camera CMOS USB 2.0 (Nikon Instruments Inc, NY), was coupled to the microscope. The videos were registered for 5 seconds at a resolution of 800 × 600 and 30 frames/s (fps) using the NIS Elements Imaging Software 3.01 (Nikon Instruments Inc, NY) and converted to avi format. Ten microliters of spermatozoa (30×10^6 sperm/mL) was put on a prewarmed slide at 38°C, covered with an 18 × 18 mm^2 coverslip, and sealed with mineral oil. Immediately after, the slide was placed over a thermal plate (38°C), and five seconds-recording were taken from 5 different fields per slide. The sperm motility percentage, curvilinear velocity, straight line velocity, and average path velocity were determined by CASA-bmg plugin using ImageJ software (version 1.46j; NIH).

2.5.2. Sperm Capacitation

After incubating spermatozoa in Biggers, Whitten and Whittingham media (0.3% bovine serum albumin and 25 mM of bicarbonate) for 45 minutes at 38.5°C with 5% CO_2, the capacitation was evaluated by two techniques: the ability of capacitated spermatozoa to undergo the pharmacologically induced acrosome exocytosis and by protein tyrosine phosphorylation.

2.5.2.1. Induced Acrosome Reaction. The percentage of spermatozoa that underwent the induced acrosome reaction was determined as an indirect indicator of the level of capacitated spermatozoa, as previously described [27]. The acrosome status was visualized by in vivo *Pisum sativum* agglutinin fluorescein staining (PSA-FITC) [28,29]. Briefly, the sperm suspension was divided into two aliquots incubated with or without 8 μM of calcium ionophore A23187 and 10 μg/mL of PSA-FITC in culture medium, for 30 minutes at 38.5°C. Then the cells were fixed in 2% formaldehyde in PBS for 20 minutes at room temperature. The sperm suspensions were washed by centrifugation in distilled water, and the pellet was let dry on a slide. The status of the acrosome was observed at 1,000× under a fluorescence microscope (Olympus BX 50; Center Valley). The acrosome-reacted spermatozoa had a green fluorescent acrosome while the acrosome-intact ones were unlabeled. The percentage of capacitated spermatozoa was determined as the difference in the percentage between induced and spontaneous acrosome-reacted spermatozoa in 200 cells.

2.5.2.2. Protein Tyrosine Phosphorylation. This parameter was determined by immunocytochemistry [30]. Spermatozoa were washed with PBS, fixed with 2% formaldehyde, smeared on slides, and air dried. Cells were permeabilized for 15 minutes in 0.2% Triton X-100 in PBS at room temperature. To block nonspecific sites, the slides were incubated for 1 hour in 5% bovine serum albumin-0.2% Triton X-100 in PBS and then incubated with the monoclonal anti-phosphotyrosine G410 mouse antibody (1:500) overnight at 4°C, in a humidified chamber. After that, the sperm were incubated with an anti-mouse Alexa-Fluo 488 antibody (1:500; Invitrogen, Carlsbad, CA). Negative controls were prepared by using a non-immune mouse immunoglobulin G instead of the anti-phosphotyrosine antibody. Finally, cells were mounted with Vectashield H-1100 (Vector Laboratories, Burlingame, CA) containing 4′,6-diamidino-2-phenylindole nuclear stain and were observed and photographed under a fluorescence microscope (Olympus, Center Valley). The state of PY was observed at 1,000× in 200 cells counted at random, in duplicated slides. The percentage of spermatozoa showing immunoreactivity on the principal piece of the flagellum was determined.

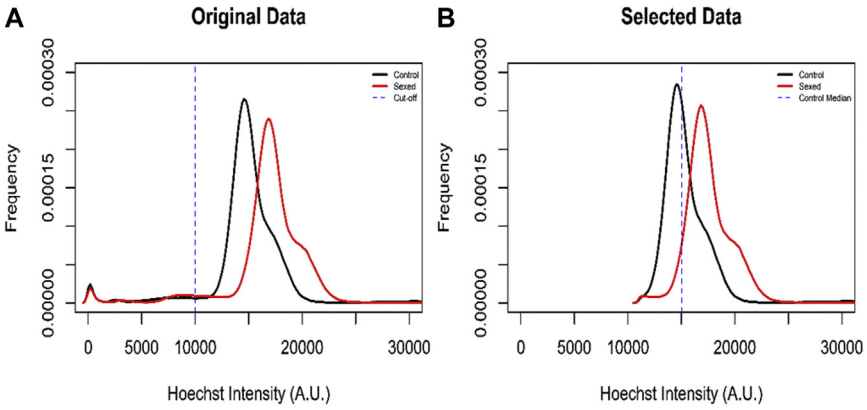

Fig. 2. Representative frequency distribution histogram of Hoechst fluorescence intensity of sexed and control samples. (A) Data collected for Cytometry show an unusual population that are not spermatozoa (data positioned before the doted blue line) and were excluded from the analysis. (B) Sperm populations showing DNA content of one representative experiment. The doted blue line represents the median of the control (black line, 50%). All cells above this value correspond to X sperm population (red line). Data correspond to 20,000 events.

2.5.3. Sperm DNA Fragmentation

The level of DNA fragmentation was evaluated by means of the sperm chromatin dispersion assay as described by Fernandez et al. [31] with minor modifications [32]. The sperm suspension was mixed with 1% low-melting point aqueous agarose at 37°C. Aliquots of 50 μL were put on a slide precoated with 0.65% standard agarose dried at 80°C. After adding a coverslip, the samples were left to solidify at 48°C for 30 minutes. The coverslips were carefully removed, and slides were immediately immersed horizontally in a tray with freshly prepared acid denaturation solution (0.08 N HCl) for 7 minutes at 22°C in the dark. DNA denaturation was stopped, and proteins were removed by a neutralizing and lysis solution 1 (0.4 M Tris, 5% 2-mercaptoethanol, 1% SDS, and 50 mM EDTA, pH 7.5) for 10 minutes at room temperature, followed by incubation in neutralizing and lysis solution 2 (0.4 M Tris, 2 M NaCl, and 1% SDS, pH 7.5) for 5 minutes at room temperature. Slides were thoroughly washed in Tris borate-EDTA buffer (0.09 M Tris-borate and 0.002 M EDTA, pH 7.5) for 2 minutes and dehydrated in sequential ethanol series. Dried sperm samples were stained with Hoechst (1 μg/mL). Images of sperm heads were captured under a fluorescence microscope (Olympus BX 50; Olympus, Center Valley, PA) coupled to a Nikon digital camera (Nikon Instruments Inc, NY), whereas at least 200 cells were analyzed per treatment. The halo area of each spermatozoon was measured using the Fiji program. Sperm were classified into different patterns according to the size of the halo (μm^2). Four halo patterns were identified: large 60 ± 10 μm^2, medium 30 ± 10 μm^2, small 10 ± 10 μm^2, and without halo. The first two patterns were considered sperm without DNA fragmentation and the other two as fragmented DNA. The percentage of DNA fragmentation was determined in sexed and control samples.

2.6. Statistical Analysis

Physiological sperm data were expressed as the mean ± standard error of mean of at least three independent experiments. Differences between treatments were determined by means of one-way analysis of variance, and a posteriori Tukey test performed with the GraphPad Prism 6.01 (La Jolla, CA) otherwise indicated, considering statistically significant differences at a level of confidence of 0.05. The parameters expressed as percentages were previously transformed to the arcsine square root of the proportion. Cytometry data were analyzed with the FACS Diva and FlowJo software, and the statistical analysis was performed using the RStudio software, v1.0143 [33]. All data were verified to accomplish the parametric assumptions of homogeneity of variances and normality.

3. Results

The efficiency of the new sex-sorting technique was verified using a flow cytometer, where 90% of the sperm population showed a significant increase in fluorescence intensity, corresponding to the spermatozoa containing the X chromosome (Table 1). Next, sperm physiology was assessed in sexed and control samples. No statistical differences were observed between sexed and control samples related to sperm viability (Table 2), motility and several velocity parameters (Fig. 3), sperm capacitation determined by the induction of the acrosome reaction and by protein tyrosine phosphorylation (Fig. 3), and the percentage of spermatozoa with DNA fragmentation (Fig. 4).

Table 1
Percentage of X spermatozoa in MNP sexed samples from three different donors.

Donkey Sample	A	B	C	% X Spermatozoa
Sexed	92	97	80	90 ± 5

MNP, magnetic nanoparticle.
Data are expressed as mean ± standard error of mean.

Table 2
Percentage of sperm viability in MNP sexed and control samples from three different donors.

Donkey Sample	A	B	C	% Viability
Sexed	18	59	20	32 ± 13.3
Control	15	44	39	33 ± 8.9

MNP, magnetic nanoparticle.
Data are expressed as a mean ± standard error of mean.

4. Discussion

In this study, we observed that those spermatozoa carrying the X chromosome can be easily isolated when the sperm sample is previously incubated with MNPs and then exposed to a magnetic field. This was the first report using flow cytometry where sperm sexing was achieved by means of the MNPs. Indeed, based on flow cytometry, 90% of the spermatozoa contained the X chromosome. Therefore, this simple technique provided a similar percentage of X spermatozoa as those achieved by means of cell sorting [34]. Additional experiments are currently being performed to confirm our findings by qPCR (cuantitative polimerase chain reaction).

However, sperm physiology (e.g., sperm membrane and DNA damage) is significantly affected by the cell sorter [7–9]. In contrast, the MNP technique provided a sperm population not only enriched in X containing spermatozoa but also viable, motile, uncapacitated spermatozoa with intact DNA similar to the control samples. Although viability and motility are relatively low in both treatments, this fact may be due to the frozen-thawed process, which is not yet standardized for donkeys. Although sperm capacitation procedure is not well defined yet for donkeys, the values are similar to those obtained for frozen-thawed equine spermatozoa (unpublished data). Although the level of sperm DNA fragmentation in sexed samples is quite low as compared to that observed in samples sexed by the cell sorter, this parameter does not differ from control samples.

It is worth noting that the embryo recovery and pregnancy rates in cows and mares artificially inseminated with frozen-thawed sexed semen by the cell sorter were significantly lower than those in unsexed samples [13,35,36]. Furthermore, the in vitro embryo production using bovine frozen-thawed sexed semen samples by the cell sorter is lower than that obtained with unsexed

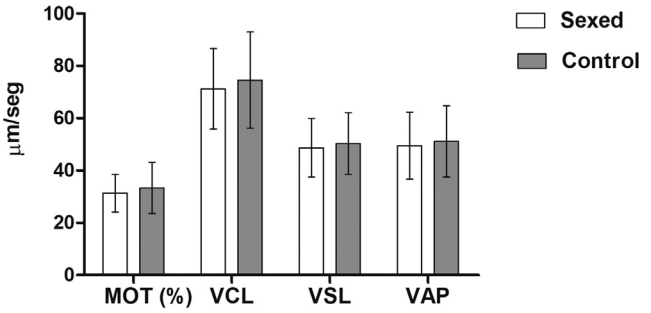

Fig. 3. Motility parameters in sexed and control donkey semen samples. Percentage of motility (MOT), curvilinear velocity (VCL), straight line velocity (VSL), and average path velocity (VAP) are represented in sexed (white bars) and control (gray bars) semen samples. Data are expressed as the mean ± standard error of mean of three independent experiments.

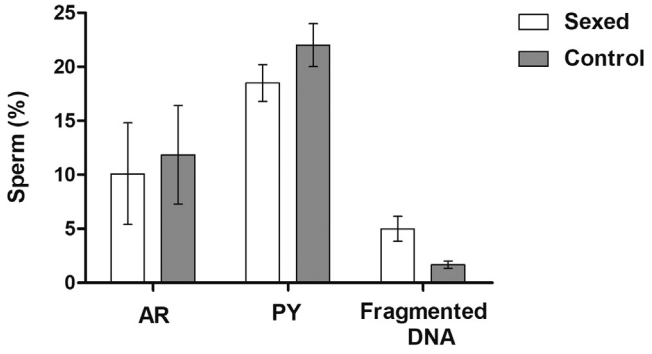

Fig. 4. Physiological sperm parameters in sexed and control donkey semen samples. Sperm capacitation defined by the induced acrosome reaction (AR) and protein tyrosine phosphorylation (PY), and sperm DNA fragmentation are represent in sexed (white bars) and control (gray bars) semen samples. Data are expressed as the mean ± standard error of mean of three independent experiments.

semen samples [37]. Preliminary experiments in stallions using spermatozoa sexed by MNPs show a pregnancy rate of 79%, and 96% of the fetus were females as determined by ultrasound [38], suggesting that the MNP technique does not significantly affect the outcome of artificial insemination. Similar experiments are currently carried out in donkeys.

5. Conclusion

In summary, the MNP technique provided an X sperm population with sperm physiological quality as the control sample. Although both techniques are similarly effective to select X spermatozoa, the MNP technique is faster and easier than the cell sorter, and it does not require high-cost equipment and specialized technicians. The new sexing technology presented here can be mainly applied to improve the donkey's milk industry.

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