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ARTICLE

Selection of High-quality Sperms by the Nanotechnological Method of Magnetic Activation in Brazilian Cervids

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ABSTRACT

Cervids show a high degree of abnormalities in their sperm cells. Thus, this study aimed to select high-quality spermatozoa using magnetic-activated sperm sorting (MASS) compared to density gradient centrifugation (DGC) by assessing the post-selection cell quality. Semen from six *Mazama* deer was collected by electroejaculation after chemical restraint. The semen was analyzed in four samples: Fresh, DGC, SEMgood - non-apoptotic fraction, and SEMpoor - apoptotic fraction. The material was analyzed for motility and vigor (light microscopy), concentration (Neubauer chamber), semen morphology (phase contrast), and supravital staining test (eosin/ nigrosine). The DGC method used 20 x 106 cells in 90% and 45% percoll® gradient. The MASS used 10 x 106 cells with 20 µl of iron nanoparticles attached to Annexin V and filtration in a magnetic separation column. Both processing methods (DGC and MASS) were effective in producing high-quality sperm samples, with a marked reduction in abnormalities from 41.83 ± 10.25 (fresh) to 14.83 ± 3.17(DGC) and 12 ± 3.01 (SEMgood), with 80.3% ± 2.06 livings cells. These findings suggest that this nanotechnological method, using nanoparticles, effectively produces high-quality semen samples in cervids for use in assisted reproduction.

1. Introduction

The preservation of a species depends on the maintenance of populations with good genetic variability, and reproduction is essential in this process. Reproductive biotechniques, such as artificial insemination, embryo transfer, *in vitro* fertilization, and semen and embryo cryopreservation, can contribute to this genetic diversity. However, the employment of these techniques is still limited in wild species, mainly due to the lack of basic knowledge in reproductive biology [1,2].

Ejaculate quality is highly variable in wild species

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when natural selection not always guarantees that males with better semen quality have more offspring, but also involves a complex process and requires several attributes so that the sperm cells may reach the fertilization site, penetrate the egg cell, and activate embryo development [3,4].

There are eight cervid species in Brazil, among them the ones belonging to the genus *Mazama*: *Mazama americana* (Erxleben, 1777), *Mazama gouazoubira* (Fischer, 1814), *Mazama nemorivaga* (Cuvier, 1817), *Mazama nana* (Hensel, 1872), and *Mazama bororo* (Duarte, 1996). The species *M. nana and M. bororo* are considered vulnerable worldwide, while the remaining species have rapidly declining populations [5].

In cervids, even in wildlife, a high degree of structural abnormalities is observed in sperm cells, with 70% and 43% of abnormalities being verified, respectively, by Assumpção [6] and Assumpção and Santos [7] in *Mazama gouazoubira*, which can be caused by natural selection mechanisms [8] or by the loss of genetic variability due to population decline [9,2]. Something similar occurs with animals in captivity, as demonstrated by Rola [10], who verified 75.5 % of sperm abnormalities in *Mazama americana*, possibly due to management stress, change in the environment, and constant coexistence with other individuals [11,12]. Poor semen quality can lead to decreased fertility in deer species, reducing the efficiency of assisted reproduction and putting at risk "ex situ" conservation programs for these animals.

The cell membrane of the spermatozoon contains phosphatidylserine (PS), a phospholipid located in the cytosolic side, and its externalization is considered a sign of apoptosis, causing membrane fluidity, decreased mitochondrial membrane potential, and DNA fragmentation[13,14,15]. The presence of this apoptotic marker is directly correlated with *in vivo* and *in vitro* fertility problems [16]. This marker (external PS) has been used to separate apoptotic cells from normal cells by techniques such as magnetic-activated cell sorting, contributing to increased motility and integrity of the sperm membrane and optimizing the cleavage and conception rates, which has modified the picture of male sub-fertility and increased the success of assisted reproduction [17].

The magnetic-activated sorting technique is a non-invasive method that uses the knowledge of the affinity of Annexin V (ANX) with the PS in the membrane. Arrighi [14] observed that PS is present in 61.4 % of sperm membranes in all regions, with more expressiveness in the head. ANX, when coupled to microspheres, attaches to the membrane-injured sperm cells and, when subjecting this set to a high-power magnetic field in a separation column, these sets will adhere to the column, while the non-apoptotic cells will freely pass through it [18,19,20]. Curti [21] and Berteli [22] verified that this type of sperm selection increases the number of cells with normal tails and increases motility and normal morphology.

The development of a sperm separation technique that does not interfere with sperm quality will allow the production of high-quality semen samples from wild animals, increasing the efficiency of assisted reproduction and species preservation. Thus, this study aimed to select high-quality spermatozoa using magnetic-activated sperm sorting (MASS) compared to density gradient centrifugation (DGC) in fresh deer semen samples by evaluating post-selection cell quality.

2. Material and Methods

Six adult male deer were used, two of each of the following species: brown brocket deer (*Mazama gouazoubira*), Amazonian brown brocket deer (*Mazama nemorivaga*), and red brocket deer (*Mazama americana*), belonging to the Deer Research and Conservation Centre (NUPECCE), Department of Animal Science of São Paulo State University, Jaboticabal, SP (IBAMA Registration nº 1/35/92/0882-5, SISBIO Registration nº 482.508). The animals were kept in individual stalls (4m x 4m), with auditory and olfactory contact between males and females, and exposed to natural fluctuations of luminosity, temperature, and relative air humidity. Feeding was provided based on the Purina® Omolene Traditional horse feed, totaling up to 500 g/animal/day, along with fresh forage, such as perennial soybean (*Neonotonia wightii*), rami (*Boehmeria nivea*), and mulberry (*Morus alba*), totaling up to 1 kg/animal/day. High-quality water was provided ad libitum.

Semen collection was performed by electroejaculation after chemical restraint. The animals received an intramuscular association of the drugs xylazine hydrochloride (1 mg/kg, Dopalen; Vetbrans Animal Health, Jacareí, Brazil) and ketamine hydrochloride (10 mg/kg, Coopazine, Mallinckrodt Vet, Cotia, Brazil). An electroejaculator (P-T Electronics®; Boring, OR; USA) coupled to a probe with 2.0 cm diameter and 28.0 cm length was used. The animals received increasing stimuli from 250 mA to 750 mA, with an average duration of three seconds and three--second intervals (total of ten stimuli per sequence). Three stimulation sequences were performed, with intervals from one to two minutes during the collection [23,24].

The analyses of sperm progressive motility (percentage) and vigor (scale from 0 to 5) were performed under light microscopy. The semen concentration was measured in a Neubauer chamber, and sperm morphology was evaluated

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in a moist chamber under phase-contrast microscopy by verifying the abnormalities individually [25]. For the supravital test (analysis of living/ dead spermatozoa), a semen smear stained with eosin-nigrosine was used (Botuvital®, Botupharma, Botucatu, SP), verifying the percentage of living and dead spermatozoa [26].

The semen was analyzed in four different samples: (1) Fresh – fresh semen; (2) DGC - density gradient centrifugation/ percoll column; (3) SEMgood – non-apoptotic fraction obtained after separation by MASS; (4) SEMpoor – apoptotic fraction obtained after the separation.

A percoll stock solution was prepared for sperm separation by DGC, composed of 90% percoll (pH 7.4; 280-290 mOsm/kg H_2O) in Eagle's medium (Sigma-Aldrich, St Louis, USA), with 0.3% bovine serum albumin (Calbiochem, Darmstadt, Germany), 10mg/L of antibiotic, and 6mM of HEPES (Sigma-Aldrich, St Louis, USA). For the percoll density gradient, 200 µl of TALP-SPERM with 200 µl of the 90% percoll solution was used in a 1.5 mL microtube (45% percoll layer), depositing 400 µl of 90% percoll below this layer. Twenty million spermatozoa were deposited over the column (Missio [27], with modifications), followed by centrifugation at 900 X G for 5 minutes, with the sperm pellet being diluted in 150 µl HEPES for cell analysis [28, 29].

The protocol adapted from Rawe [18] was used for sperm separation by MASS. To the sample containing 10 million spermatozoa, 1.5 ml of HEPES were added, followed by centrifugation at 300 X G for 10 min. The pellet was resuspended in 150 µl HEPES, and 20 µl of iron nanoparticles attached to annexin V were added (Miltenyi Biotec, Germany). After incubation for 30 minutes at ambient temperature, filtration in a proper magnetic separation column (MASS) was performed at the controlled temperature of 37^0C (filtration prototype in patent phase). The MASS comprises a column containing a matrix with ferromagnetic spheres and a high-power magnetic field in a separation column. The magnetic field effectively retains the sperms marked with the nanoparticles, and the wide matrix within the column ensures that the non-marked cells can flow easily. The filtrate or non-apoptotic fraction (SEMgood) was collected directly from the column, and the apoptotic fraction, attached to the microspheres (SEMpoor), was obtained by removing the column of the magnet and adding 300 µl of HEPES with the aid of a piston.

The values found were presented as mean ± standard error of the mean (SEM). The data were assessed for normality of residuals (Shapiro-Wilk test) and homogeneity of variances, with significance when $p<0.05$. Comparisons between treatments were performed by the MIXED procedure of the SAS software (version 9.4; SAS Institute, Inc., Cary, NC, USA). The differences between treatments were obtained by Tukey's test, with significance when $p\leq0.05$.

3. Results and Discussion

The mean progressive motility observed in the fresh semen of the deer was 75 ± 4.28%, and the vigor was 2.83 ± 0.16. The motility values observed in this study are similar to those observed in *M. americana* by Rola [10] (69.6 ± 8.92%), in *M. gouazoubira* by Perroni [30] (80%), and in *M. nana* by Abreu [11] (70 ± 8.16% and vigor 3.0 ± 0.67), but higher than in *Ozotocerus bezoarticus*, which was 45-70%[31].

Table 1 shows the mean values of progressive motility, vigor, and the types of major, minor, and total abnormalities of the sperm cells obtained in the four semen samples analyzed.

Table 1. Semen motility and vigor averages and morphological defects in sperm (%) observed in samples of Fresh semen, DGC, SEMgood, SEMbad in three species of deer *Mazama.*

	Treatments			
	Fresh	DGC	SEMgood	SEMbad
Progressive motility	75 ± 4.28^a	71.66 ± 7.49^a	18.33 ± 3.07^b	16.66 ± 2.1^b
Vigor (1-5)	2.83 ± 0.16^a	2.5 ± 0.22^a	1.66 ± 0.21^b	1 ± 0^b
Total defects	41.83 ± 10.25^a	14.83 ± 3.17^b	12 ± 3.01^b	33.83 ± 8.49^a
Major defects	20.83 ± 6.63^a	8.5 ± 2.68^a	8.5 ± 2.83^a	21.66 ± 8.04^a
Minor defects	21 ± 5.1^a	6.33 ± 1.14^{ab}	3.5 ± 0.84^b	12.16 ± 1.42^a

** Different letters on the lines indicate significant difference (p <0.05) by Tukey test.*

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The progressive motility and vigor of the spermatozoa were similar between the fresh semen and DGC samples, but low in the SEMgood and SEMpoor, maybe due to the higher sensitivity of the sperm cell in the species, the number of procedures to which the cell was subjected, and the long time spent in the analyses. Said and Lang [32] stated that all advanced methods of sperm selection involve elaborate and time-consuming manipulations and that the prolonged exposure of spermatozoa to non-physiological conditions can induce iatrogenic damage. Rawe [18] and Berteli [22] stated that the reduction in sperm motility after magnetic-activated cell sorting occurs due to the mechanic and magnetic forces within the column, which may lead to tail damage and reduction in motility, although with a high number of living cells. Rawe [18] observed that motility decreased by approximately 20% with the passage through the column since this parameter is mainly influenced by the lower number of fast progressive spermatozoa to the detriment of the higher number of slow progressive ones.

The mean total number of abnormalities verified in the fresh semen of *M. gouazoubira* was 48%, while in *M. nemorivaga* it was 61%, and 14% in *M. americana*, with a mean total of 41.83 ± 10.25%. The high number of sperm abnormalities in the fresh semen shows that semen quality is poor in most deer species and highly variable across species. Working with the same species as in this study, Rola [10] verified a higher number of pathologies in *M. americana*, with 8.37 ± 3.2% of major defects and 18.1 ± 6.5% of minor defects. Higher values were also verified by Perroni [33] in *M. nemorivaga*, with 73% of total defects. In *M. gouazoubira*, higher values compared to the ones of this study were verified by Perroni [33] (59.2% abnormalities) and Assumpção [6] (74%), while Perroni [30], Assumpção and Santos [7], and Assumpção [34] observed similar values of 46%, 43%, and 42.7% total abnormalities, respectively.

The total of abnormalities in the fresh semen was high but similar to the obtained in the SEMpoor sample. However, there was an expressive decrease of this parameter in the DGC and SEMgood samples. A high percentage of

Table 2. Individual morphological defects in sperm (%) observed in samples of Fresh semen, DGC, SEMgood, SEMbad in three species of deer *Mazama.*

	Fresh	Percoll	SEMboa	SEMruim
Abaxial/ retroaxial	0.7±0.3	0.2±0.7	0.0	1.2±0.8
Acrosome	0.2±0.1	0.0	0.0	2.3±1.4
Free normal heads	2.3±1.0	2.3±1.1	1.3±0.1	0.7±0.3
Cabeça isolada patológica	7.7±4.9 [a]	2.8±2.1 [b]	0.3±0.4 [b]	0.8±0.8 [b]
Folded tail	6.0±1.4 [a]	2.0±1.1 [b]	1.8±0.5 [b]	5.0±0.8 [a]
Curled tail	6.2±2.6 [a]	0.0 [b]	1.2±0.2 [b]	2.8±0.9 [ab]
Strongly folded and curled tail	6.8±2.0 [a]	1.3±0.2 [b]	2.7±0.0 [ab]	4.0±1.5 [a]
Abnormal contour	3.0±2.9 [ab]	2.3±0.3 [a]	1.0±0.2 [a]	6.8±2.4 [b]
Defects of midpiece	0.8±0.7	0.3±0.1	0.7±0.5	1.0±0.7
Narrow	0.0	0.0	0.8±0.6	1.2±0.5
Narrow at base	1.7±1.7	1.3±0.9	0.3±0.3	2.3±1.6
Distal cytoplasmic droplet	1.5±1.3	0.5±0.5	0.5±0.5	1.3±1.3
Proximal cytoplasmic droplet	3.3±3.3	1.2±0.9	1.3±0.8	3.7±2.9
Pouch formation	0.0	0.0	0.2±0.2	0.7±0.7

**Different letters on the lines indicate significant difference (p <0.05) by Tukey test.*

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morphological abnormalities can be considered normal in cervids, especially in species without reproductive selection [24]. Poor semen quality is a natural characteristic for wild populations that did not undergo artificial selection, domestication, and breeding [12].

When comparing the DGC and MASS techniques, we verify that both were effective in reducing sperm abnormalities, removing mainly tail and head defects. In this study, MASS showed to be very effective in selecting high-quality functional spermatozoa in these cervid species, as also verified by Faezah [26] in bovines, and by Aziz [13], Gil [35], Odhiambo [36], and Berteli [22] in human semen selected by magnetic activation.

Table 2 shows the mean values of individual abnormalities in the sperm cells obtained in the four semen samples analyzed.

Sperm abnormalities were classified according to the CBRA [25] guidelines for bovines since there is still no defect classification proposed for the studied species. We observed 14 types of sperm abnormalities, of which 4.84% were head defects, 10% of isolated heads, and 25.33% of tail and midpiece defects. As in this study, Abreu [11] also found, in *Mazama nana*, a higher incidence of tail defects (25.95 ± 6.54) than head defects (13.80 ± 6.10), as observed by Assumpção [34] in *M. gouazoubir*a, with 4% head abnormalities and 32% tail abnormalities. Peroni [33] found similar abnormalities as this study in *M. nemorivaga* and *M. gouazoubira.*

When analyzing the defects individually, it is possible to observe that the tail defects (Table 2) decreased markedly after the DGC in relation to the fresh sample. The DGC also contributed to reducing other defects in the sperm cells, but more subtly. The MASS effectively reduced head defects such as abnormal shape and slander base. Regarding other defects, such as isolated heads, both the DGC and the SEMgood reduced their numbers, showing a significant difference in the case of isolated pathological heads. When analyzing the proximal and distal cytoplasmic droplets, it is noted that the DGC and MASS techniques were not effective in reducing these types of abnormalities.

It is worth noting that the MASS also acted in reducing tail defects of the spermatozoa, such as bent tail, curled tail, and strongly bent and curled tail, in which the SEMgood showed a significant difference compared to the other samples. Arrighi [14] observed that the PS is present in 61.4% of sperm membranes in all regions (head, midpiece, and tail), thus explaining the ANX/nanoparticles attachment in the tail region, removing these defects during cell selection by magnetic activation.

Similar results to this study regarding sperm morphology were verified by Aziz [13] and Dirican [17], with a similar morphology pattern between the density gradient and the non-apoptotic fractions, which showed a low number of abnormal heads, cytoplasmic droplets, and acrosome and midpiece defects, although differing significantly from the apoptotic fraction.

The mean values obtained for the number of living cells in the supravital test of the semen samples were: fresh 80.5± 1.97 %, DGC 70.8± 2.27%, SEMgood 80.3%± 2.06, and SEMpoor 26.7± 3.67%. The number of living cells was similar (no difference) in the samples, except the SEMpoor, which decreased by 67% in relation to the SEMgood, thus reaffirming that both the DGC and the MASS techniques are effective in removing dead cells from the semen sample. Although the difference was not significant, the SEMgood showed an 11.8% increase in the number of living cells in relation to the DGC. Faezah [26], in the supravital test, observed that the magnetic separation method selects subpopulations with intact membranes, resulting in a higher number of living cells. Aziz [13] and Rawe [18], analyzing membrane integrity, showed that magnetic-activated cell sorting separates cells with an intact membrane and reduces the oxidative stress of the samples. Manuel [20] stated that this technique reduces by 60 to 70% the number of apoptotic cells. Rawe [18] and Berteli [22] stated that despite the reduction in sperm motility observed after magnetic-activated cell sorting, the number of living cells recovered after the procedures is high.

4. Conclusions

Both selection methods, density gradient centrifugation and sperm separation by magnetic activation, were effective in producing high-quality sperm samples, expressively reducing the number of abnormalities, with the subpopulation of non-apoptotic spermatozoa showing high sperm quality and a high number of living cells. These findings suggest that this nanotechnological method, using nanoparticles, is effective in producing high-quality semen samples in *Mazama* cervids.

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