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Correspondence:
Edgar A. Díaz Miranda, Department of Veterinary, Peter Henry Rolfs, University Campus, 36570-900, Viçosa, MG, Brazil.
E-mail: eddimi07@hotmail.com

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Disruption of bovine sperm functions in the presence of aplastic midpiece defect

[1]E. A. Díaz-Miranda, [1]P. P. Maitan, [1]T. P. Machado, [1]B. S. Camilo, [1]D. A. Lima, [1]D. S. Okano, [1]J. M. Penitente-Filho, [2]M. Machado-Neves, [2]L. L. de Oliveira, [3]S. E. F. Guimarães, [1]E. P. da Costa and [1]J. D. Guimarães

[1]Department of Veterinary, Universidade Federal de Viçosa, Viçosa, MG, Brazil, [2]Department of General Biology, Universidade Federal de Viçosa, Viçosa, MG, Brazil, [3]Department of Animal Science, Universidade Federal de Viçosa, Viçosa, MG, Brazil

ABSTRACT

Background: Bulls are of great importance in the productive chain and for this reason they should have a good semen quality. There is no doubt that sperm morphology is very important to bull fertility, although little is known about how exactly the abnormal morphologies may affect sperm functions.

Objectives: To detail the morphological description of the aplastic midpiece defect (AMD), as well as to understand its consequences for male fertility based on membrane and acrosome status, mitochondrial membrane potential and DNA integrity parameters.

Materials and methods: The bulls were divided into two groups: control, consisting of satisfactory potential breeders ($n = 3$); and AMD, consisting of unsatisfactory potential breeders with a high percentage of AMD ($n = 3$). Bulls were evaluated by the breeding soundness evaluation; five ejaculates were collected from each animal and analyzed by flow cytometry.

Results: Spermatozoa from AMD group exhibited lower sperm motility and vigor ($p < 0.05$). In addition, it also exhibited lower mitochondrial membrane potential ($p < 0.05$), a higher percentage of spermatozoa with DNA fragmentation ($p < 0.05$), lower acrosome and plasma membrane integrity ($p < 0.05$), and higher lipid bilayer sperm membrane disorganization ($p < 0.05$) in comparison with control bulls.

Discussion: These findings may be due to oxidative stress and a reduction of the energy production capacity in addition to an alteration in the structural composition of the sperm cell. Moreover, semen with a high percentage of AMD may also be undergoing apoptosis.

Conclusion: Bulls with a high percentage of AMD in their semen are not suitable for reproduction. Furthermore, it suggests there is a putative genetic basis for this sperm defect.

INTRODUCTION

Characterizing bull fertility is an extremely important task and should be routinely used in genetic selection to optimize the efficiency and profitability of the livestock industry (Thundathil *et al.*, 2016; Abdollahi-Arpanahi *et al.*, 2017). It is influenced by numerous factors in which sperm morphology plays an important role (Wiltbank & Parrish, 1986; Kastelic & Thundathil, 2008; Attia *et al.*, 2016). Indeed, this sperm parameter strongly reflects alterations in testicular and epididymal function (Barth & Oko, 1989; Söderquist *et al.*, 1991; Freneau *et al.*, 2010), and abnormal spermatozoa may compromise the function of morphologically normal cells due to the generation of reactive oxygen species (ROS) (Rao *et al.*, 1989; Aziz *et al.*, 2004; Chenoweth, 2007; Kastelic & Thundathil, 2008; Saacke, 2008; Aitken *et al.*, 2012). Therefore, the possible involvement of genetic factors influencing sperm morphology makes this parameter a critical objective of study (Foote, 1970; Barth & Oko, 1989; Steffen, 1997; Chenoweth, 2005; Chenoweth & McPherson, 2016).

Genetic sperm defects are classified into acrosome, head, and tail defects, as well as midpiece abnormalities (Chenoweth, 2005). The latter has been rarely reported with different effects on bull's fertility (Blom, 1959, 1966; Barth & Oko, 1989; Rocha *et al.*, 2006). Specifically, the aplastic midpiece defect (AMD) is characterized by a lack or discontinuity of the mitochondrial sheath (e.g., gap, notch, filiform). Depending on the missing segment, a dispersion of a granular substance that normally binds the mitochondria and outer dense fibers can be observed. In the end, this structural disorder may predispose sperm cells to fracture or defibrillation (Barth & Oko, 1989; Chenoweth *et al.*, 2000). It is also reported in several species (Howard *et al.*, 1991;

Rocha *et al.*, 2006; Rawe *et al.*, 2007; Veeramachaneni, 2011) and is induced by environmental effects such as by gossypol intake, selenium deficiency, viral diseases (Chenoweth & Burgess, 1972; Wallace *et al.*, 1983; Chenoweth *et al.*, 2000), and mutations (Escalier, 2006).

It is well known that the sperm midpiece consists of a mitochondrial helical sheath surrounding the axonemal complex and the nine outer dense fibers (Phillips, 1977; Zamboni, 1991). The mechanism involved in the mitochondrial sheath development, in turn, is not fully understood (Olson & Winfrey, 1986, 1990, 1992; Sun & Yang, 2010). In bovine spermatozoa, the midpiece is composed of 64 gyres with 12 μm length. Additionally, the principal arrangement of bull sperm mitochondria is a triple helix; however, in the neck region they are arranged parallel to the long axis (Phillips, 1977).

Sperm morphology is not directly related to alterations in sperm function, since morphologically abnormal spermatozoa are not always dysfunctional (Kubo-Irie *et al.*, 2004). Although the use of breeding with a high number of fertile females is the most accurate fertility test for bulls (Barth & Oko, 1989; Kastelic & Thundathil, 2008), the flow cytometry approach has become a recognized methodology for assessing fertility over the last decade (Martinez-Pastor *et al.*, 2004; Graham & Mocé, 2005; Martínez-Pastor *et al.*, 2010; Hossain *et al.*, 2011). In this context, the evaluation of sperm functional competent membranes and organelles, and an intact haploid genome are prerequisites to predict successful pregnancies (Silva & Gadella, 2006; Hossain *et al.*, 2011).

Motile spermatozoa had been considered as viable. Nevertheless, sperm viability is related to an intact plasma membrane (Hallap *et al.*, 2005) due to its intrinsical relationship to sperm functions, including maintenance of cell homeostasis and fertilizing capability (Lenzi *et al.*, 1996; Flesch & Gadella, 2000; Silva & Gadella, 2006; Rodríguez-Martínez, 2007). For instance, the acrosome membrane must remain intact until the sperm capacitation in the female reproductive tract (La Spina *et al.*, 2016), when the releasing of acrosomal enzymes allows the spermatozoa to penetrate the zona pellucida (Flesch & Gadella, 2000; Silva & Gadella, 2006). Moreover, DNA integrity is highly important for embryo development (Chenoweth, 2007; Aitken *et al.*, 2009; Aitken & Koppers, 2011). Spermatozoa with damaged DNA may fertilize an egg, but the embryo may undergo apoptosis after its first cleavages (Borini *et al.*, 2006; Fatehi *et al.*, 2006). Finally, sperm mitochondria are involved in energy production via aerobic oxidative phosphorylation (Silva & Gadella, 2006; Peña *et al.*, 2009; Hossain *et al.*, 2011; Amaral *et al.*, 2013) and play an important role in maturation (Aitken *et al.*, 2007), capacitation, and apoptosis (Aitken *et al.*, 2012; Aitken & Baker, 2013).

Therefore, we hypothesized that bulls with a high percentage of AMD are not suitable for reproduction. In the present study, we aimed to detail the morphological description of this sperm defect, and to understand the consequences on male fertility based on membrane and acrosome status, mitochondrial membrane potential and DNA integrity parameters.

MATERIALS AND METHODS

Reagents and media

Fluorescein isothiocyanate (FITC)-conjugated *Pisum sativum* (PSA) (L-0770, Sigma-Aldrich, Saint Louis, MO, EUA) (Excitation, 488 nm; Emission, 519 nm), propidium iodide (PI) (P-4170, Sigma-Aldrich) (Excitation, 488 nm; Emission, 636 nm), 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) (T4069, Sigma-Aldrich) (Excitation, 488 nm; Emission, monomers 525–530 nm and aggregates 590 nm), merocyanine 540 (M540) (M-24571, Molecular Probes Inc., Eugene, OR, EUA) (Excitation, 555 nm; Emission, 578 nm), acridine orange (AO) (A1301, Molecular Probes Inc., Eugene, OR, EUA) (Excitation, 488 nm; Emission, 515–530 nm and > 630 nm), and all the reagents which were necessary for preparation of the culture media were purchased from Sigma-Aldrich.

Ethics and animals

The experiment was conducted at a dairy cattle research facility at the Universidade Federal de Viçosa (UFV), Viçosa, Brazil. Animal care procedures and research protocols followed the Committee of Ethics on Animal Handling guidelines (CEUA/UFV, protocol no. 17/2017). Six Gyr bulls (*Bos taurus indicus*) 24 to 36 months of age obtained from the same herd and cattle management were group-fed in pens twice per day. The feed consisted of corn silage *ad libitum*, mineral mixture, and 2 kg of natural matter basis concentrate, which was administered half at 08:00 am and the remaining half at 04:00 pm. In addition, animals were allowed to graze in a pasture of *Urochloa decumbens*. The pens were 5.4 × 5.4 m, and were constructed of metal gates and cables, a concrete feed bunk, a concrete slatted floor, and an automatic water cup.

Semen collection

Thirty ejaculates were collected from six bulls by electroejaculation (five ejaculates per bull) in intervals of 3 days. Semen was stored in prewarmed, graded, conical plastic tubes that were protected (by a polystyrene cover) from light, cold shock, and rapid temperature changes. Individual ejaculates were divided into two aliquots. The first sample was used in the semen analysis from the breeding soundness evaluation and complementary routine tests, whereas the second sample was assayed through the flow cytometry.

Breeding soundness evaluation (BSE) and experimental design

In the present study, the bull selection was based on the traditional BSE, according to the standards determined by the Brazilian College of Animal Reproduction (CBRA, 2013). First, a rigorous clinical andrological examination was conducted in order to determine the normality of testicular and epididymal function, as well as of the genital organs (Rodríguez-Martínez, 2007). Further, data concerning scrotal circumference, testicular length and width were also evaluated. Immediately after semen collection, mass motility, sperm motility, and sperm vigor were subjectively assessed using light microscopy. Total sperm concentration was determined using a Neubauer hemocytometer chamber, whereas pH value was determined using pH test strips (MACHEREY-NAGEL®).

Sperm morphology was assessed by phase contrast microscopy of wet-mount semen fixed in isotonic formol saline (Hancock, 1957) using major and minor defects classification (Blom, 1973). Major abnormalities are those correlated to impaired fertility, whereas minor sperm defects are other deviating forms of minor importance. However, herein, the sperm morphology was also classified according to the anatomic site of the sperm

defect, such as the acrosome, head, midpiece, and tail, in order to represent the sperm picture of bulls with AMD. Although the AMD is characterized by segmental aplasia of the mitochondrial sheath (SAMS) to aplasia of the mitochondrial sheath (AMS), both abnormalities were described separately. Moreover, all morphological abnormalities in the sperm cell were recorded. It means that both head and midpiece abnormalities may be present in each spermatozoon.

After BSE, bulls were divided into two groups, the first group was designated as the control group (n = 3), which consisted of bulls classified as satisfactory potential breeders, while bulls from the second group were designated by the high percentage of aplastic midpiece defect (AMD), and therefore, classified as unsatisfactory potential breeders (n = 3; AMD group). Bull designated as a satisfactory potential breeder must present a healthy and sound status, with adequate scrotal circumference. Regarding sperm parameters, the bull must present sperm motility ≥60%; morphologically normal spermatozoa ≥70%; major sperm defects ≤10%; minor sperm defects ≤20%; major individual defects ≤5%; minor individual defects ≤10% (CBRA, 2013).

Complementary routine tests, such as supravital test (ST) using eosin–nigrosin staining and hypoosmotic swelling test (HOST), were performed for evaluating the sperm membrane viability and its functional competence as described by Barth & Oko (1989) and Jeyendran *et al.* (1984), respectively.

Flow cytometry

The BD FACS Verse flow cytometer (Becton Dickinson, Sunny-Vale, CA, USA) equipped with blue (488 nm, 20 mW) and red lasers (640 nm, 40 mW), as well as FL-1 (527/32 BP), FL-2 (568/42 BP), FL-3 (700/54 BP), and FL-5 (660/10 BP) filters were used for the flow cytometry analysis. Flow cytometry assessments were analyzed after non-sperm events were gated out of analyses as determined on forward and sideward scatter properties, and the quadrants used to quantify the frequency of each sperm subpopulation depended on each analysis. Data from 10,000 sperm events were recorded. Samples used for staining and flow cytometry analysis were diluted in phosphate-buffered saline (PBS) 0.1 M.

Plasma membrane and acrosome integrity (PMAI) were evaluated by dual staining with FITC-PSA and PI as described by Oliveira *et al.* (2012) with modifications. Briefly, to 150 μL of spermatozoa (5×10^6 spermatozoa/mL) 10 μL of FITC-PSA (100 μg/mL) and 3 μL of PI (0.5 mg/mL) were added. After ten minutes of incubation at 37 °C, sperm samples were diluted with 150 μL of PBS and analyzed by flow cytometer. PI is a membrane-impermeable probe that penetrates cells with a broken plasmalemma, emitting red fluorescence when binding to nucleic acids (Martínez-Pastor *et al.*, 2010). The PSA conjugated with fluorochrome FITC emits green fluorescence in reacted or deteriorated acrosomes when it binds to a component of the enzyme matrix in the lumen of the acrosome (Silva & Gadella, 2006). This dual staining assay classifies spermatozoa in four subpopulations. The first subpopulation consists of unstained spermatozoa (FnPn) with intact acrosome and plasma membrane, the second subpopulation (FpPn) consists of spermatozoa with a damaged acrosome and an intact plasma membrane. The third (FnPp) subpopulation consists of spermatozoa with an intact acrosome and a damaged plasma membrane, and the last subpopulation consists of spermatozoa that stained both (FpPp) with damaged acrosome and plasma membranes. The percentage of sperm cells presenting intact plasma membranes (Pn) and presenting intact acrosome (Fn) were also calculated.

Mitochondrial membrane potential (MMP) was assessed by using JC-1 dye as described by Ortega-Ferrusola *et al.* (2010) with modifications. In brief, to 500 μL of spermatozoa (5×10^6 spermatozoa/mL) 3 μL of JC-1 (153 μM) were added, and after 15 min of incubation at 37 °C, sperm samples were analyzed by flow cytometer. The JC-1 probe accumulates in the mitochondria as a fluorescent green monomer (inactive mitochondria), and when mitochondria exhibit high membrane potential (active mitochondria), the monomers form aggregates that shift to fluorescent orange.

Lipid bilayer sperm membrane disorganization (LBSD) was determined by dual staining with M540 and PI. To 150 μL of spermatozoa (5×10^6 spermatozoa/mL) 0.5 μL of M540 (0.5 mg/mL) and 3 μL of PI were added, and after 15 min of incubation at 37 °C, sperm samples were diluted with 150 μL de PBS. This staining aims to select a subpopulation of unstained spermatozoa considered alive and with an intact membrane; from this subpopulation, a second sperm subpopulation consisting of M540 negative cells without lipid disorganization was selected. The M540 is a lipophilic probe that binds to the plasmalemma, and when the packing order of phospholipids decrease in the membrane the probe increases its orange fluorescence, assessing membrane fluidity (Martínez-Pastor *et al.*, 2010).

For the DNA integrity assay, one aliquot of semen was centrifuged at 700 ***g*** for 5 min at room temperature. The sperm pellet was fixed in paraformaldehyde 4% for 10 min at 4 °C, washed two times in PBS 0.1 M and stored in Glycine solution (Aitken *et al.*, 2015). The sperm chromatin structure assay (SCSA) was performed as described by Evenson *et al.* (2002). To 200 μL of fixed spermatozoa diluted in TNE buffer (0.15 M NaCl, 0.01 M Tris HCl, 1 mM disodium EDTA pH 7.4, 4 °C) (5×10^6 spermatozoa/mL), 400 μL of 0.08 N HCl, 0.15 M NaCl, and 0.1% Triton X 100 pH 1.2 were mixed. After 30 sec of incubation at 4 °C, 1.20 mL of acridine orange staining solution (6 μg/mL AO; 0.037 M citric acid, 0.126 M Na_2HPO_4, 0.0011 M disodium EDTA, 0.15 M NaCl pH 6.0, 4 °C) were added, and 3 min later, sperm samples were analyzed by flow cytometer. AO is a metachromatic dye that intercalates into double-stranded DNA (native) as a green fluorescence, and shift to orange-red fluorescence when stacks on single-stranded DNA (abnormal). The percentage of spermatozoa with fragmented DNA or Cells Outside the Main Population (COMP α_t) and the majority of normal spermatozoa (main population) mean values were calculated after the debris subpopulation was excluded (Martinez-Pastor *et al.*, 2004; Love, 2005).

Statistical analysis

The experiment was conducted according to the model:

$$Y_{ij} = \mu + G_i + B_j + e_{ij}$$

where, Y_{ij} = response, μ = constant, G_i = effect of the group, B_j = random effect of the bull, and e_{ij} = error.

Statistical Analysis System (version 9.0; SAS Institute Inc., Cary, NC, USA) was used to conduct data analysis. Data were submitted to Kolmogorov–Smirnov and Bartlett tests to verify normality of errors and homogeneity of variances, respectively. Sperm concentration data were submitted to square root transformation ($Y' = \sqrt{Y + 0.5}$). Moreover, data were analyzed using MIXED procedure considering bulls as a random effect (Littell *et al.*, 2006). The LS-means were compared by Tukey–Kramer test. Data of sperm vigor and mass movement were evaluated by Kruskal–Wallis test (NPAR1WAY procedure). Overall, differences were considered significant when $p < 0.05$.

RESULTS

No significant differences were observed between groups in the physical and clinical evaluation (Fig. S1). In contrast, bulls from AMD group showed lower sperm motility when compared to control bulls ($p < 0.05$; Table 1). Particularly, some bulls from the AMD group showed no motility in several ejaculates. Furthermore, mass motility and sperm vigor were lower in bulls with AMD than the controls ($p < 0.05$; Table 1). No differences between groups were observed in volume, sperm concentration, and pH values ($p > 0.05$; Table 1). Finally, bulls from the AMD group presented a lower percentage of reacted cells to hypoosmotic and supravital tests than the control group ($p < 0.05$; Table 1).

The sperm morphology was clearly a focus of this study. In this context, bulls from AMD group presented several and high percentage of sperm midpiece abnormalities compared to the control group ($p < 0.05$; Table 2), including corkscrew, stump, Dag defect, fracture, abaxial, swelling midpiece, defibrillation, and proximal droplets (Fig. 1). In addition to mitochondrial gaps or lack of mitochondria (SAMS), bulls from the AMD group also presented a high percentage of spermatozoa without mitochondria (AMS) ($p < 0.05$), and some had an absence of the outer dense fibers, displaying a thin midpiece as the terminal piece (filiform). Furthermore, spermatozoa from bulls in the AMD group presented more than one type of cell abnormality, and occasionally showed more than one combination of midpiece abnormalities, such as swollen midpiece with mitochondrial gaps or corkscrew with defibrillation. As a consequence of the high levels of midpiece defects, bulls in the AMD group showed high levels of major defects ($p < 0.05$) as well as minor defects ($p < 0.05$; Table 3) resulting in high levels of total defects.

Table 1 Sperm parameters from fresh semen of Gyr bulls in the aplastic midpiece defect (AMD) and control groups

Sperm parameters	Control	AMD
Progressive motility (%)	72.1 ± 1.86[a]	0.1 ± 0.21[b]
Sperm vigor[1] (0–5)	2.97 ± 0.11[a]	0.04 ± 0.04[b]
Mass motility[1] (0–5)	0.5 ± 0.15[a]	0[b]
Volume (mL)	5.30 ± 0.42[a]	4.24 ± 0.38[a]
Concentration ($\times 10^6$/mL)	280.7 ± 80.7[a]	424.7 ± 72.3[a]
Total sperm concentration ($\times 10^9$)	1.52 ± 0.41[a]	1.80 ± 0.37[a]
pH	7.36 ± 0.26[a]	7.53 ± 0.26[a]
ST (%)	65.84 ± 5.48[a]	20.59 ± 5.66[b]
HOST (%)	54.83 ± 7.01[a]	19.04 ± 4.95[b]

Mean ± SE; Different letters within rows indicate significant differences $p < 0.05$ by Tukey–Kramer. HOST, hypoosmotic swelling test; ST, supravital test with eosin–nigrosin stain. [1]Kruskal–Wallis tests.

Table 2 Sperm morphology from Gyr bulls in the aplastic midpiece defect (AMD) and control groups

Sperm defects (%)[1]	Control	AMD
Acrosome	2.89 ± 0.40[a]	3.70 ± 0.25[a]
Head	4.93 ± 0.44[b]	7.95 ± 0.28[a]
Midpiece	3.35 ± 0.93[b]	87.58 ± 0.58[a]
SAMS	0[b]	17.47 ± 0.46[a]
Aplasia MS	0[b]	37.50 ± 0.86[a]
Dag defect	0[b]	10.12 ± 0.26[a]
Defibrillation	0[b]	4.06 ± 0.19[a]
Fracture	0.35 ± 0.18[b]	1.56 ± 0.11[a]
Swelling	0.06 ± 0.28[b]	3.91 ± 0.18[a]
Corkscrew	0[b]	2.53 ± 0.10[a]
Stump	0[b]	0.34 ± 0.02[a]
Abaxial	1.96 ± 0.29[b]	5.22 ± 0.18[a]
Proximal droplet	0.98 ± 0.31[b]	4.87 ± 0.19[a]
Tail	2.78 ± 0.58[b]	8.60 ± 0.36[a]

Mean ± SE; MS, mitochondrial sheath; SAMS, segmental aplasia of the mitochondrial sheath. Different letters within rows indicate significant differences $p < 0.05$ by Tukey–Kramer test. [1]Anatomic site of the sperm defect classification.

Bulls from AMD group also presented a higher percentage of sperm subpopulation with acrosome and plasma membrane damage than bulls from the control group ($p < 0.05$; Fig. 2). The latter, in turn, had a higher percentage of sperm subpopulation with an intact acrosome and plasma membrane than the AMD group ($p < 0.05$; Fig. 2). The control group showed a higher percentage of spermatozoa with an intact plasma membrane and spermatozoa with an intact acrosome ($p < 0.05$; Fig. 2). As expected, mitochondrial membrane potential was lower in bulls from the AMD group compared with the control group ($p < 0.05$; Fig. 3).

The PI-M540 assay provided further evidence among membrane status. Bulls from the AMD group presented lower (24.9%) spermatozoa with plasma membrane integrity than the control group ($p < 0.05$). Moreover, only 45.5% of these intact membranes were organized, whereas the control group ($p < 0.05$; Fig. 4) showed 76.1% spermatozoa with an organized lipid bilayer membrane. These results showed similar values to those of the complementary routine tests reinforcing the notion of membrane function and integrity from bulls with AMD. Finally, bulls from the AMD group presented a higher percentage of spermatozoa with fragmented DNA than bulls from the control group ($p < 0.05$; Fig. 5).

DISCUSSION

Our results provide pioneer information concerning functional features of bull spermatozoa in the presence of AMD and remarkable differences between fresh semen from bulls with normal and abnormal sperm morphology. Strikingly, mitochondrial abnormalities in sperm cells have received little attention (Moretti *et al.*, 2016), especially considering the importance of sperm morphology analysis in the determination of bull fertility (Chenoweth, 2005; Saacke, 2008; Enciso *et al.*, 2011).

In the present study, bulls with AMD exhibited a lack of sperm motility, in contrast to previous studies in which AMD was not linked to have a detrimental effect in motility or in fertility (Barth & Oko, 1989; Rocha *et al.*, 2006). The main differences between these earlier studies include the percentage of abnormal midpieces, which raises the issue of how little is actually known about the limit when the percentage of a sperm pathology could

Figure 1 Phase contrast and light microscopy analysis of Gyr bulls spermatozoa from the AMD group. (A) Phase contrast image of a normal spermatozoon, (B–H) spermatozoa with segmental aplasia of the mitochondrial sheath (SAMS), DAG defect, bent tail (Bt), aplasia of the mitochondrial sheath (AMS), swelling midpiece (Sw), frature (Fr), tail stump (St), pseudo droplet (Psd), free normal head (FNH), proximal droplet (Pd), and swelling acrosome (Swa). (I) Light microscopy picture from smear fixed in Carnoy's solution (methanol and glacial acetic acid in a 3:1 proportion) stained with Giemsa showing strongly foiled or coiled tail (SFCT), pouch formation (Pt), AMS, SAMS, and Swa. (J) Light microscopy picture from smear fixed in Carnoy's solution (methanol and glacial acetic acid in a 3:1 proportion) showing SAMS, Fr, Swa, and SFCT.

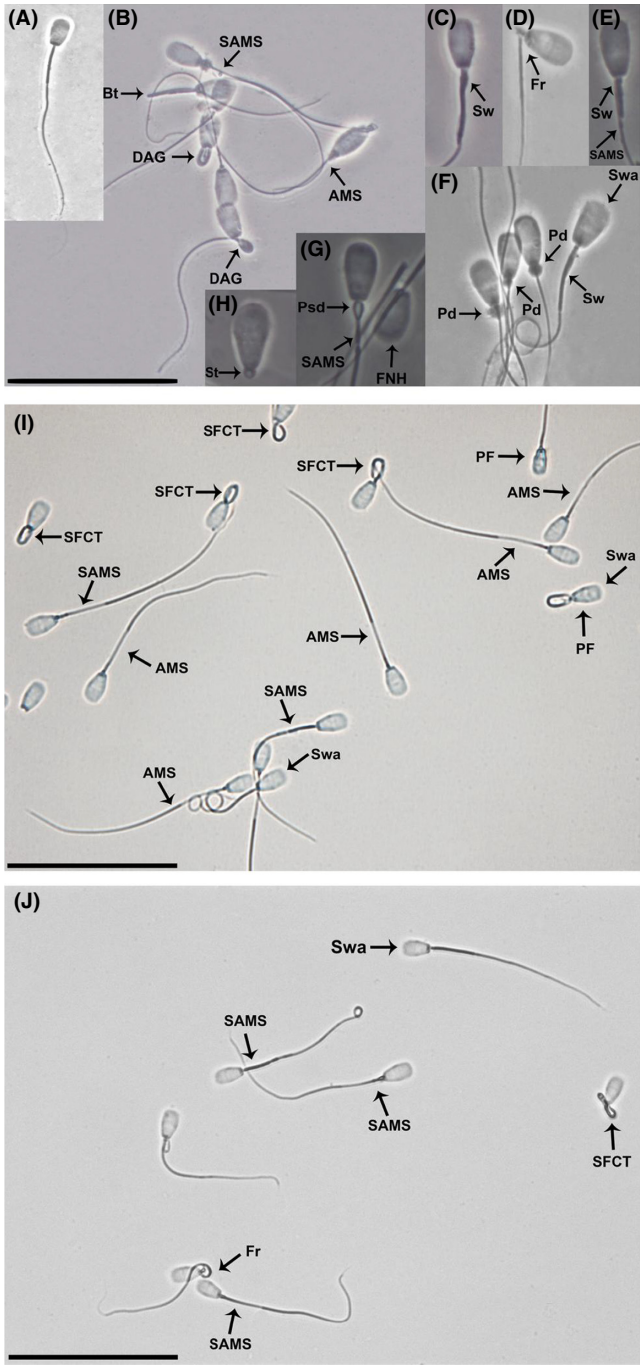


Table 3 Sperm defects from Gyr bulls in the aplastic midpiece defect (AMD) and control groups

Sperm defects (%)[1]	Control	AMD
Major	10.62 ± 0.90[b]	98.37 ± 0.56[a]
Minor	3.33 ± 0.49[b]	9.46 ± 0.31[a]
Total	13.95 ± 1.04[b]	107.83 ± 0.65[a]

Mean ± SE; Different letters within rows indicate significant differences $p < 0.05$ by Tukey–Kramer test. [1]Major and minor classification (Blom, 1973).

begin to affect the sperm function. On the other hand, the sperm pictures were not the same; for example, it is reported that in the knobbed spermatozoa the effect on the fertility depends on the severity of the defect (Thundathil *et al.*, 2000). The lack of mitochondria in the bulls from our study was higher than reported by Rocha *et al.* (2006), and it is recognized that spermatozoa without mitochondria are often degenerating (Zamboni, 1991). Moreover, two important sperm functions are suggested to be affected by midpieces defects. First, a reduction of energy production capacity suggests that mitochondria have a crucial role in bovine sperm movement by producing ATP via oxidative phosphorylation (Silva & Gadella, 2006; Peña *et al.*, 2009; Amaral *et al.*, 2013). Second, there is an alteration in the structural composition of the mitochondrial sheath that plays a supporting role during sperm motility. This structure must be elastic and strong to sustain and contain the flexion of the axoneme during sperm motility (Harris, 1976; Olson & Linck, 1977). A structurally deficient mitochondrial sheath (aplasia, swollen, gaps) may cause structural damage to the axoneme integrity, resulting in a wide variety of midpiece defects (Veeramachaneni, 2011).

In addition, fresh semen from bulls with AMD exhibited alterations in several sperm traits, such as low mitochondrial membrane potential, a high percentage of spermatozoa with DNA fragmentation, and a high percentage of damaged plasma membrane and acrosome. Likewise, half of the spermatozoa with an intact plasma membrane showed disorganization in their membrane lipid bilayer. An explanation to our findings includes the possibility that spermatozoa from bulls with AMD are undergoing oxidative stress, which is the major contributor to the defective sperm function (Aitken *et al.*, 2012). Although spermatozoa with AMD have a lack of mitochondria, they may also exhibit mitochondrial dysfunction leading to high ROS production since mitochondria are the main source of sperm-produced ROS via electron transport chain (Koppers *et al.*, 2008; Amaral *et al.*, 2013). Hence, it can affect mitochondrial integrity leading to a cycle in which ROS injures the mitochondrial membrane and the injured mitochondrial membrane enhances ROS production (Sanocka & Kurpisz, 2004; Sabeti *et al.*, 2016). Notwithstanding, there are other potential sources of ROS in bovine semen, such as spermatozoa with abnormal morphology (Rao *et al.*, 1989; Aziz *et al.*, 2004; Aitken *et al.*, 2012) and dead spermatozoa via an aromatic amino acid oxidase catalyzed reaction (Upreti *et al.*, 1998). In this study, semen from AMD bulls combined a harmful environment of a high percentage of morphologically abnormal with dead spermatozoa, suggesting that spermatozoa from this group are certainly undergoing oxidative stress.

Midpiece abnormalities were also related to high levels of lipid peroxidation, possibly due to the high membrane content associated with this abnormality, which makes midpiece region highly susceptible to free radical attack (Rao *et al.*, 1989; Lenzi *et al.*, 1996). Previous studies suggested that the majority of enzymatic antioxidant system is located in the midpiece, and its

Figure 2 Sperm plasma membrane and acrosome integrity (PMAI) from fresh semen of Gyr bulls in the aplastic midpiece defect (AMD) and control groups. Representative dot plot showing PMAI of (A) control group. (B) AMD group. (C) Percentage of each sperm subpopulations according to membranes integrity: FpPp (spermatozoa with acrosome and plasma membrane damage, UR); FnPn (spermatozoa with an intact acrosome and plasma membrane, LL); FpPn (spermatozoa with a damaged acrosome and an intact plasma membrane, UL); FnPp (spermatozoa with an intact acrosome and a damaged plasma membrane, LR). Additionally, it is also shown spermatozoa with either intact plasma membrane (Pn) or acrosome (Fn). Mean ± SE; *$p < 0.05$ by Tukey–Kramer test.

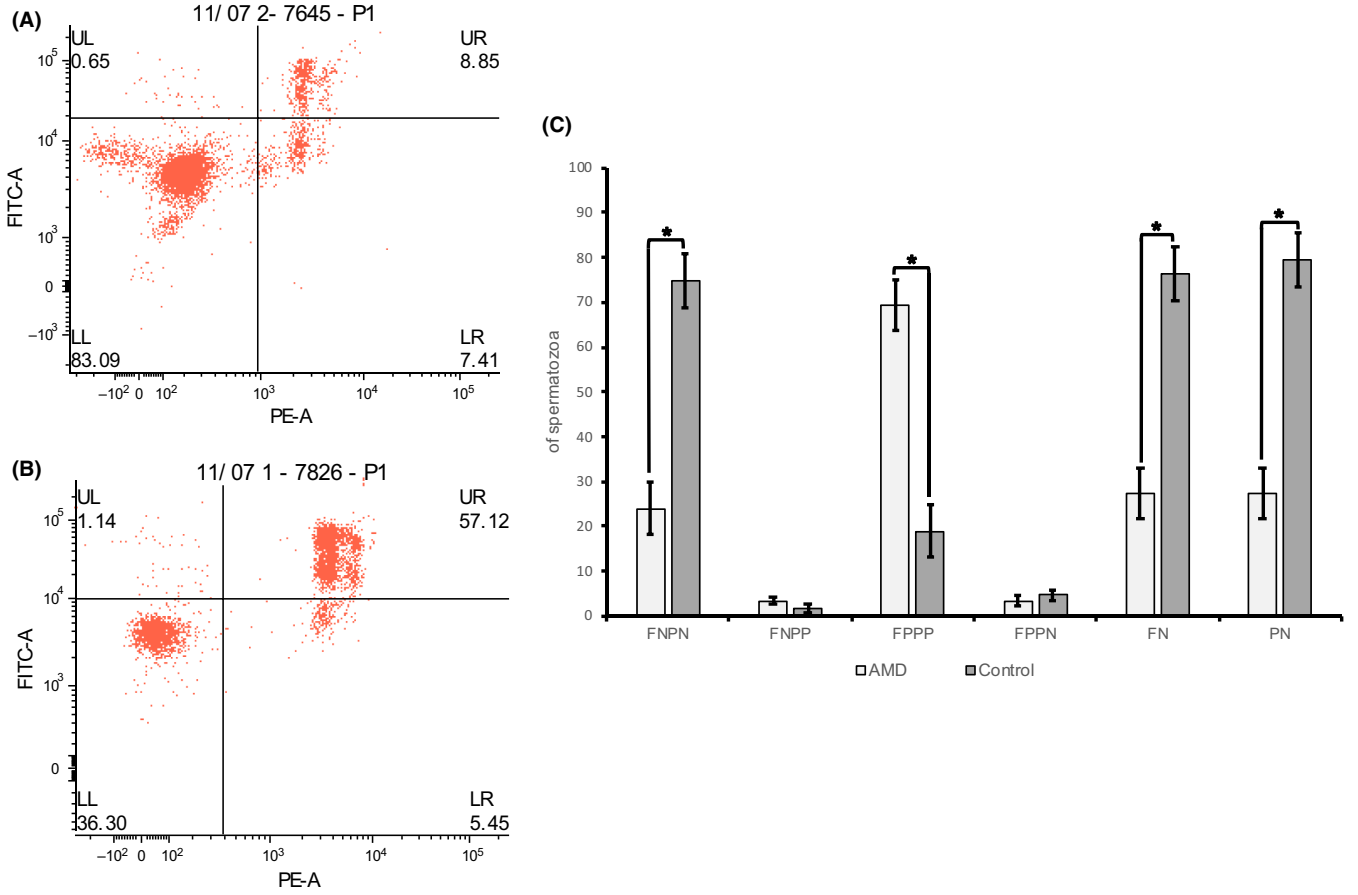
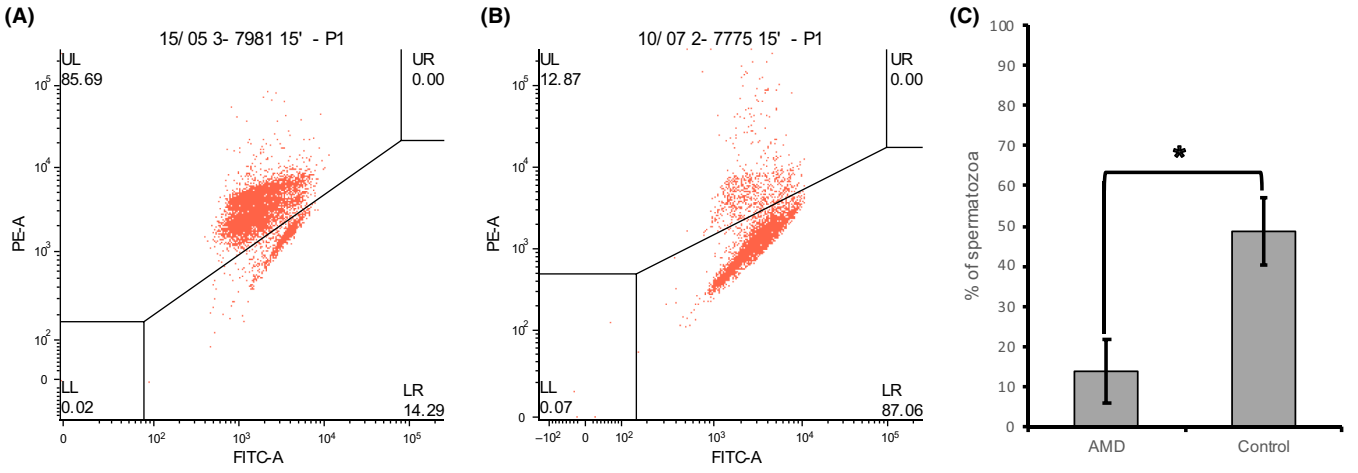


Figure 3 Mitochondrial membrane potential (MMP) from fresh semen of Gyr bulls in the aplastic midpiece defect (AMD) and control groups. Representative dot plot showing MMP of (A) control group and (B) AMD group; sperm cells subpopulations showing high MMP (UL) and low MMP (LR). (C) Percentage of sperm populations with high MMP from control and AMD groups. Mean ± SE; *$p < 0.05$ by Tukey-Kramer test.



dysfunction may be associated with morphological abnormalities in this region, favoring lipid peroxidation (Rao *et al.*, 1989; Aitken & Curry, 2011; O'Flaherty, 2014). Thus, midpiece defects such as AMD may be more vulnerable to oxidative stress. It may explain the high levels of membrane damage as well as the fluidity of the plasma membrane of the viable spermatozoa in the

AMD group. Since the acrosome acts in concert with the plasma membrane overlying the acrosome during the early events of fertilization (Abou-Haila & Tulsiani, 2000), it may share the same vulnerability to oxidative stress as the plasma membrane and similar damage as reported in the present study. Additionally, spermatozoa from AMD group had a higher percentage of acrosome damage, although they did not exhibit abnormal acrosome morphology. This fact supports the hypothesis that the acrosome damage, or early acrosome reaction, is due to the oxidative stress (El-Taieb *et al.*, 2015).

Morphologically, abnormal bull spermatozoa have poor DNA quality and damaged DNA (Khalifa *et al.*, 2008; Enciso *et al.*, 2011; Boe-Hansen *et al.*, 2018). Herein, the high levels of damaged DNA observed in spermatozoa from AMD bulls may be a result of oxidative stress occurrence. Previously, sperm midpiece defects were correlated to high levels of DNA fragmentation (Morrell *et al.*, 2008; Speyer *et al.*, 2010). In fact, chromatin compaction occurs during spermiogenesis, which makes the DNA extremely stable and difficult to damage. Inversely, it is known that poor compaction makes DNA vulnerable to injury (Aitken *et al.*, 2009; Agarwal *et al.*, 2014). Thus, the major cause of DNA damage is oxidative stress (Aitken *et al.*, 2012).

Our findings support the hypothesis that spermatozoa with AMD may be undergoing apoptosis, particularly because the sperm midpiece is where the life-death decisions for spermatozoa are made (Aitken & Baker, 2013). Thus, this sperm pathology exhibits the features of apoptosis, including DNA damage, mitochondrial dysfunction, and motility loss (Aitken & Curry, 2011; Aitken & Koppers, 2011; Aitken *et al.*, 2012). Furthermore, bulls with the AMD defect in their semen presented a high percentage of spermatozoa with lipid bilayer sperm membrane disorganization assessed by M540 probe. It was reported as suitable to detect early membrane degeneration due to the apoptotic process (Muratori *et al.*, 2004). Besides, it has been proposed that the capacitation-dependent lipid remodeling of sperm plasma membrane and the apoptotic pathway could be interconnected (Aitken, 2011; Bernabò *et al.*, 2018).

There is a growing list of sperm defects in domestic animals considered to be of genetic origin (Chenoweth & McPherson, 2014). The AMD in Gyr bulls may better fit classified under the systematic (monomorphic) and non-systematic (polymorphic, non-specific) sperm defects categorization (Chemes & Rawe, 2003). The systematic defects are suspected to be of genetic origin and the sperm pathology persists in every ejaculate (Chemes & Rawe, 2003; Moretti *et al.*, 2016). The fact that breed could have an effect in the incidence of sperm abnormalities (Söderquist *et al.*, 1991) and based on our results suggests that in this particular herd or breed, the AMD is a systematic defect with a genetic origin. However, there are no identified polymorphisms associated with heritable sperm defects (Sutovsky *et al.*, 2015).

Figure 4 Lipid bilayer sperm membrane disorganization (LBSD) from fresh semen of Gyr bulls in the aplastic midpiece defect (AMD) and control groups. Representative dot plot showing membrane integrity and LBSD of the (A, B) control group and the (C, D) AMD group. In order to restrict the assay to live spermatozoa, the PI dye was used in (A) control group and (C) AMD group showing live sperm cell subpopulation (LL) and dead sperm cells subpopulations (UL). The population of live spermatozoa was used in the (B) control Group and (D) AMD group for the M540 assay showing sperm cell subpopulation with LBSD (UL) and without LBSD (LL). (E) Percentages of spermatozoa from the control group and the AMD group with membrane integrity (PN2) and organized membranes (MN). Mean ± SE; *$p < 0.05$ by Tukey–Kramer test.

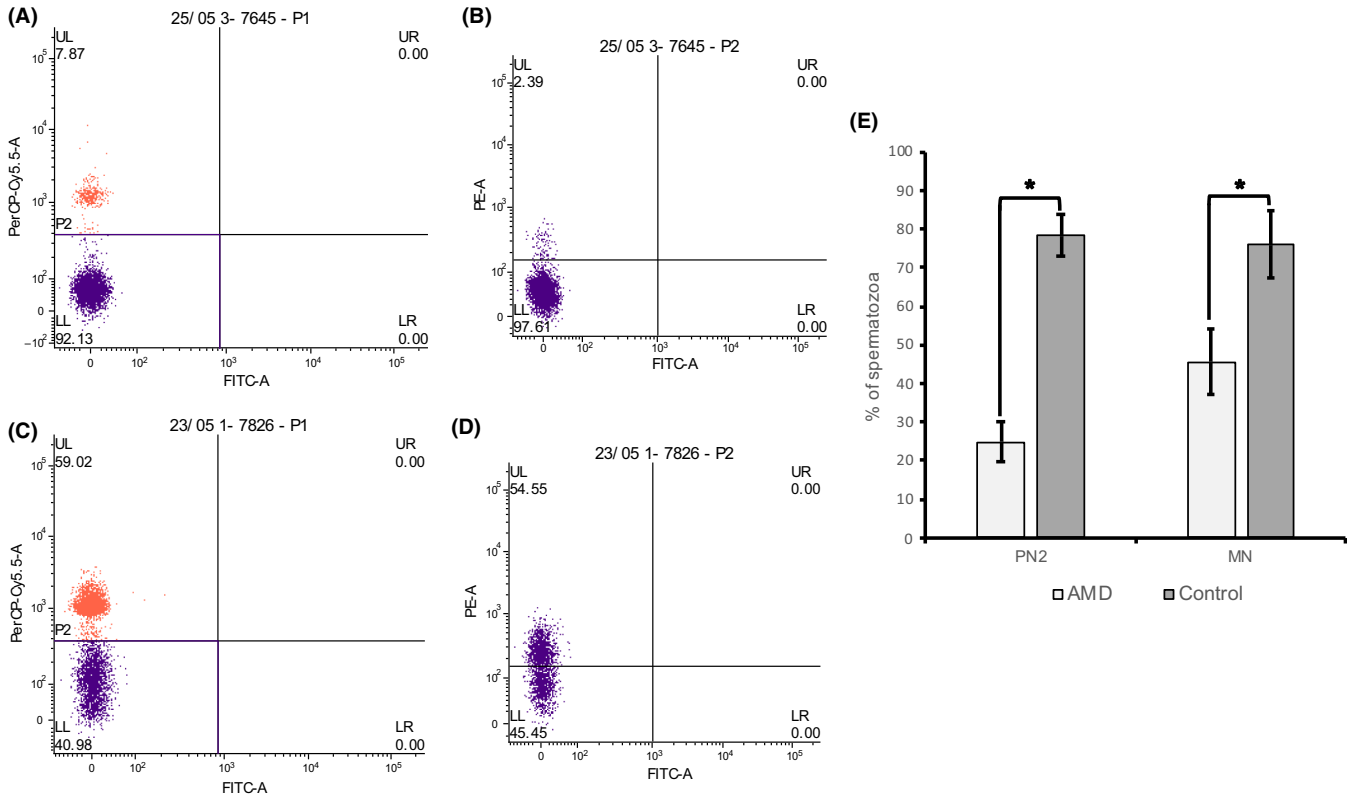
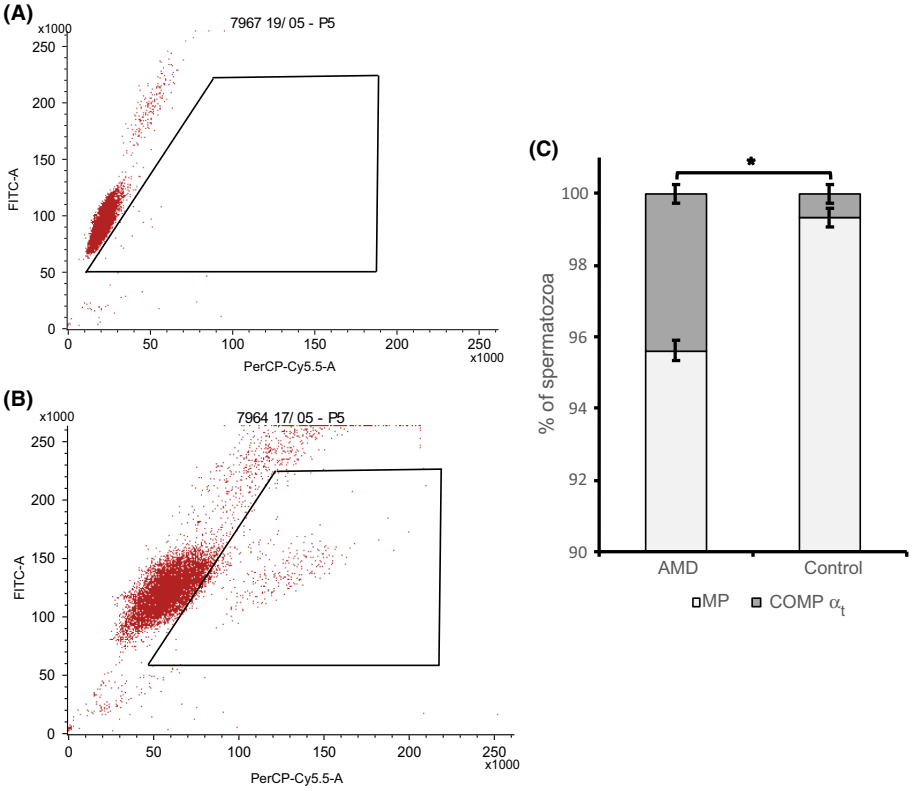


Figure 5 DNA integrity from fresh semen of Gyr bulls in the aplastic midpiece defect (AMD) and control groups. Representative dot plot showing spermatozoa with fragmented DNA or Cells Outside the Main Population ($COMP_t$) from (A) control and (B) AMD group. The Distribution of sperm cells in the scatterplot was performed as described by Love (2005). (C) Percentage of sperm populations according to COMP α_t from control and SAMS groups. Mean ± SE; *$p < 0.05$ by Tukey–Kramer test.



In conclusion, Gyr bulls with a high percentage of AMD are not suitable for reproduction due to poor motility, low mitochondrial membrane potential, a high percentage of spermatozoa with DNA fragmentation, low acrosome and plasma membrane integrity, and high membrane fluidity. These sperm alterations are exhibited possibly due to oxidative stress, resulting from the enhancement of ROS production from abnormal and dead spermatozoa in addition to a reduction of the energy production capacity and an alteration in the structural composition of the sperm cell. As a result, spermatozoa with normal morphology could be undergoing apoptosis. This is a typical semen picture from bulls with a high percentage of abnormal spermatozoa and low viability affecting the small percentage of spermatozoa with normal morphology, and it is likely they are infertile. Additionally, we highlight the importance of the sperm morphology in fertility, particularly from midpieces defects.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

EAD-M conducted the experiment, data collection, interpretation of results, and paper edition. JDG, MM-N, EPC, and SEFG supervised the study, discussed, and revised the manuscript. TPM, BSC, DAL assisted in data collection and critical revision of the manuscript. LLO, PPM, and DSO assisted with the flow cytometry analyses and critical revision of the manuscript. JMP-F contributed the statistical analysis and critically discussed and revised the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Scrotal circumference growth curve from Gyr bulls in the aplastic midpiece defect (AMD) and control groups.

