EDGAR ANDRES DIAZ MIRANDA

AVALIAÇÃO *IN VITRO* DO POTENCIAL DE FERTILIDADE DE TOUROS PORTADORES OU NÃO DE APLASIA SEGMENTAR DA BAINHA MITOCONDRIAL

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Medicina Veterinária, para obtenção do título de *Magister Scientiae*.

VIÇOSA
MINAS GERAIS – BRASIL
2018

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa

T

D542a
2018

Diaz-Miranda, Edgar Andres, 1990-
Avaliação *in vitro* do potencial de fertilidade de touros portadores ou não de aplasia segmentar da bainha mitocondrial / Edgar Andres Diaz-Miranda. – Viçosa, MG, 2018.
v, 60f. : il. (algumas color.) ; 29 cm.

Texto em português e inglês.
Orientador: José Domingos Guimarães.
Dissertação (mestrado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Touros - Reprodução - Doenças. 2. Patologia veterinária. 3. Infecundidade em animais. 4. Citologia veterinária. 5. Sêmen - Qualidade. I. Universidade Federal de Viçosa. Departamento de Veterinária. Programa de Pós-Graduação em Medicina Veterinária. II. Título.

CDD 22. ed. 636.08966692

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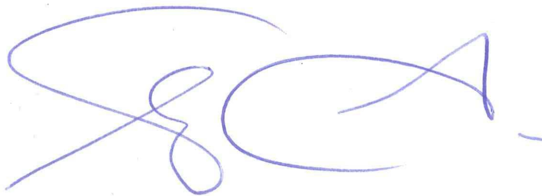
APROVADA: 23 de fevereiro de 2018.



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AGRADECIMENTOS

Agradeço primeiramente a Deus, Tudo posso naquele que me fortalece (Filipenses 4.13). Aos meus pais, Edgar e Olga, pelo exemplo, apoio e amor incondicional. Aos meus irmãos David e Mateo pela força e ajuda que sempre me ofereceram. A minha noiva pela confiança e ser o amor da minha vida e a minha família sempre me apoiando.

Ao meu orientador JD, mais que pelo aprendizado, pela amizade e confiança, sempre procurando fazer de nos primeiramente pessoas para ser profissionais. A minha coorientadora profa Mariana, pela paciência, sempre com um sorriso e me motivando e exigindo melhorar cada dia mais.

Um agradecimento imensamente importante ao Victor meu parceiro, sem palavras, devo muito a você, obrigado pela confiança e apoio.

Aos amigos que o Brasil me deu, Juliana, German e meus amigos do Lab., Breno, Paula, Thiago, Denise, Jurandy, Daniel, Daniele, Amanda, Faider, Hugo, Camilo, Juliana, Domingos, Lucas, Camila, JC, não tivesse sido o mesmo sem vocês.

Aos professores que participaram no desenvolvimento do projeto, prof. Leandro Licursi, Giancarlo Magalhães, Maria C. Baracat, Bruna Waddington. Aos meus colegas da Biologia, Marcela, Naras, Raquel, Felipe, Daniel e Ana Claudia, assim como meus colegas da Zootecnia, Carol, Ivan.

Aos meus amigos na Colômbia, Carlos, Juan Diego, Luis E., Nicolas, Jaime, Oscar, Juan Camilo, Jorge L.

Ao setor de Gado de Leite da UFV.

Ao programa de Pós-graduação do Departamento de Veterinária, especialmente a Rosi, que seria de nos sem você!

Aos membros da banca avaliadora, pelas sugestões feitas ao trabalho.

Ao Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq) pela bolsa de estudo concedida.

Agradeço imensamente à Universidade Federal de Viçosa.

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RESUMO

DIAZ MIRANDA, Edgar Andres, M.Sc., Universidade Federal de Viçosa, fevereiro de 2018. **Avaliação *in vitro* do potencial de fertilidade de touros portadores ou não de Aplasia Segmentar da Bainha Mitocondrial**. Orientador: José Domingos Guimarães. Coorientador: Eduardo Paulino da Costa.

O reprodutor tem grande importância na cadeia produtiva por possuir maior potencial de difusão do seu material genético dentro do rebanho que as fêmeas que produzem apenas uma cria por ano, em manejo de monta natural. Entretanto, este potencial somente se torna real quando os machos apresentam boa qualidade seminal, resultando em menores taxas de não retorno ao estro. Não há duvidas da importância da morfologia espermática na fertilidade de touros. Entre as anomalias espermáticas identificadas nos ejaculados de bovinos, a aplasia segmentar da bainha mitocondrial (ASBM) é classificada como defeito espermático maior, comprometendo a fertilidade dos reprodutores, pelo fato dos ejaculados apresentarem baixa motilidade e vigor espermático. Este estudo teve como objetivo estudar a fisiopatologia de ejaculados com altos porcentuais da patologia espermática ASBM, utilizando como base o exame andrológico e análise espermática por citometria de fluxo. Portanto, os touros foram divididos em dois grupos: o primeiro foi o grupo controle com morfologia espermática normal (n = 3) e o segundo foi o grupo com morfologia anormal (n = 3) com alta porcentagem de SAMS. Cinco ejaculados foram coletados de cada animal. Ejaculados de touros com ASBM apresentaram baixa motilidade ($P<0,05$), baixo potencial mitocondrial ($P<0,05$), maior porcentagem de espermatozoides com fragmentação do DNA ($P<0,05$), baixa integridade da membrana plasmática e acrossomal ($P<0,05$) e alta porcentagem de espermatozoides com desorganização da bicamada lipídica ou fluidez da membrana ($P<0,05$) em comparação aos touros com morfologia espermática normal. Além de possuir baixa porcentagem de viabilidade espermática, sêmen com alta porcentagem de ASBM pode estar sofrendo apoptose, possivelmente devido ao estresse oxidativo. Esses resultados indicam que touros com alta porcentagem desta patologia espermática são inférteis e sugere também que esta anomalia espermática seja de origem genética na raça estudada.

ABSTRACT

DIAZ MIRANDA, Edgar Andres, M.Sc., Universidade Federal de Viçosa, February, 2018. **In vitro assessment of the potential fertility from bulls with the presence of Segmental Aplasia of the Mitochondrial Sheath**. Adviser: José Domingos Guimarães. Co-Adviser: Eduardo Paulino da Costa.

Bulls are of great importance in the productive chain because they have a higher potential for diffusion of genetic material within the herd than females that produce only one calf per year in natural mating management. However, this potential is only possible when males present good seminal quality, resulting in lower rates of non-return to estrus. There is no doubt about the importance of sperm morphology in bull fertility, among the sperm anomalies identified in bovine ejaculates, the segmental aplasia of the mitochondrial sheath (SAMS) is classified as a major sperm defect, compromising the reproductive fertility; ejaculates with presence of this anomaly show low sperm motility and sperm vigor. This study aimed to understand the pathophysiology of ejaculates with high percentage of spermatozoa with SAMS, using Breeding Soundness Evaluation and sperm analysis by flow cytometry. Therefore, the bulls were divided in two groups: The first one was the control group with normal sperm morphology (n=3) and the second was the abnormal one (n=3) with high percentage of SAMS. Five ejaculates were collected from each animal. Bulls with SAMS exhibited poor motility ($P<0.05$), low mitochondrial membrane potential ($P<0.05$), a high percentage of sperm with DNA fragmentation ($P<0.05$), low acrosome and plasma membrane integrity ($P<0.05$), and high membrane fluidity ($P<0.05$) in comparison with bulls that have a normal morphology; in addition to have a low percentage of viability, sperm with a high percentage of SAMS may be undergoing apoptosis. These findings indicate that bulls with this sperm pathology are infertile; furthermore, it suggests there is a putative genetic basis for this sperm defect in this breed.

1. INTRODUÇÃO

A infertilidade no macho é tão comum como na fêmea. As patologias espermáticas podem ter origem genético, ambiental ou uma combinação de ambas (BARTH e OKO, 1989; CHENOWETH, 2005), assim, desordens genéticas são causadas por defeitos em genes ou cromossomos; aproximadamente 10 % dos genes no genoma humano estão associadas com a espermatogênese. Segundo Ferlin et al. (2006) fatores genéticos estão relacionados a 15 - 30 % dos casos de infertilidade no homem e valores semelhantes podem ser assumidos para espécies domésticas, nas quais existe maior potencial de difusão destes problemas (TURNER, 2003; CHENOWETH e LORTON, 2014; KWON et al., 2015).

As patologias espermáticas de peça intermediária são de baixa frequência, porém quando presentes no ejaculado em alta porcentagem se caracterizam por ter baixa motilidade, subfertilidade ou infertilidade (BLOM, 1959; 1966). A aplasia segmentar da bainha mitocondrial, uma patologia de peça intermediária, pode ser definida como pequenas lacunas ou ausência parcial ou total das mitocôndrias da peça intermediária, essa anomalia criada pela falta de mitocôndrias ao redor do axonema, deixa diretamente coberto pela membrana plasmática o que favorece fraturas, afetando seriamente a motilidade do espermatozoide (RAWE et al., 2007).

Compreender a fisiopatologia de espermatozoides defeituosos pode auxiliar no entendimento de como afetam a fertilidade (AITKEN et al., 2012). O teste de fertilidade mais preciso para sêmen e/ou touro é seu uso em um número suficiente de fêmeas com fertilidade comprovada (BARTH e OKO, 1989; PARKINSON, 2004; KASTELIC e THUNDATHIL, 2008; GHIRARDOSI et al., 2017). Porém, o uso de citometria de fluxo para análises de funcionalidade e qualidade seminal é agora uma ferramenta reconhecida cientificamente que se tornou rotineira na avaliação do sêmen animal destinado reprodução (MARTINEZ-PASTOR et al., 2004; 2010; GRAHAM e MOCÉ, 2005; HOSSAIN et al., 2011). Membranas competentes e funcionais, assim como organelas e um genoma haplóide intacto são pré-requisitos nos espermatozoides para a fertilidade (SILVA e GADELLA, 2006; HOSSAIN et al., 2011).

Neste contexto, este estudo teve como objetivo a avalição andrológica e analises de sêmen *in vitro* por citometria de fluxo, para entender a fisiopatologia da

patologia espermática aplasia segmentar da bainha mitocondrial e como pode afetar a fertilidade dos touros.

2. REVISÃO DE LITERATURA

As patologias espermáticas têm sido associadas com a infertilidade do macho e esterilidade na maioria das espécies. A morfologia dos espermatozoides está relacionada com a fertilidade (WILTBANK et al., 1986; PHILLIPS et al., 2004; AL-MAKHZOOMI et al., 2008; LOVE, 2011; ATTIA et al., 2016).

Segundo Chenoweth (2005) as causas de estrutura defeituosa nos espermatozoides podem ser ambiental, genética, ou combinação de ambas. Embora as causas ambientais sejam consideradas como as mais comuns e que a herdabilidade da fertilidade de touros é considerada baixa, há defeitos estruturais dos espermatozoides que são considerados de origem genética (FOOTE, 1970; CHANDLER et al., 1985; JOHNSON, 1997; STEFFEN, 1997; CHENOWETH, 2005; ENCISO et al., 2011; CORBET et al., 2013). Touros com alta consanguinidade tendem a apresentar mais anormalidades morfológicas nos espermatozoides que touros de linhagem abertas (SALISBURY e BAKER, 1966), possivelmente pela associação ao aumento de túbulos seminíferos anormais (CARROLL e BALL, 1970) resultando em subfertilidade (ELMORE et al., 1978). Assim, em touros mestiços (*Bos taurus taurus x Bos taurus indicus*) foi sugerido um possível efeito genético sobre a presença de altos índices de patologias espermáticas (BASRUR, 1986; GUIMARÃES, 1997; HORN et al., 2005).

Os defeitos espermáticos de origem genética podem ser classificados em defeitos de acrossoma, de cabeça, de peça intermediária e de cauda (CHENOWETH, 2005). Em touros, são comuns baixos percentuais (< 5 %) de defeitos de peça intermediária (BARTH e OKO, 1989), porém sua frequência pode estar relacionada a diferentes fatores, tais como anormalidades genéticas (BLOM, 1959; KOEFOED-JOHNSON et al., 1980; VIERULA et al., 1983), algumas doenças virais (CHENOWETH e BURGESS, 1972), deficiência e/ou falha no metabolismo do selênio (PALLINI e BACCI, 1979), consumo de gossipol (CHENOWETH et al., 2000), ou de origem desconhecida (idiopático). As anomalias de peça intermediária podem estar relacionadas a diferentes graus de comprometimento da fertilidade (JOHNSON, 1997; ROCHA et al., 2006).

A aplasia segmentar da bainha mitocondrial, também chamada de defeito de peça intermediária descontínua, pode ser verificada na fase final da espermiogênese e durante o trânsito epididimário, mostrando-se como um segmento ausente (lacuna) na bainha mitocondrial, além de dispersão de uma substância granular que normalmente liga à bainha e às fibras densas exteriores. Porém, os espermatozoides tornam-se móveis a medida que atravessam os epidídimos, o que predispõe a fraturas ou desfibrilação (erupção das fibras do axonema, geralmente 4, 5, 6 e 7) em função das desordens estruturais da bainha e motilidade espermática (BARTH e OKO, 1989; CHENOWETH et al., 2000).

Os componentes estruturais exteriores da cauda dos espermatozoides, ou seja, as bainhas mitocondrial e fibrosa, também desempenham papel de apoio durante a motilidade espermática. Estas estruturas devem ser elásticas e fortes para sustentar e conter a flexão do axonema durante a motilidade espermática (HARRIS, 1976; OLSON e LINCK, 1977). Deficiência estrutural da bainha mitocondrial (aplasia, edema, agenesia), pode ocasionar danos estruturais na integridade do axonema, resultando em ampla variedade de defeitos de peça intermediária, assim, a aplasia segmentar da bainha mitocondrial pode estar acompanhada de outras patologias, como o defeito *DAG*, *corkscrew*, *stump*, fraturas, desfibrilação, edema, peça intermediária curta, e dupla peça intermediária (VEERAMACHANENI, 2011).

Segundo BARTH e OKO (1989), em avaliações de rotina de sêmen bovino corado, pequeno número de espermatozoides são verificados ocasionalmente com uma ou várias pequenas lacunas aparecendo ao longo da bainha mitocondrial, geralmente com fraturas nestas lacunas, resultando em peças intermediárias de vários comprimentos. Não se sabe se estas pequenas lacunas são fenômenos transitórios ou persistentes.

2.1. Formação da bainha mitocondrial

Os espermatozoides de mamíferos são células altamente polarizadas e especializadas cujas organelas constituintes estão organizadas em locais específicos, de tal modo que funções especializadas são localizadas em regiões subcelulares específicas (FAWCETT, 1975; TURNER, 2003). Os espermatozoides são células únicas na sua morfologia celular, sua forma é particular para cada espécie, sua modulagem acontece durante a espermiogênese por meio de complexas sequências celulares e moleculares, mas sua forma final é estabelecida

durante a passagem pelos epidídimos, onde os espermatozoides sofrem modificações funcionais e morfológicas durante o processo de maturação espermática (YANAGIMACHI, 1994; SUZUKI-TOYOTA et al., 2004).

A peça intermediária dos espermatozoides de mamíferos é caracterizada por uma bainha mitocondrial helicoidal que envolve o axonema e as nove fibras densas exteriores (WOOLLEY, 1970; FAWCETT, 1975; PHILLIPS, 1977). Em diversos estudos foi relatada regularidade na organização da bainha mitocondrial em várias espécies de mamíferos (FAWCETT e ITO, 1965; PHILLIPS, 1970; PHILLIPS, 1977; BARADI e RAO, 1979). Além disso, o comprimento da peça intermediária e o número de giros da bainha mitocondrial são uniformes e de características hereditárias (WOOLLEY e BEATTY, 1967). No entanto, os mecanismos que regulam a distribuição e o desenvolvimento do padrão helicoidal da peça intermediária não são completamente compreendidos. A formação da bainha mitocondrial é fundamental para a motilidade espermática e fertilidade dos espermatozoides (OLSON e WINFREY, 1986; 1990; HO e WEY, 2007; RAWE et al., 2007; SUN e YANG, 2010; ZHANG et al., 2012).

A peça intermediária dos espermatozoides bovinos (incluindo a região da peça conectora) apresenta 12 μm de comprimento. Na região da peça conectora as mitocôndrias estão arranjadas geralmente paralelas ao eixo longitudinal dos espermatozoides. Entre a peça conectora e o *annulus*, as mitocôndrias estão dispostas principalmente numa hélice tripla, embora em algumas regiões estejam dispostas numa hélice quádrupla, a hélice é composta por cerca de 64 giros (PHILLIPS, 1977).

As mitocôndrias encontram-se exclusivamente na peça intermediária, e são responsáveis pela geração da maior parte do ATP do espermatozoide, requerido para seu metabolismo, motilidade e funcionalidade, manutenção da homeostase de Ca^{++}, geração de espécies reativas de oxigênio (ERO), além disso, desempenham papel importante na maturação, capacitação e na regulação da apoptose (TURNER, 2003; AITKEN et al., 2007; PEÑA et al., 2009; AITKEN e BAKER, 2013; AMARAL et al., 2013). Defeitos nas mitocôndrias causam disfunções fisiológicas que dão origem a muitas patologias, incluindo infertilidade (RAMALHO-SANTOS et al., 2009).

Ultraestruturalmente, as mitocôndrias caracterizam-se por terem quatro compartimentos interconectados, a membrana mitocondrial externa (OMM) e membrana mitocondrial interna (IMM), o espaço intermembranar e a matriz

mitocondrial. A IMM contém os complexos proteicos que constituem a cadeia transportadora de elétrons, a qual transporta os elétrons obtidos da oxidação do NADH e $FADH_2$ até o receptor final de elétrons, o oxigênio, originando água. Neste processo é gerado um gradiente eletroquímico pela passagem de prótons (H+) da matriz mitocondrial para o espaço intermembranar, conhecido como potencial de membrana mitocondrial (MMP), este gradiente facilita o influxo de prótons pela ATP sintetase proporcionando a energia para a síntese de ATP (RAMALHO-SANTOS et al., 2009; RODRIGUES, 2010; AMARAL et al., 2013). O MMP é um indicador da atividade das mitocôndrias e está correlacionado com a fertilidade (RODRIGUES, 2010; SOUSA et al., 2011; AMARAL et al., 2013).

As mitocôndrias dos espermatozoides são diferentes em vários aspectos daquelas observadas em células somáticas. Sabe-se que as mitocôndrias de células espermatogênicas modificam a sua organização morfológica, seu número, e sua localização durante os processos que levam à produção de espermatozoides (DOIGUCHI et al., 2002; RAMALHO-SANTOS et al., 2009; ZHANG et al., 2012). Além disso, as mitocôndrias dos espermatozoides têm sido associadas a várias proteínas ou isoformas de proteínas únicas que não são encontradas nas mitocôndrias das células somáticas (TURNER, 2003).

Com respeito à morfologia das mitocôndrias, muda do tipo chamado “ortodoxo” para o tipo “intermediário” e posteriormente para forma “condensada” durante a diferenciação das espermatogônias em espermátides haploides (De MARTINO et al., 1979; MEINHARDT et al., 1999; SUN e YANG, 2010; URIBE et al., 2017). Durante a espermiogênese, as mitocôndrias começam a se alongar e se aglutinar, logo depois migram para assumir uma forma helicoidal ao redor do flagelo, desse modo as mitocôndrias na peça intermediária perdem o aspecto morfológico condensado para o tipo intermediário (em forma de cristas crescente com menos matriz condensada), formando a bainha mitocondrial ao redor do flagelo (De MARTINO et al., 1979; MEINHARDT et al., 1999).

Parte das mitocôndrias são marcadas pela ubiquitina e quando marcadas, estas saem das espermátides em desenvolvimento e são fagocitadas pelas células de Sertoli (SUTOVSKY et al., 1999). Além disso, espermatozoides com defeitos apresentam altos índices de ubiquitinação nas mitocôndrias, sendo descartados durante a espermiogênese ou nos epidídimos e tem sido utilizada como critério para avaliação da qualidade espermática (SUTOVSKY et al., 2015; SUN e YANG, 2010).

Finalmente, as mitocôndrias arranjadas na bainha mitocondrial são encarregadas de fornecer ATP ao axonema para propulsão flagelar (DOIGUCHI et al., 2002). Além disso, há diferença ultra-estrutural na membrana externa das mitocôndrias da peça intermediária que consiste no espessamento da membrana externa (10 nm) com cinco camadas ao invés de três (ELFVIN, 1968).

De acordo com Bedford e Calvin (1974) a membrana externa das mitocôndrias dos espermatozoides de mamíferos é estabilizada por pontes de dissulfeto intermoleculares (juntamente com núcleo, fibras densas, e outros componentes), possivelmente para o fortalecimento desta estrutura que é frequentemente exposta a deformações devido ao movimento flagelar, podendo estar relacionada a sua maior resistência às condições hipotônicas e a sua impermeabilidade em relação ao citocromo C (KEYHANI e STOREY, 1973).

Tem sido demonstrado que o selênio é essencial no processo de espermatogênese, sendo ligado a uma proteína estrutural das mitocôndrias dos espermatozoides de touros que se acredita ser estabilizadora da membrana mitocondrial externa (PALLINI e BACCI, 1979). Além disso, defeitos progressivos nas mitocôndrias dos espermatozoides de ratos foram observados durante três gerações que continham deficiência de selênio (WALLACE et al., 1983). Calvin et al. (1981) identificaram um selenopolipeptidio específico associado à membrana externa queratinosa das mitocôndrias de espermatozoides de ratos, denominada cápsula mitocondrial, que é de alguma forma essencial para a montagem da bainha mitocondrial. Assim, os autores verificaram que o selênio estava associado a uma proteína estrutural rica em cisteína das cápsulas mitocondriais (SMCP), estando relacionada com algumas propriedades das mitocôndrias de espermatozoides de mamíferos, tais como manutenção da forma de crista crescente da membrana exterior, a qual é composto de três cadeias polipeptídicas (20.000, 29.000, 31.000 pm) e reticulado por pontes de dissulfeto.

Acreditava-se que a Proteína estrutural rica em cisteína da cápsula mitocondrial (SMCP) fosse a proteína mais predominante na cápsula mitocondrial, mas foi demonstrado que 50 % da cápsula é constituída pelo fosfolipídio hidroperóxido glutationa peroxidase (PHGPx), que funciona como enzima citosólica em células somáticas e como proteína estrutural enzimaticamente inativa na cápsula mitocondrial (URSINI et al., 1999).

Cada mitocôndria da peça intermediária é organizada de modo que a sua superfície alcance três organelas celulares diferentes, a membrana plasmática, mitocôndrias vizinhas e o complexo de axonema-fibras densas exteriores. Estes domínios de superfície são estruturalmente distintos. O domínio com a membrana plasmática é o mais frágil dos três domínios de membrana mitocondrial e sua função seria a importação de metabólitos utilizados para geração de energia, porém, os domínios com mitocôndrias vizinhas e axonema-fibras densas exteriores teriam função como citoesqueleto, na montagem e / ou manutenção das mitocôndrias no desenvolvimento da peça intermediária, e podem ser compostos por conjunto de proteínas que podem mediar sua adesão com estes (OLSON e WINFREY, 1992).

Segundo Olson e Winfrey (1990) existem dois mecanismos estruturais em espermatozoides que poderiam gerar e / ou manter o padrão regular da peça intermediária: no primeiro, as mitocôndrias adjacentes são unidas umas as outras por elementos com função de pontes, espaçadas regularmente, que se ligam diretamente nas membranas mitocondriais exteriores e em segundo lugar, as mitocôndrias são aderidas ao retículo submitocondrial (SMR), que está localizado exclusivamente na peça intermediária. O SMR é composto por fitas de material elétron-denso longitudinalmente orientadas, lateralmente interligadas, dispostas em distribuição espacial precisa ao redor do complexo de axonema-fibras densas exteriores subjacentes. O SMR tem a forma de malha cilíndrica que suporta a bainha mitocondrial sobrejacente. Além disso, as mitocôndrias também sofrem remodelação estrutural durante a passagem dos espermatozoides nos epidídimos (WOOLLEY, 1970; OLSON e HAMILTON, 1976).

A migração distal do *annulus* da espermátide para o flagelo define o comprimento da peça intermediária e simultaneamente com a migração do *annulus*, as mitocôndrias movem-se para assumir a sua posição ao redor dos elementos estruturais da peça intermediária (PHILLIPS, 1974; RAWE et al., 2007; CHEMES e RAWE, 2010). Então, um mecanismo estrutural para segmentar a bainha mitocondrial da peça intermediária em formação seria a falta dos elementos do SMR, visto que estes estão ligados tanto ao *annulus* como as mitocôndrias durante a posterior migração do *annulus* (OLSON e WINFREY, 1986).

A interação das mitocôndrias com os componentes do citoesqueleto é essencial para o movimento e fixação da mitocôndria dentro da célula e para o desenvolvimento da peça intermediária (OLSON e WINFREY, 1990; SUN e YANG,

2010). Sun e Yang (2010) sugeriram que na espermiogênese, os transportes intramanchete e intraflagelar poderiam estar envolvidos na migração da mitocôndria na peça intermediária. Ambos são motores moleculares associados a cargas (polaridade) e se movimentam ao longo do citoesqueleto, sendo o manchete uma estrutura transitória que desaparece quando se completa a formação da espermátide e o transporte intraflagelar consiste num transporte motor associado a microtubulos e fibras de actina, os quais utilizam proteínas adaptadoras como intermediários, tais como a Kinesina II, dineína, miosina Va, mas ainda não esta claro a interação, regulação, transporte e função especifica destes mecanismos.

2.2. Possíveis causas da aplasia segmentar da bainha mitocondrial

De acordo com OLSON et al. (2004) e HO e WEY (2007) o conhecimento da base estrutural e molecular da formação da bainha mitocondrial são essenciais para entender e identificar os potenciais fatores e mecanismos de como esta pode sofrer anormalidades.

Tem sido relatados diversos casos de deslocamento da bainha mitocondrial e uma serie de defeitos mitocondriais em espermátides e espermatozoides nos epidídimos, como em touros tratados com gossipol, (CHENOWETH et al., 2000); Ratos knockout inférteis, tais como, Gopc -/- (SUZUKI-TOYOTA et al., 2004), Nectin-2 -/- (BOUCHARD et al., 2000), Sepp1 -/- (OLSON et al., 2005), com mutações no complexo T (PILDER et al., 1997) e transgênicos KLC3 (ZHANG et al., 2012), os quais também apresentavam defeitos no desenvolvimento da bainha mitocondrial (ESCALIER, 2006). Tais fatos demonstram que estes tipos de anomalias de peça intermediária de espermatozoides podem ser de origem genética, mas a abordagem genética não será possível até que os genes envolvidos e o modo de herança sejam totalmente compreendidos (RAWE et al., 2007).

Nectin-2 é uma molécula de adesão celular codificada por um dos membros da família do gene do receptor de polivírus, sendo um componente de junções aderentes célula-célula e interage com L-afadin, uma proteína de ligação de F-actina. Alteração de ambos alelos do gene de rato Nectin-2 resultou em infertilidade. Em ratos, os espermatozoides tinham motilidade, mas eram morfologicamente anormais, com defeitos na morfologia nuclear, do citoesqueleto e na localização mitocondrial, porém, sem outra anomalia evidente (BOUCHARD et al., 2000). Nectin-2 é expresso nos testículos apenas durante as fases posteriores da

espermatocitogênese sendo expressa na membrana plasmática em espermátides, e principalmente na peça intermediária de espermatozoides. Os defeitos estruturais observados em espermatozoides com Nectin-2 -/- de camundongos sugerem um papel para esta proteína na organização e reorganização do citoesqueleto durante a espermiogênese (BOUCHARD et al., 2000).

Os camundongos Gopc -/- (*Golgi-associated PDZ- and coiled-coil motif-containing protein*) são inférteis, mostrando cabeça arredondada ou globosa, caudas enroladas e bainha mitocondrial estratificada. A função das moléculas GOPC ainda não é claramente compreendida. No entanto, a partir da sua localização e caracterização molecular, tem sido sugerido que GOPC desempenha um papel no transporte de vesículas a partir do aparelho de Golgi (YAO et al., 2001), além do papel na fusão da membrana. Espermatozoides Gopc -/- apresentaram um núcleo redondo e acrossoma ausente na maioria dos casos, o anel posterior estava ausente ou defeituoso e o núcleo era muitas vezes localizado na gotícula citoplasmática. Outra anormalidade comum era a formação da bainha mitocondrial estratificada, que resultou em desnudamento das fibras densas exteriores na peça intermediária. Uma questão era se a cauda enrolada e a separação das mitocondrias das fibras densas exteriores estão relacionados ou são independentes em ratos Gopc -/-. Mas o enrolamento da cauda não é essencial para a estratificação da bainha mitocondrial, porque esta anomalia foi vista em espermatozoides com a cauda linear (SUZUKI-TOYOTA et al., 2007).

Pilder et al. (1997) relataram que o Hst7 é um lócus relacionado a esterilidade em ratos híbridos, e foi mapeado em ligação estreita com outros quatro loci também relacionados a esterilidade em híbridos, localizados no cromossomo 17; dentro do complexo T, os quatro loci foram identificados como, Hst4, Hst5 e Hst6 na extremidade mais distal do complexo T (PILDER et al., 1993) e Hst7 na extremidade mais proximal do cromossomo (PILDER et al, 1997). O terço proximal do cromossomo 17 do rato é chamado o complexo T. Uma forma variante desta região da cromatina, chamado de haplótipo T (T) é essencialmente invertido em relação ao seu tipo livre (+) homólogo (OLDS-CLARKE, 1997). Espermatozoides de ratos S (alelo Hst7) / T apresentam nas peças intermediárias áreas ampliadas do citoplasma com camadas adicionais de mitocôndrias desalinhada. Além disso, as matrizes de algumas mitocôndrias dos espermatozoides apresentaram-se difusas e vacuolizadas, sendo característico da morfologia mitocondrial típica de células

germinais masculinas meióticas e pós-meióticas quando comparada aos espermatozoides localizados nos epidídimos (MACHADO DE DOMENECH et al., 1972; DEMARTINO et al., 1979). Deste modo, as anormalidades no desenvolvimento dos flagelos das células germinativas S / T foram detectados nas primeiras espermátides em alongamento, sugerindo, que existe interação entre Hst6 e Hst7 na formação e função do flagelo dos espermatozoides.

Segundo Suzuki-Toyota et al. (2007) duas moléculas são de interesse em relação à manutenção da bainha mitocondrial normal, SPERGEN1 (Gene 1 especifico da célula espermatogênica), GPx4 (glutationa peroxidase 4). SPERGEN1 é um forte candidato para atuar como molécula de ligação entre as mitocôndrias adjacentes na bainha, porque contém sinal de direcionamento e agregação das mitocôndrias quando é expresso artificialmente em células, estando localizada na mitocôndria de espermátides alongadas e espermatozoides maduros de ratos. Acredita-se que funciona como molécula de adesão para o arranjamento das mitocôndrias dentro da bainha mitocondrial ao redor da peça intermediária, durante o processo de espermiogênese (DOIGUCHI et al., 2002). A selenoproteína - GPx4 é conhecida por ser uma proteína multi-funcional, e possivelmente atue como peroxidase, em espermatídes em fase inicial. Em espermatozoides, a GPx4 é uma proteína estrutural que compõe mais de 50 % da cápsula mitocondrial, estabilizando a bainha mitocondrial (URSINI et al., 1999).

Recentemente, foi sugerido que a Kinesina de cadeia leve 3 (KLC3) tem um papel na formação e função de peca intermediária de espermatozoides de ratos durante a espermiogênese. As KLCs são componentes da molécula motora Kinesina I que consiste em duas kinesina de cadeias pesadas (KHCs) associadas a duas KLCs. Como o relatado anteriormente, as Kinesinas são moléculas motoras dirigidas pelos microtubulos e estão envolvidas no transporte intracelular de organelas, complexos protéicos e mRNAs para destinos específicos. A KLC3 é a única Kinesina conhecida altamente expressa em testículos e no estádio final das espermátides de ratos, momento onde se da a formação da bainha mitocondrial, além disso, *in vitro* causa agregação de mitocôndrias (ZHANG et al., 2012; AMARAL et al., 2013). Zhang et al. (2012) propuseram um modelo no qual a KLC3 se liga a uma proteína porina da membrana mitocondrial externa (Canal de ânions dependente da tensão 2, VDAC2) ou via por outra proteína adaptadora, na região TPR da KLC3, criando uma ponte molecular com as fibras densas exteriores (a qual rodeia os microtubulos do

axonema) que se ligam na região HR da KLC3 ancorando as mitocôndrias na peça intermediária em formação.

A falta ou perda da substância cimentaria, que conecta as mitocôndrias com a membrana plasmática foi relacionada à patologia seminal, aplasia mitocondrial no ápice da peça intermediária em touros, onde os espermatozoides apresentavam aplasia mitocondrial, perda da hélice mitocondrial, presença de mitocôndrias alongadas e inchadas, e aumento da espessura das fibras densas exteriores na região do ápice da peça intermediária, além da perda da substância cimentaria ao longo de toda a peça intermediária, formando extensões da membrana plasmática. Não houve efeito aparente na fertilidade dos touros, mas, ressalta-se o fato de maior percentagem de espermatozoides móveis com motilidade progressiva retilínea do que a percentagem de células com morfologia normal no ejaculado, sugerindo que pelo menos parte dos espermatozoides com esta patologia apresentaram motilidade. Entretanto, Barth e Oko (1989), verificaram que os espermatozoides apresentando esta patologia geralmente estão mortos. Contudo, os autores não verificaram a capacidade fecundante desses espermatozoides e sua possível hereditariedade. De acordo com os estudos de Rocha et al. (2006) e Rawe et al. (2007) o efeito da aplasia segmentar da bainha mitocondrial na motilidade espermática e na capacidade fecundante dos espermatozoides irá depender do grau de comprometimento ou extensão desta anomalia, que pode ser desde pequenas lacunas ou total ausência das mitocôndrias da peça intermediária, à falta da peça intermediária e agregação local das mitocôndrias com ultraestrutura normal, além, da percentagem de espermatozoides apresentando a patologia.

As patologias da peça intermediária tem sido objeto de pesquisa em muitas espécies como meio de estudo para elucidar algumas patologias relacionadas a reprodução humana. Estudos em humanos com astenospermia, a qual é uma das principais causas da infertilidade masculina humana, demonstrou que os espermatozoides de indivíduos astenozoospermicos apresentavam o comprimento de peça intermediária significativamente menor, além de menor número de giros e mitocôndrias. Nesta condição, a baixa motilidade dos espermatozoides estava relacionada a redução da capacidade de gerar energia (MUNDY et al., 1995; SUN e YANG, 2010). Em outros estudos, Guraya (1987) e Silva et al (2007) verificaram que as mitocôndrias de espermatozoides criopreservados podem apresentar desarranjo estrutural e vacuolização, e estar relacionado à baixa motilidade espermática pós

descongelamento; ressaltando a grande importância das mitocôndrias na motilidade e fertilidade dos espermatozoides.

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CAPÍTULO 1 – "DISRUPTION OF SPERM FUNCTIONS LEADS TO BULL INFERTILITY IN THE PRESENCE OF SEGMENTAL APLASIA OF THE MITOCHONDRIAL SHEATH"

Scientific paper in preparation to be submitted to the journal *Andrology*

SUMMARY

Bulls are of great importance in the productive chain and for this reason it is necessary to have good semen quality. There is no doubt about the importance of sperm morphology in bull fertility, although little is known about exactly how it affects sperm functions. In the present study we aimed to understand the pathophysiology of the abnormal morphology segmental aplasia of the mitochondrial sheath (SAMS), therefore, a flow cytometry analysis and a Breeding Soundness Evaluation were used. The bulls were divided into two groups: a control group consisting of satisfactory potential breeders with normal sperm morphology ($n = 3$) and a second group consisting of unsatisfactory potential breeders with an abnormal morphology ($n = 3$) or a high percentage of SAMS. Five ejaculates were collected from each animal. Bulls in the SAMS group exhibited poor sperm motility and vigor ($p < 0.05$), interestingly with no difference in sperm concentration ($p > 0.05$). In addition, bulls in the SAMS group also exhibited a low mitochondrial membrane potential ($p < 0.05$), a high percentage of sperm with DNA fragmentation ($p < 0.05$), low acrosome and plasma membrane integrity ($p < 0.05$), and high lipid bilayer sperm membrane disorganization ($p < 0.05$) in comparison with those with a normal morphology. 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. In conjunction with having a low percentage of viability, semen with a high percentage

of SAMS may also be undergoing apoptosis. Our results indicate that bulls with this sperm pathology are infertile; furthermore, it suggests there is a putative genetic basis for this sperm defect in this breed.

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 (Abdollahi-Arpanahi *et al.*, 2017; Thundathil *et al.*, 2017). Bull fertility is influenced by numerous factors (Kastelic & Thundathil, 2008) in which sperm morphology plays an important role (Wiltbank & Parrish, 1986; Phillips *et al.*, 2004; Al-Makhzoomi *et al.*, 2008; Attia *et al.*, 2016). Indeed, it strongly reflects alterations in testicular and epididymal function (Barth & Oko, 1989; Söderquist *et al.*, 1991; Freneau *et al.*, 2010). Moreover, abnormal spermatozoa may compromise the normal function of morphologically normal sperm (Chenoweth, 2007; Kastelic & Thundathil, 2008; Saacke, 2008) due to the generation of reactive oxygen species (ROS) (Rao *et al.*, 1989; Aziz *et al.*, 2004; Aitken *et al.*, 2012). The possible involvement of genetic factors influencing sperm morphology makes this parameter a critical objective of study (Foote, 1970; Barth & Oko, 1989; Johnson, 1997; Steffen, 1997; Chenoweth, 2005; Enciso *et al.*, 2011; Chenoweth & McPherson, 2016).

Genetic sperm defects are classified in acrosome, head, and tail defects, as well as midpiece abnormalities (Chenoweth, 2005). The latter has been rarely reported (Blom, 1959, 1966; Barth & Oko, 1989) and show different effects on bull's fertility (Rocha *et al.*, 2006). Specifically, the segmental aplasia of the mitochondrial sheath is characterized by a missing segment in the mitochondrial sheath, in addition to dispersion of a granular substance that normally binds the mitochondria and outer dense fibers. In the end, this structural disorder may predispose sperm cells to

fracture or defibrillation (Barth & Oko, 1989; Chenoweth *et al.*, 2000).

It is well known that the sperm midpiece consist of a mitochondrial helical sheath surrounding the axonemal complex and the nine outer dense fibers (Fawcett, 1975; Phillips, 1977; Zamboni, 1991). In contrast, the mechanism involved in the mitochondrial sheath (MS) development is not fully understood (Olson & Winfrey, 1986, 1990, 1992; Ho & Wey, 2007; Sun & Yang, 2010). In bovine sperm, typically, the midpiece is composed of about 64 gyres and has 12 μm length. In addition, the principal arrangement of bull sperm mitochondria is a triple helix, however, in the neck region are arranged parallel to the long axis (Phillips, 1977).

Morphologically abnormal spermatozoa are not always dysfunctional, and sperm morphology is not easily related to affect sperm function (Kubo-Irie *et al.*, 2004). Notwithstanding, the most accurate fertility test for bulls is to use breeding with enough females with proven normal fertility (Barth & Oko, 1989; Kastelic & Thundathil, 2008). Over the last decade, the use of flow cytometry for quality and functional sperm analysis becomes a recognized methodology for assessing fertility (Graham & Mocé, 2005; Martinez-Pastor *et al.*, 2004, 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 (Parks & Graham, 1992; Hallap *et al*, 2005). Sperm plasma membrane is associated with many sperm functions (Parks & Graham, 1992), including the maintenance of cell homeostasis (Silva & Gadella, 2006; Rodríguez-Martínez, 2007) and fertilizing capability (Lenzi *et al.*, 1996; Flesch & Gadella, 2000). The acrosome must remain intact until the sperm-oocyte

interaction, only then can the release and activation of acrosomal enzymes assist the sperm to penetrate the zona pellucida, which is required for mammalian fertilization (Flesch & Gadella, 2000; Silva & Gadella, 2006). Moreover, DNA integrity is highly important for embryo development (Chenoweth, 2007; Aitken *et al.*, 2009; Aitken & Koppers, 2011), sperm with damaged DNA can be functional and fertilized but cause apoptosis after the first embryonic cleavages (Borini *et al.*, 2006; Fatehi *et al.*, 2006).

There are two metabolic pathways involved in ATP production for the spermatozoa, the anaerobic glycolysis in the cytoplasm and the aerobic oxidative phosphorylation in the mitochondria (Silva & Gadella, 2006; Peña *et al.*, 2009; Hossain *et al.*, 2011; Amaral *et al.*, 2013). Remarkable differences between species exist in the main pathway involved in energy production (Turner, 2003; Storey, 2008). Nevertheless, sperm mitochondria play an important role in maturation (Aitken *et al.*, 2007), capacitation and apoptosis (Aitken *et al.*, 2012; Aitken & Baker, 2013).

In this framework, our hypothesis was that bulls with a high percentage of the sperm pathology segmental aplasia of the mitochondrial sheath are infertile. Thus, we aimed to detail the morphological description and to understand the pathophysiology of this sperm defect. Therefore, flow cytometry analysis (assessing membrane and acrosome status, mitochondrial membrane potential and DNA integrity) was used in addition to a Breeding Soundness Evaluation to identify the reproductive potential of the bull.

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, Saint Louis, MO, EUA) (Excitation, 488

nm; Emission, 636 nm), 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) (T4069, Sigma-Aldrich, Saint Louis, MO, EUA) (Excitation, 488 nm; Emission, monomers 525–530 nm and aggregates 590 nm), Merocyanine 540 (M540) (M-24571, Molecular Probes Inc., Eugene, Oregon, EUA) (Excitation, 555 nm; Emission, 578 nm), Acridine orange (AO) (A1301, Molecular Probes Inc., Eugene, Oregon, EUA) (Excitation, 488 nm; Emission, 515–530 nm and >630nm), and all the reagents which were necessary for preparation of the culture media were purchased from Sigma-Aldrich.

Ethics and Field experimental design

The experiment was conducted at dairy cattle research facility at the Universidade Federal de Viçosa (UFV), 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 h and the remaining half at 16:00 h. In addition, animals were allowed to graze in a pasture of *Brachiaria decumbens*. The pens were 5.4 x 5.4 m, and were constructed of metal gates and cables, a concrete feed bunk, a concrete slatted floor, and an automatic water cup.

Bull selection was based on the traditional Breeding Soundness Evaluation, according to the standards of the Brazilian College of Animal Reproduction - CBRA (CBRA, 2013) in which bulls are classified as satisfactory or unsatisfactory potential breeders. Bulls in the first group had a normal sperm morphology (n = 3; control group) and were classified as satisfactory potential breeders, while bulls from the

second group had Segmental Aplasia of the Mitochondrial Sheath, an abnormal sperm morphology, and were therefore classified as unsatisfactory potential breeders (SAMS) (n = 3; SAMS group).

Semen collection

Thirty ejaculates were collected from 6 bulls by electroejaculation (five ejaculates per bull) with 3 d interval. For that, semen was collected in a prewarmed, graded, conical plastic tube, protected (by a polystyrene cover) from light, cold shock, and rapid temperature changes. Individual ejaculates were divided into two aliquots. The first part was used in the semen analysis from the breeding soundness evaluation and complementary routine tests, whereas the other part was used for the flow cytometry assay.

Breeding Soundness Evaluation

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). Additionally, 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). The CBRA recommends the use of the major and minor classification of sperm morphology (Blom, 1973). However, herein, the sperm morphology was also classified according to the anatomic site of sperm defect, such as acrosome, head, midpiece and tail, in order to represent the sperm picture of bulls with SAMS.

Moreover, all morphological abnormalities in the sperm cell were recorded. It means that both head and midpiece abnormalities may be present in each spermatozoon.

Complementary routine tests, such as supravital test using eosin-nigrosin staining (ST) 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, SunnyVale, CA, USA) equipped with blue (488 nm, 20 mW) and red lasers (640 nm, 40 mW), as well as FL-1 (LP 507 nm / BP 527/32 nm), FL-2 (LP 560 nm / BP 568/42 nm), FL-3 (LP 665 nm / BP 700/54 nm), FL-4 (LP 752 nm / BP 783/56 nm), FL-5 (LP 660 nm / BP 660/10 nm), and FL-6 filters (LP 752 nm / BP 783/56 nm) was 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 for staining and flow cytometry analysis were diluted in 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 sperm (5 x 10^6 spermatozoa/mL) 10μL of FITC-PSA (100 μg/mL) and 3 μL of PI (0.5 mg/mL) were added, and after ten minutes of incubation at 37 ºC, sperm samples were diluted with 150 μL of PBS and analyzed by flow cytometer. PI is a membrane impermeable probe that penetrate cells with a broken plasmalemma, emitting red fluorescence when binding to nucleic acids (Martínez-Pastor *et al.*, 2010). The PSA conjugated with fluorochrome FITC emit

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 sperm in four subpopulations. The first is the subpopulation of unstained spermatozoa (FnPn) with intact acrosome and plasma membrane, the second is a sperm subpopulation (FpPn) with a damaged acrosome and an intact plasma membrane, the third (FnPp) is a sperm subpopulation with an intact acrosome and a damaged plasma membrane, and the last is a sperm subpopulation 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 dyes as described by Ortega-Ferrusolas *et al.* (2010) with modifications for adaptation to the bovine species. In brief, to 500 μL of sperm (5×10^6 spermatozoa/mL) 3 μL of JC-1 (153 μM) were added, and after 15 minutes of incubation at 37 ºC, 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 sperm (5×10^6 spermatozoa/mL) 0.5 μL of M540 (0.5 mg/mL) and 3 μL of PI were added, and after 15 minutes of incubation at 37 ºC, 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 minutes at 4 ºC, washed 2 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.* (2012). To 200 μL of fixed sperm diluted in TNE buffer (0.15 M NaCl, 0.01 M Tris HCl, 1 mM disodium EDTA pH 7.4, 4 ºC) (5 x 10^6 spermatozoa/mL) 400 μL of 0.08 N HCl, 0.15 M NaCl, 0.1% Triton X 100 pH 1.2 were mixed, after 30 seconds of incubation at 4 ºC, 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 ºC) were added, and 3 minutes 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 % of sperm with fragmented DNA or Cells Outside the Main Population (COMP α_t) and the majority of normal sperm (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. In contrast, bulls in the SAMS group showed lower sperm motility when compared to control bulls ($p < 0.05$; Table 1). Particularly, some bulls from SAMS group showed no motility in several ejaculates. Furthermore, mass motility and sperm vigor were lower in bulls with SAMS 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 SAMS group presented lower percentage of reacted cells to hypoosmotic and supravital tests than the control group ($p < 0.05$; Table 1).

Table 1 Sperm parameters from fresh semen of Gyr bulls in the segmental aplasia of the mitochondrial sheath (SAMS) and control groups.

Sperm parameters	Control	SAMS
Progressive motility[1] (%)	72.1 ± 1.86[a]	0.1 ± 0.21[b]
Sperm vigor[2] (0-5)	2.97 ± 0.11[a]	0.04 ± 0.04[b]
Mass motility [2] (0-5)	0.5 ± 0.15[a]	0[b]
Volume (mL)	5.30 ± 0.42	4.24 ± 0.38
Concentration (x10^6/mL)	280.7 ± 80.7	424.7 ± 72.3
Total sperm concentration (x10^9)	1.52 ± 0.41	1.80 ± 0.37
pH	7.36 ± 0.26	7.53 ± 0.26
ST[1] (%)	65.84 ± 5.48[a]	20.59 ± 5.66[b]
HOST[1] (%)	54.83 ± 7.01[a]	19.04 ± 4.95[b]

Mean ± SE; Different letters within rows indicate significant differences $p < 0.05$ by [1]Tukey-Kramer; [2]Kruskal-Wallis tests. HOST: hypoosmotic swelling test; ST: supravital test with eosin-nigrosin stain.

The sperm morphology clearly was one of the focus of this study. In this context, bulls in the SAMS group exhibit several and high percentage of midpiece abnormalities compared to the control group ($p < 0.05$), including corkscrew, stump, Dag defect, fracture, abaxial, swelling midpiece, defibrillation, and proximal droplets (Table 2). In addition to mitochondrial gaps or lack of mitochondria, bulls from SAMS group also presented high percentage of sperm without mitochondria (aplasia) ($p < 0.05$), and some had an absence of the outer dense fibers, displaying a thin midpiece as the terminal piece. Furthermore, spermatozoa from bulls in the SAMS group presented more than one type of cell abnormality, and sometimes show more than one combination of midpiece abnormalities, such as either swollen midpiece with mitochondrial gaps or corkscrew with defibrillation. As a consequence of the high

levels of midpiece defects, bulls in the SAMS 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, demonstrating a defective spermiogenesis.

Table 2 Percentage of sperm cells with abnormal morphology from Gyr bulls in the segmental aplasia of the mitochondrial sheath (SAMS) and control groups.

Sperm Defects[1]	Control	SAMS
Acrosome	2.89 ± 0.40	3.70 ± 0.25
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; Different letters within rows indicate significant differences $p < 0.05$ by Tukey-Kramer test.

1: Anatomic site of the sperm defect classification.

Table 3 Percentage of sperm defects from Gyr bulls in the segmental aplasia of the mitochondrial sheath (SAMS) and control groups.

Sperm Defects[1]	Control	SAMS
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)

Bulls from SAMS group presented higher percentage of sperm subpopulation with acrosome and plasma membrane damage (FpPp) than bulls from the control group ($p < 0.05$; Fig. 1). The latter, in turn, had a higher percentage of sperm subpopulation with an intact acrosome and plasma membrane (FnPn) than SAMS group ($p < 0.05$; Fig. 1). There were no differences between sperm subpopulation with a damaged acrosome and an intact plasma membrane (FpPn), and sperm subpopulation with intact acrosome and a damaged plasma membrane (FnPp) between the control and SAMS groups ($p > 0.05$; Fig. 1). In fact, the control group had a higher percentage of sperm with an intact plasma membrane (Pn) and sperm with an intact acrosome (Fn) ($p < 0.05$; Fig. 1). As expected, mitochondrial membrane potential was lower in bulls of the SAMS group compared to the controls ($p < 0.05$; Fig. 2).

Figure 1 Sperm plasma membrane and acrosome integrity (PMAI) from fresh semen of Gyr bulls in the segmental aplasia of the mitochondrial sheath (SAMS) and control groups. Representative dot plot showing PMAI of (A) Control group. (B) SAMS group. (C) Percentage of each sperm subpopulations according to membranes integrity:

FpPp (sperm with acrosome and plasma membrane damage, UR); FnPn (sperm with an intact acrosome and plasma membrane, LL); FpPn (sperm with a damaged acrosome and an intact plasma membrane, UL); FnPp (sperm with an intact acrosome and a damaged plasma membrane, LR). Additionally, it is also shown sperm with either intact plasma membrane (Pn) or acrosome (Fn). Mean ± SE; *p < 0.05 by Tukey-Kramer test.

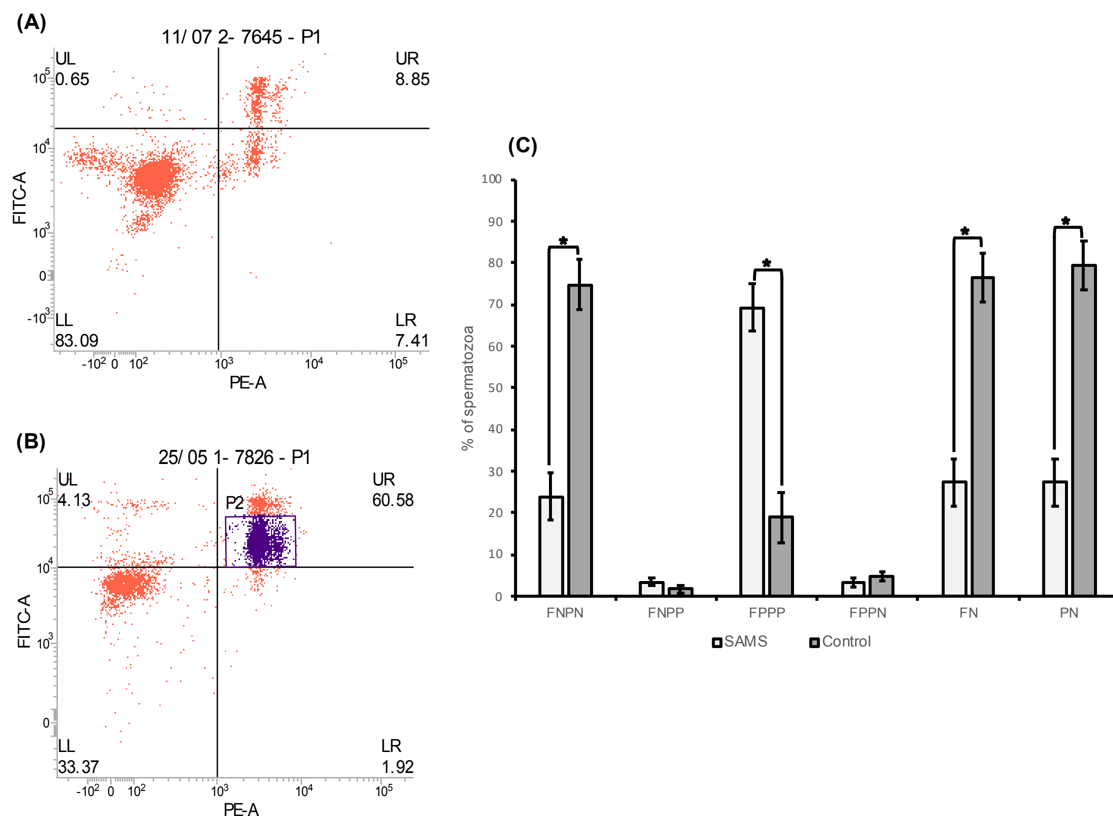
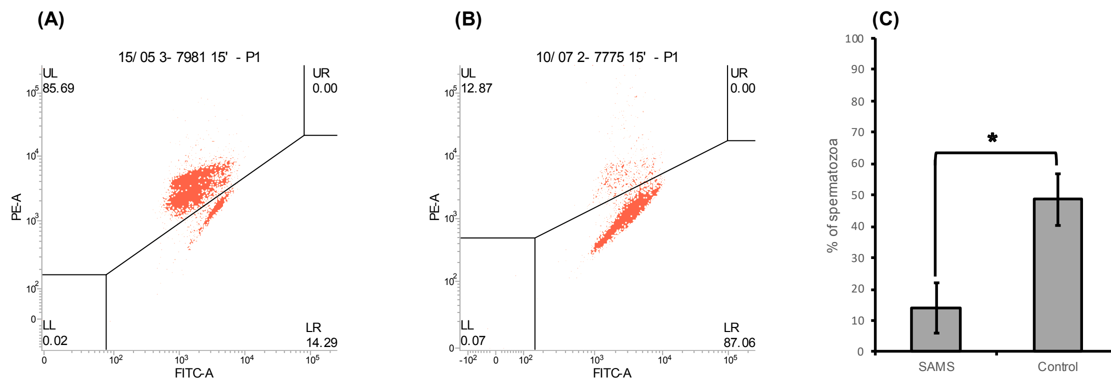


Figure 2 Mitochondrial membrane potential (MMP) from fresh semen of Gyr bulls in the segmental aplasia of the mitochondrial sheath (SAMS) control groups. Representative dot plot showing MMP of (A) control group and (B) SAMS group; sperm cells subpopulations showing high MMP (UL) and low MMP (LR). (C) Percentage of sperm populations with high MMP from control and SAMS groups. Mean ± SE; *p < 0.05 by Tukey-Kramer test.



The PI-M540 assay provided further evidence among membrane status. Bulls of SAMS group had lower (24.9%) sperm with plasma membrane integrity than the control group ($p < 0.05$); furthermore, just 45.5% of these intact membranes were organized, whereas the control group ($p < 0.05$) showed 76.1% sperm with an organized lipid bilayer membrane (Figure 3). These results showed similar values to those of the complementary routine tests reinforcing the notion of membrane function and integrity from bulls with SAMS. Finally, Bulls from SAMS group presented higher percentage of sperm with fragmented DNA than bulls from the control group ($p < 0.05$; Fig. 4).

Figure 3 Lipid bilayer sperm membrane disorganization (LBSD) from fresh semen of Gyr bulls in the segmental aplasia of the mitochondrial sheath (SAMS) and control groups. Representative dot plot showing membrane integrity and LBSD of the (A–B) control group and the (C–D) SAMS group. In order to restrict the assay to live spermatozoa, the PI dye was used in (A) Control group and (C) SAMS 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) SAMS 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 SAMS group with membrane integrity (PN2) and organized membranes (MN). Mean ± SE; *p < 0.05 by Tukey-Kramer test.

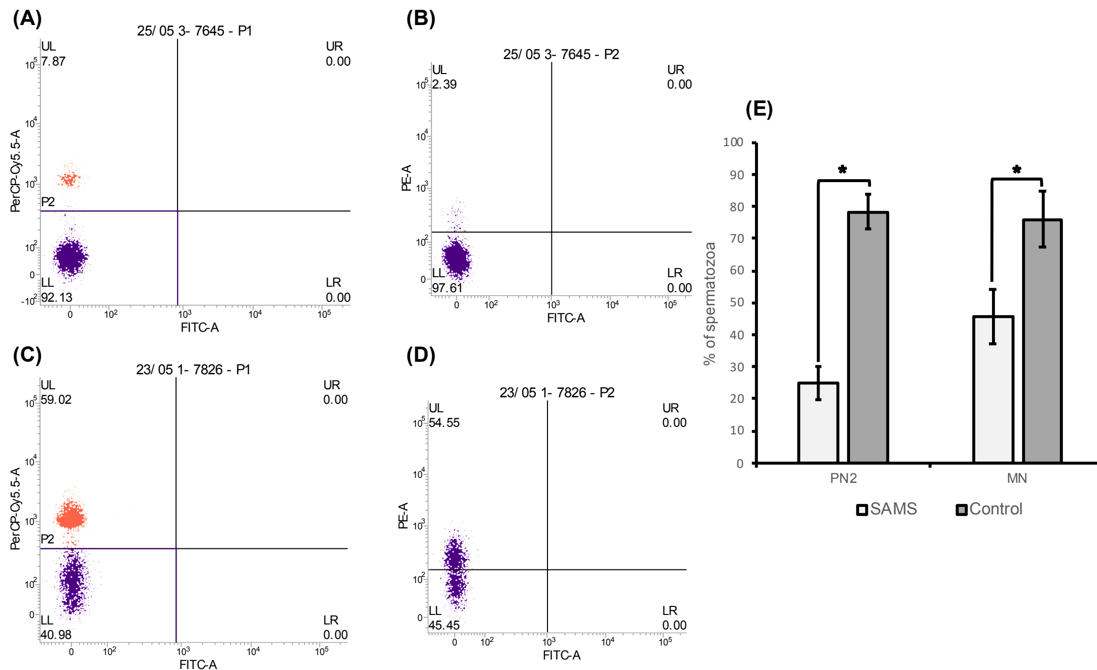
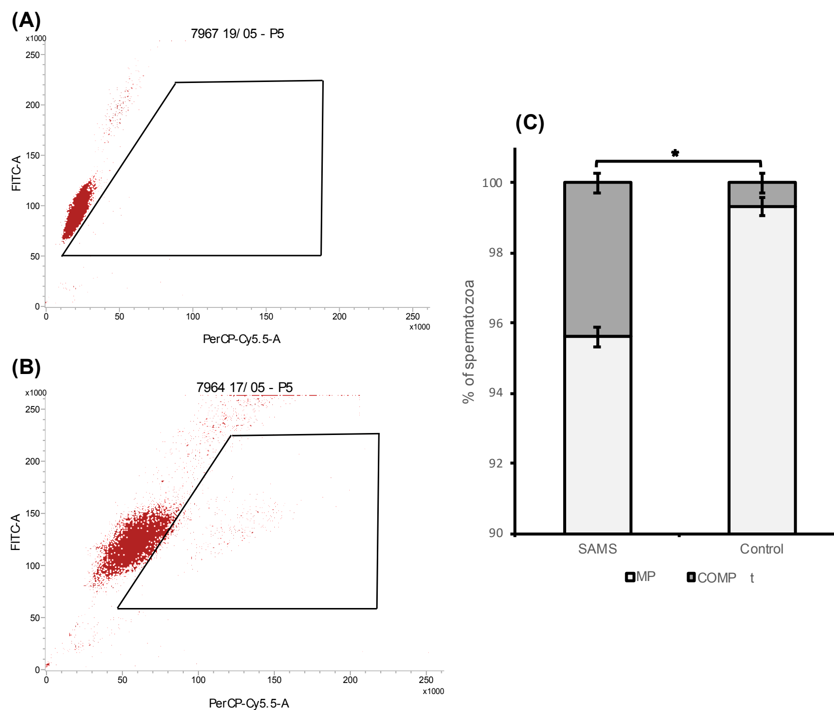


Figure 4 DNA integrity from fresh semen of Gyr bulls in the segmental aplasia of the mitochondrial sheath (SAMS) and control groups. Representative dot plot showing sperm with fragmented DNA or Cells Outside the Main Population (COMP α_t) from (A) control and (B) SAMS group. The Distribution of sperm 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.



DISCUSSION

Our results provide pioneer information concerning functional features of bull sperm in the presence of SAMS. Herein, we found remarkable differences between fresh semen from bulls with normal and abnormal sperm morphology. Mitochondrial abnormalities have received poor attention (Moretti et al., 2016). As a result, these abnormalities have frequently been described as just midpiece defects rather than identifying the specific abnormality; this makes it difficult to identify the first reported case of SAMS. In addition, SAMS is referred to in several different forms, including mitochondrial gaps, discontinuous midpiece defect and constriction or thin midpiece, among others. However, the first publications identifying SAMS in bulls may be described by Savage & Isa (1962) and Hellmén et al. (1980) in which they utilized light and electron microscopy in their methodology. Indeed, there is no doubt about the importance of sperm morphology analysis in the determination of bull fertility. Over the last decades, the results from this analysis have been contributing in bull fertility tests identifying subfertile and infertile males in many species (Chenoweth,

2005; Saacke, 2008; Enciso et al., 2011).

According to their relevance in fertility, midpiece sperm defects are classified as a major defect (Blom, 1973). In bulls, midpiece defects have been related to affect sperm motility, resulting in subfertility or infertility (Blom, 1959, 1966). However, the SAMS, a midpiece defect, has been thought to have no detrimental effect in motility or in fertility (Barth & Oko, 1989; Rocha *et al*., 2006). Our results clearly showed a lack of sperm motility in bulls with SAMS, possibly due to the reduction of energy production capacity, as well as the alteration in the structural composition of the sperm cell.

The mitochondrial sheath 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), as well as in the present study. Therefore, the SAMS may be accompanied by other pathologies, such as the Dag defect, corkscrew, stump, fractures, defibrillation, a swollen or short midpiece and sometimes decapitations. Thus, midpiece abnormalities require careful attention to identify the major and primary lesion.

Understanding the pathophysiology of sperm defects can unravel how they affect the fertility (Aitken *et al*., 2012). Bulls in the SAMS group exhibited alterations in several sperm traits including low motility, low mitochondrial membrane potential, a high percentage of sperm with DNA fragmentation, and a high percentage of damaged plasma membrane and acrosome. Furthermore, of the small percentage of sperm with an intact plasma membrane, half show disorganization of the plasma

membrane lipid bilayer.

In the context, one explanation for our findings includes the possibility that sperm from bulls in the SAMS group are undergoing oxidative stress. Due to oxidative stress is a major contributor to the defective sperm function (Aitken *et al*., 2012), and is a condition that reflects an imbalance between reactive oxygen species (ROS) production and the antioxidant defense mechanisms (Lenzi *et al*., 1996; Agarwal *et al*., 2014). Therefore, mitochondria are the main source of sperm-produced ROS via electron transport chain (Koppers *et al*., 2008; Amaral *et al*., 2013), hence an enhancement of ROS generation is related to mitochondrial dysfunction, and can affect mitochondrial integrity, starting a cycle in which ROS injures the mitochondrial membrane and the injured mitochondrial membrane enhances ROS production (Sanocka & Kurpisz, 2004; Sabeti *et al*., 2016). Abnormal sperm with SAMS have a lack of mitochondria, but may also exhibit mitochondrial dysfunction, leading to high ROS production.

However, there are other potential sources of ROS such as spermatozoa with abnormal morphology (Rao *et al*., 1989; Aziz *et al*., 2004; Aitken *et al*., 2012), in addition, it was reported that in bovine semen the generation of ROS are mainly by dead spermatozoa via an aromatic amino acid oxidase catalyzed reaction (Upreti *et al*., 1998). Bulls in the SAMS group combined a harmful environment of high percentage of morphologically abnormal and dead sperm suggesting that this group is certainly undergoing oxidative stress, which would explain the low mitochondrial membrane potential (Ghaleno *et al.*, 2014) and poor motility (De Lamirande & Gagnon, 1992). Moreover, the importance of mitochondria-derived ATP via oxidative phosphorylation for sperm motility remains in debate (Turner, 2003; Silva & Gadella, 2006; Peña *et al*., 2009; Amaral *et al*., 2013), however, the fact that sperm with

SAMS had poor or no motility suggests that mitochondria have a crucial role in bovine sperm movement.

Sperm plasma membrane is structured in an asymmetrical arrangement of lipids within the bilayer associated to peripheral membrane proteins (Parks & Graham, 1992), thus lipids are largely responsible for membrane fluidity (Lenzi *et al*., 1996). Due to the lipid composition of the plasma membrane, which consists of high levels of phospholipids, sterols, and saturated and polyunsaturated fatty acid, sperm cells are particularly vulnerable to peroxidative damage induced by excessive ROS release (Aitken *et al*., 2012; Aitken & Baker, 2013). Lipid peroxidation is an autocatalytic, self-propagating reaction, associated with cell dysfunction and loss of membrane function and integrity (Sanocka & Kurpisz, 2004). Midpiece abnormalities were related to high levels of lipid peroxidation, possibly due the high membrane content associated with such abnormality, which make this region highly susceptible to free radical attack (Rao *et al*., 1989; Lenzi *et al*., 1996). This may explain the high levels of membrane damage and fluidity of the plasma membrane of the viable sperm in the SAMS 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 could share the same vulnerability to oxidative stress as the plasma membrane and similar damage as reported in the present study.

Furthermore, the sperm cell is highly polarized and specialized functions are compartmentalized (Fawcett, 1975; Flesch & Gadella, 2000; Turner, 2003; Amaral *et al*., 2013); the sperm head plasma membrane is separated from the midpiece plasma membrane by the posterior ring, and the midpiece plasma membrane is separated from the flagellum plasma membrane by the annular ring (Parks & Graham, 1992; Flesch & Gadella, 2000). It is suggested that the majority of the enzymatic

antioxidant system is located in the midpiece (Aitken & Curry, 2011; O'Flaherty, 2014) and morphological abnormalities of this region could be associated with dysfunction of these systems, favoring lipid peroxidation (Rao *et al*., 1989). Therefore, midpiece defects as SAMS may be more vulnerable to oxidative stress.

Morphologically abnormal bull sperm have poor DNA quality and damaged DNA (Khalifa *et al*., 2008; Enciso *et al*., 2011; Boe-Hansen *et al*., 2018). In agreement with these studies, bulls in the SAMS group have higher levels of damaged DNA, possibly resulting from oxidative stress. Indeed, sperm midpiece defects are correlated to high levels of DNA fragmentation (Morrell *et al*., 2008; Speyer *et al*., 2010) and it was suggested that when low motility appears with midpiece defects, high levels of DNA fragmentation should be presumed (Speyer *et al*., 2010). The sperm chromatin is a highly organized, compact structure consisting of DNA and heterogeneous nucleoproteins (Evenson *et al*., 2002). Chromatin compaction occurs during spermiogenesis; after this process DNA is extremely stable and difficult to damage, however, poor compaction makes DNA vulnerable to injury (Aitken *et al*., 2009; Agarwal *et al*., 2014). DNA strand breakage can be induced by 2 mechanisms: through the action of nucleases or as a by-product of oxidative stress, although the major cause of DNA damage is oxidative stress (Aitken *et al*., 2012).

These findings support the hypothesis that sperm with segmental aplasia of the mitochondrial sheath may be undergoing apoptosis or spermptosis, 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, which corroborates with previous studies (Aitken & Curry, 2011; Aitken & Koppers, 2011;

Aitken *et al*., 2012). Additionally, bulls in the SAMS group had high percentage of sperm with lipid bilayer sperm membrane disorganization assessed by M540, this probe 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 remodelling 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 that are considered to be of genetic origin (Chenoweth & McPherson, 2014). The SAMS in Gyr bulls may better fit classified under the systematic (monomorphic) and non-systematic (polymorphic, non-specific) sperm defects categorization (Chemes & Rawe, 2003). Non-systematic defects are a heterogeneous combination of different abnormal sperm while systematic defects consist of a characteristic anomaly present in the majority of the spermatozoa in a semen sample. Additionally, systematic defects are suspected to be of genetic origin and the sperm pathology persists in every ejaculated (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 in our results and in the knowledge of the presence of this sperm pathology in several generations of bulls in this specific herd (unpublished data), it suggests that in this particular herd or breed, the SAMS is a systematic defect with a genetic origin.

The SAMS has also been reported in humans (Bartoov *et al*., 1980; Zamboni, 1991; Wilton *et al*., 1992; Rawe *et al*., 2007), cats (Howard *et al*., 1991), stallions (Card, 2005; Brito, 2007; Veeramachaneni, 2011), and bulls (Barth & Oko, 1989; Hoflack *et al*., 2006; Rocha *et al*., 2006), and can be induced by gossypol (Oko & Hrudka, 1982; Chenoweth *et al*., 2000; Hassan *et al*., 2004), viral diseases

(Chenoweth & Burgess, 1972), selenium deficiency (Wallace *et al*., 1983; Olson *et al*., 2004) and idiopathic. Furthermore, there are some rat knockout models and mutations with this abnormal phenotype (Doiguchi *et al*., 2002; Escalier, 2006; Zhang *et al*., 2012). The spermatogenesis is a highly sophisticated process (Chocu *et al*., 2012), future research should be dedicated to comprehend sperm midpiece pathologies in order to understand the development of the mammalian midpiece spermatozoa; understanding these pathologies may also help in therapeutic human treatment. Additionally, it was related that a proteomic approach comparing normal versus morphologically abnormal sperm is a suitable experimental model for identifying important sperm functional proteins, as well as to understand the molecular basis of functional deficiencies in sperm (Saadi *et al*., 2013). Despite the fact that there are no identified polymorphisms associated with heritable sperm defects, a genotype-to-phenotype approach could be another interesting tool (Sutovsky *et al*., 2015) and can assist animal science programs guiding future crossings (Genetic Breeding Programs) to eliminate this anomaly of the herd.

In conclusion, this is a typical semen picture from high percentages of abnormal sperm, with low viability affecting the small percentage of sperm with normal morphology. Semen from bulls with SAMS is characterized by poor motility, low mitochondrial membrane potential, high percentage of sperm with DNA fragmentation, low acrosome and plasma membrane integrity, and high membrane fluidity, possibly due to oxidative stress, resulting from the enhancement of ROS production from abnormal and dead sperm in addition to a reduction of the energy production capacity and an alteration in the structural composition of the sperm cell. Furthermore, sperm with normal morphology should be undergoing apoptosis. Finally, we highlight the importance of the sperm morphology in fertility, particularly

from midpieces defects.

ACKNOWLEDGEMENTS

This work was supported by CNPq, CAPES and FAPEMIG. The authors thank Prof. Leandro Licursi and the Nucleus of Microscopy and Microanalysis at the Universidade Federal de Viçosa, (http://www.nmm.ufv.br/) for providing the equipment and technical support for experiments involving flow cytometry. In addition, the authors also thank the dairy cattle research facility for housing the animals.

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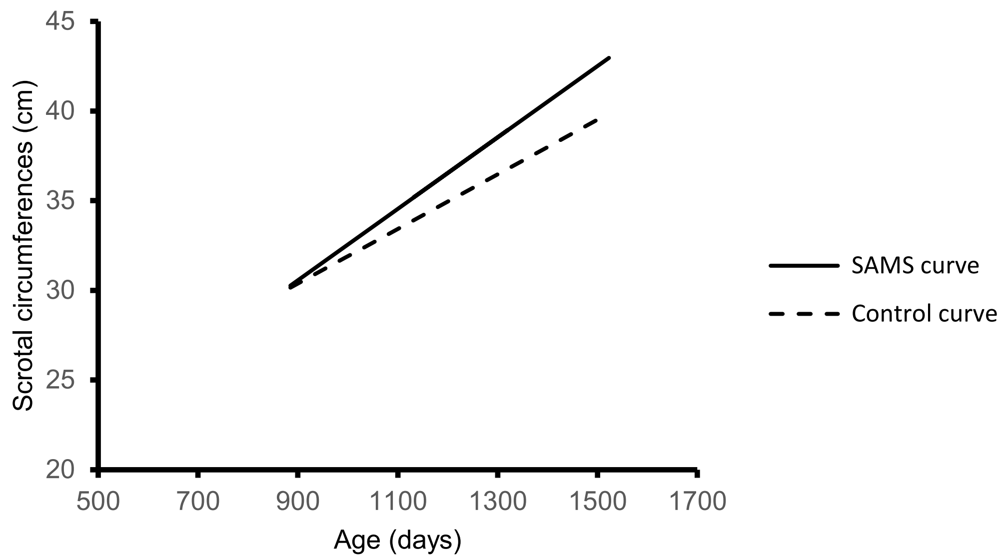
SUPPORTING INFORMATION

Data of scrotal circumference related to age were analyzed by linear regression analysis (REG procedure). Scrotal circumference growth curves are presented in Fig. 1 with a linear behavior; the equations were: Control $\hat{Y} = 16.67596 + 0.01523x$ ($p < 0.05$; $R^2 = 0.86$), SAMS $\hat{Y} = 12.65503 + 0.01990x$ ($p < 0.05$; $R^2 = 0.93$).

Figure 1 Scrotal circumference growth curve from Gyr bulls in the segmental aplasia of the mitochondrial sheath (SAMS) and control groups.



With regard to the scrotal circumference both groups have similar mean values in the age range to what has previously been reported as normal in the breed (Martinez et al., 2000; Folhadella *et al*., 2006; Martins *et al*., 2011)**.**