LAURA NATALY GARCIA OLIVEROS

**Produção de espécies reativas ao oxigênio no sêmen de touros
Nelore com degeneração testicular**

Pirassununga

2019

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**Produção de espécies reativas ao oxigênio no sêmen de touros Nelore com
degeneração testicular**

Dissertação apresentada ao Programa de Pós-Graduação em Reprodução Animal da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para a obtenção do título de Mestre em Ciências

Departamento:

Reprodução Animal

Área de concentração:

Reprodução Animal

Orientadora:

Profa. Dra. Eneiva Carla Carvalho Celeghini

De acordo: _____

Orientadora

Pirassununga

2019

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1. Espermatozoides. 2. Espermatogênese. 3. Maturação espermática. 4. Peroxidação
lipídica. 5. Morfologia. I. Título.

CERTIFICADO

Certificamos que a proposta intitulada "Produção de espécies reativas ao oxigênio no sêmen de touros Nelore com degeneração testicular", protocolada sob o CEUA nº 5162170616, sob a responsabilidade de **Eneiva Carla Carvalho Celeghini e equipe**; *Laura Nataly Garcia Oliveros ; Leonardo Batissaco ; Vinicius José Nogueira ; Vitor Hugo Guilger Gonzaga* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 15/08/2016.

We certify that the proposal "Production of reactive oxygen species in the semen of Nelore bulls with testicular degeneration", utilizing 24 Bovines (24 males), protocol number CEUA 5162170616, under the responsibility of **Eneiva Carla Carvalho Celeghini and team**; *Laura Nataly Garcia Oliveros ; Leonardo Batissaco ; Vinicius José Nogueira ; Vitor Hugo Guilger Gonzaga* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 08/15/2016.

Finalidade da Proposta: [Pesquisa](#)

Vigência da Proposta: de [07/2016](#) a [07/2017](#) Área: [Reprodução Animal](#)

Origem: [Prefeitura do Campus da USP de Pirassununga](#)

Espécie: [Bovinos](#)

sexo: [Machos](#)

idade: [30 a 34 meses](#)

N: [24](#)

Linhagem: [Nelore](#)

Peso: [545 a 660 kg](#)

Resumo: A degeneração testicular é caracterizada como uma afecção de grande impacto na fertilidade bovina, tendo como causa principal o estresse térmico. Uma das consequências desse estresse é a alteração do balanço na quantidade de espécies reativas ao oxigênio, culminando no estresse oxidativo. Com isso, o presente projeto visa avaliar o estresse oxidativo, fragmentação de DNA e atividade enzimática de touros Nelore com degeneração testicular. Para tal serão utilizados 24 touros Nelore distribuídos em dois grupos: grupo controle (CON, n=12) e grupo com degeneração testicular (n=12). A degeneração testicular será induzida pela colocação de bolsas insuladoras nos testículos dos touros. Serão realizadas colheitas de sêmen uma vez por semana nos períodos: duas semanas antes da colocação das bolsas (S-2 e S-1); no dia da colocação (imediatamente antes, D0) e da retirada (imediatamente após, D3); e durante 11 semanas após (S+1 a S+11). A colheita de sêmen será feita por eletroejaculador e as colheitas de sangue serão feitas por punção da veia caudal. Além disso, o sêmen será avaliado quanto ao volume, concentração, turbilhonamento, motilidade, morfologia, integridade da membrana plasmática e acrossomal, função mitocondrial, estresse oxidativo, atividade enzimática, peroxidação lipídica e fragmentação do DNA. A análise dos dados obtidos será realizada utilizando o programa estatístico Statistical Analysis System, (SAS 2004) por meio da análise de variância adicionando o fator medidas repetidas no tempo MIXED. O nível de significância considerado será de 5%. Palavras-chave: Degeneração testicular, Estresse oxidativo, Antioxidantes, Fragmentação de DNA.

Local do experimento: Centro de Biotecnologia de Reprodução (CBRA-VRA) Campus Pirassununga

São Paulo, 16 de agosto de 2016

Profa. Dra. Denise Tabacchi Fantoni
Presidente da Comissão de Ética no Uso de Animais

Roseli da Costa Gomes
Secretaria Executiva da Comissão de Ética no Uso de Animais

FOLHA DE AVALIAÇÃO

Autor: GARCIA-OLIVEROS, Laura Nataly

Título: Produção de espécies reativas ao oxigênio no sêmen de touros Nelore com degeneração testicular

Dissertação apresentada ao Programa de Pós-Graduação em Reprodução Animal da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para a obtenção do título de Mestre em Ciências

Data: ____/____/____

Banca Examinadora

Prof. Dr.: _____

Instituição: _____ Julgamento: _____

Prof. Dr.: _____

Instituição: _____ Julgamento: _____

Prof. Dr.: _____

Instituição: _____ Julgamento: _____

DEDICATORIA

Dedico esta dissertação a minha família, minha mãe Maria Sandra, meu pai Carlos Daniel, meu irmão Juan Daniel, minha mãezinha Mery. Vocês são meu maior tesouro, minha casa, minha paz e o meu porto seguro. São a mais linda benção. Minha família Garcia e Oliveros, tenho sorte e sou abençoada de tê-los em cada momento da minha vida. Obrigada sempre pelas orações, pelo apoio, pelas palavras lindas, pelos bons dias e boas noites todos os dias.

Obrigada aos meus anjos da guarda, a saudade se sente na terra mas a companhia se leva no coração, sei que vocês estão comigo sempre ☐.

Dedico este trabajo a mi familia, mi mama Maria Sandra, mi papa Carlos Daniel, mi hermano Juan Daniel, mi mamita Mery. Ustedes son mi mas grande tesoro, mi casa, mi paz, mi puerto seguro. Son mi mas grande bendicion. A mi familia Garcia y Oliveros, soy afortunada y bendiciada de tenerlos en cada momento de mi vida. Gracias por las oraciones, por apoyarme siempre, por las palabras lindas, por los buenos dias y las buenas noches todos los dias.

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A Deus e a minha família por ser minha guia, meu apoio incondicional e minha roca.

A Profa. Dra. Eneiva Carla Carvalho Celeghini por ter me acolhido, por ter confiado em mim e nas minhas capacidades, mesmo inexperiente para iniciar um projeto de pesquisa, você sempre teve fé em que podia fazer as coisas. Obrigada pela paciência com o idioma, obrigada pelo respeito, por esse jeito carinhoso, pelo apoio e exemplo como profissional e como pessoa, pelo ensino constante e a ética, pelas chamadas de atenção e preocupação no momento que mais precisava, obrigada por querer continuar me orientando, MILLONES DE GRACIAS SIEMPRE POR TODO.

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RESUMO

GARCIA-OLIVEROS, L.N. **Produção de espécies reativas ao oxigênio no sêmen de touros Nelore com degeneração testicular.** 119 f. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2019.

O estresse térmico testicular causa degeneração testicular em touros, pois provoca alterações no processo da espermatogênese e maturação espermática, caracterizada pela queda da qualidade seminal. No entanto, a cronologia desses efeitos, desde o aparecimento das primeiras alterações até a recuperação da espermatogênese, ainda não é totalmente compreendida, e é fundamental para fornecer informações que auxiliem no diagnóstico, tratamento e prognóstico desta alteração. Neste sentido, foram realizados 2 experimentos; o experimento 1 objetivou avaliar e definir a cronologia de efeitos do estresse térmico testicular sobre a qualidade das diferentes características estruturais e funcionais de espermatozoides bovinos, especificamente nos primeiros 28 dias pós estresse térmico testicular para dar diagnóstico precoce da degeneração testicular. Foram utilizados 20 touros da raça Nelore e distribuídos em 2 grupos: controle e estresse térmico testicular (bolsas testiculares/96 horas) e submetidos a colheitas de sêmen e sangue semanais, iniciando-se 14 dias antes da colocação das bolsas e se mantendo até 28 dias após a retirada das bolsas testiculares. Todas as amostras de sêmen foram avaliadas quanto à motilidade, morfologia, integridade das membranas plasmática e acrossômica, potencial da membrana mitocondrial, peroxidação lipídica e fragmentação do DNA. Os plasmas seminal e sanguíneo foram avaliados para a peroxidação lipídica. No experimento 2, objetivou-se avaliar as cronologicamente as lesões do estresse térmico testicular sobre a qualidade das mesmas características espermáticas estruturais e funcionais e do sangue mencionadas e avaliadas no experimento 1, assim como avaliar o predomínio de defeitos maiores e menores, e a susceptibilidade do sêmen à peroxidação lipídica em cada estágio específico da espermatogênese, maturação espermática e recuperação do epitélio seminífero. Para isso foi proposto dividir os achados em 5 períodos: 1 (14 e 7 dias antes do insulto), 2 (dia da retirada do insulto/D0 até 7 dias após), 3 (14, 21, 28, 35 e 42 dias após), 4 (49, 56 e 63 dias após) e 5 (considerado como o período de recuperação do epitélio seminífero: 70 e

77 dias após). Foram utilizadas e distribuídos 10 touros Nelore em 2 grupos: controle e estresse térmico testicular (bolsas testiculares/96 horas) e, submetidos também a colheitas de sêmen e sangue semanais, iniciando-se 14 dias antes da colocação das bolsas e esta vez, se mantendo até 77 dias após a retirada. Quanto aos resultados, no primeiro estudo foi possível observar principalmente, o aumento de defeitos morfológicos 7 dias após o estresse térmico, e fragmentação do DNA 28 dias depois. Já no segundo estudo, notou-se que no período 1 os grupos de touros foram homogêneos antes do insulto. O período 2 mostrou efeitos do estresse térmico testicular sobre a maturação espermática no epidídimo, evidenciados pelo aumento nos defeitos espermáticos e diminuição da integridade das membranas. No período 3 houve aumento da peroxidação lipídica dos espermatozoides e diminuição do potencial de membrana mitocondrial, motilidade espermática, integridade da membrana plasmática e DNA. Durante o período 4, os defeitos continuaram aumentando e o potencial mitocondrial diminuindo. No período 5, houve a recuperação de todas as características espermáticas avaliadas. Desta forma, com os 2 estudos pode-se concluir que a cronologia dos efeitos da degeneração testicular por estresse térmico começa com o aumento dos defeitos espermáticos e da peroxidação lipídica, sugerindo estresse oxidativo do sêmen e alterando em primeiro lugar a função das mitocôndrias, refletindo sobre a motilidade espermática, a integridade da membrana plasmática e, mais tardiamente, na integridade do DNA. Assim, estes achados permitiram afirmar que, embora a peroxidação lipídica espermática aumente somente durante 14 a 21 dias após o estresse térmico, a motilidade e morfologia espermática e, a integridade das membranas permanecem diminuídas até 63 dias após o estresse. Nesse sentido, o conhecimento da cronologia dos efeitos da degeneração testicular por estresse térmico testicular, em cada fase da espermatogênese e maturação espermática, trouxeram informações valiosas sobre o status da espermatogênese e ajudarão no embasamento dos achados clínicos para a realização do diagnóstico da degeneração testicular em touros, proporcionando melhor prognóstico e intervenções terapêuticas.

Palavras chave: Espermatozoides. Espermatogêneses. Maturação espermática. Peroxidação lipídica. Estresse térmico.

ABSTRACT

GARCIA-OLIVEROS, L.N. **Production of reactive oxygen species in the semen of Nellore bulls with testicular degeneration.** 119 s. Dissertation (Master in Science) - School of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, 2019.

Testicular heat stress is known to cause testicular degeneration in bulls, as it causes alterations in the spermatogenesis and sperm maturation process, characterized by a decrease in seminal quality. However, the chronology of these impairments, from the appearance of the first alterations until the recovery of spermatogenesis, is still not fully understood, and it is fundamental to provide information to intervene adequately in the diagnosis, treatment and prognosis of this alteration. In this sense, two studies were performed; the study 1 aimed to evaluate and define the chronology of the effects of testicular heat stress on the quality of different structural and functional characteristics of bovine sperm, specifically, during the first 28 days after the insult to give an early diagnosis of the testicular degeneration. Twenty Nellore bulls were distributed in the control and testicular heat stress groups (scrotal bags/96 hours) and submitted to weekly blood and semen collections, starting 14 days before the placement of the bags and remaining up to 28 days after. All semen samples were evaluated for sperm motility, morphology, plasma and acrosomal membrane integrity and mitochondrial membrane function, lipid peroxidation, and DNA fragmentation. Semen and blood plasma were also evaluated for lipid peroxidation. In the second study, the objective was to evaluate chronologically the impairments of the testicular heat stress on the quality of the same structural and functional sperm characteristics and blood mentioned and evaluated in the previous study, as well as to evaluate the predominance of major and minor defects, and the susceptibility from semen to lipid peroxidation at each specific stage of spermatogenesis, sperm maturation and recovery of the seminiferous epithelium. For this, it was proposed to divide the findings into 5 periods: 1 (7 and 14 days before the insult), 2 (day of removal of the insult / D0, up to 7 days before the insult), 3 (14, 21, 28, 35 and 42 days before the insult), 4 (49, 56, 63 days before the insult) and 5 (considered as the recovery period / 70 and 77 days before the insult). Ten Nellore bulls were distributed in the control and testicular heat stress groups (scrotal bags/96 hours)

and submitted to weekly blood and semen collections, starting 14 days before the placement of the bags and remaining up to 77 days after. Regarding the results, in the first study it was possible to observe specially, the increase of morphological defects 7 days after the heat stress, and DNA fragmentation 28 days later. In the second study, during period 1 it was noticed that the groups of bulls were homogeneous before the insult. Period 2 showed effects of testicular heat stress on sperm maturation in the epididymis, evidenced by the increase in spermatic defects and decrease of membrane integrity. In period 3 there was an increase in sperm lipid peroxidation and a decrease in mitochondrial membrane potential, sperm motility, plasma membrane and DNA integrity. During period 4, the defects continued to increase and the mitochondrial potential decreased. In period 5, there was recovery of all evaluated sperm characteristics. Thus, the chronology of the effects of testicular heat stress begins with the increase of sperm defects and lipid peroxidation, suggesting oxidative stress of the semen and altering in the first place the function of the mitochondria, reflecting on the sperm motility, membranes integrity and, later, on DNA integrity. Therefore, these findings allowed us to affirm that, although sperm lipid peroxidation increases for 14 to 21 days after heat stress, sperm motility, morphology and membranes integrity remain decreased up to 63 days after stress. In this sense, the knowledge of chronology of the testicular heat stress affections during each phase of spermatogenesis and sperm maturation, bringing valuable information of the status of spermatogenesis and will aid in the achievement of accurate diagnoses, better prognosis and therapeutic interventions.

Keywords: Spermatozoa. Spermatogeneses. Sperm maturation. Lipid peroxidation. Heat stress.

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Introdução

1 INTRODUÇÃO

No cenário mundial do rebanho bovino, o Brasil destaca-se como o segundo maior produtor de carne bovina, com 232.310 mil cabeças e 9,47 milhões de toneladas de carcaça (ANUALPEC, 2017). Contudo, o crescimento da população brasileira e, ao mesmo tempo da demanda de proteína animal, leva à constante necessidade de melhorar a produtividade do rebanho nacional, adotando medidas com o objetivo de expandir as exportações. A produtividade do rebanho bovino depende principalmente do potencial genético e da fertilidade, sendo ambos os fatores determinantes para a produção eficiente (MORAIS, 2011; CELEGHINI et al. 2017). Com o intuito de promover o melhoramento do potencial produtivo e reprodutivo, as biotécnicas ligadas à reprodução bovina vêm se desenvolvendo e expandindo. Verificou-se que no ano 2018, o mercado da inseminação artificial em tempo fixo (IATF) cresceu 16,1% em relação ao ano anterior. Também, em 2017 foram comercializados 11.416.196 protocolos, comparados aos 13.259.690 protocolos comercializados em 2018. Isto indica que 86% das inseminações no Brasil foram realizadas por IATF, demonstrando a consolidação dessa biotécnica no mercado da inseminação artificial (BARUSELLI, 2019).

A maior parte das IATFs realizadas no Brasil emprega o sêmen criopreservado, fazendo com que essa associação gere taxas médias de prenhez em torno de 52% (BARUSELLI et al., 2018). Nos rebanhos nacionais, sabe-se que o touro é responsável por 70% ou mais do melhoramento genético de uma população (MENEGASSE et al., 2011). Em 2017, o Brasil exportou sêmen para mais de nove países (ANUALPEC, 2017). No primeiro semestre de 2019, as vendas de sêmen gerais cresceram 19,1%, sendo comercializadas 6.090.104 doses contra as 5.114.601 doses verificadas na primeira metade de 2018. A maior elevação foi registrada nas raças de corte, com 3.757.526 de doses vendidas, aumento de 27,9% (ASBIA, 2019).

Diante deste cenário, é evidente a importância da manutenção da eficiência reprodutiva dos touros para garantir o sucesso do potencial reprodutivo. Contudo, a capacidade reprodutiva dos touros depende de vários fatores que podem influenciar na qualidade do sêmen, e consequentemente, no potencial reprodutivo.

As alterações testiculares são bastante comuns em diversas espécies, e podem ser ocasionadas por fatores extrínsecos ao animal, como a elevação da

temperatura ambiente. As células da linhagem germinativa são altamente susceptíveis à ação do calor (REECE, 2006; HANSEN, 2009). Desta forma, no Brasil, o estresse térmico é a principal causa de degeneração testicular em touros, devido ao fato de a eficiência do processo de espermatogênese depender da correta termorregulação testicular. Por este motivo, a degeneração testicular por estresse térmico caracteriza-se como a causa mais comum de subfertilidade e infertilidade de touros que servem a campo (VALE FILHO et al., 1974; CELEGHINI et al., 2017).

A patogenia da degeneração testicular ainda não está completamente elucidada. Entretanto, sabe-se que o aumento de temperatura testicular gera aumento do metabolismo celular, o qual não é acompanhado pelo suporte sanguíneo dos testículos, gerando quadro de hipóxia celular no tecido testicular (PAUL et al., 2009), desbalanço entre a quantidade de espécies reativas de oxigênio e os mecanismos antioxidantes, o que culmina em estresse oxidativo (YUE et al., 2010). O estresse oxidativo leva a danos na estrutura e função do espermatozoide, devido à diminuição da concentração e motilidade espermática, bem como aumento de defeitos morfológicos (RAHMAN et al., 2018) e da fragmentação do DNA, levando à apoptose das células dos túbulos seminíferos (PÉREZ-CRESPO et al., 2008; PAUL et al., 2009). Um dos componentes fundamentais do espermatozoide, necessário para a formação normal dos embriões, é a existência do núcleo com cromatina e matriz nuclear intacta (WARD et al., 1999). As alterações da morfologia espermática influenciam na motilidade e cromatina espermática, dificultando o processo de fertilização. De igual forma, alterações na cromatina podem ainda gerar interferências no desenvolvimento embrionário (BELETTI, 2013).

Muitos pesquisadores demonstraram diferentes maneiras de explorar a fisiopatogenia da degeneração testicular (BARTH e BOWMAN, 1994; KASTELIC et al., 1997; BRITO et al. 2003; RAHMAN et al. 2011; DAS et al. 2016; RAHMAN et al. 2018); porém, a cronologia dos eventos ainda não é totalmente compreendida, e tal compreensão é fundamental para auxiliar adequadamente no diagnóstico, tratamento e prognóstico da degeneração testicular. Desta forma, a fim de estabelecer a cronologia das alterações na estrutura e viabilidade espermática durante a degeneração testicular, o presente trabalho busca avaliar diferentes características espermáticas como motilidade,

morfologia, integridade das membranas plasmática e acrossomal, potencial mitocondrial, susceptibilidade à peroxidação lipídica e integridade do DNA; além disso, avaliar o predomínio de defeitos maiores e menores e a ocorrência de peroxidação lipídica do plasma seminal e sanguíneo, utilizando o estresse térmico testicular como modelo. Para isso, o estudo está apresentado em dois artigos. O primeiro artigo teve como objetivo avaliar as características espermáticas durante os primeiros 28 dias após o estresse térmico testicular. Neste estudo foi hipotetizado que nos 28 dias após o estresse, as fases mais sensíveis da espermatogênese são afetadas e os danos ao DNA já são evidentes. No segundo artigo foram considerados cinco períodos, a fim de ampliar as mesmas análises da viabilidade e estrutura espermática, desta vez, avaliando as diferentes fases do ciclo espermático, maturação espermática e a recuperação do epitélio seminífero.

Capítulo 1: Revisão de Literatura

2 REVISÃO DE LITERATURA

2.1 ESPERMATOGÊNESE EM TOUROS

Na maioria dos animais mamíferos, os testículos exercem duas funções principais, a exócrina e a endócrina. A função exócrina envolve a produção de espermatozoides mediante o processo de espermatogênese. Este é um processo contínuo e finamente regulado de multiplicação e diferenciação de células germinativas, que leva à produção dos gametas masculinos (BANKS et al., 2005). Estes são liberados no lúmen dos túbulos seminíferos e armazenados no epidídimo. A função endócrina é realizada pelas células de Leydig, localizadas no compartimento intersticial, fora dos túbulos seminíferos e tem como papel principal a produção de hormônios esteroides (STAUB e JOHNSON, 2018). No bovino, a espermatogênese pode ser dividida em três fases: espermatocitogênese, meiose e espermiogênese, as quais levam aproximadamente 21, 23 e 17 dias, respectivamente (JOHNSON et al., 2000). Durante a espermatocitogênese, as células germinativas primordiais dividem-se por mitose na parte basal dos túbulos seminíferos para formar espermatogônias tipo A1 ou células-tronco. Uma parte importante desta fase é a renovação de algumas espermatogônias tipo A1, permitindo a constante formação de novas células-tronco (HOCHEREAU-DE REVIERS, 1976). As outras espermatogônias tipo A1 passam pelo processo de divisão mitótica para formar espermatogônias tipo A2, A3 e A4. Posteriormente, as espermatogônias tipo A4 dividem-se para formar espermatogônias intermediárias (Int), e em seguida, espermatogônias tipo B. Estas por sua vez, ao se dividirem por mitose dão origem aos espermatócitos primários, os quais iniciam a meiose I (STAUB e JOHNSON, 2018). Os espermatócitos primários, na fase de pré-leptóteno/leptóteno (prófase I da meiose I) atravessam a barreira hemato-testicular, onde terminam a meiose I, originando os espermatócitos secundários, o qual prosseguirá na meiose II. Ao final da segunda divisão meiótica os espermatócitos secundários darão origem às espermátides redondas (STAUB e JOHNSON, 2018).

As divisões meióticas ocorrem no compartimento adluminal dos túbulos seminíferos (WROBEL et al., 1995). Durante a meiose I, os espermatócitos primários duplicam seu DNA e sofrem mudanças nucleares progressivas na

prófase I (leptóteno, zigóteno, paquíteno e diplóteno) (AMANN, 1983). Cada um desses estágios representa um passo diferente na progressão da síntese e replicação do DNA. Os espermátócitos primários devem progredir a partir da interfase (imediatamente após sua divisão) por meio desta série de mudanças, antes que a primeira divisão meiótica possa ocorrer. O evento importante da fase de pré-leptóteno é a replicação completa do DNA, formando tétrades sem separação. Essas tétrades se fundem em pontos aleatórios conhecidos como quiasmas, onde ocorrerá o *crossing-over* do DNA. O termo *crossing-over* refere-se à troca de segmentos de um cromossomo para o cromossomo homólogo no momento da separação das cromátides, resultando numa variedade aleatória de diferentes segmentos de cada cromossomo. Deste modo, a prófase da primeira divisão meiótica assegura que a heterogeneidade genética permaneça e, que cada espermátócito secundário e espermátide sejam geneticamente únicos. A prófase da primeira divisão meiótica é o processo mais longo da espermatogênese, de forma que o tempo de vida do espermátócito primário é maior em comparação aos outros tipos de células germinativas. Este ocupa cerca de 30% do tempo necessário para todo o processo espermatogênico, ou seja, de 18 a 19 dias no touro. Enquanto, a fase de espermátócitos secundários leva de 1,1 a 1,7 dias (SENGER, 2003). A primeira divisão meiótica é também conhecida como a divisão redutora, pois ocorre redução do número de cromossomos e separação de cromossomos homólogos. Enquanto, que a segunda divisão meiótica é descrita como a divisão equacional, ocorrendo a separação das cromátides filhas (YAN CHENG e MRUK, 2012; STAUB e JOHNSON, 2018).

Posteriormente, as espermátides redondas passam pelo processo de espermiogênese, durante o qual estas se transformam gradualmente no espermatozoide (STAUB e JOHNSON, 2018). Durante a espermiogênese, as espermátides redondas tornam-se espermátides alongadas, e passam por condensação da cromatina nuclear, formação do flagelo ou cauda do espermatozoide, e o desenvolvimento da capa acrossomal (CLERMONT et al., 1985; TANII et al., 1992; YOSHINAGA et al., 2001). Esta fase também ocorre no compartimento adluminal dos túbulos seminíferos, onde as espermátides alongadas resultantes movimentam-se perto do lúmen dos túbulos seminíferos. Por último, são liberadas no lúmen dos túbulos seminíferos, enquanto passam por um processo de transformação adicional conhecido como espermição, antes de

passar para o epidídimo para a maturação final e armazenamento (YOSHINAGA et al., 2003). Os espermátócitos e/ou espermátides sofrem extensa remodelação durante as fases meiótica e espermogênica do desenvolvimento: inicialmente as histonas núcleo-proteicas são substituídas por proteínas de transição não-histônicas e finalmente por protaminas (GAUCHER et al., 2010). As protaminas estão envolvidas no processo de empacotamento da cromatina espermática, de tal forma que o genoma paterno permanece funcionalmente inerte e protegido, e uma notável redução no volume nuclear é alcançada (RATHKE et al., 2007; WARD, 2010; JOHNSON et al., 2011).

Uma vez formados dentro dos túbulos seminíferos, os espermatozoides, sem motilidade, são liberados no líquido tubular seminífero e transportados para o epidídimo, onde ganham parte da capacidade de se movimentar e fertilizar os ovócitos (YANAGIMACHI et al., 1994). O epidídimo está dividido em três partes: cabeça, corpo e cauda. Em touros, o trânsito dos espermatozoides através do epidídimo geralmente leva de 8 a 11 dias (VOGLER et al., 1991). Para atingir a capacidade de fertilização, os espermatozoides sofrem muitas mudanças durante seu trânsito (YANAGIMACHI et al., 1994). Estas incluem alterações nos lípidos da membrana plasmática, proteínas e glicosilação, alterações na membrana acrossomal externa, alterações morfológicas no acrossomo e ligação cruzada de protaminas nucleares, proteínas da fibra densa externa e bainha fibrosa. A cauda do epidídimo e a porção proximal do ducto deferente são as regiões onde os espermatozoides são armazenados antes da ejaculação (TURNER et al., 1995; JONES, 1999). Durante a ejaculação, os espermatozoides armazenados e o fluido circundante são misturados com as secreções alcalinas das glândulas sexuais acessórias; desta forma, o sêmen resultante é depositado durante a cópula na vagina na espécie bovina (RAHMAN et al., 2018).

2.2 TERMORREGULAÇÃO EM TOUROS

A eficiência da produtividade do rebanho bovino de corte está diretamente relacionada à manutenção dos animais dentro na zona de termoneutralidade (ZTN) (MAGALHÃES et al., 2000; SILVA, 2000; McMANUS et al., 2009). Para que a espermatogênese ocorra de forma normal nos mamíferos, geralmente os testículos requerem uma temperatura de 2 a 6 °C abaixo da temperatura corporal,

sendo de 33 a 34°C a temperatura testicular ideal para espermatogênese em bovinos (WILDEUS e ENTWISTLE, 1983; BARTH e BOWMAN, 1994; PERUMAL et al., 2017). A manutenção dessa temperatura ocorre, na maioria dos mamíferos, pela localização dos testículos no escroto, fora da cavidade abdominal. Além disso, os testículos contam com diferentes mecanismos para controlar a temperatura dos tecidos, o que permite o sucesso da termorregulação testicular. Em touros, a pele escrotal é fina, desprovida de gordura subcutânea e dependendo da raça, de pêlos, mas rica em glândulas sudoríparas (SENGER, 2003). Diferenças individuais no número de glândulas sudoríparas da superfície escrotal, além dos próprios padrões morfológicos contribuem para as diferenças entre animais taurinos e zebuínos. Isto determina a susceptibilidade ao calor e também a adaptabilidade, o que separa esses animais entre termossensíveis ou termorresistentes (MARRA, 2007). Desta forma, os zebuínos são mais tolerantes ao calor em relação aos taurinos, principalmente, devido, a sua maior densidade de glândulas sudoríparas na superfície escrotal (DOWLING, 1955; GODINO e CARDOSO, 1980).

Há dois músculos importantes: a *túnica dartos* e o músculo cremaster, os quais desempenham um papel fundamental na termorregulação. A túnica dartos é uma fina camada de músculo liso, localizada abaixo da pele escrotal, a qual possibilita o posicionamento do escroto em direção ao abdômen ou longe dele, em resposta a ambientes frios e quentes, respectivamente (SETCHELL, 1978). Devido à natureza lisa da túnica darlos, o posicionamento do escroto pode ser mantido por um período prolongado, especialmente em ambiente frio. A função do músculo cremaster também é aproximar os testículos do abdome após a contração. No entanto, devido à natureza estriada deste músculo, a contração não pode ser mantida por um período prolongado de tempo. Os músculos lisos nas arteríolas cutâneas do escroto são inervados por neurônios simpáticos. A estimulação desses neurônios pelo frio causa vasoconstrição e, por outro lado, o calor provoca vasodilatação dessas arteríolas. Desse modo, ocorre diminuição ou aumento do suprimento sanguíneo para o escroto (SETCHELL, 1978; RAHMAN et al., 2018).

Além dessas contrações musculares, o sistema vascular também é um dos principais responsáveis pelo resfriamento dos testículos. A artéria testicular traz sangue quente do centro do corpo para os testículos, e está intimamente envolvida

por uma rede venosa complexa, chamada de plexo pampiniforme. Toda a estrutura (rede venosa e artéria) é denominada cone vascular testicular (HESS et al., 1984). Stabenfeldt e Edqvist (1996) *apud* (SOUZA, 2004) afirmam então, que existem dois mecanismos fundamentais e especializados na regulação da temperatura testicular: o primeiro, o resfriamento dos testículos pelo chamado sistema de “contra-corrente” onde há troca de calor com o sangue venoso no plexo pampiniforme localizado externamente e cranialmente aos testículos. As extensas circunvoluções e anastomoses dos vasos arteriais e venosos permitem a transferência do calor do lado arterial para o lado venoso mais frio, que drena os testículos. O segundo, a atividade dos músculos cremaster externo e *túnica dartos*, permitindo o movimento dos testículos por meio de contração e relaxamento muscular. Além disso, as glândulas sudoríparas também têm papel importante no controle da temperatura testicular, uma vez que a densidade das glândulas sudoríparas é maior na pele escrotal do que nas outras regiões do corpo dos touros (BLAZQUEZ et al., 1988).

2.3 DEGENERAÇÃO TESTICULAR POR ESTRESSE TÉRMICO

Segundo Pachauri e Reisinger (2007), as mudanças climáticas têm chamado a atenção do mundo nas últimas décadas, sendo mais visíveis nas regiões tropicais e subtropicais (RAHMAN et al., 2018). Os efeitos adversos da mudança climática estão influenciando de distintas formas a produção animal: afetando diretamente a saúde e a reprodução animal (GAULY et al., 2013). As altas temperaturas do ambiente causam estresse térmico, sendo este considerado como um dos fatores mais importantes que ocasionam subfertilidade em bovinos nos países tropicais ou subtropicais (TAKAHASHI, 2012; DAS et al., 2016). Em touros, o estresse térmico durante o verão tem sido relatado como uma das principais preocupações devido ao efeito deletério para a qualidade do sêmen (MATHEVON et al., 1998; BALIC et al., 2012; RAHMAN et al., 2018). Isto porque geralmente, os testículos atuam no limite da hipóxia, ou seja, o aumento da temperatura aumenta o metabolismo testicular, com uma necessidade concomitante de oxigênio para manter o metabolismo aeróbico. Porém, o fluxo sanguíneo testicular não consegue acompanhar esse incremento de metabolismo e acaba aumentando muito pouco em resposta à elevação da temperatura testicular

e, conseqüentemente, os testículos se tornam mais hipóxicos (SETCHELL, 1978). Os testículos são altamente sensíveis às mudanças da temperatura do ambiente, levando a alterações degenerativas, caracterizadas pela diminuição do tamanho dos testículos, alteração na sua consistência e baixa produção de sêmen (MCENTEE, 1990). Além disso, o estresse térmico também pode reduzir a secreção do hormônio luteinizante (LH), que é essencial para a produção de espermatozoides, especialmente na espermatogênese (GILAD et al., 1993).

Conceitualmente, a degeneração testicular é o conjunto de alterações do parênquima testicular que causam disfunções bioquímicas e variações estruturais nas células da linhagem germinativa, levando o tecido normal a um estado funcional menos ativo (GARCIA, 2017). Em bovinos, esta é uma alteração adquirida, pode ocorrer nos dois ou em apenas um dos testículos, podendo ser em parte ou em todos os túbulos seminíferos, permanecendo produção espermática parcial, ou em casos mais graves, resulta em interrupção total da espermatogênese, levando à queda da libido após afetar as células de Leydig. A degeneração testicular pode ser temporária ou permanente, e sua gravidade vai depender do tipo, severidade e duração do insulto (SETCHELL, 1998; NASCIMENTO et al. 2010). As causas podem ter diversas origens: ação de agentes infecciosos específicos ou inespecíficos do sistema reprodutor (ADAMU et al., 2007), estresse térmico (BARTH e BOWMAM, 1994; KASTELIC et al., 1997; MATHEVON et al., 1998; BRITO, 2003; RAHMAN et al., 2011; TAKAHASHI, 2012; DAS et al., 2016; CELEGHINI et al., 2017; GARCIA, 2017; RAHMAN et al., 2018), desequilíbrios nutricionais (CHENOWETH et al., 2000), alterações vasculares como varicocele (KOIVISTO et al., 2002), excesso de gordura no funículo espermático (ADEEL et al., 2009), causas medicamentosas (HORN et al., 1999), irradiação (HERRID et al., 2013), distúrbios hormonais, fatores autoimunes, intoxicações, toxemia, obstrução da cabeça do epidídimo, agentes químicos, traumas e dermatites (NASCIMENTO et al. 2010).

Atualmente, a literatura nacional não faz distinção entre as diferentes causas da degeneração testicular em animais mantidos a campo. Contudo, tendo em vista que a maior parte dos animais de produção é criada em regiões de clima tropical, pode-se considerar que no Brasil as condições de estresse pelo calor assumam a principal causa de degeneração testicular (BARROS et al., 2015; GARCIA,

2007). Clinicamente, a degeneração testicular caracteriza-se inicialmente por flacidez dos testículos, assim como pela discreta diminuição do tamanho, sendo que, em etapas mais avançadas, estes podem se tornar atrofiados, fibróticos e com consistência firme à palpação. Histologicamente, observa-se que nos túbulos seminíferos ocorre diminuição da espermatogênese, devido à morte das células da linhagem germinativa. As espermatogônias apresentam citoplasma vacuolizado e núcleo picnótico, as espermatídes se degeneram e há aparição de células gigantes multinucleadas, resultantes da fusão de várias espermatídes. Adicionalmente, alguns túbulos podem mostrar ausência total do epitélio seminífero, membrana basal espessa e hialinizada, bem como proliferação de tecido conjuntivo fibroso, que invade os túbulos em casos mais avançados (VAN CAMP, 1997; NASCIMENTO; SANTOS, 2003).

O ejaculado de touros com degeneração testicular, geralmente, apresenta baixa concentração espermática, baixa motilidade e número moderado de células com defeitos morfológicos, como gotas protoplasmáticas proximais, diadema, vacúolos e outros defeitos nucleares (BLOM, 1972; BARTH e BOWMAM, 1994.; KASTELIC et al., 1997.; BRITO, 2003.; RAHMAN et al., 2011). Em casos mais severos, além da oligozoospermia e da astenozoospermia, pode ser detectado também aumento no número de espermatozoides com anomalias morfológicas, especialmente com defeitos de peça intermediária e cabeça (GARCIA, 2004). A presença de células primordiais da linhagem espermatogênica, células gigantes, medusas ou células germinativas multinucleadas, assim como a presença de espermatozoides com formas teratológicas em grande quantidade, sugere danos severos ao epitélio seminífero (VAN CAMP, 1997; NASCIMENTO; SANTOS, 2003) e da espermatogênese (JOHNSON, 1997). Segundo Wenkoff (1988) *apud* (GARCIA, 2017), devido ao fato de os espermatócitos primários se dividem por meiose, são os primeiros a sofrer os efeitos e são mais sensíveis do que as espermatogônias tipo A e B. Os efeitos sobre os espermatócitos primários apresentam-se como distúrbios na formação do fuso nas células em divisão. Em casos mais leves de degeneração testicular, os espermatócitos secundários podem alcançar o estágio de anáfase antes do distúrbio acontecer. Apesar das células passarem pelo processo de divisão, ocorrem alterações nos cromossomos e no núcleo das células filhas, o que resulta no aumento de alterações de cabeça, além da diminuição da motilidade e

aparência de gotas protoplasmáticas proximais. Nos casos moderados de degeneração, os cromossomos das células em prometáfase são espalhados no interior da célula e lisados pelo citoplasma, o que resulta no aparecimento de células conhecidas como “células-fantasma”. Estas são pouco frequentes e aparecem em pequenas quantidades. Além dessas alterações, também pode se observar espermatozoides com gota protoplasmática proximal, “pouch formation” e outros tipos de vacúolos celulares e defeitos nucleares. De igual forma, nos casos mais severos e/ou prolongados de degeneração testicular, os distúrbios acometem as células em metáfase. Aqui, os cromossomos permanecem próximos ao pólo equatorial condensando-se e formando a alteração conhecida como núcleo picnótico. A ocorrência de várias destas estruturas conjuntamente formam um sincício conhecido como “células gigantes”. Em casos de azoospermia, o dano ao tecido testicular impossibilita a reposição das espermatogônias (SOUZA, 2004).

Experimentalmente, a degeneração testicular pode ser induzida por diversos métodos, sendo o aumento local de temperatura por insulação escrotal, a técnica ideal e mais comumente utilizada para o estudo das alterações testiculares e do ejaculado. Esta permite o aumento da temperatura testicular, interrompendo a termorregulação testicular e também interferindo na transpiração escrotal, podendo causar danos na qualidade do sêmen, afetando principalmente aqueles espermatozoides que estavam na fase de divisão meiótica e espermiogênese no momento da insulação escrotal (RAHMAN et al., 2018).

2.4 DIAGNÓSTICO DA DEGENERAÇÃO TESTICULAR

O diagnóstico da degeneração testicular é realizado durante o exame andrológico, tomando por base os achados clínicos, desde informações da anamnese, alterações testiculares e do espermograma (HOPPER, 2015). Durante o exame clínico do aparelho reprodutivo, é possível notar, pela palpação testicular, a consistência alterada, podendo variar de consistência normal, em casos mais leves, a consistência flácida em casos mais graves de degeneração (BEZERRA et al. 2008; NASCIMENTO et al. 2010).

Dependendo do grau de degeneração testicular, o espermograma mostra diminuição da motilidade e algumas vezes, da concentração espermática e aumento de espermatozoides com alterações morfológicas. Os defeitos de cabeça

e cauda, cabeças isoladas e gotas citoplasmáticas proximais, são os mais comumente encontrados neste distúrbio (VAN CAMP, 1997; NASCIMENTO; SANTOS, 2003). Neste sentido, para identificar a origem do defeito, é importante conhecer o período de passagem dos espermatozoides pela cabeça, corpo e cauda do epidídimo, assim como a duração da espermatogênese. Isto, devido ao fato de que, por exemplo, as alterações do epidídimo serão as primeiras a aparecer no ejaculado, e a recuperação da espermatogênese vai depender da duração do ciclo espermático de cada espécie (MOREIRA et al., 2001; ALVARENGA; PAPA, 2009; RAHMAN et al., 2011). Entretanto, apenas a avaliação da motilidade, concentração e morfologia espermática, não são suficientes para a conclusão do diagnóstico, assim como também não oferecem uma avaliação mais detalhada das alterações espermáticas durante a degeneração. As espécies reativas de oxigênio (ERO), formadas em grande quantidade durante este distúrbio, geram um desbalanço com os agentes antioxidantes, ocasionando estresse oxidativo, que, por sua vez, leva à peroxidação da membrana plasmática espermática e à fragmentação de DNA espermático (GUTHRIE; WELCH, 2012).

Diante deste cenário, se faz necessário que durante a avaliação do sêmen, outros compartimentos e/ou funções do espermatozoide sejam verificados. A avaliação da integridade da membrana plasmática, integridade da membrana acrossomal e do potencial de atividade mitocondrial por meio de sondas fluorescentes, tornou-se importante para o diagnóstico da atividade e função espermática (CELEGHINI et al., 2007), já que a membrana plasmática está diretamente relacionada com a viabilidade celular (BORGES et al., 2011), o acrossomo com a capacidade fecundante (BRENER et al., 2002) e a mitocôndria com a produção de energia celular (GRAVANCE et al., 2001). Tendo em vista que o estresse oxidativo e a fragmentação do DNA são outras alterações da célula espermática que podem ser verificadas durante a degeneração testicular, a análise da produção de espécies reativas e/ou a quantificação da peroxidação lipídica e da integridade do DNA, podem acrescentar informações importantes para o diagnóstico e prognóstico de um touro com degeneração testicular. Por outro lado, outros exames clínicos complementares, como por exemplo, a ultrassonografia e a termografia testiculares, podem ser ferramentas valiosas no diagnóstico da degeneração testicular e na identificação de seus diferentes graus e fases (COULTER et al., 1988; ARTEAGA et al., 2005; CAPRARO et al., 2008;

DUDEA et al., 2010; NETO et al., 2011).

2.5 ESTRESSE OXIDATIVO

O oxigênio é uma molécula essencial para a vida aeróbica das plantas e animais, capaz de produzir energia suficiente para garantir a sobrevivência e manutenção do metabolismo celular por meio da oxidação de moléculas biológicas. Porém, o oxigênio também é potencialmente tóxico às células (IWASAKI e GAGNON, 1992). Qualquer célula aeróbica, incluindo o espermatozoide, está constantemente enfrentando o chamado "Paradoxo do Oxigênio": o oxigênio é crucial para manter a vida aeróbica, mas, ao mesmo tempo, é inerentemente ameaçador para a existência das células. O metabolismo aeróbio normal, vem acompanhado com a geração de subprodutos chamados espécies reativas de oxigênio (EROs) (BOVERIS; CHANCE, 1973; CHANCE et al., 1979; SIES, 1993), que são essenciais para as funções celulares normais (MAKKER et al., 2009). Dentre elas estão os radicais livres (RL), os quais podem conter um ou mais elétrons desemparelhados (HALIWELL e GROOTVELD, 1987). As EROs incluem todas as moléculas que contêm pelo menos um átomo de oxigênio. Estas podem ser classificadas quimicamente em radicais e não radicais. Dentro dos radicais, os mais importantes são o radical ânion superóxido ($O_2^{\cdot-}$), o radical hidroxila ($OH^{\cdot}$) e o óxido nítrico (NO). E, dentro dos não radicais, o peróxido de hidrogênio (HERNÁNDEZ-MATOS et al., 2010). Essas moléculas de vida curta, instáveis e altamente reativas, podem "roubar" elétrons das estruturas adjacentes para atingir um estado fundamental e fazer com que outras moléculas se tornem radicais livres. Essa reação em cadeia amplifica o grau de alteração nas estruturas celulares vizinhas (GOLDFARB, 1999). Em um corpo saudável, mecanismos pró-oxidantes (EROs), e mecanismo de defesa antioxidantes permanecem em equilíbrio. No entanto, se os níveis das EROs aumentam, seja devido à sua superprodução intrínseca ou extrínseca, ou pelos baixos níveis de mecanismos de defesa antioxidante, pode ocorrer o que se conhece como estresse oxidativo (EO) (SIES, 1993), tornando-se prejudicial à sobrevivência das células espermáticas, devido aos danos significativos que ocasionam nas biomoléculas, como lipídios, proteínas, ácidos nucleicos e açúcares (AGARWAL e PRABAKARAN, 2005). Foi relatado que o estresse térmico afeta

a síntese de proteínas, DNA e RNA (RHOADS et al., 2013). Concomitantemente, o estresse térmico induz a ativação de fatores de transcrição de choque térmico (HSFs), principalmente HSF1, que possibilita a produção de proteínas de choque térmico (HSPs) para reduzir os efeitos prejudiciais da carga de calor (COLLIER et al., 2008). Se o estresse térmico continuar, a produção fisiológica de HSPs é alterada, levando ao aumento da produção de espécies reativas de oxigênio (ERO) (RHOADS et al., 2013).

2.5.1 Espécies reativas de oxigênio (EROs) e o espermatozoide

Durante a espermatogênese, são desencadeados diferentes e importantes processos biológicos, como as mudanças nos lipídios da membrana plasmática do espermatozoide, a peroxidação lipídica (POL) e a apoptose das células germinativas não competentes. Durante o trânsito do espermatozoide pelo epidídimo, são incorporados lipídios à estrutura da sua membrana, como por exemplo, fosfatidilcolina e ácidos graxos insaturados. Estas mudanças estão relacionadas com o aumento da atividade flagelar e, por conseguinte, a motilidade espermática (LLAMOS, 2006; HERNÁNDEZ-MATOS et al., 2010). As espécies reativas de oxigênio podem ter efeitos prejudiciais ou benéficos sobre as funções espermáticas, porém, isto vai depender da natureza e a concentração das EROs involucrada, assim como do momento e o lugar de exposição (AITKEN e BAKER, 1995).

Fisiologicamente, as EROs desempenham um papel crucial na ativação das vias intracelulares responsáveis pela maturação, capacitação, hiperativação, reação acrossômica e fusão do espermatozoide com o gameta feminino (AITKEN et al., 1989; GAGNON et al., 1991). Contudo, ao mesmo tempo que as EROs são necessárias no processo em que o espermatozoide adquire a capacidade de fertilização, a célula espermática é altamente susceptível aos danos causados pela sua elevação. Isto, devido à alta quantidade de ácidos graxos poli-insaturados (AGPI) presentes na membrana plasmática, e ao seu reduzido citoplasma, o que faz com que as concentrações de enzimas antioxidantes sejam menores (ALVAREZ e STOREY, 1995; DE LAMIRANDE e GAGNON, 1995). Assim, altos níveis de EROs podem lesar a membrana espermática, resultando em baixa motilidade, capacitação e reação acrossômica prematura, comportamento anormal

dos canais iônicos de transporte e comunicação, alterações na translocação da fosfatidilserina, anormalidades morfológicas e comprometimento da fusão espermatozoide-oócito (AITKEN et al., 1989; AITKEN et al., 1991; WHITTINGTON e FORD, 1998). De igual forma, as reações oxidativas em cadeia podem levar à peroxidação lipídica (POL), degradação proteica, alterações nas vias de sinalização intracelular e apoptose. Além disso, o estresse oxidativo tem sido associado a danos no DNA do espermatozoide, o que pode resultar em pobre embriogênese, aborto espontâneo e infertilidade (ALVES et al., 2015; SERAFINI et al., 2016; PRIETO, 2018).

A POL pode ser definida como uma cascata de eventos bioquímicos resultantes da ação dos RL sobre os lípidos insaturados das membranas celulares, levando à destruição de sua estrutura, falência dos mecanismos de troca de metabólitos e, numa condição extrema, à morte celular (BENZIE, 1996). A POL é um evento citotóxico primário que desencadeia sequência de lesões na célula. As alterações nas membranas levam a transtornos da permeabilidade, alterando o fluxo iônico e o fluxo de outras substâncias, o que resulta na perda da seletividade para entrada e/ou saída de nutrientes e substâncias tóxicas à célula, alterações do DNA, oxidação das lipoproteínas de baixa densidade (LDL) e, comprometimento dos componentes da matriz extracelular (proteoglicanos, colágeno e elastina) (VACA et al, 1988; BABER e HARRIS, 1994). Basicamente, a POL consiste na incorporação de oxigênio molecular a um AGPI para produzir um hidroperóxido lipídico (LOOH) como produto primário inicial. Nos sistemas biológicos a POL pode ocorrer principalmente por duas vias: (1) uma via enzimática envolvendo as ciclooxigenases e lipoxigenases na oxigenação dos AGPI e (2) a peroxidação não enzimática, que envolve a participação de ERO, espécies reativas de nitrogênio (RNS), metais de transição e outros radicais livres (AL MEHDI et al., 1993; PORTER et al., 1995).

Gomez et al. (1996) realizaram um estudo no qual encontraram uma correlação significativa ($r = 0,4883$; $P < 0.01$) entre o excesso de citoplasma residual na peça intermediária e a alta geração de EROs. Contudo, a relação entre outros defeitos morfológicos espermáticos e produção de EROs espermáticos ainda não foi estudada (AZIZ et al., 2004).

2.5.2 Métodos para detecção de espécies reativas de oxigênio

A resposta dos espermatozoides às flutuações das EROs vai depender da intensidade, duração e contexto da sinalização (DU PLESSIS et al., 2015). Como os altos níveis de EROs tem sido fortemente associados a falhas no sucesso da reprodução masculina, medir os níveis de EROs no sêmen deve ser considerada uma parte importante da avaliação inicial, bem como o acompanhamento de homens subférteis e inférteis (AGARWAL et al., 2014; KO EY et al., 2014). Em humanos, os métodos iniciais de detecção e quantificação de EROs se basearam especialmente em medições de absorbância. No entanto, com valores de referência milimolares, essas medições baseadas na absorbância forneciam apenas informação em contextos clínicos. Apesar disso, a descoberta de que as EROs agem como mensageiras e reguladoras intracelulares, fez com que surgissem novas ferramentas analíticas, tendo em mente os requisitos de detecção micromolar. Estas ferramentas são primariamente baseadas em fluorescência, e mais recentemente, foram introduzidas ferramentas luminométricas. Atualmente, existem mais de 30 testes diferentes para avaliar e estudar o estresse oxidativo seminal. Geralmente, os testes se baseiam na interação de superóxido ou outras EROs com algum outro composto para criar um resultado mensurável (TREMELLEN, 2008; AGARWAL et al., 2008). A quimioluminescência é a técnica mais comumente usada para diagnosticar a infertilidade, mas não diferencia as EROs produzidas nos espermatozoides e as EROs produzidas pelos leucócitos (ALVES et al., 2015). Por outro lado, uma técnica amplamente utilizada para medir o estresse oxidativo, é a medição do malondialdeído com a técnica de substâncias reativas ao ácido tiobarbitúrico (TBARS) em espectrofotometria (AITKEN et al., 1992; NICHII et al., 2006).

Para a avaliação da POL em sistemas biológicos, as metodologias utilizadas medem a formação de produtos gerados durante as diferentes fases deste processo. Portanto, é importante utilizar várias técnicas disponíveis, pois a sua escolha dependerá do propósito do investigador, ou seja, da fase do processo de POL que se pretenda avaliar. Um método considerado ideal na mensuração da POL deve: (1) identificar e quantificar produtos derivados especificamente do processo de peroxidação; (2) ter baixo coeficiente de variação, quando analisadas amostras diferentes de um mesmo indivíduo; (3) não estar sujeito a interferências

de outras biomoléculas; (4) empregar técnicas robustas e, portanto, mais confiáveis; (5) não sofrer interferência direta da dieta e (6) ter sensibilidade para mensurar níveis basais dos produtos (GUTTERIDGE, 1995; HALLIWELL, 2000; ABUJA e ALBERTINI, 2001).

Por outro lado, além das técnicas que mensuram os produtos gerados durante a POL, atualmente existem várias sondas fluorescentes que estão sendo utilizadas para análise de estresse oxidativo. Alguns exemplos são a sonda C11-BODIPY (COLLIER et al., 2008), a MitoSOX Red® (AITKEN et al., 2013) e H2DCF (GUTHRIE e WELCH, 2006). A sonda C11-BODIPY tem afinidade pela membrana plasmática do sêmen, desta forma atua como um ácido graxo poliinsaturado e, conseqüentemente, é peroxidado pelas EROs. Nessa condição, a fluorescência vermelha da sonda C11-BODIPY é alterada para verde (PAP et al., 1999; GUTHRIE e WELCH, 2007). Portanto, os valores de peroxidação obtidos com essa técnica são indiretos porque expressam a suscetibilidade da célula ao estresse oxidativo. A sonda Mito SOX Red® e H2DCF detectam os compostos das EROs. Elas são capazes de detectar o ânion superóxido e o peróxido de hidrogênio, respectivamente (GUTHRIE e WELCH, 2006; KOPPERS et al., 2008).

2.5.3 Teste de substâncias reativas ao ácido tiobarbiturico (TBARS)

São necessárias mais alternativas para a análise do estresse oxidativo no espermatozoide, levando em consideração que eles devem ser métodos fáceis de aplicar e interpretar (ALVES et al., 2015). O estresse oxidativo pode ser avaliado pela quantificação direta das espécies reativas de oxigênio (VERNET et al., 2004) e pelos produtos resultantes da cascata de estresse oxidativo, ou seja, a quantificação indireta das EROs. Os métodos indiretos mais utilizados para aferir as EROs e, conseqüentemente, as lesões oxidativas, são os espectrofotométricos e cromatométricos, que medem a atividade enzimática (SOD, catalase, GSH-Px e GSH-Rd) e/ou a concentração de tripeptídeos (GSH, GSSG) e aldeídos (Malondialdeído) (FERRERIA; MATSUBARA, 1997). Estas medidas podem ser realizadas em tecidos, sangue e outros fluidos. Entretanto, o método mais usado para monitorar a peroxidação lipídica das membranas e, por sua vez o estresse oxidativo, é o método do malondialdeído (MDA) (FERRERIA; MATSUBARA,

1997; HSIEH et al., 2006; LYKKESFELDT, 2007; HAMILTON et al., 2016; HAMILTON et al., 2016; WAGNER et al., 2017). Este é um dialdeído secundário formado durante a oxidação de ácidos graxos poliinsaturados (AGPI) por cisão *beta* dos AGPI peroxidados, principalmente o ácido araquidônico (LIMA e ABDALLA, 2001). Em condições apropriadas de incubação (meio ácido e aquecimento), reage eficientemente com uma variedade de agentes nucleofílicos para produzir cromógenos com alta absorvidade molar no espectro visível (JANERO, 1990; BENZIE, 1996). A sua condensação com o ácido tiobarbitúrico (TBA) forma produtos, que podem ser determinados por absorção ou por fluorescência (JANERO, 1990). A formação de produtos derivados do MDA usualmente requer pH baixo e temperaturas elevadas (80-100 °C). A reação não específica do MDA com grupos amino primários ocorre em várias biomoléculas (proteínas, ácidos nucleicos, amins e fosfolípidos) (JANERO, 1990).

Esta metodologia foi introduzida por Kohn e Liversedge, em 1944, sendo bastante popular já que é simples e rápida, porém inespecífica. Consiste na medida de um cromógeno róseo formado pela reação do MDA com duas moléculas de ácido tiobarbitúrico, em meio ácido e alta temperatura. Essa reação, chamada de “teste das substâncias reativas com ácido tiobarbitúrico (TBARs)”, representa múltiplos métodos que utilizam o TBA, formando o complexo MDA:TBA (LIMA; ABDALLA, 2001).

2.6 INTEGRIDADE DO DNA ESPERMÁTICO

A cabeça do espermatozoide é constituída pelo acrossoma, núcleo e, algumas estruturas do citoesqueleto e citoplasma. O núcleo contém ácido desoxirribonucleico (DNA), o qual possui proteínas denominadas histonas. As histonas são proteínas nucleares básicas e ricas em lisina, que durante a espermiogênese, são total ou parcialmente substituídas por protaminas (BELETTI, 2013). Desta forma, forma-se o complexo DNA-protamina ou cromatina, bem condensada, em função da neutralização do esqueleto fosfodiéster do DNA, ocorrida pela interação dos grupos fosfatos procedentes da fita de DNA e amina oriunda das proteínas (BALHORN, 1982; BELETTI e MELLO, 1996). Antes da meiose, a estrutura da cromatina no núcleo do espermatócito é organizada de forma semelhante ao núcleo de todas as células somáticas. Como citado

anteriormente, as proteínas predominantes nesta fase são as histonas, as quais aumentam o volume da cromatina (BALHORN, 2011; SHARMA e AGARWAL, 2011). As protaminas são proteínas específicas dos espermatozoides, as quais possuem caráter mais básico em razão da abundância em arginina (BLOCH, 1969; HOFMANN, HILSCHER, 1991).

Existem dois tipos de protamina, a P1 e P2, sendo a primeira encontrada no espermatozoide de todos os mamíferos (BALHORN, 2011). São as proteínas mais abundantes no núcleo espermático de mamíferos (WARD e COFFEY, 1991; ZINI e AGARWAL, 2011), que permitem a organização, condensação e compactação da cromatina, protegendo o genoma paterno durante o transporte através do trato reprodutivo do macho e da fêmea, e assim garantindo a chegada ao ovócito em boas condições (SHARMA; AGARWAL, 2011), permitindo a fusão dos genomas, favorecendo o desenvolvimento embrionário e a correta expressão da informação genética (SASSONI-CORSI, 2002; ERENPREISS et al., 2006). A protaminação deficiente é consequência da espermatogênese anormal, que por sua vez pode estar associada a altos índices de defeitos morfológicos do espermatozoide (ENCISO et al., 2011; OLIVA e CASTILLO, 2011). Estudos mostram que a permanência de histonas somáticas ou anormalidades nas protaminas podem ocasionar distúrbios na condensação da cromatina, tornando-a frouxa, refletindo na morfologia da célula (BELETTI; MELLO, 2004) e, por sua vez, prejudicando a fertilidade in vivo (EVENSON et al., 1980). Gledhill (1966) identificou espermatozoides com má compactação, ou seja, com alterações no complexo DNA-protamina em touros subférteis (KIPPER, 2014). Segundo Britto e Mello (1988), touros com espermograma normal, pelas avaliações de rotina, podem apresentar intervalos de subfertilidade inexplicáveis, sendo as alterações na compactação da cromatina uma possível explicação (FONSECA, 2014).

Devido ao fato do DNA do espermatozoide contribuir com metade do material genético para a progênie, é imprescindível que esta célula esteja saudável para que exista a fertilização, o desenvolvimento do embrião, o desenvolvimento fetal e pós-natal correto. Porém, em qualquer estágio do processo de espermatogênese, os danos ao DNA podem ser produzidos, sendo este um fenômeno multifatorial e não completamente delimitado (PRIETO, 2018). O estudo do potencial de fertilidade do macho nas diferentes espécies animais vem se baseando tradicionalmente na análise de sêmen, por meio de um conjunto de

avaliações de acordo com as normas do Manual para Exame Andrológico e Avaliação de Sêmen Animal (CBRA), 2013. Entre eles estão as avaliações de motilidade, morfologia, e alguns não inclusos no manual, como a atividade metabólica e integridade das membranas espermáticas (CELEGHINI et al., 2007; ARRUDA et al., 2011; OLIVEIRA et al., 2014). Contudo, quando feitas isoladamente, essas técnicas de avaliação, acabam se tornando ineficientes em prever a fertilidade. Alguns espermatozoides com anormalidades na cromatina podem fecundar oócitos *in vivo* e *in vitro*, mas o defeito no DNA pode persistir durante o período embrionário, induzindo apoptose, desestruturação embrionária e aborto (ELLINGTON et al., 1998; TWIGG, et al., 1998). Nesse contexto, destaca-se o DNA como fator importante para o sucesso da fertilização e desenvolvimento embrionário, sendo responsável pela transmissão das informações genéticas paternas (LOPES et al., 1998; ALVES et al., 2015; SERAFINI et al., 2016).

2.6.1 Estresse térmico como mecanismo de alteração do dna espermático

O estresse térmico tem sido reportado como uma das principais causas de comprometimento da integridade do DNA do espermatozoide (BANKS et al., 2005; PEREZ-CRESPO et al., 2008; CARRETERO et al., 2012; RAHMAN et al., 2011; WRIGHT et al., 2014). Embora as EROs sejam necessárias para a função fisiológica normal dos espermatozoides, o estresse oxidativo excessivo pode causar maior suscetibilidade a danos no DNA, levando potencialmente não apenas à infertilidade, mas também à perda recorrente de gestação ou mutações geneticamente hereditárias (DINESH et al., 2012). Devido ao fato de o conteúdo de ácidos graxos insaturados dos espermatozoides serem altamente susceptíveis ao estresse oxidativo, podem ocorrer danos extensos à membrana.

Como foi elucidado anteriormente, uma vez que a oxidação lipídica tenha sido iniciada, as ligações duplas de um ácido graxo insaturado são atacadas por um radical livre para criar um radical peróxido lipídico. O radical peróxido lipídico reage com a molécula lipídica vizinha desencadeando uma reação em cadeia que pode levar a mais de 50% de oxidação da membrana plasmática do espermatozoide (KOTHARI et al., 2010). Os subprodutos da oxidação lipídica incluem moléculas mutagênicas e genotóxicas malondialdeído (MDA) e 4 hidroxí-nonenal (4-HNE), as quais levam indiretamente a danos no DNA (BISHT

et al., 2017). Os radicais livres têm a capacidade de danificar diretamente o DNA do espermatozoide, atacando as bases purinas e pirimidinas (GEVA et al., 1998). As EROs causam danos por meio de quebras de DNA de cadeia simples e dupla, ligações cruzadas e rearranjos cromossômicos (BISHT et al., 2017). A maior parte do genoma do espermatozoide (~ 85%) está ligada a nucleoprotaminas centrais que a protegem dos danos causado por radicais livres (BISHT et al., 2017). Os homens inférteis frequentemente tem protaminação deficiente, o que pode tornar o DNA espermático mais vulnerável a danos causados por EROs (OLIVA, 2006). Outro mecanismo de dano espermático ao DNA é a apoptose iniciada por radicais livres, levando à destruição de DNA mediada por caspase (TREMELLEN, 2008). Os espermatozoides têm capacidade limitada de reparar o DNA e só podem realizar reparos durante estágios específicos da espermatogênese (MARCON e BOISSONNEAULT, 2004; WRIGHT et al., 2014).

Demonstrou-se que a diminuição da motilidade é devido à peroxidação mediada por H₂O₂, induzida por EROs, principalmente de lipídios na membrana do espermatozoide, diminuindo a flexibilidade e inibindo os mecanismos de motilidade. A redução na motilidade espermática é proporcional à quantidade de peroxidação lipídica (MORIELLI et al., 2015). O dano do DNA mitocondrial induzido por EROs leva à diminuição da disponibilidade de ATP e energia, impedindo a motilidade espermática (LAMIRANDE e GAGNON, 1992). Estudos mostraram níveis mais elevados de EROs em pacientes com oligospermia (AGARWAL et al., 2014). Altos níveis de EROs interrompem as membranas mitocondriais, levando à ativação de caspases e, finalmente, à apoptose (AGARWAL et al., 2008). A liberação do citocromo C durante a via apoptótica aumenta ainda mais os níveis de EROs, promovendo danos ao DNA e fragmentação, e aumentando potencialmente o ciclo apoptótico (THOMSON et al., 2009).

2.6.2. Avaliação do DNA espermático

Amostras de sêmen que tiveram resultados normais no espermiograma foram avaliadas como amostras com baixo potencial de fertilidade quando analisadas por ensaio de fragmentação de DNA (GIWERCMAN et al., 2003). Desta forma, a fim de agregar mais acurácia para predizer o potencial de

fertilidade de determinada amostra de sêmen ou determinado touro, o desenvolvimento de técnicas mais sofisticadas continua sendo uma prioridade (ALKMIN et al., 2013).

Atualmente, várias metodologias são utilizadas para identificação das alterações na estabilidade da cromatina espermática. Estas podem ser classificadas em técnicas de avaliação da compactação da cromatina do DNA espermático, as quais avaliam o processo de protaminação, entre elas estão o azul de toluidina, azul de anilina e a cromomicina A3 (ALVES et al., 2015); além disso, em técnicas de avaliação direta da fragmentação do DNA espermático, que por sua vez estão divididas em dois grupos: 1) fundamentado na prévia lise das membranas plasmática e nuclear com o objetivo de expor o DNA e realizar a marcação deste com sondas fluorescentes ou corantes. Exemplos destas são o ensaio Cometa e o teste de dispersão da cromatina (SCD), modificado ou não (DONNELLY et al., 2000; ENCISO et al., 2006); 2) fundamento na adição de nucleotídeos modificados e marcados com moléculas fluorescentes às fitas de DNA fragmentadas, como a técnica com a sonda laranja de acridina, o SCSA e a técnica de TUNEL (KRAYEVSKY et al., 2000). Entretanto, algumas destas técnicas não são rotineiramente empregadas devido ao alto custo, à laboriosidade exigida pelos testes ou pelo desconhecimento do funcionamento das mesmas (ALVES et al., 2015).

A técnica do laranja de acridina (LA) é prática e pode ser utilizada na microscopia de fluorescência e na citometria de fluxo. A sonda é classificada como metacromática e, dessa forma, pode emitir fluorescência em 530nm com a coloração verde ou em 640nm com a coloração vermelha. Quando o DNA espermático está íntegro, a sonda se intercala entre os ácidos nucleicos e emite fluorescência verde. O contrário ocorre quando há fragmentação, onde a sonda se liga aos grupos fosfatos das fitas simples e emite fluorescência vermelha (MCMASTER; CARMICHAEL, 1977; TEJADA et al., 1984). Pode ser considerada como uma técnica de avaliação da descondensação do DNA espermático ou de avaliação da integridade do DNA espermático. A técnica LA, na década de 80, apresentou-se como uma importante ferramenta para indicar o potencial de fertilidade humano (TEJADA et al., 1984). Na mesma década, estudos mostraram que a presença de espermatozoides positivos à sonda, ou seja, aqueles corados em vermelho apresentavam correlação significativa com

espermatozoides morfolologicamente anormais ($P \leq 0,001$) (IBRAHIM; PEDERSEN, 1988). Da mesma forma, a integridade de DNA detectada pela técnica apresentou correlação positiva com a motilidade espermática e quantidade de espermatozoides morfolologicamente normais (STERZIK et al., 1989). Em pacientes submetidos a estresse térmico, a técnica apresentou correlação positiva ($r = 0,418$; $P < 0,01$) com a técnica azul de anilina (ROUX e DADOUNE, 1989). Lymberopoulos e Khalifa (2010) verificaram também, que as células positivas à técnica laranja de acridina influenciaram nas taxas de fertilidade.

***Capítulo 2: Heat stress effects on
bovine sperm cells: a chronological
approach to early finding***

Heat stress effects on bovine sperm cells: a chronological approach to early findings

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Abstract

Testicular heat stress affects sperm quality and fertility. However, the chronology of these effects is not yet fully understood, and it is fundamental to provide information to intervene properly. This study aimed to establish the early sequential effects of heat stress in bull sperm quality. Semen and blood samples of *Nellore* breed bulls were collected and distributed into control and testicular heat stress (scrotal bags/96 hours) groups. Semen samples were evaluated for sperm motility, abnormalities, plasma and acrosome membranes integrity, mitochondrial membrane function, lipid peroxidation and DNA fragmentation. Blood and seminal plasma were also evaluated for lipid peroxidation. Testicular heat stress chronological effects began 7 days later, starting with an increase in sperm abnormalities. After 14 days, sperm lipid peroxidation increased and mitochondrial membrane function, sperm motility and plasma membrane integrity decreased. At 21 days after heat stress, these sperm findings were still in progress. As a late effect, after 28 days, there was an increase in sperm DNA fragmentation. Thus, effects of heat stress began increasing sperm abnormalities and lipid peroxidation, suggesting oxidative stress in the semen, and first altering mitochondrial function, then sperm motility, plasma membrane integrity and, belatedly, DNA fragmentation. Although sperm abnormalities persisted and increased through over time, sperm lipid peroxidation, in turn, increased only 14 and 21 days after heat stress. In this regard, knowledge of the chronological effects of heat stress will provide valuable insights about spermatogenesis status and help in performing precise diagnostics, providing better prognosis and treatment interventions.

Key-words: Spermatozoa; Seminal plasma; Testicular degeneration; Lipid peroxidation; TBARs

Introduction

Spermatogenesis process depends on testicular thermoregulation that is responsible to maintain testicular temperature 2 to 6° C below corporal temperature (Alves et al. 2016). In bulls, spermatogenesis can be divided into three phases: spermatocytogenesis, meiosis and spermiogenesis, which take approximately 21, 23 and 17 days, respectively. The duration of the seminiferous epithelium cycle is 13.5 days and the complete spermatogenesis adds a total of 61 days, approximately, which is 4.5 times of duration of the cycle of seminiferous epithelium (Staub and Johnson, 2018). Heat stress interferes in the testicular thermoregulation process, being considered as one of the most important factors to predispose bulls to testicular degeneration in tropical or subtropical countries (Takashi 2012). Usually, cause decline in sperm quality, characterized by low sperm concentration and motility and high sperm abnormalities (Barth and Bowman 1994; Kastelic et al. 1996; Brito et al. 2003; Rahman et al. 2011; Das et al. 2016; Rahman et al. 2018).

Increase in testicular temperature results in higher cellular metabolism, which increases reactive oxygen species (ROS) production and, consequently, causes oxidative stress (Setchell 1998). ROS are small, oxygen-based molecules that participate in the sperm physiology through the capacitation processes, acrosome reaction, hyperactivation and sperm binding with the zona pellucida of the oocyte (Blumer et al. 2008). However, the sperm cell is highly susceptible to ROS damage due to the large amount of polyunsaturated fatty acids present in the plasma membrane and low concentrations of antioxidant enzymes in its reduced cytoplasm (Nichi et al. 2017). All of this is involved in the cascade of lipid peroxidation, which, if not interrupted, can affect the integrity of many classes of molecules, including lipids, proteins, nucleic acids (DNA, RNA) and carbohydrates; which cause, for example, injuries in the sperm chromatin and increase the frequency of breaks in the DNA (Aikten and Krausz 2001).

It is already known that heat stress negatively impacts male reproduction, disturbing multiple stages of spermatogenesis, and causing a general reduction in sperm motility and normal morphology (Dada et al. 2003; Hansen 2009; Zhang et al. 2015). Furthermore, other studies have suggested that also relatively moderate heat stress can induce DNA fragmentation within the testis (Gao et al. 2012; Li et

al. 2013; Wechalekar et al. 2016). During the spermatogenesis process the most heat sensitive stages of germ cell development correspond to the spermatids and the pachytene spermatocytes (Setchell 1998; Zhu et al. 2004). Also, both of these germ cell populations exhibit elevated levels of DNA damage in response to heat stress, which can also impair the development of spermatocytes into spermatids (Wettemann and Desjardins 1979). In this regard, the most sensitive phases of spermatogenesis are meiosis and spermiogenesis because during these phases, replication of genetic material and morphological differentiation of sperm cells occur (Senger, 2003).

However, even though testicular degeneration is an important cause of sperm quality decline in bulls, there is not yet an efficient diagnosis method established. Usually, the diagnosis of testicular degeneration caused by testicular heat stress, is made through semen evaluation during the breeding soundness evaluation (Barth and Bowman 1994; Brito et al. 2003; Fernandes et al. 2008; Rahman et al. 2011; Rahman et al. 2018). Still, most of the spermatogenesis alterations may have occurred some time earlier and may not have even been recognized until the testicular degeneration is picked up on a routine semen evaluation. Also, despite the existing studies, it should be noted that the chronology of events that occur after heat stress is not yet fully known, and this knowledge can contribute to defining the pathophysiology of testicular degeneration, as well as, its early diagnosis.

Considering this scenario, it is hypothesized the chronological effect of the injuries to the development of sperm cells and, thereafter, their structure, mostly, during the first 28 days after testicular heat stress. When, the most sensitive phases of spermatogenesis will be affected, as well as when damage to the DNA will begin to be evident. In this sense, the present study aimed to evaluate sperm motility, morphological abnormalities, plasma and acrosome membranes integrity, mitochondrial membrane potential and sperm DNA integrity, as well as sperm susceptibility to lipid peroxidation, and blood lipid peroxidation, during the first 28 days after heat stress. In order to observe the first changes in sperm characteristics during a case of testicular degeneration, as well as to diagnosing it early and subsequently prevent the economic losses that it brings.

Material and methods

Animals and experimental design

Twenty healthy *Nellore* breed bulls with an average age of 30 ± 6.3 months and body weight of 584 ± 98.78 kg were used. Animals were housed in paddocks located at the University of São Paulo, *Campus* of Pirassununga. Hay and concentrate were provided in accordance with National Research Council (NRC 2011) requirements. Environment temperature and humidity were evaluated in every analysis throughout the experiment by data logger (OPUS 20 THI,8120.00; Lufft, Germany). During the study, the daily average temperature was $26.27 \pm 4.43^{\circ}\text{C}$ and the mean relative humidity was $65.30 \pm 18.19\%$.

Bulls were divided into two groups: 1) control (CO, $n = 10$) and 2) submitted to testicular heat stress (HS, $n = 10$). Testicular heat stress was induced by scrotal insulation method using scrotal bags made especially for bulls and maintained for 96 hours. Scrotal bags are made according to the model proposed by Alves et al. (2016).

The study was divided into two periods. During the first period, pre-heat stress period, analyses were used as biological leveling to evaluate similarity between the animals before the induction of insult, and consisted of collecting semen and blood once a week 14 and 7 days before the testicular heat stress (-14 and -7 days). The second period, the post-heat stress period, involved the same collections, but this time, once a week from the day of removal of scrotal bags (day 0) to 28 days afterward (7, 14, 21, and 28 days).

Semen collection

Unless otherwise mentioned, the reagents and chemical used were purchased from Sigma-Aldrich (St. Louis, MO, USA). Also, the same trained technician performed all the evaluations during all the different periods of the experiment.

Before semen collection, bulls were maintained in a containment trunk, where the prepuce was washed with 0.9% NaCl solution and cleaned with paper towel to remove debris and dry liquids. Then, semen was collected by electroejaculation (Autojac V2, Neovet, Uberaba, Brazil) in pre-warmed tubes of

15 mL (37°C). Semen collection was performed on days -14, -7, 0, 7, 14, 21 and 28 in relation to heat stress, according to the health and safety criteria established by the Brazilian College of Animal Reproduction (CBRA 2013).

Semen conventional analysis

After collection, semen analysis consisted of sperm motility (%) and morphology (%). Motility was evaluated using a phase contrast microscope (Eclipse E200, Nikon) at 100x magnification. Sperm abnormalities (%) were analyzed fixing semen in 4% formaldehyde (previously warmed to 37 °C) using the wet chamber technique, classifying the defects as major, minor and totals, as described by Blom (1973) by differential interference contrast microscopy (DIC, Nikon 80i model) at 1,000x magnification, in a total of 200 cells.

Evaluation of the plasma, acrosome and mitochondrial membranes

To evaluate sperm plasma, acrosome and mitochondrial membranes integrity, 150 µL of diluted semen in modified Tyrode's medium (sperm-TALP; herein called TALP) (25×10^6 sperm/mL) were used, adding fluorescent probes: 2 µL of Hoescht 33342 (0.5mg/mL, Molecular Probes), 3 µL of propidium iodide (0.5 mg/mL), 50 µL agglutinin obtained from *Pisum sativum* conjugated to fluorescein isothiocyanate (FITC-PSA, 100 µg/mL) and 2 µL 5,5', 1,1',6,6'tetrachloro' 3,3'tetraetilbenzimidazolil carbocyanine iodide (JC-1, 153 µM) prepared as described by Celeghini et al. (2007) and incubated 8 min at 37°C. Then, 200 cells were evaluated by epifluorescence microscopy (model 80i, Nikon, Tokyo, Japan) with magnification of 1,000x using the triple filter (D/F/R, C58420). The presenting sets were UV-2E/C (excitation 340-380nm and emission 435-485nm), B-2E/C (excitation 465-495nm and emission 515-555nm) and G-2E/C (excitation 540-525nm and emission 605-655nm), and classified in sperm with plasma membrane integrity (PMI), sperm with acrosome membrane integrity (AMI), sperm with high mitochondrial membrane potential (HMMP) and sperm with simultaneously intact plasma membrane integrity, acrosome membrane integrity, and with high mitochondrial membrane potential (PIAIH).

Sperm lipid peroxidation

Evaluation of sperm susceptibility to lipid peroxidation was performed using the technique of induced thiobarbituric acid reactive substances (TBARSs assay) according to Alves et al. (2016). The induction was executed by the incubation of 200 μ L of unprocessed semen with 50 μ L of iron sulfate (4 mM), and 50 μ L vitamin C (20 mM), both incubated at 37°C in a dry-water bath for 90 min with the tube cover open. Then, 600 μ L of cold trichloroacetic acid 10% was added the sample, and centrifuged at 19.700 g for 10 min. Next, 1% of thiobarbituric acid was added to the sample in a 1:1 proportion (600 μ L of the sample + 600 μ L of TBA) and incubated well closed at 100°C for 15 min in a water bath, then placed on ice to stop the reaction. Samples were analyzed by a spectrophotometer (Ultrospec 2200 Pro Amersham Biosciences; Piscataway, NJ, USA) at a wavelength of 532 nanometers. Results were expressed in nanograms of TBARS/ 10^6 spermatozoa.

Sperm DNA Fragmentation

Sperm DNA fragmentation was evaluated by the acridine orange chromatin structure assay methodology previously described by Tejada et al. (1984), Celeghini et al. (2008) and Acevedo (2001). Based on this protocol, which measures the susceptibility of sperm nuclear DNA to acid-induced denaturation *in situ* by quantifying the metachromatic shift of acridine orange (AO) from green to orange, 500 μ L of semen were centrifuged two to three times at 755xg for 10 min and washed with 500 μ L of distilled water between each centrifugation. After the last centrifugation, the pellet was resuspended to a concentration of 25×10^6 sperm/mL with distilled water. Subsequently, 7 μ L of each sample was put on a slide and air-dried for 60 min at room temperature. Immediately after, the slides were immersed and fixed in a DTT solution [2.9% (11.6 g) of Sodium Citrate Tribasic Dihydrate, S4641] + [9 mM (1.344 g) of Ethylenediaminetetraacetic acid, E6758] + [1 mM (0.00616 g) of 1% Dihydrotreitol D063] diluted in 400 mL of MiliQ water for 30 min. Then, slides were immersed in a buffer solution (equal amounts of sodium citrate and ethylenediaminetetraacetic acid) for 20 min to remove excess DTT solution and to test chromatin stability. Lastly, each smear

was fixed overnight in Carnoy's solution, (300 mL of Methanol 99.9%, 34860 + 100 mL of Glacial Acetic Acid P.A.A.S., A1019.01.BJ, SYNTH). After fixation, the slides were air-dry for 10 min and then stained with 3 mL of acridine orange stain prepared as follows: 10 mL of stock solution of Acridine Orange [1g of Acridine Orange hemi (zinc chloride) salt A6014], diluted into 1000 mL of distilled water + 40 mL of citric acid stock solution [Citric Acid Monohydrate, 1.00244.0500, 500G, MERCK, at 0.1 M (5.25 g of citric acid to 1000 ml of distilled water)] + 2.5 mL of di-Sodium hydrogen phosphate anhydrous [1.06559.0500, 500G, MERCK, to 0.3 M (20.10 g of disodium phosphate to 250 mL of distilled water)] for 5 min. Slides were then carefully washed with sodium citrate solution [2.9% Sodium citrate S464] in 400mL MiliQ water, to remove background staining; while still wet, they were covered with coverslips and allowed to airdry for 10 min. For the evaluation, 500 cells were analyzed by epifluorescence microscopy in a 1,000x magnification, using a filter (D/F/R, C58420). Sperm with normal DNA content present a green fluorescence, whereas sperm with abnormal DNA content emit a varying of a yellow-orange fluorescence. The percentage of sperm with intact chromatin was calculated by dividing the number of sperm stained green by the total number of sperm (green + orange) and multiplying the result by 100.

Seminal plasma lipid peroxidation

To evaluate the susceptibility to lipid peroxidation on seminal plasma was used the spontaneous TBARS assay, according to Alves et al. (2016). Seminal plasma was obtained from centrifugation of the total ejaculate at 200xg for 15 min. The method was executed adding 10% of trichloroacetic acid (TCA) to the sample in a 1:2 proportion (500 µL of the seminal plasma sample + 1000 µL of TCA). Then, the sample was centrifuged at 20,817xg for 15 to 5°C. Afterward, 1% of thiobarbituric acid was added to both samples in a 1:1 proportion (600 µL of the sample + 600 µL of TBA) and incubated well closed at 100°C for 15 min in a water bath, then placed on ice to stop the reaction. Reactions of samples were analyzed by a spectrophotometer (Ultrospec 2200 Pro Amersham Biosciences; Piscataway, NJ, USA) at a wavelength of 532 nanometers. Results were expressed in nanograms of TBARS/mL.

Blood plasma lipid peroxidation

Blood samples were collected from the coccygeal vein using Vacutainer® EDTA (anticoagulant) blood collection tubes, on days -14, -7, 7, 14, 21 and 28 in relation to heat stress. To evaluate the susceptibility to lipid peroxidation on blood plasma was used the spontaneous TBARS assay, according to Alves et al. (2016). Blood plasma was obtained from centrifugation of the total blood at 200xg for 15 min. Then, blood spontaneous TBARS was performed according to method previously described for seminal plasma spontaneous TBARS. The results were expressed in nanograms of TBARS/mL.

Statistical analysis

In this experiment, two treatments (control x heat stress) and different times were considered for statistical analyses performed on Statistical Analysis Software (SAS 9.3). Data were previously submitted to the evaluation of normality by the Shapiro-Wilk test. The variables that did not meet the assumptions were submitted to the removal of outliers, transformation (logarithmic, quadratic or inverse) or non-parametric tests (spermatozoa lipid peroxidation, seminal plasma lipid peroxidation and sperm DNA fragmentation). When necessary, the nonparametric test used was Kruskal Wallis. The variables that met the normality (sperm motility, plasma membrane, acrosomal membrane and mitochondrial membrane potential and blood plasma lipid peroxidation) were then submitted to the MIXED procedure, adding the time factor using the REPEATED command. The best matrix (lowest AICC) was previously tested for each variable. For this analysis, post-heat stress period (0, 7, 14, 21 and 28 days) and two groups (control and testicular heat stress) were considered. The isolated effects of treatment and time were only considered when there was no interaction time* treatment. For pre-heat stress period (7 and 14 days before insulation), two groups (control and testicular heat stress) were considered and the variables that met the normality were submitted to the MIXED procedure to verify similarity between the groups. For all analyses, adjusted p-value was considered and significance level was of $P \leq 0.05$, and was considered tendency when $P > 0.05$ and < 0.1 .

Additionally, were made correlations considering the whole set of data, times (D0, D7, D14, D21, D28), treatments (CO and HS), and interaction time x treatment through the CORR proc. These correlations were considered in the discussion.

Results

The results corresponding to the pre-heat stress period (table 1) showed that the animals of CO and HS groups were similar for all the characteristics evaluated: sperm motility, morphology (major, minor and total defects), membranes (PMI, AMI, HMMP and PIAIH), spermatozoa lipid peroxidation (induced TBARS) and DNA fragmentation, as well as seminal and blood plasma lipid peroxidation (spontaneous TBARS).

In concern to the post-heat stress period, results showed that there were not effects of heat stress on the day the scrotal bag was removed (D0), for all sperm characteristic. However, 7 days after heats stress, the chronology of the effects of heat stress began to be highlighted, with sperm morphology; increasing the major defects (Fig.1b), minor defects (Fig.1c) and total defects (Fig.1d) in the animals of the HS group ($P=0.001$; $P=0.004$; $P=0.008$, respectively). Also, at the same day, it was already possible to notice a tendency ($P=0.07$) to decrease the percentage of cells with high mitochondrial potential on the same group of animals (Fig. 2c). Fourteen days after heat stress, the negative effects on sperm morphology continued in the animals of the HS group (major: $P<0.0001$; minor: $P=0.001$ and total defects: $P<0.0001$). Likewise, the same animals started to show high concentrations of spermatozoa lipid peroxidation (Fig. 3), compared with the animals of the CO group ($P=0.04$; 6.95 ± 2.45 , 8.03 ± 0.82 , respectively). Differently to the previous 7 days, by this time, it was already possible to notice the decrease in the percentages of cells with high mitochondrial membrane potential (Fig. 2c: $P=0.01$, 66.20 ± 7.29), as well as, the percentages of sperm motility (Fig. 1a: $P=0.01$: 61.00 ± 3.39) and the number of cells with plasma membrane integrity (Fig.2a: $P=0.04$, 63.80 ± 7.86). However, there was a tendency for interaction treatment x time (Fig. 2d: $P = 0.07$) for PIAIH cells ($P=0.02$: 61.15 ± 7.58). Additionally, regarding the correlation results, it was possible to evidence a positive correlation ($r=0.53$; $P=0.01$) between high percentages of

major defects obtained 7 days after the testicular bags removal and high spermatozoa lipid peroxidation 14 days after heat stress.

After twenty-one days after the removal the testicular bags, the animals continued showing high percentages of major (Fig. 1b: $P<0.0001$), minor (Fig. 1c: $P=0.001$) and total defects (Fig. 1d: $P<0.0001$), as well as, high percentages of spermatozoa lipid peroxidation (Fig.3: $P=0.05$). Sperm motility, plasma membrane integrity and mitochondrial membrane potential of the HS group continued decreasing their percentages (Fig. 1a: $P=0.007$; Fig. 2a: $P=0.01$ and Fig. 2c: $P=0.009$; and Fig. 2d: $P=0.01$: 52.11 ± 9.66 , respectively), and the tendency for interaction treatment x time ($P = 0.07$) also continued evidencing the decrease in the percentage PIAIH cells ($P=0.01$). Correlations were equally found between spermatozoa lipid peroxidation and plasma membrane integrity ($r=-0.49$; $P=0.02$), mitochondrial membrane potential ($r=-0.49$; $P=0.02$) and PIAIH cells ($r=-0.46$; $P=0.02$). Twenty-eight days after the heat stress, animals of the HS group continued showing a decrease in sperm motility (Fig.1a: $P=0.001$), plasma membrane integrity (Fig. 2a: $P=0.002$), and mitochondrial membrane potential (Fig. 2c: $P=0.0009$). The number of PIAIH cells also continued to showed the decrease ($P=0.003$) with the tendency to interaction treatment x time ($P=0.07$), and as a late effect, DNA integrity was also affected (Fig. 4). This is evidenced by the interaction treatment x time ($P=0.007$), and the high percentages of DNA fragmentation ($P=0.008$; 2.1 ± 1.00) in the animals of the HS group. Besides, was found a positive correlation ($r=0.54$; $P=0.02$) between spermatozoa lipid peroxidation at day 21 and DNA fragmentation at day 28. Testicular heat stress did not affected negatively acrosome membrane integrity (Fig. 2b), neither the seminal and blood plasma (Figure 5a and b).

Discussion

This study aimed to evaluate the integrity of the sperm motility, morphology, membranes and sperm DNA, as well as their susceptibility to lipid peroxidation, during the first 28 days of insult. We sought to establish chronologically how testicular heat stress acts and what the negative effects are on the sperm cycle of bulls. These analyses are important because, to the best of our knowledge, there are no previous studies describing the chronological effects of heat stress on

sperm features as showed here.

Starting with scrotal pre-insulation period, our results show that the animals used were homogeneous before the induction of testicular heat stress. Regarding testicular heat stress, scrotal insulation is commonly used to cause inconsistencies in the testicular thermoregulation and induced testicular degeneration, reflecting on semen quality and affecting mostly those sperm cells under spermiogenesis and meiotic stages of development at the time of testicular heat stress (Rahman et al. 2018) (Fig. 6c, d and e). Testicular degeneration commonly manifests with no general clinical changes, but shows some alterations in reproductive system, as low motility and moderate number of abnormal sperm cells (Brito et al. 2003).

It is important to note that the chronology of the appearance of damage on sperm characteristics in the ejaculate depends on the severity of the testicular heat insult, considering intensity and time of duration of insult, which reflect on the stages of sperm cell development during heat stress (Fig. 6). Thus, we were able to observe that there were no effects on sperm characteristics immediately after scrotal bag removal (day 0), when the spermatozoa were presumably residing in the epididymis at the moment of insult (Houston et al. 2018).

Seven days after heat stress induction, we observed an increase of abnormal sperm cells (major, minor and total), as well as a tendency for decreased sperm mitochondrial membrane potential. However, no other alterations were noticed at this time. At this time, sperm were also in the tail of the epididymis and ampulla of deferens ductus (Amann et al. 1983) (Fig. 6b). When stored at the tail of epididymis and ampulla, the head and nuclear sperm structures can be more compact and resistant, even though the epididymal ambient can also be altered by heat stress (Karabinus et al. 1997). Likewise, Januskauskas et al. (1995) found that scrotal insulation applied to sperm cells present in the epididymis and rete testis (sperm cells obtained between 2 and 9 days after insulation) did not produce significant differences on motility, morphology, and sperm membrane integrity.

After 14 days of heat stress, there were high concentrations of lipid peroxidation in spermatozoa (Fig. 6c), resulting in a significant decrease in mitochondrial membrane potential, plasma membrane integrity and sperm motility, as well as a more pronounced increase in sperm abnormalities, evidenced by higher percentages of minor defects. Other authors also reported that the effects of testicular heat stress on sperm motility and sperm morphology were noticed 14

days after the insult (Fernandes et al. 2008; Rahman et al. 2011), because germ cells are exposed at this time to testicular heat stress as round spermatids (Pérez-Crespo et al. 2008).

Despite the high percentage of lipid peroxidation in the spermatozoa at 14 days, for lipid peroxidation in the seminal plasma, a non-significant increase in the values was noticed. In this sense, Shiva et al. (2011) reported that the deficiency of the antioxidant defense system in infertile men is associated with an accumulation of seminal plasma MDA, which is a biomarker of lipid peroxidation, and also negatively correlated with sperm motility and morphology. Differently, in this study, there were no correlations between seminal plasma lipid peroxidation and sperm characteristics. Nevertheless, the authors also confirmed that in male infertility, excessive ROS generation interferes with the antioxidant defense in the seminal plasma, leading to a condition of oxidative stress, which negatively affects sperm quality. Those results may explain the increase in abnormal sperm and decreasing sperm motility, plasma membrane integrity and mitochondrial potential, due to the deficiency of the antioxidant system in the seminal plasma caused by high ROS production and oxidative stress, as evidenced by high concentrations of lipid peroxidation in spermatozoa. Gong et al. (2017) reported that exposure of sperm cells to hyperthermia causes a dephosphorylation and activation of glycogen synthase kinase 3 (GSK3), which binds to proteins of the outer mitochondrial membrane and regulates its permeability, leading to a reduction in its capacity to produce energy. Thus, loss of mitochondrial membrane potential and, consequently, decrease in energy production lead to decreased sperm motility (Vitorino et al. 2006). It is hypothesized that morphologically abnormal spermatozoa could be a potential source of ROS. Subramanian et al. (2018) found that participants with teratozoospermia and oligoteratozoospermia had elevated MDA levels. The study showed a positive correlation between major defects obtained 7 days after heat stress and spermatozoa lipid peroxidation 14 days after heat stress ($r=0.53$; $P=0.01$), supporting the hypothesis of the potential source of ROS due to the high percentage of morphological defects.

After 21 days (Fig 6d), lipid peroxidation reached the highest concentrations in the spermatozoa, causing even more significant insults to sperm morphology. In fact, the highest percentages of major defects were observed at this time, as well as the lowest percentages of sperm motility. Similarly, Kastelic et al. (2017)

found that the increase in abnormal sperm cells occurred 21 days after heat stress. In addition, authors found that plasma membrane integrity, mitochondrial membrane potential, and PIAIH cells numbers continued to decrease. Studies founded that heat stress alters mitochondrial activity, leading to ROS generation and stimulating a state of oxidative stress (Hawkins and Warner 2017). The results can also be justified by the correlations found in this period between spermatozoa lipid peroxidation and minor defects ($r = 0.53$; $P = 0.02$), plasma membrane integrity ($r = -0.49$; $P = 0.02$), mitochondrial membrane potential ($r = -0.49$; $P = 0.02$) and PIAIH cells ($r = -0.46$; $P = 0.03$). Despite the damage of lipid peroxidation on the above characteristics, the acrosome membrane was not affected by heat stress, which can suggest that this membrane is more resistant than the others.

Twenty-eight days later, the percentage of morphological abnormalities continued to increase, sperm motility, plasma membrane integrity, mitochondrial membrane potential and PIAIH cell number reached the lowest percentages. Also at this time, testicular heat stress caused injures on sperm DNA, evidenced by a rise in the percentage of DNA fragmentation (Fig. 6e). However, there were no longer signs of lipid peroxidation. This could be explained because the imbalance during oxidative stress produces base damage and breaks in the double stranded DNA, inducing sperm DNA fragmentation (Aikten and Roman 2008). During spermiogenesis, the replacement of histones by protamines and the compaction of the genome take place. Consequently, the DNA damage may be present in the testicular sperm, epididymal sperm or the ejaculated sperm. Accordingly, it is possible that heat stress may be influencing protamination process and causing damage to sperm chromatin, as demonstrated by some studies (Rahman et al. 2011; Wright et al. 2014; Hamilton et al. 2018). This corroborated the results of DNA fragmentation 28 days after heat stress, when the spermatocytes were probably under meiosis. Both in vitro and in vivo studies have shown that there is a negative correlation between sperm DNA integrity and fertility (Fleming et al. 2004; Evenson et al. 2006), relating that DNA damage is associated with poor embryo development, decreased implantation and poor pregnancy outcome in humans (Fernández-Gonzales et al. 2008), which justify the importance of studies relating oxidative stress and DNA integrity.

Correlations were made between characteristics of different days to explain a

temporal effect, in which one change at one point may reflect in another characteristic of the subsequent period. Thus, we found a positive correlation ($r = 0.54$; $P = 0.02$) between sperm lipid peroxidation at day 21 and DNA fragmentation at day 28. Other positive correlations were found between spermatozoa lipid peroxidation 21 days after heat stress and major ($r = 0.49$; $P = 0.04$), minor ($r = 0.54$; $P = 0.01$), and total ($r = 0.49$; $P = 0.04$) defects 28 days after heat stress. A negative correlation was found between sperm lipid peroxidation at 21 days and plasma membrane integrity ($r = -0.50$; $P = 0.02$) and mitochondrial membrane potential ($r = -0.49$; $P = 0.02$) at 28 days after heat stress. Spermatozoa lipid peroxidation at 21 days showed a positive correlation with total defects ($r = 0.49$; $P = 0.04$) at 28 days. Negative correlations were also found between sperm lipid peroxidation at 21 days with mitochondrial membrane potential ($r = -0.48$; $P = 0.04$) and sperm motility ($r = -0.45$; $P = 0.05$) at 28 days. Still, the high concentrations of lipid peroxidation 14 and 21 days after testicular heat stress appear to have been sufficient to overcome the antioxidant defense capacity provided by seminal plasma (Atig et al. 2012).

In conclusion, this study reports the chronological effect of testicular heat stress on the characteristics and functionality of sperm cells. In this way, testicular heat stress first causes increase of the morphological defects that persist until spermiogenesis and the first phase of meiosis. This is followed by an increase of sperm lipid peroxidation, which affects sperm motility, plasma membrane, mitochondrial membrane potential and sperm DNA integrity.

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TABLES

Table 1 Mean $\pm$ standard error of sperm characteristics: total motility, sperm membranes, sperm morphology, lipid peroxidation (spermatozoa, blood and seminal plasma), and sperm DNA fragmentation of the pre-heat stress period of bulls from Control and Heat stress groups.

<i>Sperm characteristic</i>	<i>Treatments</i>		<i>P value</i>
	<i>Control</i> (<i>n</i> =9)	<i>Heat stress</i> (<i>n</i> =9)	
<i>Sperm kinetics</i>			
Total motility (%)	69.75±1.38	71.11±1.37	0.4
<i>Sperm membranes</i>			
PIAIH ¹ (%)	75.22±2.71	75.56±2.79	0.93
PMI ² (%)	82.77±2.68	81.51±2.33	0.53
HMMP ³ (%)	81.40±2.28	83.88±2.51	0.46
AMI ⁴ (%)	88.92±1.55	91.15±1.35	0.55
<i>Sperm morphology</i>			
Major defects (%)	4.10±0.58	3.5±0.50	0.47
Minor defects (%)	1.40±0.26	0.97±0.28	0.15
Total defects (%)	5.95±0.84	6.02±1.35	0.63
<i>Lipid peroxidation</i>			
Spermatozoa (ng TBARS/10 ⁶ sperm)	4.96±1.31	5.77±2.61	0.79
Seminal plasma (ng TBARS/10 ⁶ sperm)	114.80±7.24	124.56±9.88	0.53
Blood plasma (ng TBARS/10 ⁶ sperm)	240.86±17.02	265.15±30.79	0.49
<i>Sperm DNA</i>			
Sperm DNA fragmentation+ (%)	5.95±0.84	6.02±1.32	0.88

¹PMI: plasma membrane integrity, ²AMI: acrosome membrane integrity, ³HMMP: high mitochondrial membrane potential and ⁴PIAIH: sperm with intact plasma membrane integrity, acrosome membrane integrity, and with high mitochondrial membrane potential.

FIGURES

Fig. 1 Mean $\pm$ standard error of **a.** sperm motility (%), **b.** percentages of major defects (%), **c.** percentages of minor defects (%) and **d.** percentages of total defects (%) of bulls submitted to testicular heat stress (HS, n=9) and control bulls (CO, n=9), 0 (scrotal bag remove), 7, 14, 21 and 28 days after scrotal insulation. ^{a, b}: Lowercase letters indicate difference between treatments within the same time. ^{A, B, C}: Capital letters indicate time effect within each treatment. Black letters indicate difference in HS group. Gray letters indicate difference in CO group. (P $\leq$ 0.05). (*) Asterisk indicates tendency.

Fig. 2 Mean $\pm$ standard error of **a.** cells with plasma membrane intact (PMI) (%), **b.** cells with acrosomal membrane intact (AMI) (%), **c.** cells with high mitochondrial membrane potential (HMMP) and **d.** cells with plasma membrane intact, acrosome membrane intact and high mitochondrial membrane potential (PIAIH) of bulls submitted to testicular heat stress (HS, $n=9$) and control bulls (CO, $n=9$), 0 (scrotal bag remove), 7, 14, 21 and 28 days after scrotal insulation. ^{a, b}: Lowercase letters indicate difference between treatments within the same time. ^{A, B, C}: Capital letters indicate time effect within each treatment. Black letters indicate difference in HS group. Gray letters indicate difference in CO group. ($P \leq 0.05$). (*) Asterisk indicates tendency.

Fig. 3 Mean $\pm$ standard error of spermatozoa lipid peroxidation (ng of TBARS/ 10^6 spermatozoa) 7, 14, 21 and 28 days after scrotal insulation, of bulls submitted to testicular heat stress (HS, n=9) and control bulls (CO, n=9). ^{a, b}: Lowercase letters indicate difference between treatments within the same time. ^{A, B, C}: Capital letters indicate time effect within each treatment. Black letters indicate difference in HS group. Gray letters indicate difference in CO group. ($P \leq 0.05$). (*) Asterisk indicates tendency.

Fig. 4 Mean $\pm$ standard error of percentages of sperm DNA fragmentation (%), 0 (scrotal bag remove), 7, 14, 21 and 28 days after scrotal insulation, of bulls submitted to testicular heat stress (HS, n=9) and control bulls (CO, n=9). ^{a, b}: Lowercase letters indicate difference between treatments within the same time. ^{A, B, C}: Capital letters indicate time effect within each treatment. Black letters indicate difference in HS group. Gray letters indicate difference in CO group. ($P \leq 0.05$). (*) Asterisk indicates tendency.

Fig. 5 Mean $\pm$ standard error of lipid peroxidation of **a.** seminal plasma (ng of TBARS/mL) and **b.** blood plasma (ng of TBARS/mL) of bulls submitted to testicular heat stress (HS, n=9) and control bulls (CO, n=9) 7, 14, 21 and 28 days after scrotal insulation. ($P \leq 0.05$). (*) Asterisk indicates tendency.

Fig. 6 Representation of the chronological effects of testicular heat stress period (0, 7, 14, 21 and 28 days) on the spermatic cell of bulls: **a.** Sperm cells with plasma membrane intact, acrosome membrane intact, high mitochondrial membrane potential and integrity of DNA; **b.** Beginning of the increase of morphological defects; sperm cells with plasma membrane intact, acrosome membrane intact, tendency to decrease mitochondrial membrane potential, and integrity of DNA, (*) Asterisk indicates the tendency to decrease HMMP; **c.** Beginning of the increase of sperm motility, peak in percentage of minor defects; sperm cells with acrosome membrane intact, beginning of the decrease of plasma membrane integrity, mitochondrial membrane potential and PIAIH cells; beginning of the increase of spermatozoa lipid peroxidation; integrity of DNA; **d.** Greater decrease in sperm motility; peak in percentage of major defects; low plasma membrane integrity and low mitochondrial membrane potential, intact acrosome membrane; peak of lipid peroxidation in spermatozoa; integrity of DNA; **e.** Greater decrease in sperm motility; high percentage of morphological defects; sperm cell with damaged plasma membrane, intact acrosome and low mitochondrial membrane potential; high percentage of morphological defects; high DNA fragmentation.

Capítulo 3: Characterization and differentiation of the morphofunctional impairments of the sperm cell during each spermatogenesis stages of Nellore bulls

Characterization and differentiation of the morphofunctional impairments of the sperm cell during each spermatogenesis stages of Nellore bulls

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Abstract

Spermatozoa are complex cells that can be damaged when spermatogenesis and sperm maturation are affected, leading to the production of altered spermatozoa. In addition, the specific time that the spermatogenesis and sperm maturation after an insult occurs, as well as when recovery occurs, as they are not yet fully understood. Thus, this study aimed to calculate the prevalence of major and minor sperm defects, and all the impairments related to sperm quality in every specific stage of the spermatogenesis and sperm maturation, as well as, to confirm the recovery of the spermatogenic cycle. *Nellore* breed bulls were separated into control and heat stress (scrotal bags/96 hours) groups and submitted to semen and blood samples collect. Findings were divided into 5 periods, and evaluated for sperm motility, abnormalities, plasma and acrosome membranes integrity, mitochondrial membrane potential, sperm lipid peroxidation and DNA fragmentation. Seminal and blood plasma were evaluated to lipid peroxidation. Period 1 showed the homogeneity of the entire sperm cells line. During period 2 some sperm features started to impair, being more affected during period 3, where spermatocytes, round and elongated spermatids were the most susceptible cells of the sperm line. Acrosome and mitochondrial membrane are the most sensitive structures to damage during spermatogenesis, due to the constant appearance of defects related to acrosomal integrity and to the late recovery of the mitochondrial membrane potential. Head defects, such as loose abnormal head were the only defect that persisted even during the recovery period. In this regard, knowledge about how every stage of spermatogenesis and sperm maturation is affected, and sperm cycle recovery will provide valuable insights about spermatogenesis status and help in performing precise diagnostics, providing better prognosis and treatment interventions.

Key-words: Spermatozoa; Spermatogenesis; Spermatocytogenesis; Meiosis; Spermiogenesis

INTRODUCTION

The relationship between semen quality and fertility is one of the most important aspects of cattle production system (Januskauskas et al., 2001). Fertilization involves a complex process, which, in order for the sperm cell to be able to complete its function, needs to reach the fertilization site, penetrate the oocyte and activate embryo development (Ôura and Toshimori, 1990; Braundmeier and Miller, 2001). For this, the sperm cell needs some morphological and functional characteristics, which over the years, has become the focus of many studies that aim to understand the fertility variations of the bulls. In the Manual of the Brazilian College of Animal Reproduction (CBRA, 2013) are presented some minimal values of progressive motility, vigor, morphological defects and sperm concentration that a bovine semen ejaculate should have to be considered appropriate for reproduction. Most of the time, due to the alteration in these characteristics, some ejaculates result in fertility failures. On the other hand, some ejaculates when used in AI show a deficiency in fertility potential, which can be attributed to changes in structures or sperm functions not contemplated in the spermogram.

The mammalian sperm has two major components, the head and the flagellum or tail, joined by the connecting our middle piece, and the plasma membrane covering the entire cell (Ôura and Toshimori, 1990; Eddy and O'Brien, 1994; Mortimer, 1997). The plasma membrane plays a fundamental role in the survival of the spermatozoid in the female reproductive tract and in the maintenance of its fertilizing capacity, since it securities cellular homeostasis and is essential to maintain viability (Flesh and Gadella, 2000). The spermatozoid head consists of the acrosome, nucleus, and some structures of the cytoskeleton and cytoplasm. The nucleus contains deoxyribonucleic acid (DNA), which contributes half of the genetic material to the progeny. However, at any stage of the spermatogenesis process, DNA damage can be produced, a phenomenon that is multifactorial and not completely delimited (Fernández-Gonzales et al. 2008; Beletti, 2013). The mitochondria, located in the intermediate part of the spermatozoa, are responsible for the production of ATP, which serves as an energy source for the flagellar beating. On the other hand, the acrosome is indispensable for fertilization (Flesh and Gadella, 2000). To have fertilizing

ability, spermatozoa must acquire a combination of features, such as intact plasma membranes, acrosome integrity, and high mitochondrial membrane potential for the production of adenosine triphosphate (ATP)-generating energy for flagellar beat and motility (Celeghini et al., 2004). Chromatin integrity is another important factor because DNA fragmentation impairs fertility (Benchai et al., 2003). Combining the results of various sperm function tests improves the reliability of fertility estimation. The evaluation of the sperm morphology is of great importance, since abnormal spermatozoa are more susceptible to oxidative stress (Carreira et al., 2012), and since it can detect changes in the spermatid structure that can be related to dysfunctions in spermatogenesis, spermiogenesis and sperm maturation, among other processes (Barth and Oko, 1989; Arruda et al., 2015).

In this way, spermatozoa are complex cells that undergo injuries when one of several biochemical or morphological factors is affected (Siqueira et al., 2007). Historically, several studies have associated the scrotal instillation model in order to mimic the damage produced by heat stress to the sperm cell (Barth and Bowman, 1994; Januskauskas et al., 1995; Kastelic et al., 1996; Brito et al., 2003; Fernandes et al., 2008; Rahman et al., 2011; Houston et al. 2018). However, although it has been well documented that heat stress negatively impacts male reproduction, apparently affecting multiple stages of spermatogenesis, and consequently driving an overall reduction in sperm count, motility and normal morphology, as well as oxidative stress induction, which also causes DNA damage (Pérez-Crespo, 2008; Hansen, 2009; Zhang et al., 2015).

There are no studies evaluating which injuries and at what specific time point of the spermatogenesis and sperm maturation, all the impairments related to motility, morphology, membrane and DNA integrity, as well as the production of lipid peroxidation occur. Thus, using the heat stress model as inductor of injuries, the objective of this study was to calculate the prevalence of major and minor defects, in every specific stage of the spermatogenesis and sperm maturation. At the same time, the study aimed to evaluate all the impairments related to motility, morphology, membrane and DNA integrity, as well as the production of lipid peroxidation in every point of the spermatogenesis and sperm maturation, as well as to confirm the time of recovery of the spermatid cycle, divided in five periods, based on the model proposed and validated previously by Rahman et al. (2011).

MATERIAL AND METHODS

Animals and experimental design

Nine healthy *Nellore* breed bulls with an average age of 30 ± 6.3 months and body weight of 584 ± 98.78 kg were used. It was approved by the Ethical Committee on Animals Use of the School of Veterinary Medicine and Animal Science of the University of São Paulo, (16 August 2016, CEUA 5162170616). Animals were housed in paddocks located at the University of São Paulo, *Campus* of Pirassununga. Hay and concentrate were provided in accordance with National Research Council (NRC, 2011) requirements. Environment temperature and humidity were evaluated in every analysis throughout the experiment by data logger (OPUS 20 THI, 8120.00; Lufft, Germany). During the study, the daily average temperature was $26.27 \pm 4.43^{\circ}\text{C}$ and the mean relative humidity was $65.30 \pm 18.19\%$. Bulls were divided into two groups: 1) control (CO, $n = 4$); and 2) submitted to testicular heat stress (HS, $n = 5$). Testicular heat stress was induced by scrotal insulation method using scrotal bags made especially for bulls maintained during 96 hours. Scrotal bags are made according the model proposed by (Alves et al., 2016).

In order to observe and compare the susceptibility of spermatozoa, seminal and blood plasma to testicular heat stress during the different stages of spermatogenesis, the ejaculates and blood samples were divided to five periods, according to the model described in (Rahman et al., 2011): Period 1 (pre-heat stress period): collections once a week, fourteen and seven days before the testicular heat stress (-14 and -7 days). Those analyses were used as biological leveling, to evaluate similarities between the animals before the induction of insult. Subsequent 3 periods were considered as post-heat stress periods. They involve the same collections, but in different moments on the sperm cycle. Period 2: once a week from the day the scrotal bags were remove (day 0) to seven days afterwards (+ 7 d= period when spermatozoa were presumably present in the epididymis at the time of testicular heat stress induction), period 3: once a week from 14 to 42 days after the HS (+ 14, + 21, + 28, + 35 and + 42 days = period when cells were presumably undergoing spermiogenesis and meiosis stages at the time of HS induction), period 4: once a week from 49 to 63 after the HS (+ 49, +

56 and + 63 days = period when cells presumably were undergoing mitosis or spermatocytogenesis stage at the time of HS induction. And, period 5 (recovery period): once a week from 70 to 77 days after the HS (+ 70 and + 77 days) (Figure 1).

Unless otherwise mentioned, the reagents and chemical used were purchased from Sigma-Aldrich (St. Louis, MO, USA). Also, the same trained technician performed all the evaluations during all the different periods of the experiment.

Semen collection

Before semen collection, bulls were maintained in a containment trunk, where the prepuce was washed with 0.9% NaCl solution and cleaned with paper towel to remove debris and dry liquids. Then, semen was collected by electroejaculation (Autojac V2, Neovet, Uberaba, Brazil) in pre-warmed tubes of 15 mL (37°C). As mentioned before, semen collection was performed 14 days before testicular heat stress, and 77 days after testicular heat stress, according to the health and safety criteria established by the Brazilian College of Animal Reproduction (CBRA, 2013).

Semen conventional analysis

After collection, semen analysis consisted of, sperm motility (%) and abnormalities. Motility was evaluated using a phase contrast microscope (Eclipse E200, Nikon) at 100x magnification. Sperm abnormalities (%) were analyzed fixing semen in formaldehyde 4% (previously warmed to 37 °C) using the wet chamber technique, classifying the defects as major, minor and totals, as described in (Blom, 1973) by differential interference contrast microscopy (DIC, Nikon 80i model) at 1,000x magnification, in a total of 200 cells. All major and minor defects were also analyzed separately.

Sperm plasma, acrosome and mitochondrial membranes potential evaluation

To evaluated sperm plasma, acrosome and mitochondrial membranes integrity, 150 μL of diluted semen in modified Tyrode's medium (sperm-TALP; herein called TALP) (25×10^6 sperm/mL) were used, adding fluorescent probes: 2 μL of Hoescht 33342 (0.5mg/mL, Molecular Probes), 3 μL of propidium iodide (0.5 mg/mL), 50 μL agglutinin obtained from *Pisum sativum* conjugated to fluorescein isothiocyanate (FITC-PSA 100 $\mu\text{g/mL}$) and 2 μL 5,5', 1,1', 6,6'tetracloro' 3,3'tetraetilbenzimidazolil carbocyanine iodide (JC-1, 153 μM) prepared as described by (Celeghini et al., 2007) and incubated 8 min at 37°C. Then, 200 cells were evaluated by epifluorescence microscopy (model 80i, Nikon, Tokyo, Japan) with magnification of 1,000x using the triple filter (D/F/R, C58420). The presenting sets were UV-2E/C (excitation 340-380nm and emission 435-485nm), B-2E/C (excitation 465-495nm and emission 515-555nm) and G-2E/C (excitation 540-525nm and emission 605-655nm), and classified in sperm with plasma membrane integrity (PMI), sperm with acrosome membrane integrity (AMI), sperm with high mitochondrial membrane potential (HMMP) and sperm with simultaneously intact plasma membrane integrity, acrosome membrane integrity, and with high mitochondrial membrane potential (PIAIH).

Sperm lipid peroxidation

Evaluation of sperm susceptibility to lipid peroxidation was performed using the technique of induced thiobarbituric acid reactive substances (TBARSs assay) according to Alves (Alves et al., 2016). The induction was executed by the incubation of 200 μL of unprocessed semen with 50 μL of iron sulfate (4 mM), and 50 μL vitamin C (20 mM), both incubated at 37°C in a dry-water bath for 90 min with the tube cover open. Then, 600 μL of cold trichloroacetic acid 10% was added the sample, and centrifuged at 19.700 g for 10 min. Next, 1% of thiobarbituric acid was added to the sample in a 1:1 proportion (600 μL of the sample + 600 μL of TBA) and incubated well closed at 100°C for 15 min in a water bath, then placed on ice to stop the reaction. Samples were analyzed by a spectrophotometer (Ultrospec 2200 Pro Amersham Biosciences; Piscataway, NJ, USA) at a wavelength of 532 nanometers. Results were expressed in nanograms of TBARS/ 10^6 spermatozoa.

Sperm DNA Fragmentation

Sperm DNA fragmentation was evaluated by the acridine orange chromatin structure assay methodology previously described in (Tejada et al., 1984; Acevedo, 2001; Celeghini et al., 2008). Based on this protocol, which measures the susceptibility of sperm nuclear DNA to acid-induced denaturation *in situ* by quantifying the metachromatic shift of acridine orange (AO) from green to orange, 500 µL of semen were centrifuged two to three times at 755xg for 10 min and washed with 500 µL of distilled water between each centrifugation. After the last centrifugation, the pellet was resuspended to a concentration of 25×10^6 sperm/mL with distilled water. Subsequently, 7 µL of each sample was put on a slide and air-dried for 60 min at room temperature. Immediately after, the slides were immersed and fixed in a DTT solution [2.9% (11.6 g) of Sodium Citrate Tribasic Dihydrate, S4641] + [9 mM (1.344 g) of Ethylenediaminetetraacetic acid, E6758] + [1 mM (0.00616 g) of 1% Dihydrotreitol D063] diluted in 400 mL of MiliQ water for 30 min. Then, slides were immersed in a buffer solution (equal amounts of sodium citrate and ethylenediaminetetraacetic acid) for 20 min to remove excess DTT solution and to test chromatin stability. Lastly, each smear was fixed overnight in Carnoy's solution, (300 mL of Methanol 99.9%, 34860 + 100 mL of Glacial Acetic Acid P.A.A.S., A1019.01.BJ, SYNTH). After fixation, the slides were air-dry for 10 min and then stained with 3 mL of acridine orange stain prepared as follows: 10 mL of stock solution of Acridine Orange [1g of Acridine Orange hemi (zinc chloride) salt A6014], diluted into 1000 mL of distilled water + 40 mL of citric acid stock solution [Citric Acid Monohydrate, 1.00244.0500, 500G, MERCK, at 0.1 M (5.25 g of citric acid to 1000 ml of distilled water)] + 2.5 mL of di-Sodium hydrogen phosphate anhydrous [1.06559.0500, 500G, MERCK, to 0.3 M (20.10 g of disodium phosphate to 250 mL of distilled water)] for 5 min. Slides were then carefully washed with sodium citrate solution [2.9% Sodium citrate S464] in 400mL MiliQ water, to remove background staining; while still wet, they were covered with coverslips and allowed to airdry for 10 min. For the evaluation, 500 cells were analyzed by epifluorescence microscopy in a 1,000x magnification, using a filter (D/F/R, C58420). Sperm with normal DNA content present a green fluorescence, whereas sperm with abnormal DNA content emit a varying of a yellow-orange

fluorescence. The percentage of sperm with intact chromatin was calculated by dividing the number of sperm stained green by the total number of sperm (green + orange) and multiplying the result by 100.

Seminal and blood plasma lipid peroxidation

To evaluate the susceptibility to lipid peroxidation on seminal and blood plasma, we used spontaneous TBARS, as described in (Alves et al., 2016). Blood samples were collected from the coccygeal vein using Vacutainer® EDTA (anticoagulant) blood collection tubes, during the same days in which semen was collected. Samples were stored at -80 °C. Seminal and blood plasma was obtained from centrifugation of the total ejaculate and total blood at 200xg for 15 min. The method was executed adding 10% of trichloroacetic acid (TCA) to the samples in a 1:2 proportion (500 µL of the seminal and blood plasma samples + 1000 µL of TCA). Then, the samples were centrifuged at 20,817xg for 15 to 5°C. Afterward, 1% of thiobarbituric acid was added to both samples in a 1:1 proportion (600 µL of the sample + 600 µL of TBA) and incubated well closed at 100°C for 15 min in a water bath, then placed on ice to stop the reaction. Reactions of samples were analyzed by a spectrophotometer (Ultrospec 2200 Pro Amersham Biosciences; Piscataway, NJ, USA) at a wavelength of 532 nanometers. Results were expressed in nanograms of TBARS/mL.

Statistical analysis

In this experiment, two treatments (control x heat stress) and different times/periods were considered for statistical analyses performed on Statistical Analysis Software (SAS 9.3). Data were previously submitted to the evaluation of normality by the Shapiro-Wilk test. The variables that did not meet the assumptions were submitted to the removal of outliers, transformation (logarithmic, quadratic or inverse) or non-parametric tests. When necessary, the nonparametric test used was Kruskal Wallis. The variables that meet the normality were then submitted to the MIXED procedure adding the time factor using the REPEATED command to consider the period effect. The best matrix (lowest AICC) was previously tested for each variable. The isolated effects of treatment

and time/period were only considered when there was no interaction time* treatment. For all analysis, adjusted p-value was considered and significance level was of $P \leq 0.05$.

RESULTS

The percentage of sperm motility, major, minor and total morphological defects (mean $\pm$ SD) to five periods of semen and blood characteristics (period 1 to 5) for CO and HS groups of Nellore bulls are shown in Figure 2. These results showed that sperm motility (Figure 2a) decreased in period 3 ($P < 0.0001$) to HS group. In concern to sperm morphology, major defects (Figure 2b) increased their percentages in the HS group compared with the CO group at periods 3 ($P < 0.0001$) and 4 ($P = 0.004$). Minor defects (Figure 2c) also showed an increase in the percentages in the HS group during period 3 ($P < 0.0001$). As well as major defects, percentages of total defects (Figure 2d) increased in the HS group during period 3 ($P < 0.0001$) and 4 ($P = 0.004$).

Analyzing the major defects separately (Figure 3) was possible to observe that the HS group showed high percentages different defects when compared with CO group: acrosome defects (Figure 3a) during periods 3 ($P = 0.03$) and period 4 ($P = 0.03$); proximal cytoplasmic droplet defect (Figure 3b) during the period 3 ($P = 0.004$); microcephalic head (Figure 3c) during period 3 ($P = 0.004$); loose abnormal head (Figure 3d) during periods 3 ($P = 0.0003$) and 5 ($P = 0.01$); tail coiled around the head (Figure 3e) in periods 3 ($P = 0.001$) and 4 ($P = 0.03$); tapered head (Figure 3f) during period 4 ($P = 0.02$); irregular shape (Figure 3g) during period 3 ($P = 0.0006$); pouch formation (Figure 3h) in period 3 ($P = 0.0008$); strongly bent tail (Figure 3i) during period 3 ($P = 0.004$). As well as major defects, minor defect were analyzed separately (Figure 4a and b). High percentages of loose abnormal head (Figure 4a) were found in the HS group during periods 2 ($P = 0.008$), 3 ($P < 0.0001$) and 4 ($P = 0.05$). Equally, there were high percentages of abaxial tail (Figure 4b) during period 3 ($P = 0.0004$).

Regarding sperm membranes (Figure 5), the HS group showed a decrease in the percentage of cells with plasma membrane integrity (Figure 5a). Acrosome membrane (Figure 5b) in the HS group showed a decrease in the percentage of cells with acrosomal membrane integrity during period 2 ($P = 0.003$).

Mitochondrial membrane potential (Figure 5c) also decreased in the HS group compared during periods 2 ($P = 0.001$), 3 ($P < 0.001$) and 4 ($P = 0.04$). There were lower PIAIA cells (Figure 5d) in periods 2 ($P = 0.01$), 3 ($P < 0.001$) and 4 ($P = 0.03$). Observing the production of lipid peroxidation in the spermatozoa, Figure 6a shows that during period 3 the HS group had a tendency to increase ($P = 0.09$). Figure 6b shows that there were high percentages of DNA fragmentation in bulls of the HS group, during period 3 ($P = 0.001$). Seminal plasma lipid peroxidation results did not show treatment ($P = 0.72$) and period ($P = 0.35$) effects. Only showed a tendency to interaction period x treatment ($P = 0.07$). For seminal blood lipid peroxidation were not treatment effect ($P = 0.72$), period effect ($P = 0.35$) and, interaction treatment x period ($P = 0.07$).

DISCUSSION

The experimental design of this study was based on the model proposed by Rahman et al. (2011). In that model, the authors divided the findings in 4 different periods (from 7 to 63 days after heat stress) in order to compare the susceptibility of the sperm cell to heat stress during the stages of spermatogenesis. This study proposed to distribute the time in relationship to heat stress from 14 days before the induction of heat stress to 77 days after in 5 different periods. As a result, was possible to characterize and divide all phases of spermatogenesis, as well as the recovery of the sperm cycle after heat stress insult. Thus, the objective was to evaluate the prevalence of major and minor morphological defects, and to evaluate injures to sperm motility, membrane and DNA integrity, and the production of lipid peroxidation, in every stage of the spermatogenesis and sperm maturation, as well as to confirm the time of recovery of the spermatic cycle. These analyses are important because, to the best of our knowledge, there are no previous studies describing the prevalence of morphological defects during spermatogenesis of bulls with testicular heat stress, as well as its effects on sperm features, as showed here. Starting with period 1, which covers the 14 and 7 days before HS, was possible to observe that the animals used were homogeneous before the induction of testicular heat stress, due to the harmlessness of all structural and functional characteristics.

In bulls, the duration of the seminiferous epithelium cycle is 13.5 days. The total duration of spermatogenesis is 61 days, which is 4.5 times the duration of the cycle of the seminiferous epithelium. Spermatogenesis is generally divided into spermatocytogenesis (also called mitotic proliferation), meiosis, and spermiogenesis (also called cytodifferentiation), which take approximately 21, 23 and 17 days, respectively (Staub and Johnson, 2018). Once formed within the seminiferous tubules, the immotile spermatozoa are released into the seminiferous tubular fluid and transported to the epididymis, where they gain the ability to move and fertilize the ova. The epididymis is generally divided into three parts: caput, corpus, and cauda. In bulls, the transit of spermatozoa through the epididymis usually takes 8 to 11 days (Vogler et al., 1991; Yanagimachi, 1994; Johnson et al., 2000). In this sense, the first involved exposure period was the second one (Period 2). This involves the day of removal of the scrotal bag (day 0), and the first 7 days after heat stress. Although, it is important to note that the chronology of the appearance of damage on sperm characteristics in the ejaculate depends on the severity of the testicular heat insult, considering intensity and time of duration of insult, which reflect on the stages of sperm cell development during heat stress (Garcia-Oliveros et al., 2019: unpublished data, Capítulo 2). During this period, no differences were observed between groups for sperm motility, sperm plasma membrane integrity, DNA integrity, spermatozoa production of lipid peroxidation and, percentages of major, minor and total defects. This can be justified because at this time point, sperm were in the tail of the epididymis and ampulla of deferens ductus (Amann et al. 1983), where the head and nuclear sperm structures can be more compact and resistant, even though the epididymal ambient can also be altered by heat stress (Karabinus et al. 1997).

Still, regarding to minor defects, was evident an increased in the percentages of loose normal head. A decreased of the number of cells with high mitochondrial membrane potential and the numbers of cells with acrosomal membrane integrity was observed. This can be justified because to attain the fertilizing capacity, spermatozoa undergo many maturational changes during maturation and during their transit through the epididymal duct, including including changes in plasma membrane lipids, proteins and glycosylation, and also, alterations in the outer acrosomal membrane, gross morphological changes in the acrosome, and cross-linking of nuclear protamines, proteins of the outer dense fiber and fibrous sheath

(Yanagimachi, 1994; Arruda et al., 2015). Or even, the increase in the proportion of isolated heads is often seen as a transient phenomenon associated with testicular degeneration, inflammatory conditions of the vesicular glands, ampullae and epididymis, increased testicular temperature, either by experimental insulation, disease or pain that causes the bull will lay down for a long time will increase the proportion of isolated heads in semen (Blom, 1950; Wright, 1974; Cooper and Peet, 1984; Barth and Oko, 1989). Overall, compared with other studies, authors also did not report significant changes during this period (Januskauskas et al. 1995; Rahman et al., 2011).

After several proliferations of the last spermatogonia during the mitotic divisions, the pre-leptotene spermatocytes are produced, and cross the blood-testis barrier to engage in meiotic prophase. During the meiotic prophase, primary spermatocytes differentiate successively into different stages (leptotene, zygotene, pachytene, diplotene) before undergoing the two meiotic divisions that originates secondary spermatocytes. So, the first meiotic division is the reductional division (reduction of chromosome number, separation of homologous chromosomes), while the second meiotic division, which originates secondary spermatocytes, is the equational division (separation of the daughter chromatids). Meiosis therefore allows the production of round haploid spermatids (Staub and Johnson, 2018). Subsequent, spermiogenesis stage starts. This consists of the differentiation of round spermatids into spermatids at various degrees of elongation and finally into spermatozoa. In this way, contrary to the previous period, was possible to observe that the sperm cells collected within period 3 suffered more impairment. Probably, because at the moment of insult, spermatozoa were at spermiogenesis or meiosis stage (Senger, 2003; Rahman et al., 2011).

Furthermore, although no significant difference was found, there was a tendency to increase the spermatozoa lipid peroxidation production, which probably was enough to cause a decrease the number of cells with integrated plasma membrane and mitochondrial potential. Thus, loss of mitochondrial membrane potential and, consequently, decrease in energy production lead to decreased sperm motility (Vitorino et al. 2006), as evidenced in this study, where sperm motility also decreased in this period. Due to the stage the germ cells were exposed at this time to testicular heat stress, round spermatids and spermatocytes (Pérez-Crespo et al. 2008), there were an increase in the percentage of DNA

fragmentation, major, minor and total defects, even when evaluated separately.

Acrosomal defects, proximal cytoplasmic droplet, loose normal and abnormal head, pouch formation and, strongly bent tail, were the major and minor defects that had the highest percentages, when compared to the other major and minor defects. High percentages of acrosomal defects during this period may have occurred because biosynthesis of acrosome starts in late spermatogenesis, by the formation of proacrosomal vesicles in the perinuclear region near the Golgi apparatus of pachytene spermatocytes or, primary spermatocytes (Sotomayor and Handel, 1986). Proximal cytoplasmic droplets are originating in the final stages of spermiogenesis and refer to the maintenance of residual bodies from the organelles that made up the cytoplasm of spermatids (Hermo et al., 1994). Previous studies have related some spermatozoa injuries with high percentages of proximal cytoplasmic droplets (PCDs) in humans and bovines (Zini et al., 1998; Carreira et al., 2012). Abnormal spermatozoa are more susceptible to oxidative stress, due to the fact that the production of reactive oxygen species (ROS) by spermatozoa with PCDs can induce oxidative stress (Said, Agarwal and Sharma, 2005). What results in a cellular dysfunction by a number of mechanisms, among them membrane lipid peroxidation (Aitken and Krausz, 2001). As found in our results, there was an increase of the percentages of spermatozoa with proximal cytoplasmic droplet; however, it seems that this amount was not enough to produce high concentrations of lipid peroxidation, due to the results found on lipid peroxidation. Conversely, the high percentages of spermatozoa with DNA fragmentation, damaged plasma membrane and with low mitochondrial potential, as observed here in the animals of the HS group, may have been due to the high PCDs and consequently ROS production, resulting in oxidative damage. DNA results could be explained because the imbalance during oxidative stress produces base damage and breaks in the double stranded DNA, inducing sperm DNA fragmentation (Aitken and Roman 2008). During spermiogenesis, the replacement of histones by protamines and the compaction of the genome take place. Consequently, the DNA damage may be present in the testicular sperm, epididymal sperm or the ejaculated sperm, but was only observed on spermatocytes, round and elongated spermatids (spermiogenesis and meiosis).

Lagerlöf (1934) *apud* (Arruda et al., 2015) established that the presence of proximal cytoplasmic droplet couldn't exceed 3% in normal bulls. In this study

7.86 % of proximal cytoplasmic droplet was found for bulls of the HS group. Spermatozoa with pouch formation or nuclear vacuoles present some type of alteration in their DNA (chromatin defects). These defects appear to be associated with mild testicular degeneration related to stress, since other defects and findings consistent with mild degeneration are frequently present (Wenkoff, 1988), as founded in this study. Indeed, there are some studies in humand and bovines in which vacuoles and diadem defects (pouch formation) were not correlated with sperm nuclear integrity, and hence, these are likely physiological features that may not directly affect sperm chromatin configuration (Fortunato et al., 2016; Boe-Hansen, Fortes and Satake, 2018). The alterations of strongly bent tail occur during sperm maturation, in transit through the epididymis, where the tails fold after forming, due to some alteration that may be osmotic shock. Similarly, this alteration is present in degenerative processes of the testes or sexual immaturity, and the epididymal environment is altered (Koefoed-Johnsen et al., 1980; Arriola et al., 1985 *apud* Arruda et al., 2015).

Impairments in the spermatic head, as normal, are associated with transitory or permanent alterations in spermiogenesis, due to testicular trauma or heat stress (Chenoweth, 2005). The occurrence of a pathologically isolated head originates from the fragility of the attachment of the intermediate piece, which detaches the tail with the onset of motility, along with another alteration, such as abnormal contour or acrosome defects, due to altered spermatogenesis (Wenkoff, 1988; Oko, 1989). It is emphasized that head defects are closely associated with abnormalities in the condensation of sperm chromatin. They participate in the reduction of temporary or permanent fertility (subfertility) and are associated with the low rate of cleavage and the lower embryonic development (Amaral et al., 2009). Tail coiled around the head defects, occurs after sperm formation, during sperm maturation, in the epididymis. When the epididymal environment presents osmotic conditions different from those of the testicular tubules, osmotic shock occurs in the spermatozoon, which responds by wrapping the tail over the head (Wenkoff, 1988). However, during this period, the spermatozoa are presumably passing through the meiosis and spermiogenesis phase, not through the epididymal phase.

During spermatocytogenesis, the primordial germ cells divide by mitosis in the basal part of the seminiferous tubules to form type A1 spermatogonia or stem

cells. An important part of this phase is the renewal of some type A1 spermatogonia, allowing the constant formation of new stem cells. The other type A1 spermatogonia pass through the process of mitotic division to form type A2, A3 and A4 spermatogonia. Later, type A4 spermatogonia divide to form intermediate spermatogonia (Int), and then, type B spermatogonia. These, in turn, continue the process of mitotic division, once or twice, until they form primary spermatocytes or spermatocytes in pre-leptoten phase (Staub and Johnson, 2018). In this sense, it was expected, that sperm abnormalities returned to period 1 level at period 4, since the germ cells at the stage of spermatocytogenesis during heat stress apparently are not adversely affected (Ross and Entwistle, 1979). However, there were still high numbers of sperm cells with low mitochondrial membrane potential and, high percentages of major defects, including acrosomal, tail around the head and tapered head defects. Contrary, minor defects started to attended lower percentages, evidenced by only a tendency to increase, and the presence only of loose normal heads. During period 5, was possible to observe the recovery of most of the sperm cells and, subsequently, their features. Still, head defects continued to appear. The only defect that was found was a high percentage of loose abnormal head.

CONCLUSION

Spermatocytes, round and elongated spermatids seem to be the more susceptible cells to injuries during spermatogenesis, because it is the moment when extensive chromatin remodeling of the cells takes place. Acrosome and mitochondrial membrane seems to be the most sensitive structures to damage during spermatogenesis, due to the constant appearance of defects related to acrosomal integrity and to the late recovery of the mitochondrial membrane potential. Head defects, such as loose abnormal head were the only defect that persisted even during the recovery period. This indicates that studies related to sperm head defects are few and necessary.

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CONFLICT OF INTEREST

The author declare no conflicts of interests.

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FIGURES

Figure 1. Schematic representation of the spermatogenic cycle divided in the different experimental periods. Period 1: 14 and 7 days before the heat stress; Period 2: from the day of the removal of the scrotal bag (D0) until 7 days after the heat stress; Period 3: 14, 21, 28, 35 and 42 days after the heat stress; Period 4: 49, 56 and 63 days after the heat stress; Period 5 (recovery period): 70 and 77 after the heat stress. Epididymal maturation (EM); Epididymal storage (EST); Primary spermatocytes (PS); Secondary spermatocytes (SS); Round spermatids (RS); Spermatozoa; From spermatogonia type A₁ to spermatogonia type int (A₁- int); Spermatogonia type B (A_B).

Figure 2. Mean $\pm$ standard error of the percentage of **a.** sperm motility, (%), **b.** percentages of major defects (%), **c.** percentages of minor defects (%) and **d.** percentages of total defects (%), among five periods of semen collection (period 1 to 5) in Nellore bulls. Period 1: 14 and 7 days before the heat stress; Period 2: from the day of the removal of the scrotal bag (D0) until 7 days after the heat stress; Period 3: 14, 21, 28, 35 and 42 days after the heat stress; Period 4: 49, 56 and 63 days after the heat stress; Period 5 (recovery period): 70 and 77 after the heat stress. ^{a, b}: Lowercase letters indicate difference between treatments within the same time. ^{A, B, C}: Capital letters indicate time effect within each treatment. Black letters indicate difference in HS group. Gray letters indicate difference in CO group. ($P \leq 0.05$).

4. Capítulo 3: Characterization and differentiation of the morpho/functional impairments of the sperm cells during each spermatogenesis stages of Nellore bulls (Figure 3)

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Figure 3. Mean $\pm$ standard error of the percentage of **a.** acrosomal defects (%), **b.** proximal cytoplasmic droplet (%), **c.** microcephalic head (%) and **d.** loose abnormal head (%), **e.** tail coiled around the head, **f.** tapered head, **g.** irregular shape, **h.** pouch formation, **i.** strongly bent tails among five periods of semen collection (period 1 to 5) in Nellore bulls. Period 1: 14 and 7 days before the heat stress; Period 2: from the day of the removal of the scrotal bag (D0) until 7 days after the heat stress; Period 3: 14, 21, 28, 35 and 42 days after the heat stress; Period 4: 49, 56 and 63 days after the heat stress; Period 5 (recovery period): 70 and 77 after the heat stress ^{a, b}: Lowercase letters indicate difference between treatments within the same time. ^{A, B, C}: Capital letters indicate time effect within each treatment. Black letters indicate difference in HS group. Gray letters indicate difference in CO group. ($P \leq 0.05$). The image used to represent tail coiled around the head (e) was adapted from the Brazilian College of Animal Reproduction (CBRA, 2013).

Figure 4. Mean $\pm$ standard error of the percentage of **a.** loose normal head (%), **b.** abaxial tails (%) among five periods of semen collection (period 1 to 5) in Nellore bulls. Period 1: 14 and 7 days before the heat stress; Period 2: from the day of the removal of the scrotal bag (D0) until 7 days after the heat stress; Period 3: 14, 21, 28, 35 and 42 days after the heat stress; Period 4: 49, 56 and 63 days after the heat stress; Period 5 (recovery period): 70 and 77 after the heat stress. ^a, ^b: Lowercase letters indicate difference between treatments within the same time. ^A, ^B, ^C: Capital letters indicate time effect within each treatment. Gray letters indicate difference in CO group. ($P \leq 0.05$). The image used to represent loose normal head defect (a), were adapted from the Brazilian College of Animal Reproduction (CBRA, 2013).

Figure 5. Mean $\pm$ standard error of the percentage of **a.** cells with plasma membrane intact (PMI) (%), **b.** cells with acrosomal membrane intact (AMI) (%), **c.** cells with high mitochondrial membrane potential (HMMP) and **d.** cells with plasma membrane intact, acrosome membrane intact and high mitochondrial membrane potential (PIAIH) among five periods of semen collection (period 1 to 5) in Nellore bulls. Period 1: 14 and 7 days before the heat stress; Period 2: from the day of the removal of the scrotal bag (D0) until 7 days after the heat stress; Period 3: 14, 21, 28, 35 and 42 days after the heat stress; Period 4: 49, 56 and 63 days after the heat stress; Period 5 (recovery period): 70 and 77 after the heat stress ^{a, b}: Lowercase letters indicate difference between treatments within the same time. ^{A, B, C}: Capital letters indicate time effect within each treatment. Black letters indicate difference in HS group. Gray letters indicate difference in CO group. ($P \leq 0.05$).

Figure 6. Mean $\pm$ standard error of the percentage of **a.** spermatozoa lipid peroxidation (ng of TBARS/10⁶ spermatozoa), **b.** DNA fragmentation (%) among five periods of semen collection (period 1 to 5) in Nellore bulls. Period 1: 14 and 7 days before the heat stress; Period 2: from the day of the removal of the scrotal bag (D0) until 7 days after the heat stress; Period 3: 14, 21, 28, 35 and 42 days after the heat stress; Period 4: 49, 56 and 63 days after the heat stress; Period 5 (recovery period): 70 and 77 after the heat stress. ^{a, b}: Lowercase letters indicate difference between treatments within the same time. ^{A, B, C}: Capital letters indicate time effect within each treatment. Black letters indicate difference in HS group. Gray letters indicate difference in CO group. ($P \leq 0.05$).

Figure 7. Representation of sperm quality impairments by heat stress model among five periods of semen collection (period 1 to 5) in Nellore bulls. Period 1 (14 and 7 days before the heat stress): normal sperm cell; Period 2 (from the day of the removal of the scrotal bag (D0) until 7 days after the heat stress): sperm cell with motility, plasma membrane intact, damage acrosome membrane, high mitochondrial membrane potential and integrity of DNA, high percentages of loose normal head; Period 3 (14, 21, 28, 35 and 42 days after the heat stress): sperm cell with low motility, low plasma membrane integrity, low mitochondrial membrane potential, acrosome membrane intact, high DNA fragmentation, high percentages of major defects (high percentages of acrosomal defects, proximal cytoplasmic droplet, microcephalic head, tail coiled around the head, tapered head, irregular shape, pouch formation and strongly bent tail), high percentages of minor defects (high percentages of loose normal head and abaxial tail), tendency to increase spermatozoa lipid peroxidation; Period 4 (49, 56 and 63 days after the heat stress): sperm cell with motility, plasma membrane intact, acrosome membrane integrity, low mitochondrial membrane potential and integrity of DNA, high percentages of major defects (high percentages of acrosomal defect, tapered head and tail coiled around the head), tendency to increase minor defects (high percentages of loose normal head); Period 5 (recovery period, 70 and 77 after the heat stress): sperm cell with motility, plasma membrane intact, acrosome membrane intact, high mitochondrial membrane potential, integrity of DNA and, high percentages of loose abnormal head. The images used to represent some sperm cells stages were adapted from the smart servier medical art (smart.servier.com, June 10,19). The images used to represent tail coiled around the head and, loose normal head defects, were adapted from the Brazilian College of Animal Reproduction (CBRA, 2013). Red and orange lowercase letters indicate the sperm defect of result (graphics).

3 CONSIDERAÇÕES

Este estudo relata o efeito cronológico do estresse térmico testicular sobre as características e funcionalidade dos espermatozoides. Dessa forma, o estresse térmico testicular causa primeiro o aumento dos defeitos morfológicos que persistem até a espermiogênese e a primeira fase da meiose. Isto é seguido por um aumento da peroxidação lipídica dos espermatozoides, que afeta a motilidade espermática, a membrana plasmática, o potencial de membrana mitocondrial e a integridade do DNA espermático. De igual forma, pode-se concluir que os ospermatócitos, espermátides redondas e alongadas, parecem ser as células mais suscetíveis a lesões durante a espermatogênese, visto que é nesta fase que ocorre o remodelamento extensivo da cromatina das células. O acrossoma e a membrana mitocondrial, também, parecem ser as estruturas mais sensíveis a serem danificadas durante a espermatogênese, devido ao constante aparecimento de defeitos relacionados à integridade acrossomal e à recuperação tardia do potencial de membrana mitocondrial. Por último, a cabeça isolada anormal, foi o único defeito que persistiu mesmo durante o período de recuperação. Isso indica que os estudos relacionados aos defeitos de cabeça do espermatozoide são poucos e necessários.

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