GLÁUCIA ELOISA MUNHOZ DE LION SIERVO

EFEITOS DA RESTRIÇÃO DE SONO DURANTE A PERIPUBERDADE SOBRE O DESENVOLVIMENTO TESTICULAR E EPIDIDIMÁRIO DE RATOS

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Dissertação apresentada ao Programa de Pós-Graduação em Patologia Experimental, da Universidade Estadual de Londrina, como requisito parcial para a obtenção do título de Mestre.

Orientadora: Profa. Dra. Glaura Scantamburlo Alves Fernandes.
Co-orientador: Profa. Dra. Monica Levy Andersen.

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BANCA EXAMINADORA

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Londrina, 11 de março de 2016.

Aos meus pais, Glauco e Eloisa, pelo apoio infinito e incondicional...

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“The only way to do a great work is to love what you do. If you haven’t found it yet, keep looking. Don’t settle. As with all matters of the heart, you’ll know when you find it.”

Steve Jobs

SIERVO, Gláucia Eloisa Munhoz de Lion. **Efeitos da restrição de sono durante a peripuberdade sobre o desenvolvimento testicular e epididimário de ratos**. 2016. 88 f. Dissertação de Mestrado – Programa de Pós-Graduação em Patologia Experimental – Universidade Estadual de Londrina, Londrina, 2016.

RESUMO

A puberdade representa uma fase complexa do desenvolvimento, cuja finalidade é a maturação sexual e obtenção da capacidade reprodutiva. Alterações no sono afetam o controle neuroendócrino reprodutivo e modulam o balanço redox do organismo. O objetivo deste trabalho é avaliar se a restrição de sono durante o período peripuberal pode prejudicar o desenvolvimento pós-natal testicular e epididimário de ratos. Foram utilizados 30 ratos machos Wistar, com 40 dias pós-natal (DPN 40). O grupo Restrição de Sono (RS) foi submetido a 21 dias de restrição de sono pelo método da plataforma múltipla modificada (18h de restrição e 6h de sono, por dia). O grupo Controle (C) foi mantido em gaiolas-moradia durante todo o período experimental. No DPN 62, os animais foram pesados, anestesiados e sofreram eutanásia. Os testículos e epidídimos foram retirados, pesados e utilizados para contagem espermática, determinação de marcadores de estresse oxidativo, parâmetros histopatológicos e estereológicos ou morfométricos, bem como para avaliação do perfil inflamatório (atividade de MPO e NAG). Espermatozoides do ducto deferente foram utilizados para avaliação da morfologia e motilidade espermática. O peso corporal final dos animais do grupo RS foi menor assim como o peso absoluto do ducto deferente. Os pesos do epidídimo, testículo e vesícula seminal foram semelhantes entre os grupos, enquanto o peso relativo do testículo e do epidídimo foi maior no grupo RS. A peroxidação lipídica (QL), a capacidade antioxidante total do tecido (TRAP) e as concentrações de GSH mostraram-se aumentadas na região da cabeça do epidídimo no grupo restrito de sono. Na cauda epidimária houve aumento da peroxidação lipídica e de GSH no grupo RS, sendo a TRAP semelhante entre os grupos. No testículo, houve aumento da peroxidação lipídica nos animais restritos de sono, enquanto a capacidade antioxidante total permaneceu inalterada. No grupo RS foi observado um remodelamento das regiões 2A e 5A/B epididimárias, com diminuição das porções luminais e epiteliais, respectivamente, seguidas por um aumento do estroma. No testículo, a altura do epitélio germinativo mostrou-se diminuída após a restrição de sono, enquanto o diâmetro do túbulo seminífero não sofreu alteração. O número de células de Sertoli foi menor no grupo RS. O número de neutrófilos foi diminuído na cabeça/corpo do epidídimo do grupo RS, enquanto o número de neutrófilos na cauda e o número de macrófagos em ambas as regiões epididimárias foi semelhante entre os grupos, assim como o número de neutrófilos e macrófagos no testículo. A porcentagem de espermatozoides móveis foi menor no grupo restrito de sono. O número de espermatozoides no testículo e epidídimo, a produção diária espermática e o tempo de trânsito dos espermatozoides não sofreram alterações. A análise histopatológica testicular e epididimária, a dinâmica da espermatogênese e a morfologia espermática não foram afetadas pelo período de restrição de sono. Conclui-se que a restrição de sono durante a peripuberdade afeta o desenvolvimento pós-natal epididimário e testicular bem como a motilidade espermática, com papel crucial do aumeto do estresse oxidativo.

Palavras-chave: Sono. testículo. epidídimo. espermatozoide. puberdade.

SIERVO, Gláucia Eloisa Munhoz de Lion. **Effects of sleep restriciton during peripuberty on development of testis and epididymis of rats**. 2016. 88 p. Master's Degree Dissertation – Experimental Pathology Postgraduation Program – State University of Londrina, Londrina, 2016.

ABSTRACT

Puberty represents a period of complex sexual development, which leads to sexual maturation and reaching reproductive capability. Sleep alterations affect the neuroendocrine reproductive control and redox balance. The aim of this study is to evaluate whether sleep restriction during the peripubertal period could impair epididymal and testicular postnatal development in rats. Thirty male Wistar rats (postnatal day - PND 40) were used. The Sleep Restriction (SR) group was exposed to 21 days of sleep restriction by the modified multiple-platform method (18h of sleep restriciton and 6h of sleep, per day). The Control group (C) was maintained in their home-cages during all the experiment. At PND 62, the rats were weighed, anesthetized and euthanized. Testis and epididymis were removed, weighted and used to sperm count, oxidative stress markers, histological, esteriological and morphometric parameters as well as inflammatory profile evaluation (MPO and NAG activity). Spermatozoa from vas deferens were used to sperm morphology and motility. The final body weight of SR animals were decreased as well as absolute weight of vas deferens. The epididymal, testicular and seminal vesicles absolute weights were similar among the groups, while the relative testicular and epididymal weights were increased in SR group. The lipid peroxidation, total antioxidant capacity (TRAP) and GSH levels were increased in the caput epididymis in sleep restricted group. In the cauda epididymis, there was an increase in lipid peroxidation and GSH levels in the SR group, while the toal antioxidant capacity was similar between the groups. In testis, there is na increase in lipid peroxidation in sleep restricted animals, while the total antioxidant capacity remains unchanged. In the SR group there was a remodeling in 2A and 5A/B epididymal regions, with a decrease in lumial and epithelial portions, respectively, followed by a stromal augment. In testis, the seminiferous epithelium height were decreased after sleep restriction period, and the tubular seminiferous diameter did not change. The Sertoli cells number were decresed in SR group. The neutrophil number was diminished in caput/corpus epididymis in SR group, while the neurophil and macrophages number in cauda epididymis and macrophages number in both regions were similar between the groups, as well as the macrophages and neutrophil number in testis. The percentage of mobile spermatozoa decreased in the sleep restricted group. The sperm number in testis and epididymis, the daily sperm production and sperm transit time were not altered. The histopathological analysis in testis and epididymis, spermatogenesis kinetics and sperm morphology were not affected by sleep restriction period. We conclude that sleep restricion during peripuberty affects epididymal and testicular postnatal development as well as sperm motility, with a crucial role of oxidative stress.

Keywords: Sleep. testis. epididymis. spermatozoa. puberty.

LISTA DE ILUSTRAÇÕES

LISTA DE ABREVIATURAS E SIGLAS

$^{-1}O_2$	oxigênio singlet
-OH	radical hidroxila
DPN	dia pós-natal
ERO	espécies reativas de oxigênio
FSH	hormônio folículo-estimulante
GnRH	hormônio liberador de gonadotrofinas
GSH-Px	glutationa peroxidase
H_2O_2	peróxido de hidrogênio
HO_2	hidroperoxila
IL-1β	interleucina-1β
IL-10	interleucina-10
LH	hormônio luteinizante
LOOH	hidroperóxidos lipídicos
MDA	malondialdeído
NADPH	nicotinamida adenina dinucleótido fosfato
NO	óxido nítrico
NREM	não-movimento rápido dos olhos
NSQ	núcleo supraquiasmático
O_2^-	ânion superóxido
PAMPs	padrões moleculares associados a patógenos
PLOOH	hidroperóxidos fosfolipídicos
REM	movimento rápido dos olhos
SOD	superóxido diamutase
TGF-β	fator transformador do crescimento beta
TLR	receptores do tipo Toll
TNF	fator de necrose tumoral
T_{reg}	linfócitos T reguladores

SUMÁRIO

1 INTRODUÇÃO

1.1 SISTEMA GENITAL MASCULINO

O sistema genital masculino humano, assim como em roedores, é composto por testículos (gônadas), epidídimos, ductos deferentes, glândulas sexuais acessórias e órgão copulador (Figura 1).

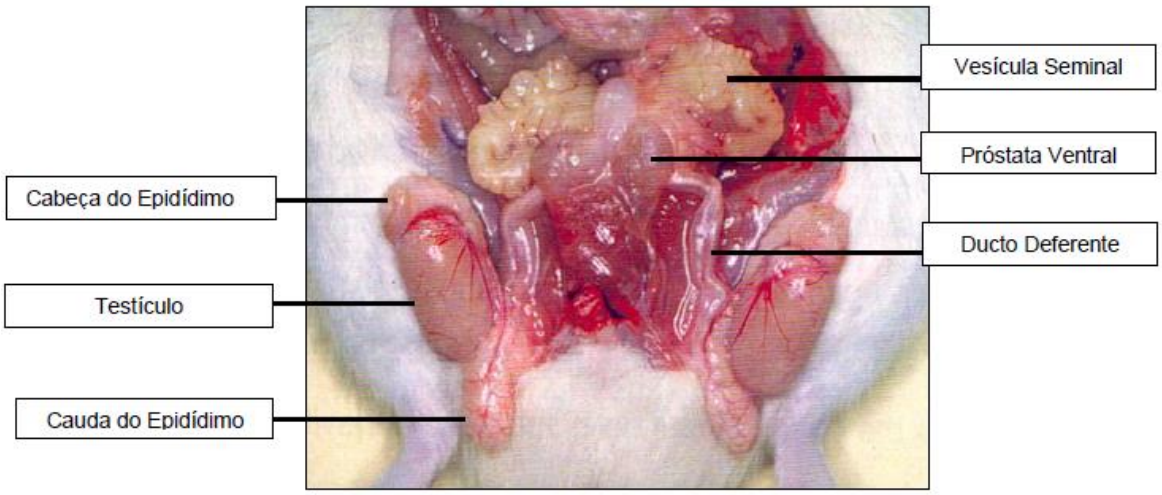

Figura 1 – Aspecto macroscópico do sistema genital masculino do rato (PEROBELLI, 2012).

Os testículos são órgãos pares localizados no interior do escroto, revestidos externamente por uma cápsula de tecido conjuntivo denso - a túnica albugínea. Externamente e adjacente à túnica albugínea encontra-se uma camada de peritônio visceral, a túnica vaginal, que também reveste a superfície interna da bolsa escrotal (KOMÁREK et al., 2000). Morfologicamente, os testículos são compostos por túbulos seminíferos e tecido intersticial, responsáveis pela espermatogênese e esteroidogênese, respectivamente (RODRIGUEZ; FAVARETTO, 1999).

Em humanos, o parênquima testicular é formado por uma série de lóbulos de tecido conjuntivo que compartimentalizam os túbulos seminíferos. No rato adulto, cada testículo apresenta em média 20 túbulos seminíferos, uma quantidade escassa de tecido conjuntivo e não apresenta lóbulos. No final de cada túbulo seminífero, observa-se um epitélio de transição, denominados túbulos retos, que conectam os túbulos seminíferos a uma rede de canais anastomosados, denominada rede testicular (FOLEY, 2001). Em animais adultos, os túbulos seminíferos são

constituídos por epitélio germinativo, composto por células somáticas de Sertoli e células germinativas (espermatogônias, espermatócitos primários, secundários e espermátides) que estão organizadas em camadas concêntricas (Figura 2).

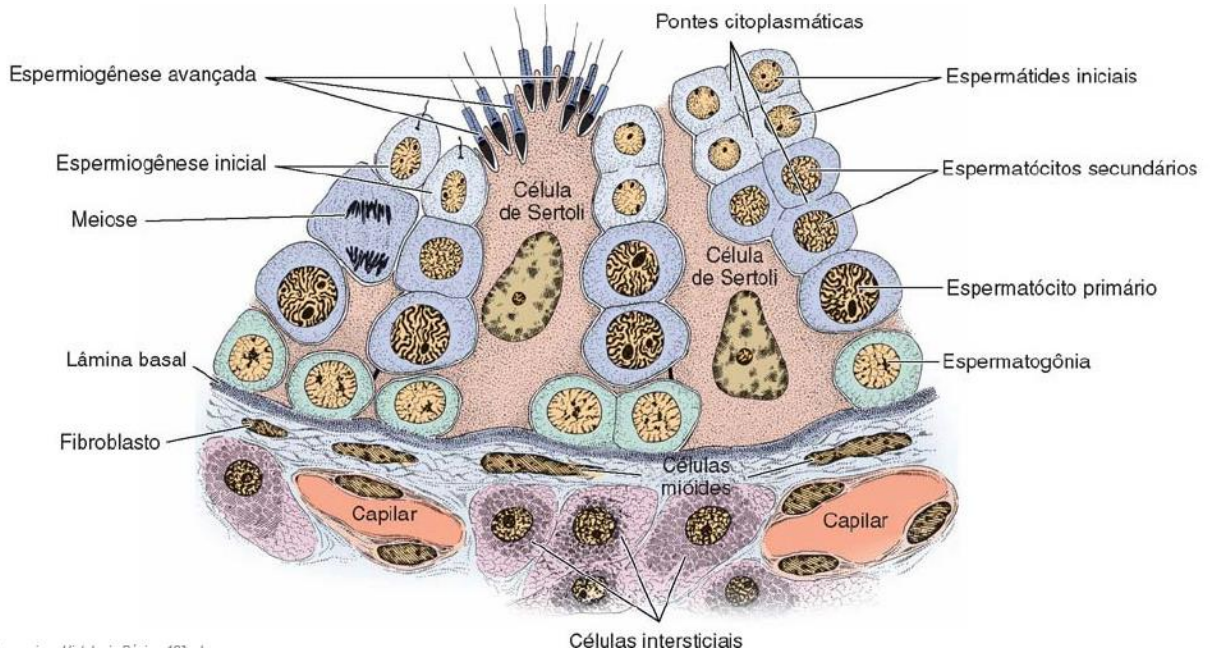

Figura 2 – Esquema de túbulo seminífero e da área intersticial. Os túbulos seminíferos são cercados por membrana basal, células peritubulares mioides e pelo tecido intersticial que contém células intersticiais (de Leydig), capilares sanguíneos e fibroblastos. Espermatogônias estão localizados na região basal do túbulo seminífero. Espermatócitos e espermátides e espermatozoides maduros estão localizados na porção apical às junções oclusivas das células de Sertoli, as quais compõe a barreira hemato-testicular. As células de Sertoli se projetam da lâmina basal basal até o lúmen do túbulo (JUNQUEIRA; CARNEIRO, 2004).

O interstício testicular é composto por tecido conjuntivo, vasos sanguíneos, linfáticos, nervos, macrófagos residentes e células de Leydig, responsáveis pela produção de andrógenos, substrato para uma variedade de outros hormônios esteroides, como a testosterona (RUSSEL et al., 1990).

A célula de Sertoli é uma célula somática presente nos túbulos seminíferos que se estende desde a lâmina basal até a luz. Possui funções variadas que são importantes no processo espermatogênico, como suporte nutricional e estrutural às células germinativas em desenvolvimento, a formação da barreira hematotesticular, fagocitose e a secreção de fluido e hormônios (FOLEY, 2001). Dessa forma,

alterações no número, estrutura e função deste tipo celular podem resultar no comprometimento da espermatogênese (BOEKELHEID et al., 2005).

O processo espermatogênico é dividido em três fases: proliferativa, meiótica e espermiogênica (CLERMONT, 1972; RUSSEL et al., 1990). A fase proliferativa ou mitótica é caracterizada pela proliferação de células tronco espermatogoniais com a finalidade de aumentar a população destas células (espermatogônias tipo A). Partes destas espermatogônias, as espermatogônias tipo B, ligadas entre si por pontes citoplasmáticas, diferenciam-se e formam os espermatócitos primários (AMANN, 1986; RUSSEL et al., 1990). Na fase meiótica, os espermatócitos primários (diploides) sofrem meiose I originando os espermatócitos secundários (haploides), que por sua vez sofrem meiose II formando espermátides arredondadas (haploides). As espermátides arredondadas passam por um processo de citodiferenciação, no qual ocorre condensação do material genético, formação do acrossoma, reposicionamento das mitocôndrias, perda de citoplasma e formação do flagelo, originando espermátides tardias, que permanecem presas ao epitélio germinativo. Este processo de citodiferenciação é denominado espermiogênese. Uma vez liberadas na luz dos túbulos seminíferos, as espermátides tardias passam a ser denominados espermatozoides (CLERMONT, 1972).

A duração total da espermatogênese em mamíferos, baseada em 4,5 ciclos espermatogênicos, é de aproximadamente 30 a 75 dias (SHARPE, 1993), sendo geralmente constante dentro de uma espécie (FRANÇA et al., 2005). Cada geração de células germinativas está em um mesmo estágio de desenvolvimento, ou seja, foram produzidas aproximadamente ao mesmo tempo e de maneira sincronizada. As várias gerações destas células formam associações celulares de composição fixa, denominadas estágios do ciclo da espermatogênese. No rato, o número de estágios da espermatogênese são 14, mas este número varia de acordo com a espécie (CLERMONT, 1972).

Este processo é diretamente regulado pelo eixo hipotalâmico-hipofisário-gonadal e inicia-se a partir da puberdade. A hipófise, por influência hipotalâmica, secreta dois hormônios sexuais que exercem o controle da função testicular, o LH (hormônio luteinizante) e o FSH (hormônio folículo-estimulante) (RUSSEL et al., 1990). O FSH atua sobre as células de Sertoli, estimulando suas funções sobre a espermatogênese, e o LH sobre as células de Leydig, estimulando a produção de andrógenos, principalmente a testosterona (CHRISTENSEN; MASON, 1965;

LIPSETT, 1976). Em roedores pré-púberes, tanto a testosterona quanto o FSH podem aumentar o número de cálulas de Sertoli, sendo este último mais efetivo (SMITH; WALKER, 2015). Em ratos, o FSH apresenta atuação específica sobre o estágio inicial de desenvolvimento espermatogonial (MEACHEM et al., 1999), enquanto a testosterona regula principalmente a espermiogênese (KERR et al., 1992). Estes hormônios atuam em cooperação nos demais estágios da espermatogênese, estimulando o início do processo meiótico e promovendo o desenvolvimento de espermatócitos e a produção de espermátides arredondadas (KERR et al., 1992) (Figura 3).

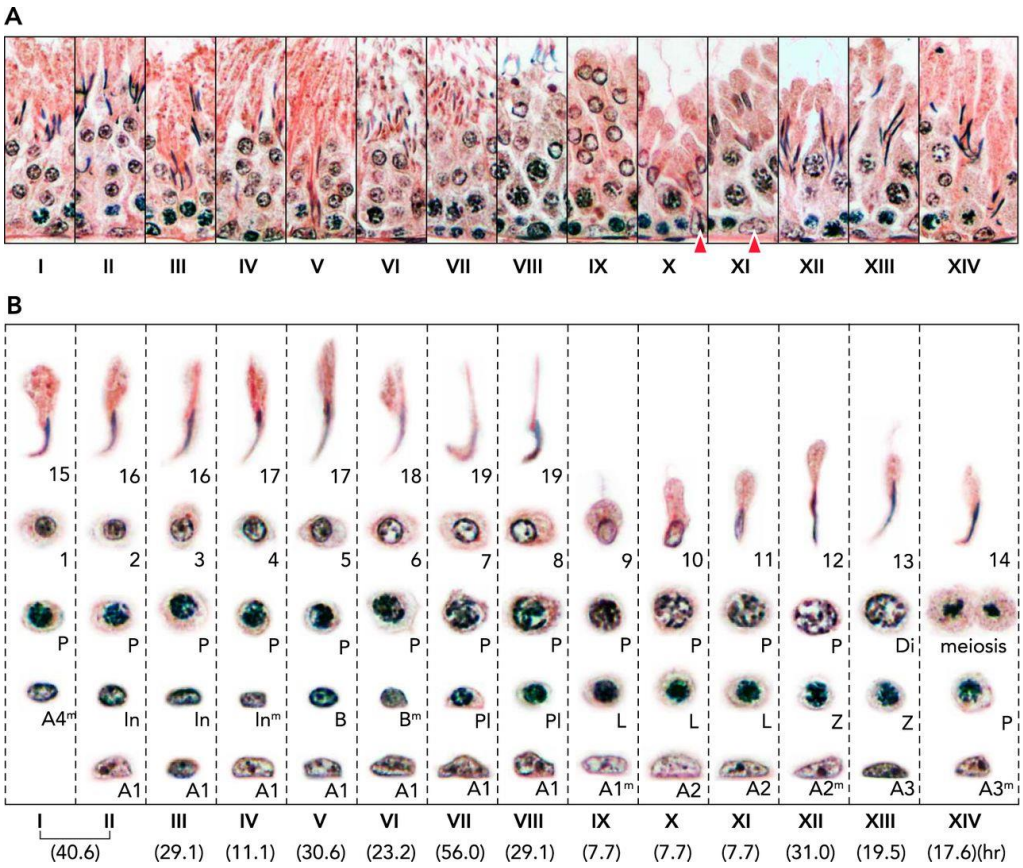

Figura 3 – O ciclo do epitélio seminífero da espermatogênese. (A) Cada fase do ciclo do epitélio seminífero ilustra a associação única de células germinativas específicas com a célula de Sertoli (ponta de seta vermelha). (B) Mostra os diferentes tipos de células germinativas que são encontrados em cada fase do ciclo. Por exemplo, na fase VIII, com duração de aproximadamente 29,1 horas no rato, espermátides 19 alinham-se próximo ao lúmen tubular para a espermiação. Todo o ciclo de I a XIV leva aproximadamente 12,9 dias para se completar. Porém, para que uma espermatogônia A tipo II se torne uma espermátide 19, são necessários cerca de 4,5 ciclos, o que leva por volta de 58 dias (adaptado de XIAO et al., 2014).

Nos testículos, os túbulos seminíferos se convergem e formam a rede testicular, a qual se continua com os ductulos eferentes. Por sua vez, estes ductulos convergem para formar um ducto único e altamente enovelado, o epidídimo, que vai se ligar através de sua porção terminal ao ducto deferente. O comprimento do ducto epididimário varia de acordo com a espécie, sendo 3 metros em ratos e aproximadamente 6 metros no homem (ROBAIRE et al., 2006).

Em roedores, este órgão é dividido em segmento inicial, cabeça, corpo e cauda (ROBAIRE et al., 2015). Utilizando os mesmos critérios, o epidídimo humano é dividido em apenas três regiões, denominadas cabeça, corpo e cauda. A ausência de segmento inicial se deve ao fato da maior parte da cabeça do epidídimo humano ser constituída por ductos eferentes (TURNER, 2008). Estas porções são histologicamente subdivididas em zonas, sendo: 1A, 1B, 2A, 2B, 3A e 3B para a região da cabeça; 4A e 4B para a região do corpo; 5A, 5B, 6A e 6B para a região da cauda (MILLER; KILLIAN, 1987). As zonas são designadas de acordo com a altura do epitélio e distribuição e quantidade dos seus seis tipos de células (REID; CLEAND, 1957) os quais são: basais, principais, estreitas, halo, claras e apicais (HERMO; ROBAIRE, 2002).

Quando os espermatozoides deixam os testículos são morfologicamente completos, mas ainda não exibem atividade móvel e são incapazes de fecundar um ovócito II (DACHEUX; DACHEUX, 2015). Neste contexto, além do transporte, o epidídimo tem papel crucial sobre a maturação espermática, regulando o desenvolvimento da motilidade, da capacidade de sofrer reação acrossômica e de reconhecer e fundir-se com o ovócito, além de ser sítio de importantes modificações da membrana plasmática dos espermatozoides. Em adição, o epidídimo protege os espermatozoides de espécies reativas de oxigênio (ROS) e estoca os gametas maduros na região da cauda (COSENTINO; COCKETT, 1986; HERMO; ROBAIRE, 2002; ROBAIRE et al., 2006). Estas funções são executadas dentro dos diferentes ambientes luminais presentes ao longo do ducto epididimário (RODRIGUEZ et al., 2002).

A maturação espermática no ducto epididimário parece depender de uma interação altamente regulada entre a lâmina própria, células epiteliais e fluido luminal que banha os espermatozoides (BEDFORD, 1975; ORGEBIN-CRIST et al., 1975; KEMPINAS; KINEFELTER, 2010). Este processo consiste em um intenso remodelamento da membrana do espermatozoide, na qual proteínas de origem

testicular são removidas ou modificadas, e proteínas epididimárias são secretadas e adsorvidas à membrana espermática ou apenas interagem com o gameta, a fim de que este adquira sua capacidade funcional (CUASNICÚ et al., 2002; DA ROS et al., 2004; BARRIOS et al., 2005). Cada etapa de maturação, ainda pouco elucidada, é determinante para a qualidade espermática e não está sob o controle genômico das células germinativas (DACHEUX et al, 2005; ROBERTS, 2010). O microambiente intraluminal do epidídimo é rigorosamente regulado pela atividade secretora e absortiva das células epiteliais (ROBAIRE et al., 2006). O processo de maturação do epidídimo é dependente da ação de andrógenos (EZER; ROBAIRE, 2002).

O ducto deferente é um órgão par que liga o epidídimo à uretra prostática. Durante muito tempo foi considerado um simples condutor dos espermatozoides no momento da ejaculação, no entanto novos estudos têm mostrado importantes funções do epitélio do ducto em relação aos espermatozoides, como término do processo de maturação espermática (KOMÁREK et al., 2000). O ducto deferente se insere na próstata e se abre na uretra prostática. O seguimento que entra na próstata é chamado de ducto ejaculatório, cuja mucosa é semelhante a do ducto deferente, porém não é envolta por músculo liso (JUNQUEIRA; CARNEIRO, 2004).

Em roedores, as glândulas sexuais são a glândula seminal, próstata, glândula coaguladora, glândula bulbouretral e glândula prepucial. Elas estão localizadas ao longo do trajeto que os espermatozoides fazem pela uretra, ou seja, do ducto deferente ao pênis (HASCHEK; ROUSSEAUX, 1998). As funções das glândulas sexuais acessórias estão principalmente relacionadas à produção de secreções, que contribuem para a nutrição e suporte dos espermatozoides pós-ejaculação, ou seja, fora do sistema genital masculino. Estas funções são dependentes da ação de andrógenos e por isso refletem mudanças do estado endócrino e/ou da função testicular (MANN, 1974; CLEGG et al., 2001).

Nos mamíferos, o sêmen é depositado diretamente no trato genital feminino pelo órgão copulador, o pênis. O corpo do pênis do rato é formado por dois corpos cavernosos penianos e um corpo cavernoso uretral, ou seja, dois ramos do pênis, um corpo esponjoso e a uretra (CHIASSON, 1969). Histologicamente, o corpo cavernoso é constituído por tecido erétil com auréolas calibrosas, revestidas por uma espessa camada de tecido conjuntivo denso, a túnica albugínea (MURAKAMI; MIZUNO, 1986). Além disso, o pênis do rato apresenta um osso peniano localizado centralmente na glande. Este osso inicia-se na transição do corpo para a glande,

como uma continuação do corpo cavernoso do pênis (HEBEL; STROMBERG, 1976). No homem, o pênis é composto por três colunas de tecido erétil, sendo duas colunas chamadas de corpos cavernosos, localizadas dorsalmente e uma coluna chamada de corpo esponjoso, localizada ventralmente e onde está inserida a porção peniana da uretra. Cada qual está contida dentro de uma capsula de tecido conjuntivo fibroso, a túnica albugínea. A região distal do corpo esponjoso termina em uma porção bulbosa dilatada, a glande do pênis, que é perfurada em sua extremidade formando o óstio da uretra (GARTNER; HIATT, 2007).

1.2 PUBERDADE E DESENVOLVIMENTO PÓS-NATAL DO SISTEMA GENITAL MASCULINO

O período de desenvolvimento do rato pode ser dividido em cinco fases: pré-natal, neonatal, infantil, puberdade e adulto (SHARPE, 2010). Todas as fases são reguladas por mecanismos relacionados com estados hormonais e genéticos do organismo (DAMGAARD et al., 2002).

Na fase da puberdade, ocorrem eventos dinâmicos e complexos do desenvolvimento sexual, que envolvem mudanças físicas, comportamentais e hormonais, através das quais a maturação sexual ocorre e a capacidade reprodutiva é obtida (GOLUB et al., 2008). Estas mudanças são estabelecidas devido à ocorrência de uma cascata de eventos que levam à maturação do eixo hipotalâmico-hipofisário-gonadal e consequentemente ao início do ciclo reprodutivo da espécie (OJEDA; URBANSKI, 1994). Biologicamente, a puberdade é definida como o tempo em que a primeira espermatogênese completa todo o ciclo (KLINEFELTER et al., 1997).

Durante a puberdade ocorrem mudanças no perfil hormonal, especialmente na síntese e secreção de esteroides (testosterona) em resposta ao aumento da pulsatilidade do GnRH (hormônio liberador de gonadotrofinas) e ao aumento da síntese e secreção de LH e FSH (OJEDA; URBANSKI, 1994).

Na fase peripuberal ocorrem importantes eventos tais como: leve redução na proliferação e desenvolvimento das células germinativas, e um aumento nos níveis de testosterona e proliferação de células de Sertoli em humanos (SHARPE, 2010; O'SHAUGHNESSY, 2015). No entanto, esta fase foi ainda relativamente pouco estudada (PEROBELLI, 2014)

Os mecanismos que regem o início da puberdade no rato macho ainda não são totalmente compreendidos, porém já existem relações entre os elementos do eixo hipotalâmico-hipofisário-gonadal que iniciam a sua interação nas primeiras semanas de vida pós-natal (ROBB et al., 1978). Após o nascimento, o desenvolvimento é classificado em quatro fases: neonatal (dia pós-natal – DPN 1 – 7), Infantil (DPN 8-21), juvenil (DPN 22-35), peripuberal (DPN 36 – 55 ou 65) e adulto, onde atinge sua maturidade sexual (OJEDA et al., 1980).

As primeiras espermátides maduras no testículo são encontradas no DPN 40, enquanto nos epidídimos os espermatozoides são observados apenas no DPN 50. Com base nestes relatos, o rato macho pode atingir a puberdade por volta de 50 dias de idade (ROBB et al., 1978).

Ratos com 75 dias de idade apresentam a máxima produção de espermatozoides no testículo e aos 100 dias, a máxima concentração de espermatozoides armazenados na cauda do epidídimo, atingindo neste período a maturidade sexual plena (ROBB et al., 1978; ZANATO et al., 1994).

Como a espermatogênese e a esteroidogênese ainda não estão totalmente estabelecidas durante a peripuberdade, esta pode ser uma fase crítica do desenvolvimento reprodutivo (JOHNSON et al., 1997). Assim, qualquer alteração nos eventos característicos que ocorrem nesta fase pode acarretar sérios prejuízos e comprometer a reprodução do indivíduo na vida adulta.

1.3 SONO E FALTA DE SONO

O sono é um fenômeno biológico que ocupa cerca de um terço da vida de um ser humano e em outros mamíferos, como roedores, essa proporção chega até em dois terços. Esse comprometimento de tempo é um dos indicadores de seu papel crucial no organismo (SIEGEL, 2008; TUFIK et al., 2009). Biologicamente, caracteriza-se pela adoção de uma postura específica da espécie, abolição prontamente reversível da consciência, elevação do limiar de respostas ao meio ambiente, imobilidade e pela geração de padrões característicos na atividade eletroencefalográfica (SIEGEL, 2008).

Alternando-se de forma rítmica com o estado de vigília em mamíferos, o sono se desenrola em diversas fases consecutivas, sendo composto por duas fases principais: movimento rápido dos olhos (rapid eye moviment – REM) e não-REM (NREM). As fases 1 e 2 são as fases mais leves do sono NREM e ocorrem

primeiramente, muitas vezes alternando-se com breves episódios de vigília. Os estágios mais profundos (fases 3 e 4) do sono NREM ocorrem predominantemente no início da noite, enquanto o sono REM aparece em intervalos de aproximadamente 90 minutos. Normalmente, ocorrem de 4 a 6 destes ciclos a cada noite, com episódios de sono REM se tornando mais longos no decorrer da noite e episódios de sono NREM se tornando mais curtos e mais leves (ANDERSEN et al., 2011).

Embora seja usualmente visto como uma condição passiva, o sono é um processo ativo que requer a ativação e desativação sincronizada de muitos centros nervosos localizados no hipotálamo e tronco cerebral, com modificação direta da atividade elétrica cortical. O sono NREM é caracterizado pela supressão quase completa da liberação de acetilcolina e uma redução na concentração de noradrenalina e serotonina (MCGINTY; SZYMUSIAK, 2001). É chamado também de sono de ondas lentas devido à presença de atividade eletroencefalográfica de alta voltagem e baixa frequência, tônus muscular diminuído e movimento lento dos olhos (GÓMEZ-GONZÁLEZ et al., 2013). No sono REM ocorre uma diminuição da liberação de noradrenalina e serotonina e um aumento na liberação de acetilcolina na ponte; no hipotálamo, neurônios histaminérgicos e orexinérgicos são silenciados durante o sono REM em comparação com a vigília (REINOSO-SUÁREZ et al., 2001). É observada ainda a atividade elétrica cerebral de alta frequência e baixa voltagem, acompanhado pelo rápido movimento dos olhos e atonia muscular nos músculos esqueléticos (GÓMEZ-GONZÁLEZ et al., 2013). Além das mudanças observadas na liberação de neurotransmissores, uma íntima relação recíproca entre o sono e o sistema endócrino foi descrita em modelos humanos e animais. Tem sido demonstrado que a secreção hormonal da hipófise ocorre durante determinadas fases de sono nos humanos e, reciprocamente, a perda de sono modifica suas concentrações plasmáticas (STEIGER, 2007). O sono também é capaz de influenciar os níveis de citocinas plasmáticos tanto em condições normais quanto patológicas (IMERI; OPP, 2009).

O relógio biológico é sincronizado pelo núcleo supraquiasmático (NSQ), localizado no hipotálamo. Os neurônios do NSQ são estimulados pela luz proveniente do ambiente que chega até a retina, e quando há diminuição na quantidade de luz recebida, os neurônios respondem estimulando a glândula pineal, que produz e libera o hormônio melatonina. O aumento de melatonina está

associado com um aumento da sonolência (GANZ, 2012). Além de modular os níveis plasmáticos hormonais e o sistema imune, existe uma estreita interação entre o ciclo sono-vigília com a temperatura central e a pressão arterial (ZISAPEL, 2007).

Um conjunto considerável de evidências clínicas sugere ainda que o sono insuficiente aumenta o risco de hipertensão, aterosclerose e resistência à insulina, bem como a remodelação das regiões cerebrais que participam na regulação da memória e ansiedade (CHANG et al., 2008). Os mecanismos detalhados para estas alterações sistêmicas ainda não estão totalmente esclarecidos, porém foi proposto que a restrição do sono pode aumentar o gasto energético bem como a atividade metabólica. O metabolismo celular exacerbado pode perturbar a homeostasia e resultar na geração de grandes quantidades de espécies reativas de oxigênio (ERO) com consequente aumento do estresse oxidativo (FRISARD; RAVUSSIN, 2006; MCEWEN, 2006). Várias teorias sugerem que a privação de sono leva ao dano oxidativo em áreas distintas do cérebro e que o sono diminui o estresse oxidativo, removendo as espécies oxidantes que são produzidas durante os momentos de vigília (VOLLERT et al., 2011). Dessa forma, a privação crônica de sono diminui o mecanismo antioxidante de defesa cerebral, contribuindo para o aumento do estresse oxidativo nesta região (ALZOUBI et al., 2012).

Embora a duração do sono diminua nos primeiros 10 anos de vida no ser humano, o tempo de sono parece não diminuir durante a adolescência (em média 9 horas por noite) (JENNI; CARSKADON, 2012). O padrão de sono característico dos adolescentes é a tendência em dormir mais tarde do que as crianças mais novas, mesmo quando os tempos vigília são diminuídos por atividades escolares ou pelo trabalho (HAGENAUER; LEE, 2013).

A prevalência de distúrbios do sono tem aumentado nas sociedades modernas, onde a exposição constante à luz artificial e atividades interativas, como televisão e internet, combinam com as pressões sociais e econômicas para encurtar o tempo do sono (TUFIK et al., 2009). Dessa forma, muitos indivíduos podem estar cronicamente privados de sono em virtude de seu estilo de vida (MIRÓ; CANO-LOZANO; BUELA-CASAL, 2002).

Atualmente, acredita-se que as crianças não estão dormindo o suficiente e que o seu tempo total de sono vem diminuindo (MATRICCIANI et al., 2012). A literatura mostra que acontecem profundas mudanças na regulação do ciclo sono-vigília e na biologia do ritmo circadiano durante a adolescência. Fatores externos,

por exemplo, a influência dos pais e os horários escolares precoces também contribuem para o sono insuficiente em adolescentes (OWENS, 2014). Além disso, segundo a National Sleep Foundation (2014), 8% das crianças entre 6 e 11 anos e mais de 29% das crianças de 12 a 14 anos dormem menos de 8h por noite.

1.4 Relação entre sono e sistema genital masculino

A testosterona é capaz de afetar a duração e a qualidade do sono. Da mesma forma, a restrição de sono tanto em homens quanto em animais experimentais está associada a concentrações de andrógenos reduzidas e, consequentemente, podem afetar funções dependentes deste hormônio (ANDERSEN et al., 2011).

A literatura científica relata que os ritmos circadianos são regulados por um relógio interno presente no NSQ do hipotálamo, e falhas nessa regulação pode levar a efeitos graves para a saúde (KARATSOREOS; SILVER, 2007). No NSQ, hormônios androgênicos agem via receptores de andrógenos (AR) e modulam a resposta à entrada de luz e, portanto, a regulação da atividade circadiana em machos (DUFFY et al., 2011). Os padrões de sono mudam ao longo da vida, sugerindo que a ascensão e queda de hormônios sexuais durante toda a vida pode influenciar o tempo dos ciclos de sono-vigília (ROENNEBERG et al., 2004).

A produção e liberação de testosterona mostram ritmos circadianos e ultradianos (BORST; MULLIGAN, 2007). A variação circadiana consiste em um pico de testosterona durante a manhã com constante queda até o período noturno. O ritmo ultradiano é cíclico, onde a concentração de testosterona oscila a cada 90 minutos aproximadamente (ANDERSEN et al., 2011; BORST; MULLIGAN, 2007). Durante o início do sono, as concentrações de testosterona sobem e chegam a um platô no início do sono REM, cerca de 90 minutos mais tarde (ANDERSEN et al., 2011). Foi relatado ainda que a liberação cíclica de testosterona noturna está relacionada aos ciclos de sono profundo e REM/NREM (LUBOSHITZKY et al., 2001). Sabe-se que o sono REM não desencadeia a produção de testosterona, porém parece haver uma ligação entre o estado neurofisiológico do sono REM e os mecanismos regulatórios envolvidos na síntese de testosterona (VELDHUIS et al., 2000).

Além da testosterona, a liberação de LH e FSH também apresenta influência circadiana e seus níveis séricos começam a aumentar antes do início da puberdade (MITAMURA et al., 2000).

Dessa forma, os mecanismos de produção e liberação de hormônios sexuais, importantes para o desenvolvimento pós-natal adequado do sistema genital, estão diretamente ligados ao sono.

1.5 SISTEMA GENITAL MASCULINO E ESTRESSE OXIDATIVO

O estresse oxidativo é considerado uma importante condição fisiopatológica que pode promover uma variedade de desordens celulares, inclusive morte celular (MELCHIORRI *et al.,* 1996). Esta condição provém de um desequilíbrio entre espécies oxidantes, como as espécies reativas de oxigênio (ERO), e as espécies antioxidantes.

As ERO, também conhecido como radicais livres, possuem pelo menos um elétron desemparelhado. São agentes oxidantes gerados como subprodutos do metabolismo do oxigênio. Devido ao seu elétron desemparelhado eles são capazes de formar moléculas altamente reativas (HENKEL, 2011; MIRANDA-VILELA et al., 2010). As ERO representadas principalmente pelo oxigênio singlet ($^{-1}O_2$), radical hidroxila (-OH), ânion superóxido (O_2-), hidroperoxila (HO_2), peróxido de hidrogênio (H_2O_2), hidroperóxidos lipídicos (LOOH) e hidroperóxidos fosfolipídicos (PLOOH). Os elementos antioxidantes podem ser endógenos não-enzimáticos (vitamina E ou α-tocoferol, vitamina C ou ácido ascórbico, vitamina A, carotenos, bilirrubinas, ácido úrico e albumina) e enzimáticos, como superóxido diamutase (SOD), glutationa peroxidase (GSH-Px) e catalase (DAMASCENO, et al. 2002; NAZIROĞLU, 2003).

Em altas concentrações, as ERO são extremamente tóxicas para as células, causando danos na molécula de DNA, peroxidação lipídica, degradação proteica (SUN, 1990), podendo afetar diversos tipos de moléculas biológicas (DAMASCENO, et al. 2002). Porém, em condições fisiológicas as ERO estão envolvidas em processos bioquímicos normais, como controle da proliferação e sinalização celular (FINKEL, 1998).

A geração de ERO nos espermatozoides pode ocorrer de duas maneiras: (1) o sistema nicotinamida adenina dinucleótido fosfato oxidase (NADPH oxidase) ao nível da membrana plasmática do espermatozoide; e (2) a reação oxido-redutase dependente de NADPH a nível mitocondrial. O último mecanismo se mostra como a principal fonte de ERO espermática (AGARWAL et al., 2014; VERNET et al., 2001).

O ânion superóxido é formado a partir do oxigênio molecular, pela adição de um elétron, de forma espontânea, pincipalmente pela membrana mitocondrial, através da cadeia respiratória (NORDBERG & ARNÉR, 2001). É uma das principais ERO geradas pelos espermatozoides (AITKEN, 1995).

Os espermatozoides expressam uma série de enzimas redox durante todo seu desenvolvimento no testículo e epidídimo, e utilizam sua capacidade antioxidante nos diferentes ambientes pelo qual percorrem, desde o epidídimo até o trato genital feminino. Além disso, o plasma seminal está entre os fluidos corporais mais enriquecidos em antioxidantes de baixo peso molecular (CONRAD et al., 2014).

Durante o processo de capacitação, os espermatozoides inevitavelmente passam por um processo de estresse oxidativo, devido ao aumento na produção de ERO, como o ânion superóxido e o peróxido de hidrogênio (LEWIS; AITKEN, 2001). Essa produção tem um papel fisiológico importante e desempenha controle tanto da capacitação espermática quanto da reação acrossômica (GRIVEAU et al., 1995). Acredita-se que as ERO induzam a fosforilação da tirosina, que é um evento bioquímico chave para os processos de capacitação espermática (LEWIS; AITKEN, 2001). Em humanos, o estímulo na produção de ERO melhora a fosforilação da tirosina, enquanto uma redução nesta produção teve efeito oposto (AITKEN et al., 1995). Porém, a geração excessiva de ERO pelos espermatozoides é associada com prejuízos na função destes e com danos ao DNA da linhagem de células germinativas (SHARMA; AGARWAL, 1996; AITKEN, 1999).

Além disso, a restrição do sono também pode alterar o balanço redox do organismo por aumentar o gasto energético e a atividade metabólica. O metabolismo celular exacerbado perturba a homeostasia e resulta na geração de grandes quantidades de ERO, com consequente aumento do estresse oxidativo (FRISARD; RAVUSSIN, 2006; MCEWEN, 2006). Várias teorias sugerem que a falta de sono leva ao dano oxidativo em áreas distintas do cérebro e que o sono diminui o estresse oxidativo, removendo as espécies oxidantes que são produzidas durante os momentos de vigília (VOLLERT et al., 2011). No sistema genital masculino, foi relatada que a restrição de sono aumenta a peroxidação lipídica testicular (malondialdeído - MDA) (AKINDELE et al., 2014).

1.6 SISTEMA GENITAL MASCULINO E INFLAMAÇÃO

A inflamação é uma resposta natural do organismo a uma variedade de danos teciduais, incluindo aqueles mediados por patógenos, substâncias, disfunção celular e trauma físico. Esta resposta envolve a ação de múltiplos tipos celulares, residentes e recrutados. O local afetado sofre modificações fisiológicas, resultantes das alterações na perfusão vascular, na permeabilidade e na produção de mediadores locais de inflamação, que irão promover o influxo de células imunes. A resposta inflamatória, quando bem sucedida, resulta na resolução do dano inicial, seguida da restauração da homeostase tecidual (KARALIS et al., 2009).

É bem estabelecido que infecções sistêmicas podem ter efeitos inibidores sobre a função reprodutora masculina. De maneira indireta, os mediadores inflamatórios como interleucina-1**β** (IL-1**β**), óxido nítrico (NO), fator de necrose tumoral (TNF) e ERO podem afetar o eixo hipotalâmico-hipofisário-testicular e reduzirem a produção de hormônios androgênicos (HEDGER, 2011).

Durante infecções, uma das vias que desencadeia a inflamação e a ativação do sistema imune é o reconhecimento de padrões moleculares associados a patógenos (PAMPs), presente em bactérias, vírus, fungos e protozoários. Este reconhecimento é mediado por receptores específicos, sendo os receptores do tipo Toll (TLR) o melhor caracterizado. Os TLRs são encontrados principalmente em células mieloides (monócitos, macrófagos, células dendríticas), mas também são expressos por células epiteliais como as células de Sertoli testiculares e células do epitélio epididimário (KAWAI; AKIRA, 2010; HEDGER, 2011)

No testículo normal, as células imunes são encontradas exclusivamente no tecido intersticial, zona peritubular e cápsula testicular. Estas células incluem macrófagos residentes, células dendríticas e linfócitos circulantes, com presença variável de mastócitos e eosinófilos, dependendo da espécie (WANG et al., 1994; ANTON et al., 1998). A maioria dos macrófagos residentes no testículo tem uma atividade pró-inflamatória reduzida, liberando preferencialmente citocinas de caráter anti-inflamatório (TGF-**β** e IL-10) e expressam marcadores de superfície compatíveis com o fenótipo alternativamente ativado ou M2 (BRYNIARSKI et al., 2004; MARESZ et al., 2008). Estes macrófagos exercem um importante papel na regulação das respostas aos antígenos no ambiente testicular, juntamente com os linfócitos T reguladores (T_{reg}), também presentes em quantidade significativa neste órgão (HEDGER, 2011).

Ao contrário do que se observa no testículo, macrófagos e linfócitos são frequentemente observados tanto no epitélio quanto no tecido intersticial epididimário, sendo comumente identificados em cortes histológicos como células halo (FLICKINGER et al, 1997; SERRE; ROBAIRE, 1999). Os linfócitos intra-epiteliais presentes no epidídimo de ratos, camundongos e humanos são predominantemente células T $CD8^{+}$, e o aumento no número destes linfócitos pode estar relacionado com distúrbios espermáticos (SERRE; ROBAIRE, 1999). Além disso, é descrito que os macrófagos presentes no interior do ducto epididimário possuem função de fagocitar espermatozoides malformados ou senescentes (HEDGER, 2011; ROBAIRE; HINTON; ORGEBIN-CRIST, 2015)

O papel do plasma seminal em relação ao sistema imunológico é contraditório. Por um lado, a presença de sêmen no sistema genital feminino, no prazo de algumas horas, desencadeia uma maciça infiltração leucocitária e subsequente fagocitose de espermatozoides senescentes. Por outro lado, o plasma seminal possui propriedades imunossupressoras que inibem a resposta imunológica materna contra espermatozoides (AITKEN; BAKER, 2013).

A considerável diferença entre a distribuição de células imunes no testículo e no epidídimo indica claramente a existência de um microambiente imunológico característico em cada órgão, sendo que a manutenção deste microambiente é fundamental para a qualidade espermática adequada.

2 JUSTIFICATIVA

Alterações no estilo de vida ocasionadas pela modernidade, como distúrbios no sono, vêm aumentando nos últimos anos. Uma das consequências disto é a diminuição da fertilidade masculina pelo declínio da qualidade espermática e da funcionalidade dos órgãos genitais. Neste contexto, a má qualidade do sono destaca-se pelo fato de alterar a homeostase metabólica e endócrina. Indivíduos do sexo masculino expostos à restrição de sono podem ter alterações reprodutoras endócrinas, morfológicas e fisiológicas na idade adulta, mas principalmente durante o desenvolvimento dos órgãos do sistema genital masculino (período pré-natal ou pós-natal) onde as células ainda não estão totalmente diferenciadas. As principais alterações no sistema genital derivadas da má qualidade e quantidade do sono são modificações na produção e secreção de hormônios sexuais (LH, FSH e testosterona). Embora as alterações descritas acima sejam evidenciadas, os estudos na literatura até o presente não avaliam com exatidão os impactos da restrição de sono no desenvolvimento e funcionalidade do sistema genital masculino.

Foi observado ao longo dos últimos anos que alterações no sono são bastante comuns em muitas sociedades, inclusive entre crianças e adolescentes. Essas alterações devem-se principalmente aos horários escolares, considerados por muitos especialistas neste assunto como inadequados, uma vez que a tendência de crianças e adolescentes é dormir mais tarde e por mais tempo. Outro fator que afeta o tempo e o padrão de sono são as novas tecnologias, que contribuem para esse atraso na hora de dormir e para a fragmentação do sono, uma vez que aparelhos eletrônicos podem emitir sons e luzes durante a noite. Dessa forma alterações nos níveis hormonais, tais como a testosterona, são esperadas em indivíduos na puberdade.

O presente estudo possui grande aplicabilidade ao avaliar se a restrição de sono durante o desenvolvimento pós-natal do sistema genital masculino trará danos celulares, teciduais ou fisiológicos para este sistema, especificamente para os testículos e epidídimos, em ratos peripuberais.

3 OBJETIVOS

3.1 GERAL

A partir da falta de informações específicas na literatura especializada e da relevância clínica e social do assunto, o objetivo deste estudo foi avaliar se restrição de sono durante o período peripuberal traz prejuízo ao desenvolvimento testicular e epipidimário de ratos.

3.2 ESPECÍFICOS

- Avaliar a morfologia do testículo através de análises histopatológicas e morfométricas;
- Avaliar a morfologia epididimária pelas análises estereológica e histopatologia;
- Analisar a fisiologia testicular a partir de análises da produção diária de espermatozoides;
- Analisar o tempo de transito espermático no epidídimo;
- Avaliar a qualidade espermática através de análises de morfologia e motilidade;
- Quantificar os marcadores de estresse oxidativo no testículo e epidídimo;
- Realizar análises de perfil inflamatório no testículo e epidídimo;
- Contribuir com dados sobre os efeitos e mecanismos de ação da restrição de sono no desenvolvimento pós-natal do testículo e epidídimos de ratos.

4 ARTIGO I

Restrição de sono em ratos *Wistar* prejudica o desenvolvimento pós-natal do epidídimo e a motilidade espermática em associação com o estresse oxidativo

Sleep restriction in *Wistar* rats impairs epididymal postnatal development and sperm motility in association with oxidative stress

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Sleep restriction in *Wistar* rats impairs epididymal postnatal development and sperm motility in association with oxidative stress

Running tittle: Impairment of epididymis by sleep restriction

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Abstract

Good sleep quality has a direct effect on the activity of neuroendocrine reproductive control axis and on oxidative stress. Thus, the aim of this study was to evaluate whether sleep restriction (SR) during the peripubertal period can impair the postnatal development of the epididymis in *Wistar* rats. For this, after 21 days of SR (18h per day) epididymides were collected at PND 62 for evaluation of oxidative stress markers, inflammatory profile, sperm count, and histopathological and stereological analysis and spermatozoa from vas deferens was used for sperm motility. The results showed that SR significantly increased lipid peroxidation and glutathione (GSH) levels in the caput and cauda epididymis, and total radical-trapping antioxidant parameter (TRAP) levels only in the caput epididymis. The neutrophil migration to caput/corpus epididymis was decreased by SR as well as the luminal compartment in the 2A region and the epithelial compartment in the 5A/B region. In these regions, the interstitial compartment was increased. The percentage of immobile sperm was higher in SR group. We conclude that SR affects epididymal postnatal development as well as sperm motility in association with an increase in oxidative stress and a decrease in the epithelial compartment in the cauda.

Keywords: sleep, puberty, epididymis, lipid peroxidation, inflammation, spermatozoa, rat

Introduction

During puberty a complex sexual development occurs, which involves physical, hormonal and behavioral changes, sexual maturation, and reproductive capability (Golub *et al.* 2008). In childhood and peripubertal phases, some important events occur, such as slow germ cell proliferation compared to prenatal period and subsequent development, and increase in testosterone levels and Sertoli cell proliferation (specifically at the prepubertal period) (Perobelli 2014; O'Shaughnessy 2015). In male rats, postnatal sexual development is divided into four stages: neonatal period (postnatal day - PND 0 to PND 7); infantile period (PND 8 to PND 21); juvenile period (PND 22 to PND 35); and peripubertal period (PND 36 to PND 55 – 60), at which time mature sperm appear in the vas deferens (Prevot 2015). The postnatal development of epididymis includes the undifferentiated period (PND 1 to PND 15), the period of differentiation (PND 16 to PND 44) and the period of expansion (PND 45 to adult) (Sun and Flickinger, 1979).

In rodents, the sleep need is around 12 h per day (Yasenkov and Deboer, 2012). In humans, although sleep duration decreases in the first 10 years of life, the need for sleep does not seem to decline in the course of adolescence (an average of 9 h) (Jenni and Carskadon 2012) and perhaps the best known characteristic of teenage sleep patterns is the tendency to stay up late (Hagenauer and Lee 2013). According to the National Sleep Foundation (2014), 8% of 6- to 11-year-old children and up to 29% 12- to 14-year-old teenagers sleep less than 8 h per night. Early school hours could be the cause of insufficient sleep (Dexter *et al.* 2003, Owens 2014), in addition to many modern social and technological features (Van den Bulck 2003; Hagenauer and Lee 2013; Owens 2014).

In this context, good sleep quality has a direct effect on the activity of neuroendocrine reproductive control axis, allowing the optimal regulation of hormonal homeostasis (Andersen *et al.* 2011), and reactivation of the reproductive axis during puberty is closely related to sleep (Shaw *et al.* 2015). Despite this consensus, it remains to be determined the effect of sleep restriction (SR) on epididymal functions or development.

Furthermore, sleep disorders are commonly associated with various metabolic disturbances (McEwen 2006). These disturbances are frequently related to oxidative stress (McEwen 2006; Chang *et al.* 2008), mainly in some regions of the central nervous system (CNS) (Vollert *et al.* 2011). The reactive oxygen species (ROS) produced during the wake cycle are removed during sleep (Alzoubi *et al.* 2012). Under high concentrations, ROS are extremely toxic to cells leading to lipid peroxidation, DNA damage and protein degradation as well as damage to many other biological molecules (Sun 1990).

Thereby, due to the few studies which analyzed the impact of sleep loss on the male reproductive system, especially during epididymal development, and the relevance of this condition in modern life, the aim of this study was to evaluate whether SR during the peripubertal period can impair the postnatal development of the epididymis.

Materials and Methods

Ethical approval

Experimental procedures were in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and were approved by the Ethics Committee on Animal Use of State University of Londrina (CEUA/UEL protocol number 3467.2014.86).

Experimental protocol

Peripubertal male *Wistar* rats with 30 days old (PND 30) and body weight of approximately 125 g, were supplied by Animal Facilities House, Biological Sciences Centre, State University of Londrina (CCB - UEL) and were acclimated in the new environment (Laboratory of Toxicology and Metabolic Dysfunctions of Reproduction) during 10 days before the beginning of experimental period. Rats were randomly distributed into 2 groups (15 animals/group), and submitted to experimental period between PND 40 and 61. Animals from Control group were placed into polypropylene cages (43 × 30 × 15 cm) with laboratory grade pine shavings as bedding during all the experiment. Rats of Sleep Restriction (SR) group were exposed to SR by the modified multiple-platform method (Zager and Andersen 2007). These rats were placed inside a water tank (140 x 60 x 30 cm) containing 20 circular platforms (each 6.5 cm in diameter) with the water level within 3 cm of the upper surface. The animals could move within the tank by jumping from one platform to another. When they reached the paradoxical phase of sleep, muscular atonia caused them to fall into the water and awake. Rats were kept on the water tank for 18 hours (beginning at 16:00) and allowed to sleep for 6 hours in polypropylene cages (from 10:00 to 16:00) every day for 21 days, which enabled partial compensation for the sleep loss (Machado *et al.* 2005). Throughout the study, the experimental room was maintained at a controlled temperature (23 ± 2 ºC) with a 12h light-dark cycle (lights on at 07:00 and off at 7:00). Rats had free access to food and water located on a grid on top of the tank. The water in the tank was changed daily. All animals began their SR period in the dark phase of the light-dark cycle (16:00) because we elected not to invert the light-dark cycle. Thus, the light-dark cycle was maintained as usual.

Body, epididymis and vas deferens weights

Rats were weighed, anesthetized with a combination of ketamine (Quetamina®, Louveira, Brazil) and xylazine (Anasedan®, Paulínia, Brazil) and killed by cardiac puncture at the 22nd experimental day (PND 62). Epididymis and vas deferens were removed and their weights (absolute and relative to body weights) were determined. Epididymis was used for sperm counts (n=5/group), oxidative stress status assay (n=10/group), inflammatory profile (n=5/group) and histological processes (n=5/group). Spermatozoa from right vas deferens were used for sperm motility analysis (n=10/group).

Oxidative stress status assay

Tissue Preparation

The left epididymis was divided into caput and cauda and was prepared according to the method described by Zimiani *et al.* (2005). The tissue was homogenized for 45 seconds in an Ultra-turrax® homogenizer containing 50 mg/ml of tissue in 30 mM KH_2PO_4/K_2HPO_4 buffer and 120 mM KCl at pH 7.4. The supernatant from total homogenate was obtained by centrifugation at 11,000 g for 15 min at 4 ºC and used for the following analysis:

Measurement of Tert-Butyl Hydroperoxide–Initiated Chemiluminescence

Reaction mixtures were placed in luminescence tubes containing the following: homogenate from caput or cauda epididymis (50 mg/ml); 30 mM KH_2PO_4/K_2HPO_4 buffer (with 120 mM KCl, pH 7.4); and 6 mM tert-butyl hydroperoxide, in a final volume of 1 ml. The tert-butyl hydro-peroxide–initiated chemiluminescence (CL) reaction was assessed by luminometer (Promega®) with a response range of 300 – 650 nm (Gonzalez Flecha *et al.* 1991; Oliveira and Cecchini 2000). The tubes were kept in the dark until the moment of assay, which was carried out at a room temperature of 30 ºC. For each animal, a 40-min curve was obtained by interpolation (each point represented the differential smoothing of 600 readings). The results were expressed in relative light units per gram of tissue (RLU/g tissue). For cauda epididymis, a final curve was determined by Gaussian fit. The entire curve was used to determine the lipid hydroperoxides present in the sample.

Reduced glutathione (GSH) measurement

Reduced glutathione (GSH) was measured according to the method described by Tietze (1969). GSH levels were determined with 5,50-dithiobis 20-nitro benzoic acid on caput and cauda epididymis homogenate supernatant (50 mg/ml for both epididymis portions), and evidenced by a yellow color formation. GSH levels were measured at 412 nm, and the results expressed in μM/mg of protein.

Total radical-trapping antioxidant parameter (TRAP)

Total antioxidant capacity of caput and cauda epididymis was measured by chemiluminescence (Promega®), according to the method used by Repetto *et al.* (1996). The method measures hydrosoluble low-molecular weight antioxidants and Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, Sigma-Aldrich®), a soluble analogue of α-Tocopherol, is used as a standard for antioxidant capacity comparison. For this, 40 μL of supernatant caput and cauda homogenate (50 mg/mL) was used. The reaction medium also contained 20 μM 2-azo-bis-(2-amidinopropane) and 200 μM luminol (Sigma-Aldrich®) at 30 °C. Total quenching time for the supernatant was compared with trolox quenching and the results expressed as μM trolox.

Inflammatory profile

Myeloperoxidase (MPO) activity

MPO is an enzyme abundantly present in the azurophilic granules of neutrophils that has been used as a biochemical marker of neutrophil infiltration into various tissues; thus, MPO colorimetric assay was used to assess neutrophil migration into the caput and cauda regions. Frozen samples were homogenized as described above and centrifuged (16,100 g, 2 min, 4 ºC). The resulting supernatant was assayed spectrophotometrically for MPO activity determination at 450 nm. Briefly, 5 μL of the supernatant was mixed with 200 μL 50 mM phosphate buffer (pH 6.0), containing 0.167 mg/mL o-dianisidine dihydrochloride and 0.015% hydrogen peroxide (Bradley *et al.*, 1982; Valério *et al.*, 2009). The results of MPO activity are expressed as the number of neutrophils per mg of tissue by using a standard curve of neutrophils (196 – 400,000 cells).

N-acetyl-β-D-glucosaminide (NAG) activity

NAG activity was determined by an adapted colorimetric method previously described by Horinouchi *et al.* (2013). Briefly, 20 µL of supernatant (caput and cauda), previously described in MPO activity, was placed in a 96-well plate, followed by the addition of 80 µL of 50 mM phosphate buffer, pH 6.0. The reaction was initiated by the addition of 2.24 mM 4-nitrophenyl N-acetyl-β-D-glucosaminide. The plate was incubated at 37 ºC for 10 min, and the reaction was stopped by the addition of 100 µL of 0.2 M glycine buffer, pH 10.6. The enzymatic activity was determined spectrophotometrically at 400 nm (Multiskan GO Microplate Spectrophotometer, Thermo Scientific®, Vantaa, Finland). The results of NAG activity are expressed as the number of macrophages per mg of tissue by using a standard curve of macrophages (196 – 400,000 cells).

Sperm number and transit time through the epididymis

Right epididymis was weighed and homogenized as described previously by Robb *et al.* (1978), with the modifications described by Fernandes *et al.* (2007). After dilution of the homogenate, a small sample was transferred to Neubauer chamber (4 fields per animal) for counting spermatozoa. To calculate sperm transit time through the epididymis, the number of sperm in each portion was divided by daily sperm production in testis. To calculate the daily production of sperm, the concentration of spermatids per testis is divided by 6.1 (number of days in which mature spermatids are present in the seminiferous epithelium).

Sperm motility

This analysis was performed as described by Siervo *et al.* (2015). The left vas deferens was rinsed with 1.0 ml of modified HTF medium with gentamicin (Human Tubal Fluid, Irvine Scientific®), at 34 º – 36 ºC, to obtain sperm. At the same temperature, a Makler counting chamber (Sefi-Medical®, Haifa, Israel) was loaded with a small aliquot of sperm solution (10 ml). Sperm motility was assessed by visual estimation (100 spermatozoa per animal) under a light microscope (Motic®) at 100X magnification, and was done by the same person throughout the study. Spermatozoa were classified as: mobile or immobile.

Histological processing

The left epididymis (5 per group) was removed, fixed in Metacarn (60% methanol, 30% chloroform, 10% acetic acid), embedded in Paraplast® and sectioned at 5 µm. The sections were stained with hematoxylin and eosin (HE) and examined for general histopathological and stereological analysis as described to Favareto *et al.* (2011). For

histopathological analysis, epididymal cross-sections (caput and cauda) were qualitatively evaluated using an Opton® microscope (100× and 400× magnification). In stereological analysis, 10 random epididymal cross-sections per animal of caput (2A) and cauda (5A/B) (Miller and Killian 1987) were captured using a photomicroscope (Opton®) and BELView Software (version 6.2.3.0 for Windows) in 400× magnification and analyzed. This analysis was performed by means of Weibel's multipurpose graticulate with 168 points (Weibel 1963) to compare the relative proportion among the epididymal components (epithelium, stroma and lumen) in the experimental groups (50 sections/group for each epididymal region).

Statistical analyses

The variance between the CL curves was compared using Two-Way ANOVA with the *post hoc* Bonferroni´s test. For other analysis, Unpaired t test or the non-parametric Mann–Whitney was used depending on data distribution. Differences were considered significant when $p<0.05$. The statistical analyses were performed by GraphPad Prism (version 5.0).

Results

Body, epididymis and vas deferens weights

The body (initial and final), epididymis and vas deferens weights (absolute and relative) are shown in Table 1. The body weight at the end of the experimental period ($p<0.001$) and epididymal relative weight were significantly incresed in SR group in relation to Control group ($p<0.05$). Furthermore, SR induced a significant decrease of the vas deferens absolute weight compared to Control group ($p<0.05$). However, the absolute weight of epididymis and relative weight of vas deferens showed no significant difference after SR period.

Oxidative stress status assay

In relation to antioxidant system defense, sleep loss increased the GSH levels in epididymal caput and cauda compared to Control group (Fig 1, B and E), and the TRAP levels only in caput epididymis (Fig 1, C and F).

Inflammatory profile

The SR exposure significantly decreased the neutrophil recruitment in caput/corpus epididymis as shown in Table 2. In epididymal cauda, there was no significant difference

between SR and Control groups, as well as macrophages migration in caput/corpus or cauda of epididymis.

Sperm number and transit time though the epididymis

The sperm numbers in caput/corpus (Control= 46.75 ± 1.54; SR= 45.40 ± 6.20 – $x10^6$) and cauda (Control= 30.46 ± 4.87; SR= 29.20 ± 4.67 – x 10^6) epididymis were similar between groups as well as spermatic transit time though the caput/corpus (Control= 2.60 ± 0.20; SR= 1.98 ± 0.43 days) and cauda epididymis (Control= 1.64 ± 0.19; SR= 1.29 ± 0.34 days).

Sperm motility

The SR significantly impaired the sperm motility since the percentage of immobile sperm increased in SR group compared to Control group ($p<0.05$). The values are shown in Table 3.

Histopathological analysis

The histopathological analysis of epididymal tissue including the epithelium, stroma and lumen did not show any differences between control and the SR group.

Stereological analysis

The values are shown in Table 3. The SR group presented a significant increase in stromal compartment followed by a decrease in luminal compartment from 2A region of caput epididymis compared to Control group ($p<0.01$). On the other hand, SR induced a significant increase in stromal compartment in 5A/B region of epididymal cauda ($p<0.01$) and a decrease in epithelial compartment compared to Control group ($p<0.05$).

Discussion

When spermatozoa leave the testis, they are morphologically complete but immobile and unable to fertilize an oocyte. In mammals, the fertilization ability of the spermatozoon is a result of the discrete post-gonadal differentiation stages that occur during transit along the epididymal duct (Dacheux and Dacheux 2014). Peripubertal perturbations may have broad consequences that can be noticeable during the pubertal phase, as well as during later adult

life (Mantovani and Fucic 2014). Therefore, any interference in epididymal postnatal development could impair fertility.

A relationship between CL and lipid peroxidation was previously demonstrated, as well as a positive correlation between CL and tissue damage (Wright *et al.* 1979; Llesuy *et al.* 1990). The enhancement in CL curves (entire and Gaussian fit) corresponds to a preceding peroxidative attack of tissue by ROS, inducing the consumption of antioxidants and augmenting the formation of lipid hydroperoxides, resulting in increased photon emission (Oliveira and Cecchini 2000; Zimiani et al. 2005). Furthermore, the different patterns of CL curves between caput and cauda epididymis can be explained by the different biochemical microenvironment of these tissues. The present study shows that the increase of TRAP and GSH levels in the caput would suggest an attempt to restore the optimal reduction-oxidation (redox) balance impaired by sleep loss since lipid peroxidation levels (CL curve) were significantly increased in this epididymal region. On the other hand, the cauda region did not show an augmentation in TRAP levels; as the CL curve and GSH levels were increased, the same hypothesis could be applied. It is reasonable to assume that the entire epididymis experiences oxidative injury, but different regions experiences different intensities. Experimental animal SR models have revealed a decrease in antioxidants and an increase in oxidative stress in the entire brain (Kumar and Singh 2008), the hippocampus (Alzoubi *et al.* 2012; Zhang *et al.* 2013), the cerebral cortex (Zhang *et al.* 2013) and plasma (She *et al.* 2014). Oxidative DNA damage in the liver, lung, and small intestines has also been noted (Everson *et al.* 2014). Although many studies assess the oxidative stress status after sleep loss, none has evaluated this parameter in the male reproductive system, specifically the epididymis.

The most visible and quantifiable change in the epididymal sperm is the development of motility (Dacheux and Dacheux 2014). Free radicals ensure the morphological reshaping of spermatozoa by activating intracellular pathways that lead to the development of motility; however, excessive amounts of ROS have pathological effects on spermatozoa (Du Plessis *et al.* 2015). In this context, alterations observed in sperm motility in our study could be related to disturbances in redox balance. Previous studies in our laboratory already showed that increased oxidative stress is capable of interfering with sperm motility, decreasing the number of mobile spermatozoa (Siervo *et al.* 2015). Other studies have also shown an association between increased lipid peroxidation and impairment of sperm motility (Aitken *et al.* 2006; Hosseinzadeh Colagar *et al.* 2013; García-Díaz *et al.* 2015). Recently, Alvarenga *et al.* (2015) observed that adult male rats showed significantly fewer spermatozoa with faster movement after 96 continuous hours of sleep deprivation (SD) or after 18 hours of SR per day for 21

days. Sperm count parameters were not affected by SR in the present study, similar to the findings of Alvarenga *et al.* (2015), which showed no significant changes in sperm concentration after 96 hours of SD or 21 days of SR.

The alterations observed in the epithelial, luminal and interstitial compartments after the period of SR, from the 2A and the 5A/B regions of the caput and cauda epididymis, respectively, infer that SR is capable of impairing epididymal development. These events can alter the sperm maturation process and consequently, the fertile capacity of the gamete. In this context, the function of the caput and corpus regions is to carry out early and late sperm maturational events (Cornwall 2009). Meanwhile, the major function of the distal segments of the epididymis is to store mature sperm, as well as to recognize, neutralize and destroy the abnormal appearing and dead spermatozoa (Robaire *et al.* 2015). The cells that carry out this function are mainly present in the epithelial compartment. Thus, the reduction in the epithelial compartment in the 5A/B region in SR rats could also relate to an inability in recognizing and destroying these abnormal sperm, increasing the number of immobile sperm.

According to the inflammatory profile assay, SR caused a decrease in neutrophil migration to the caput/corpus epididymis, while neutrophil migration to the cauda and macrophages in the cauda and caput/corpus did not change. These data corroborate with the histopathological analysis findings, in which no significant inflammatory infiltrate or immune cells in the lumen were found in the caput or cauda epididymis. Zager *et al.* (2007) reported that rats after SR (21 days) had a decrease in circulating leukocytes and lymphocytes, but not on circulating neutrophils. These authors suggested that chronic SR, like that observed in a modern population on a daily basis, caused impairment of the systemic circulation of leukocytes, and quite possibly increases the susceptibility to infectious diseases. SR could thus impair neutrophil recruitment to the epididymis, thereby making this organ more susceptible to infectious processes.

The decrease in final body weight observed in our study corroborates with previous data using adult male rats after 21 days of SR (Rodrigues *et al.* 2015) or after 27 and 99 continuous hours of SD (Wallingford *et al.* 2014). According to Barf *et al*. (2012), adult male rats that underwent SR for 8 days of 20 hours per day had attenuation of the weight gain as a consequence of chronic SR. In control rats, energy intake was higher than energy expenditure, resulting in weight gain. On the other hand, in SR rats, energy expenditure was significantly increased, resulting in an attenuation of weight gain compared to controls. This possible mechanism could also be applied to our study.

The SR is unable to affect the absolute weight of epididymis; however, the relative organ weight increased because of the reduced body weight. Maia *et al.* (2011) reported maintenance in body weight and in absolute and relative epididymides weight in adult mice after 15 days of SR. The diminished absolute vas deferens weight after the experimental period showed that this organ was more susceptible to the effects of sleep loss. Thus, the relative vas deferens weight was maintained due to the decrease in final body weight.

It is reasonable to point that the stress induced by the SR protocol results in stressful stimuli beyond the stress of SR alone. Despite the methodological rigor, it is impossible to eliminate all stressors, thus, this is a limitation of the study with SR animal model (Mônico-Neto *et al*. 2015).

Conclusion

The present data demonstrate that SR impairs postnatal epididymal development and sperm motility with a crucial participation of lipid peroxidation and altered reduction-oxidation balance. Importantly, the present study unveils previous unrecognized consequences of SR in the epididymal postnatal development and further studies are necessary to elucidate the possible mechanisms that are responsible for these alterations.

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Table 1

Body, epididymis and vas deferens weights (n=15/group)

	Control	Sleep Restriction
Initial body weight (g)	124.38 ± 2.95	125.14 ± 1.81
Final body weight (g)	238.42 ± 4.99	202.27 ± 7.41 **
Epididymis (g)	0.254 ± 0.01	0.243 ± 0.01
Epididymis/body weight ratio (g/g)	0.107 ± 0.00	0.122 ± 0.01 *
Vas deferens (g)	0.073 ± 0.00	0.063 ± 0.00 *
Vas deferens/body weight ratio (g/g)	0.031 ± 0.00	0.031 ± 0.00

Values expressed as mean ± SEM. Unpaired t test. * $p<0.05$ ** $p<0.001$

Table 2

Inflammatory profile (n=5/group)

	Control	Sleep Restriction
Number of neutrophils/mg caput and corpus (MPO activity)	1692.90 ± 366.37	811.55 ± 118.07 *
Number of neutrophils/mg cauda (MPO activity)	2279.60 ± 1202.00	1928.3 ± 478.39
Number of macrophages/mg caput and corpus (NAG activity)	100316.40 ± 2496.80	92352.2 ± 6027.20
Number of macrophages/mg cauda (NAG activity)	79247.30 ± 17954.00	74681.6 ± 4953.40

Values expressed as mean ± SEM. Mann-Whitney test. *$p<0.05$

Table 3

Sperm motility and stereological analysis (%)

	Control	Sleep Restriction
Sperm motility (n=10/group)		
Mobile sperm	62.00 [58.75 – 69.00]	54.00 [53 – 55.75] *
Immobile sperm	38.00 [31.00 – 41.25]	45.5 [42.75 – 47.00] *
Caput (2A) (n=5/group)		
Epithelium	25.5 [21.9 – 25.4]	25.3 [18.7 – 29.2]
Stroma	20.8 [14.9 – 25.4]	27.9 [18.7 – 34.1] **
Lumen	53.6 [59.0 – 126.0]	46.8 [40.6 – 53.5] **
Cauda (5A/B) (n=5/group)		
Epithelium	25.1 [20.4 – 30.3]	22.0 [17.8 – 25.0] *
Stroma	27.3 [21.0 – 32.1]	33.9 [26.8 – 37.3] **
Lumen	47.6 [41.7 – 54.8]	44.1 [36.3 – 53.7]

Values expressed as median [Q1 – Q3]. Mann-Whitney test. *p<0.05 **p< 0.01

Figure

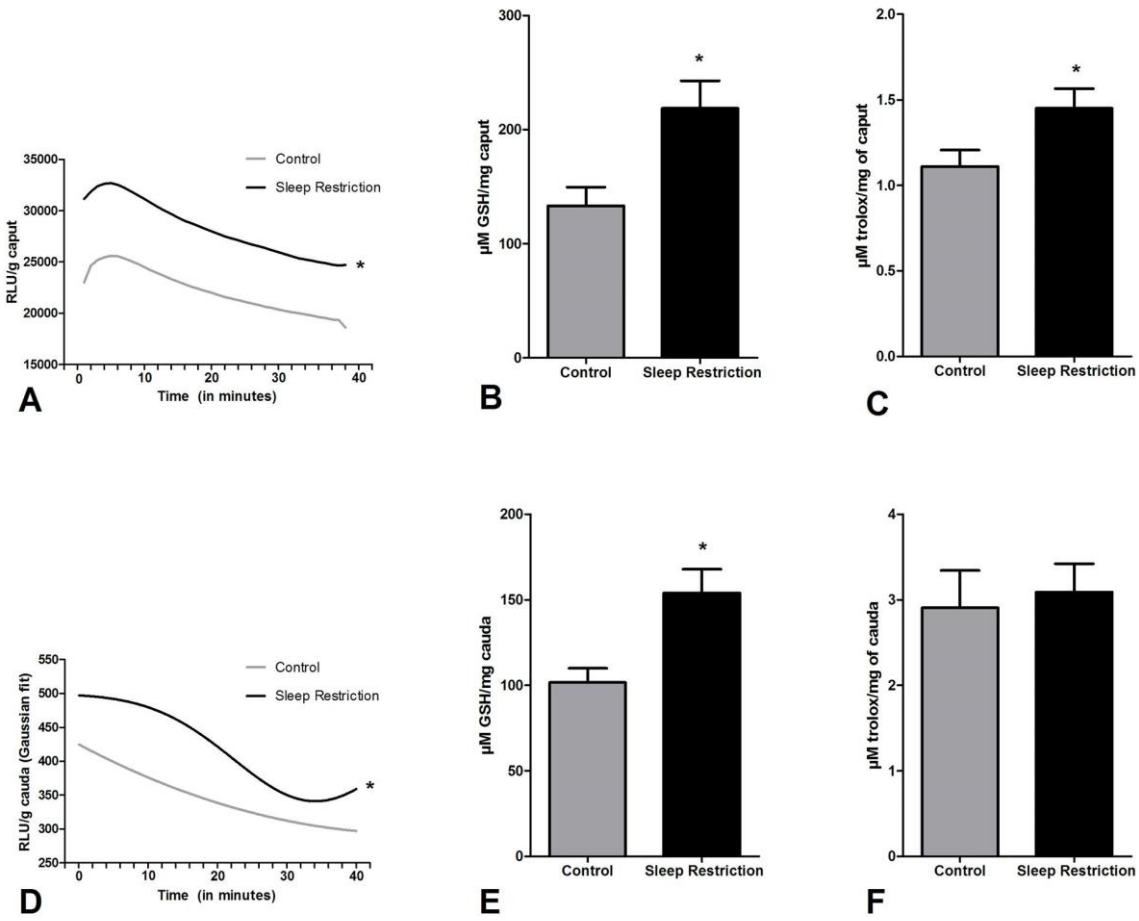

Figure 1 Oxidative stress status assay in caput (A – C) and cauda (D – F) epididymis (n=10/group). (A and D) The *tert*-butyl hydroperoxide–initiated chemiluminescence was monitored continuously during 40 min. (A) represents an entire curve of the mean of 2400 readings of emitted light in caput epididymis and (D) represents a Gaussian fit curve of the mean of 2400 readings of emitted light in cauda epididymis. The entire curves were employed to perform statistical comparison by two-way ANOVA followed by Bonferroni's test. *$p<0.05$. (B and E) GSH levels in caput and cauda epididymis, respectively. Results are expressed in µM/mg tissue. (C and F) Total antioxidant capacity (TRAP) in supernatant of caput and cauda, respectively. Results are expressed in µM trolox/mg tissue. Unpaired t test was performed for GSH and TRAP analysis. *$p<0.05$.

5 ARTIGO II

Prejuízo no desenvolvimento testicular de ratos submetidos à restrição de sono durante a peripuberdade

Impairment of testicular development in rats submitted to sleep restriction during peripuberty

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Impairment of testicular development in rats submitted to sleep restriction during peripuberty

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Abstract

Sleep directly affects the activity of neuroendocrine reproductive axis and its impact on health and medical conditions remains partially unrecognized. Children and adolescents may be chronically sleep deprived due early school hours and constant exposure to artificial light and interactive activities. The aim of this study was to evaluate whether sleep restriction (SR) could affect testicular postnatal development and sperm morphology in peripubertal rats. For this, 30 male *Wistar* rats (postnatal day – PND 40) were distributed into 2 groups: Control group was maintained in their home-cages during all the experiment and Sleep Restriction group was submitted to SR during 18 hours per day, for 21 days, by modified multiple-platform method. At PND 62, testes and seminal vesicle were collected and weighted. Testes were used for evaluation of oxidative stress markers, inflammatory profile, sperm count, spermatogenesis kinetics, Sertoli cells count, histopathological and morphometric analysis. Spermatozoa from vas deferens were used for sperm morphology. The results showed that SR significantly increased lipid peroxidation in the testis and decreased the number of Sertoli cells and the testicular epithelium height. The total antioxidant capacity (TRAP), neutrophil and macrophages activity, sperm parameters, histopathological analysis, spermatogenesis kinetics and tubular diameter did not change due SR. We conclude that the sleep restriction impairs postnatal testicular development and the lipid peroxidation may be the main factor.

Keywords: Sleep restriction, Testis, Lipid peroxidation, Sertoli cells, Puberty.

Ethical approval

Experimental procedures were conducted in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and were approved by the Ethics Committee on Animal Use of State University of Londrina (CEUA/UEL protocol number 3467.2014.86).

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1 INTRODUCTION

Sleep occupies approximately one-third of a person's life, but its impact on health and medical conditions remains partially unrecognized (Tufik et al., 2009). In children and adolescents, early school hours and constant exposure to artificial light and interactive activities, such as television and the internet could be the cause of insufficient sleep (Dexter et al., 2003; Owens, 2014; Tufik et al., 2009). In USA, 8% of 6- to 11-year-old children and up to 29% 12- to 14-year-old teenagers sleep less than 8 h per night (National Sleep Foundation, 2014).

Studies show that good sleep quality has a direct effect on the activity of neuroendocrine reproductive control axis and hormonal homeostasis (Andersen et al., 2011; Shaw et al., 2015), even during puberty, when several events which leads to maturation of hypothalamic-hypofisary-gonadal axis occurs and thus the beginning of specie's reproductive cycle (Ojeda; Urbanski, 1994). In addition, the lack of sleep is detrimental to sexual behavior and could interfere in the reproductive system of male rats, the latter being clearly reflected in the reduction of spermatic viability (Alvarenga et al., 2015).

Sleep disturbances are also commonly associated with some metabolic disturbances due oxidative stress (Chang et al., 2008; McEwen, 2006). It could be related with the removing of reactive oxygen species (ROS) that are produced in the wake cycle during sleep (Alzoubi et al., 2012). In adult sleep deprived rats, the testicular redox balance where altered (Akindele et al., 2014).

Postnatal sexual development is divided into four stages in male rats: neonatal period (postnatal day - PND 0 to PND 7); infantile period (PND 8 to PND 21); juvenile period (PND 22 to PND 35); and peripubertal period (PND 36 to PND 55–60), at which time mature sperm appear in the vas deferens (Prevot, 2015). All these phases are regulated by mechanisms related with hormonal and genetic conditions (Damgaard et al., 2002). During peripubertal

phase there is a slow germ cell proliferation and subsequent development, and increase in testosterone levels (Perobelli, 2014; Sharpe, 2010).

There are few studies which analyzed the alterations on the male reproductive system after sleep loss, especially during postnatal development of this system. Thus, the aim of the present work was to evaluate whether sleep restriction (SR) during the peripubertal phase could impair the testicular postnatal development.

2 MATERIALS AND METHODS

2.1 Experimental protocol

Peripubertal male *Wistar* rats with 30 days old (PND 30) and body weight of approximately 125g, were supplied by Animal Facilities House, Biological Sciences Centre, State University of Londrina (CCB - UEL) and were acclimated in the new environment (Laboratory of Toxicology and Metabolic Dysfunctions of Reproduction) during 10 days before the beginning of experimental period. Rats were randomly distributed into 2 groups (15 animals/group), and submitted to experimental period between PND 40 and 61. Animals from Control group (C) were maintained into polypropylene cages (43 × 30 × 15 cm) with laboratory grade pine shavings as bedding during all the experiment. Rats of Sleep Restriction (SR) group were exposed to SR by the modified multiple-platform method (Zager and Andersen, 2007). These rats were placed inside a water tank (140 x 60 x 30cm) containing 20 circular platforms (each 6.5 cm in diameter) with the water level within 3 cm of the upper surface. The animals could move within the tank by jumping from one platform to another. When they reached the paradoxical phase of sleep, muscular atonia caused them to fall into the water and awake. Rats were kept on the water tank for 18 hours (beginning at 16:00) and allowed to sleep for 6 hours in polypropylene cages (from 10:00 to 16:00) every day for 21

days, which enabled partial compensation for the sleep loss (Machado et al., 2005). Throughout the study, the experimental room was maintained at a controlled temperature (23±2ºC) with a 12h light-dark cycle (lights on at 07:00 and off at 7:00). Rats had free access to food and water located on a grid on top of the tank. The water in the tank was changed daily. All animals began their SR period in the dark phase of the light-dark cycle (16:00) because we elected not to invert the light-dark cycle. Thus, the light-dark cycle was maintained as usual.

2.2 Body, testis, seminal vesicle and vas deferens weights

Rats were weighed, anesthetized with a combination of ketamine (Quetamina®, Louveira, Brazil - 10mg/kg, intramuscular) and xylazine (Anasedan®, Paulínia, Brazil - 2mg/kg, intramuscular) and killed by cardiac puncture at the 22nd experimental day (PND 62). Testes and seminal vesicles (without the coagulating gland) were removed and their weights (absolute and relative to body weights) were determined. Testis was used for sperm counts (n=5/group), oxidative stress status assay (n=10/group), inflammatory profile (n=5/group) and histological processes (n=5/group). Spermatozoa from left vas deferens were used for sperm morphology analysis (n=10/group).

2.3 Oxidative stress status assay

2.3.1 Tissue Preparation

Left testes were prepared according to the method described by Zimiani et al 2005 (adapted from skeletal muscles homogenization). The tissue was homogenized for 30 seconds in an Ultra-turrax® homogenizer containing 50 mg/ml of tissue in 30 mM KH_2PO_4/K_2HPO_4 buffer and 120 mM KCl at pH 7.4. The supernatant from total homogenate was obtained by centrifugation at 11,000 g for 15 min at 4 ºC and used for the following analysis:

2.3.2 Measurement of *Tert*-Butyl Hydroperoxide–Initiated Chemiluminescence

Reaction mixtures were placed in luminescence tubes containing the following: homogenate from testis (50 mg/ml); 30 mM KH_2PO_4/K_2HPO_4 buffer (with 120 mM KCl, pH 7.4); and 6 mM tert-butyl hydroperoxide, in a final volume of 1 ml. The tert-butyl hydroperoxide–initiated chemiluminescence (CL) reaction was assessed by luminometer (Promega®) with a response range of 300 – 650 nm (Gonzalez Flecha et al., 1991; Oliveira and Cecchini, 2000). The tubes were kept in the dark until the moment of assay, which was carried out at a room temperature of 30 ºC. For each animal, a 40-min curve was obtained by interpolation (each point represented the differential smoothing of 600 readings). The results were expressed in relative light units per gram of tissue (RLU/g tissue).

2.3.3 Total radical-trapping antioxidant parameter (TRAP)

Total antioxidant capacity of testis was measured by chemiluminescence (Promega®), according to the method used by Repetto et al (1996). The method measures hydrosoluble low-molecular weight antioxidants and Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, Sigma-Aldrich®), a soluble analogue of α-Tocopherol, is used as a standard for antioxidant capacity comparison. For this, 40 μL of supernatant testis homogenate (50 mg/mL) was used. The reaction medium also contained 20 μM 2-azo-bis-(2-amidinopropane) and 200 μM luminol at 30 °C. Total quenching time for the supernatant was compared with trolox quenching and the results expressed as μM trolox.

2.4 Inflammatory profile

2.4.1 Myeloperoxidase (MPO) activity

MPO is an enzyme abundantly present in the azurophilic granules of neutrophils that has been used as a biochemical marker of neutrophil infiltration into various tissues; thus,

MPO colorimetric assay was used to assess neutrophil migration to testis. Frozen samples were homogenized as described above and centrifuged (16,100 g, 2 min, 4 ºC). The resulting supernatant was assayed spectrophotometrically for MPO activity determination at 450 nm. Briefly, 5 µL of the supernatant was mixed with 200 µL 50 mM phosphate buffer (pH 6.0), containing 0.167 mg/mL o-dianisidinedihydrochloride and 0.015% hydrogen peroxide (Bradley et al., 1982; Valério et al., 2009). The results of MPO activity are expressed as the number of neutrophils per mg of tissue by using a standard curve of neutrophils (196 – 400,000 cells).

2.4.2 N-acetyl-β-D-glucosaminide (NAG) activity

NAG activity was determined by an adapted colorimetric method previously described by Horinouchi et al (2013). Briefly, 20 µL of supernatant, previously described in MPO activity, was placed in a 96-well plate, followed by the addition of 80 µL of 50 mM phosphate buffer, pH 6.0. The reaction was initiated by the addition of 2.24 mM 4-nitrophenyl N-acetyl-β-D-glucosaminide. The plate was incubated at 37ºC for 10 min, and the reaction was stopped by the addition of 100 µL of 0.2 M glycine buffer, pH 10.6. The enzymatic activity was determined spectrophotometrically at 400 nm (Multiskan GO Microplate Spectrophotometer, Thermo Scientific®, Vantaa, Finland). The results of NAG activity are expressed as the number of macrophages per mg of tissue by using a standard curve of macrophages (196 – 400,000 cells).

2.5 Sperm parameters

For sperm count, right decapsulated testes were weighed and homogenized as described previously by Robb et al (1978), with the adaptations described by Fernandes et al (2007). After dilution of the homogenate, a small sample was transferred to Neubauer

chamber (4 fields per animal) for counting homogenization-resistant spermatids (stage 19 of the spermatogenesis). To calculate the daily production of sperm (DSP), the concentration of spermatids per testis is divided by 6.1, which is the number of days in which mature spermatids are present in the seminiferous epithelium.

For sperm morphology, contents of the vas deferens were removed by internal rinsing with 1.0 mL of saline formol 10%. Smears into histological slides were prepared from this solution and observed in a photomicroscope (Opton®) at 400X magnification. Two hundred spermatozoa were analyzed per animal. Morphological analysis was classified into three general categories: normal morphology, head abnormalities (without characteristic curvature or isolated form, i.e., no tail attached) and tail abnormalities (broken, rolled into a spiral and isolated, i.e., no head attached). This analysis was performed as described by Fernandes et al (2007).

2.6 Histological processing

The left testis (5 per group) was removed, fixed in Metacarn (60% methanol, 30% chloroform, 10% acetic acid), embedded in Paraplast® and sectioned at 5 μm. The sections were stained with hematoxylin and eosin (HE) and examined for the following analysis:

2.6.1 Histopathological and morphometric analysis

One hundred random testicular cross-sections per animal were observed for histopathological analysis, using an Opton® microscope (100X and 400X magnifications). The seminiferous tubules were divided in normal or abnormal, according to the alterations present in the seminiferous tubules.

Seminiferous tubule diameters were measured using Opton® photomicroscope (400X magnification) and BELview software (version 6.2.3.0 for Windows). For this, ten random

testicular cross-sections, per animal, in stage IX of the seminiferous epithelium cycle, were examined. Likewise, the seminiferous epithelium height was measured using the same tubules and methodology mention above. In each seminiferous tubule the mean of four measures for diameters and height was calculated and used in the statistical analysis.

2.6.2 Spermatogenesis kinetics and Sertoli cells number

For evaluation of spermatogenesis kinetics, one hundred random tubular sections per animal were classified into four categories: stages I–VI, VII–VIII, IX–XIII and XIV of the seminiferous epithelium cycle, according to Leblond and Clermont (1952) under a light microscope (Opton®) at 100X and 400X magnifications.

To evaluate the process of proliferation of Sertoli cells, nuclei were counted in histological cuts from the testis of rats in 20 seminiferous tubules per rat at stage VII of spermatogenesis.

2.7 Statistics

The variance between the CL curves was compared using Two-Way ANOVA with the *post hoc* Bonferroni´s test. For other analysis, Unpaired t test or the non-parametric Mann–Whitney was used depending on data distribution. Differences were considered significant when $p<0.05$. The statistical analyses were performed by GraphPad Prism (version 5.0).

3 RESULTS

3.1 Body, testis, seminal vesicle and vas deferens weights

The evolution of body weight is shown in Fig 1. The body weight at the end of the experimental period ($p<0.01$) was significantly reduced in SR group in relation to C group. The relative testis weight was increased ($p<0.01$) in SR group compared to Control group.

However, the absolute weights of testis and seminal vesicle (full and empty) and relative weights of seminal vesicle (full and empty) did not change after SR period (Table 1).

3.2 Oxidative stress status assay

SR was not capable to affect the antioxidant system defense, which is shown by the similar levels between Control and SR groups (Fig 2A). On the other hand, SR induced a significant increase in the CL curves after SR, compared to Control group (Fig 2B).

3.3 Inflammatory profile

The neutrophil recruitment to testicular tissue was not affected by SR (C= 2199.43 ± 478.61; RS= 1897.95 ± 102.76 – neutrophil number, mean ± SEM) as well as the macrophages migration (C= 18726.23 ± 3609.00; RS= 15043.88 ± 1000.20 – macrophages number).

3.4 Sperm parameters

The sperm number in testis (C = 111.74 ± 6.99; RS= 114.50 ± 4.97 – $x10^6$– mean ± SEM) were similar between groups as well as DSP (C = 18.32 ± 1.15; RS= 18.77 ± 0.81 – $x10^6$).The SR did not alter sperm morphology since the number of head and tail malformation were similar between the groups (data not shown).

3.5 Testicular morphometric and histopathological analysis

The values are shown in Table 2. The SR group presented a significant decrease in seminiferous epithelium height ($p<0.01$). On the other hand, seminiferous tubular diameter was not affected by SR. The histopathological analysis of the testicular tissue evidenced that both groups had similar number of normal tubules.

3.6 Spermatogenesis kinetics and Sertoli cells number

The percentage among spermatogenesis kinetics stages was statically equal between the groups. However, the Sertoli cells number were decreased by SR ($p<0.01$). The values are represented in table 3.

4 DISCUSSION

The CL curves corresponds to a preceding peroxidative attack of tissue by ROS, inducing the consumption of antioxidants and augmenting the formation of lipid hydroperoxides, resulting in increased photon emission (Oliveira and Cecchini, 2000; Zimiani et al., 2005). In our study, the augment in CL curves corroborates with a previous study that showed that sleep deprivation increased testicular malondialdehyde (MDA) (Akindele et al., 2014). On the other hand, the total antioxidant capacity did not change in our study in contrast with the results of Akindele et al. (2014), in which superoxide dismutase (SOD) and catalase levels increased compared with control. Unpublished studies from our group showed that SR (18h of SR per day, during 21 days) increases the lipid peroxidation, the total antioxidant capacity (TRAP) and reduced glutathione (GSH) levels in caput epididymis, and lipid peroxidation and GSH levels in cauda epididymis. These data demonstrate that the male reproductive system is affected by SR with an increase in oxidative stress, which leads to oxidative injury in both organs.

It is well established that basic levels of ROS may function as signals to promote cell proliferation and survival, whereas increase of ROS can induce autophagy and apoptosis by damaging cellular components (Zhang et al., 2015). In this context, the present work shows a decrease in Sertoli cells number in SR rats, which could be related with the increase in testicular lipid peroxidation (CL curves) and consequent Sertoli cells death. In rodents, Sertoli

cell number increases continuously after initial testis differentiation, until early puberty, with no further change thereafter. On the other hand, data from human suggest that there is renewed proliferation of the cells and a marked increase in Sertoli cell number in beginning of puberty (O'Shaughnessy, 2015). The reduction in epithelium height after SR could also be related with increased lipid peroxidation, since the excessive amount of ROS and defective reduction/oxidation balance can impair the proliferation of germinative cells or lead them to cell death pathways.

Despite de reduction in seminiferous epithelium height, the tubular diameter did not change after 21 days of sleep restriction. Previous unpublished studies in our laboratory show that SR is capable to interfere in epididymal postnatal development, after the same SR protocol used in the present work.

SR did not affect the absolute weight of testis and absolute and relative weights of seminal vesicle. Nevertheless, the relative testis weight was increased due to reduced body weight. This reduction corroborates with previous data using adult male rats after 21 days of SR or after 27 and 99 continuous hours of sleep deprivation (SD) (Rodrigues et al., 2015; Wallingford et al., 2014). We can infer that energy expenditure was significantly increased in SR rats in comparison with Control group, which results in an attenuation of weight gain (Barf et al., 2012). In relation to seminal vesicle weight, Mathur and Chattopadhyay (1991) found that sleep deprived rats had a decrease in relative weight of this organ, both full and empty. These authors suggest that the reductions in testosterone and prolactin levels are related with the decreased weights.

The normal values for sperm number and DSP in testis and sperm morphology observed in the present study agree with previous data by Alvarenga et al. (2015), in which adult male rats have no alteration in sperm concentration, morphology and viability, after 21 days of SR or 96h of continuous SD. It demonstrates that sleep loss cannot affect the

spermatogenesis process, since the number of spermatozoa in testis, sperm morphology and spermatogenesis kinetics were not impaired in our work.

The present study shows no alterations in macrophages and neutrophil activity, which are in accordance with the normal aspect of seminiferous epithelium observed in histopathological analysis since no immune cells in lumen or inflammatory infiltrate were found. This result agrees with previous study by Zager and Andersen (2007), in which rats after SR (21 days) had no alterations on circulating neutrophils.

CONCLUSION

This is the first study that evaluates the effects of sleep restriction on the testicular development during the peripubertal period. We conclude that these experimental conditions, the sleep restriction affects the postnatal testicular development and lipid peroxidation is main responsible event.

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Conflict of interest

Authors have no conflicts of interest.

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Table 1. Testis and seminal vesicle weights (n=10/group)

	Control	Sleep Restriction
Testis (g)	1.22 ± 0.03	1.24 ± 0.04
Testicular weight/body weight ratio (g/g)	0.52 ± 0.02	0.62 ± 0.02 *
Seminal vesicle (full) (g)	0.67 ± 0.05	0.60 ± 0.03
Seminal vesicle's (full) weight/body weight ratio (g/g)	0.28 ± 0.02	0.30 ± 0.01
Seminal vesicle's (empty) (g)	0.38 ± 0.03	0.33 ± 0.02
Seminal vesicle's (empty) weight/body weight ratio (g/g)	0.16 ± 0.01	0.17 ± 0.01

Values expressed as mean ± SEM. Unpaired t test. *$p<0.01$

Table 2. Morphometric and histopathological analysis (n=5/group)

	Control	Sleep Restriction
Seminiferous tubular diameter (µm)	286.02 ± 3.10	282.19 ± 4.06
Seminiferous epithelium height (µm)	94.69 ± 2.07	87.16 ± 1.67 *
Normal tubules	88.20 ± 2.73	85.20 ± 4.22
Abnormal tubules	11.80 ± 2.73	14.80 ± 4.22

Values expressed as mean ± SEM. Unpaired t test. *$p<0.01$

Table 3.Spermatogenesis kinetics and Sertoli cells number (n=5/group)

	Control	Sleep Restriction
I - VI (%)	30 [28 – 31]	33 [33 – 36]
VII – VIII (%)	37 [32 – 49]	32 [30 – 40]
IX - XIII (%)	22 [20 – 34]	31 [18 – 32]
XIV (%)	05 [03 – 06]	04 [03 – 05]
Sertoli cells number	26 [23 – 28]	19 [17 – 22]*

Values expressed as median [Q1 – Q3]. Mann-Whitney test. *$p<0.01$

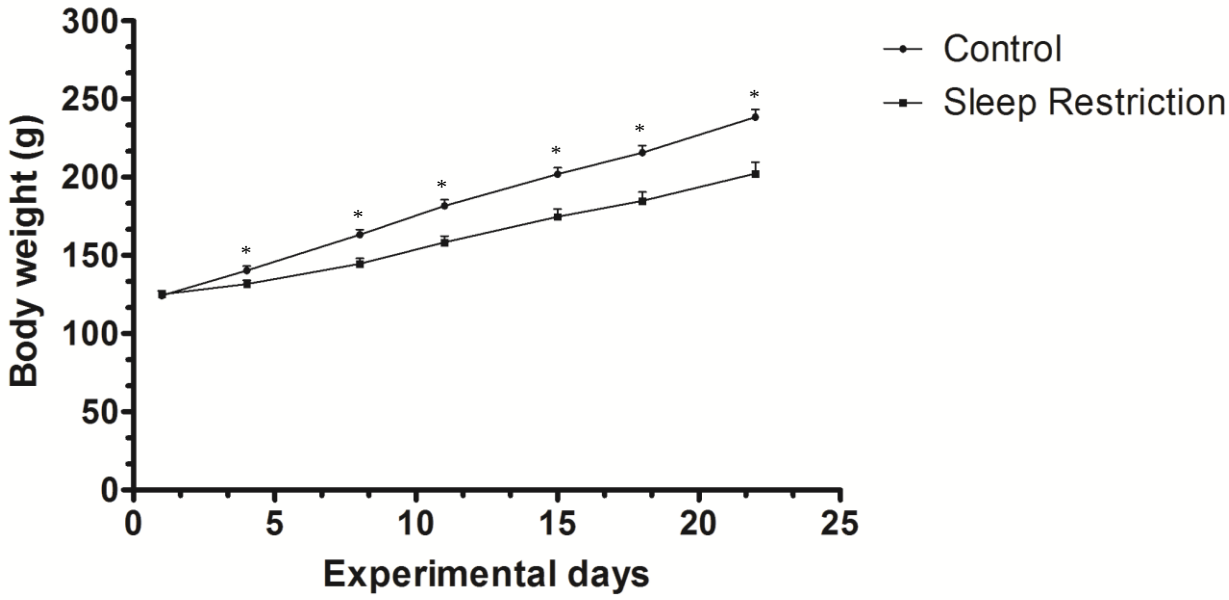

Figure 1. Body weight evolution during experimental days (n=15/group). Unpaired t test, *p<0.05.

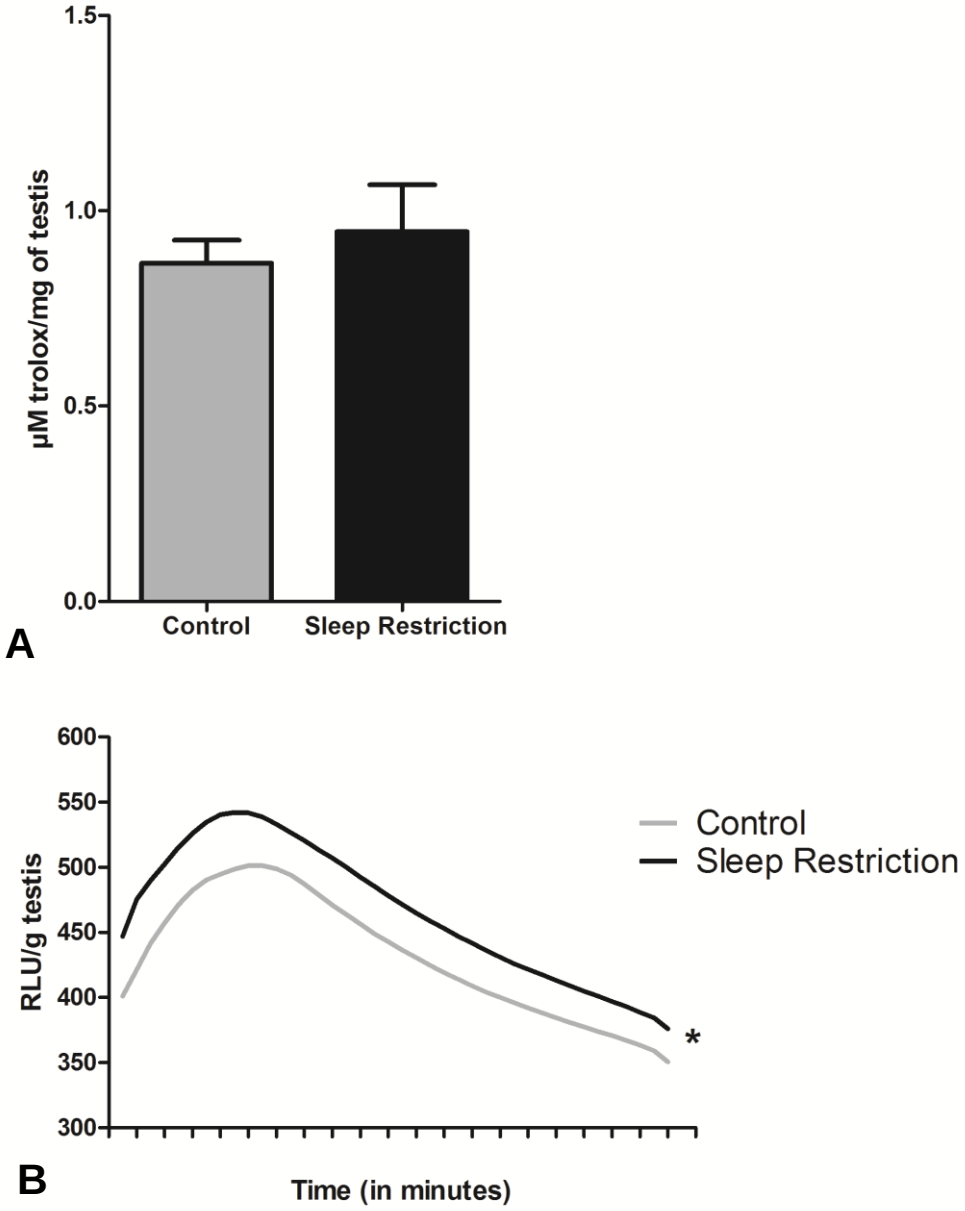

Figure 2. Oxidative stress status assay in testis (n=10/group). (A) Total antioxidant capacity (TRAP) in supernatant of testis. Results are expressed in µM trolox/mg tissue. Unpaired t test. (B) The *tert*-butyl hydroperoxide–initiated chemiluminescence was monitored continuously during 40 min. The entire curves of the mean of 2400 readings of emitted light were employed to perform statistical comparison by two-way ANOVA followed by Bonferroni's test, * $p<0.05$.

6 CONSIDERAÇÕES FINAIS

Nestas condições experimentais, concluímos que a restrição de sono durante a peripuberdade afeta o desenvolvimento pós-natal do sistema genital masculino, sendo que o aumento do estresse oxidativo participa deste processo. Além disso, alterações em parâmetros reprodutivos durante a fase de maturação sexual podem ter consequências para a fertilidade do indivíduo.

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