FERNANDA MITHIE OGO

EFEITOS DA ALTERAÇÃO DO CICLO CIRCADIANO MATERNO SOBRE O DESENVOLVIMENTO DO SISTEMA REPRODUTOR DA PROLE NA VIDA ADULTA EM RATOS

Londrina
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Exame de defesa apresentado ao Programa de Pós-Graduação em Patologia Experimental, da Universidade Estadual de Londrina, como requisito para a obtenção do título de Doutor.

Orientadora: Profa. Dra. Glaura Scantamburlo Alves Fernandes

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BANCA EXAMINADORA

_____
Orientadora: Profa. Dra. Glaura Scantamburlo A. Fernandes
Universidade Estadual de Londrina – UEL

_____
Profa. Dra. Alessandra Lourenço Cecchini
Universidade Estadual de Londrina – UEL

_____
Profa. Dra. Daniela Cristina Ceccatto Gerardin
Universidade Estadual de Londrina – UEL

_____
Profa. Dr. Fábio Goulart Andrade
Universidade Estadual de Londrina – UEL

_____
Profa. Dra. Marli Cardoso Martins Pinge
Universidade Estadual de Londrina – UEL

Londrina, 29 de março de 2019.

DEDICO,

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Meu eterno amor e gratidão!

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RESUMO

Alterações no ciclo circadiano são conhecidas por causar distúrbios fisiológicos nos eixos hipotálamo-hipófise-adrenal e hipotálamo-hipófise-gonadal em indivíduos adultos. Portanto, o presente estudo teve como objetivo avaliar se a exposição de ratas prenhas à luz constante pode alterar o desenvolvimento do sistema reprodutor da prole masculina e feminina. As mães foram divididas em dois grupos: um grupo claro-escuro (LD) que de ratas prenhes foram expostas a um fotoperíodo claro-escuro (12h/12h), e um grupo claro-claro (LL) que de ratas prenhes foram expostas a um fotoperíodo de luz constante durante o período de gestação. Após o nascimento, os filhotes de ambos os grupos permaneceram no fotoperíodo claro-escuro normal (12h/12h) até a idade adulta. Um filhote (macho e fêmea) de cada ninhada foi selecionado e, na idade adulta (PND 90), o sangue do tronco foi coletado no PND90 mensuração dos níveis plasmáticos de testosterona, testículos e epidídimo para contagem de espermatozoides, estresse oxidativo e análises histopatológicas, e os espermatozoides dos vasos deferentes, para realizar as análises morfológicas e de motilidade em machos e na prole feminina, foi coletado na fase de estro, para análise dos níveis plasmáticos de hormônio luteinizante (LH) e progesterona, e o útero e os ovários foram colhidos para estresse morfométrico, histológico e oxidativo avaliações. Os resultados das análises realizadas em machos mostraram que um fotoperíodo de luz constante causou diminuição nos níveis de testosterona, peso do epidídimo e contagem de espermatozóides no epidídimo, diâmetro do túbulo seminífero, número de células de Sertoli e número de espermatozóides normais. Danos histopatológicos também foram observados nos testículos, e alterações estereológicas, no grupo LL. Enquanto os resultados da prole feminina observaram-se uma redução do LH adulto e aumento dos níveis plasmáticos de progesterona, além de lesões uterinas como aumento do número de glândulas endometriais e redução dos níveis de enzimas antioxidantes, como glutationa redutase e glutationa S-transferase. Em conclusão, a exposição à luz constante durante o período gestacional prejudica o sistema reprodutivo da prole tanto masculina quanto feminina na idade adulta.

OGO, Fernanda Mithie. **Effects of the alteration of the maternal circadian cycle on the development of the reproductive system of the offspring in the adult life in rats.** 2019. 103 p. Thesis (Doctorate in Experimental Pathology) - State University of Londrina, Londrina, 2019.

ABSTRACT

Alterations in the circadian cycle are known to cause physiological disorders in the hypothalamic-pituitary-adrenal and the hypothalamic-pituitary-gonadal axes in adult individuals. Therefore, the present study aimed to evaluate whether exposure of pregnant rats to constant light can alter reproductive system development of male offspring. The dams were divided into a light-dark group (LD) of pregnant rats exposed to a light-dark photoperiod (12h/12h), and a light-light group (LL) of pregnant rats exposed to a photoperiod of constant light during the gestation period. After birth, offspring from both groups remained in the normal light-dark photoperiod until adulthood. The trunk blood was collected at PND90 to measure plasma testosterone levels, testes and epididymis for sperm count, oxidative stress and histopathological analyses, and the spermatozoa from the vas deferens, to perform the morphological and motility analyses. Results showed that a photoperiod of constant light caused a decrease in testosterone levels, epididymal weight and sperm count in the epididymis, seminiferous tubule diameter, Sertoli cell number and normal spermatozoa number. Histopathological damage was also observed in the testes, and stereological alterations, in the LL group. In conclusion, exposure to constant light during the gestational period impairs the reproductive system of male offspring in adulthood.

LISTA DE FIGURAS

ARTIGO 2

LISTA DE TABELAS

ARTIGO 1

ARTIGO 2

SUMÁRIO

1. INTRODUÇÃO

1.1 SISTEMA CIRCADIANO

Os ritmos circadianos são responsáveis pela organização dos sistemas fisiológicos em uma ordem temporal, assim como seu alinhamento em ciclos ambientais de 24 horas (MAN *et al*., 2017). O termo “circadiano” é derivado do latim *circa*, que significa “ao redor” e *diem*, que significa “dia”. Dessa forma, o sistema circadiano pode ser definido como o processo biológico que exibe uma oscilação endógena de cerca de 24 horas em um ambiente sem influencias externas (MAN *et al*., 2017).

O ritmo circadiano está envolvido em vários processos fisiológicos, incluindo os processos de sono / vigília (DIJK; DUFFY, 1999), regulação da temperatura corporal (VAN SOMEREN, 2000), secreção hormonal (COPINSCHI; VAN CAUTER, 1995), reparo tecidual (JANICH; MENG; BENITAH, 2014) e função cardiovascular (COOKE-ARIEL, 1998). Ademais, está presente em quase todos os organismos vivos, incluindo plantas, espécies não mamíferas e mamíferas (MAN *et al*., 2017).

Além de se apresentar distribuído em todos os ramos evolutivos, o sistema circadiano também pode estar relacionado com respostas imunes e inflamatórias. Variações circadianas interferem na taxa de sobrevivência de camundongos infectados com doses letais de bactérias (HALBERG *et al*., 1960; SHACKELFORD; FEIGIN, 1973). Estudos com a infecção murina por espécies bacterianas mostraram letalidade aumentada no final da fase de repouso, aproximadamente duas horas antes do início da atividade. Como os roedores são espécies noturnas, o início da atividade imunológica ocorre quando no ciclo escuro. Embora esse processo seja complexo e ainda especulativo, ele coincide com o período de redução da indução de citocinas pró-inflamatórias, juntamente com a redução da depuração e da letalidade das bactérias (HALBERG *et al*., 1960; SHACKELFORD; FEIGIN, 1973; MAN et al., 2017).

A alteração de processos circadianos está relacionada também com a expressão diferencial de genes relacionados à resposta imune. Esta variação pode levar a aumentos da resposta inflamatória a componentes bacterianos como LPS (ELLA *et al*., 2016); atenuação do recrutamento de monócitos (GAGNIDZE *et al*., 2016); ritmos alterados dos fatores imunológicos das células natural killers (NK),

assim como diminuição da função desse tipo celular (LIU *et al*., 2006; LOGAN *et al*., 2013) e aumento da expressão de citocinas inflamatórias (KELLER *et al*., 2009).

Nos mamíferos, os ritmos circadianos são controlados por um relógio central localizado no núcleo supraquiasmático (NSQ) do hipotálamo (DIBNER; SCHIBLER; ALBRECHT, 2010), atuando através de uma rede coordenada de relógios circadianos moleculares em células individuais para gerar ritmos de 24 horas. A captação de estímulos luminosos pela retina é o principal mecanismo de marca-passo central, estando diretamente relacionada como a capacidade de sincronização do relógio circadiano interno com o ciclo geofísico dos períodos de claro e escuro (LEE *et al*., 2013; MAN *et al*., 2017).

A partir do momento da percepção da luz por células sensoriais presentes na retina, o estímulo é conduzido através do trato retino-hipotalâmico. Os neurônios dessa região se projetam da região ocular para o núcleo supraquiasmático (NSQ), fazendo a sinalização através de AMP cíclico, modulando assim o relógio molecular para atingir o pico / fase das oscilações, permitindo a sincronização com o meio ambiente (GOOLEY *et al*., 2001; PANDA *et al*., 2002; LEE *et al*., 2013). O processo de regulação baseia-se em uma série de loops de *feedback* de transcrição e tradução por um grupo de genes circadianos conhecidos como “*clock genes*” ou “genes de relógio”.

Do ponto de vista molecular, a família de *CLOCK genes* inclui os fatores de transcrição BMAL1 e CLOCK; as proteínas codificadas pelos genes PER1, PER2, PER3, CRY1, CRY2 e a enzima casein-quinase-1 Épsilon (CK1ε) (DUNLAP, 1999). Em mamíferos, um loop de feedback típico de transcrição e tradução consiste nos dois ativadores transcricionais (Bmal1 e Clock) que formam hetrodímeros no citoplasma e entram no núcleo, onde se ligam às sequências de E-box nos promotores de Per (Período) 1, 2 e Cry (Cryptochrome) 1, 2 ativando sua expressão. No citoplasma, as proteínas Per e Cry interagem umas com as outras e entram no núcleo para inibir a atividade dos complexos Bmal / Clock. Os níveis de transcritos Per e Cry e suas respectivas proteínas, portanto, diminuem. Acredita-se que Bmal e Clock contribuam para a ativação da atividade de transcrição de outros *clock genes* através de uma série de modificações das histonas (associadas à atividade da histona acetil transferase), fosforilação e desfosforilação (HARDIN; YU, 2006), atuando, portanto, de maneira epigenética. *CLOCK genes* são expressos em quase

todos os tipos de tecido, incluindo o coração, fígado, músculos, e na glândula adrenal (VALENZUELA *et al*., 2008).

A sincronização circadiana dentro da célula e entre diferentes sistemas corporais é crucial para a manutenção da saúde, e a quebra deste relógio de 24 horas pode levar a condições patológicas envolvendo os sistemas neurológico, metabólico, cardiovascular, endocrinológico e gastrointestinal (RICHARDS; GUMZ, 2012). Hipotetiza-se que a evolução dos *clock genes* nos mamíferos esteja relacionada como a capacidade de antecipar as mudanças ambientais relacionadas ao fotoperíodo e aos ciclos sazonais (EDGAR et al., 2012). Isso permite que o corpo se adapte e responda a vários sinais ambientais, incluindo o aumento do estresse oxidativo (IVASHKIV, 2013; MAN; LOUDON, CHAWLA, 2016).

O eixo hipotalâmico-hipofisário é um importante fator que também está sob controle circadiano, afetando o momento da ovulação e a secreção hormonal. A desregulação dos ritmos circadianos pela exposição inadequada à luz ou a manipulação do relógio biológico afeta negativamente a implantação e o sucesso da prenhez em animais (SUMMA; VITATERNA; TUREK, 2012). Trabalhos demonstram que a interrupção do relógio circadiano pelo trabalho em turnos pode resultar em infertilidade, desregulação menstrual e aborto espontâneo (STOCKER *et al*., 2014; FERNANDEZ *et al*., 2016).

Em modelos murinos, o desenvolvimento do NSQ se inicia a partir do 12° dia do desenvolvimento embrionário e a ritimicidade só se inicia ao final da gestação, como isso, o sistema circadiano materno é o responsável pela maior parte da regulação que ocorre na prole durante esse período, e tal regulação ocorre principalmente pelo fotoperíodo e a produção de melatonina (SLÁDEK *et al*., 2004; MENDEZ *et al*., 2016).

Um importante fator amplamente descrito para a regulação circadiana é a melatonina. Também conhecida como N-acetil-5-metoxitriptamina, a melatonina é um hormônio neuroendócrino produzido pela glândula pineal (HARDELAND; PANDI-PERUMAL; CARDINALI, 2006), placenta (OKATANI et al., 1998) e ovário (CUTLER; GARCIA, 1980) e é considerado um hormônio “cronobiótico” com um sinal fotoperiódico universal, e uma molécula com função fisiológica diversa (BRZEZINSKI, 1997). Sua secreção é regulada por estímulos claro / escuro, o que por sua vez influencia o ritmo circadiano. Em humanos, o pico e a baixa dos ritmos circadianos para diferentes variáveis fisiológicas, incluindo a pressão arterial e o

ciclo sono / vigília, ocorrem em diferentes momentos do relógio. Sob ciclos normais de luz e escuridão, a concentração de melatonina alcançaria um pico em torno das duas horas da manhã (DUBOCOVICH *et al*., 2010).

Em mamíferos, a melatonina é um potente imunomodulador (MILLER *et al.*, 2006) em termos de regulação circadiana na proliferação de linfócitos (KUHLWEIN; IRWIN, 2001) aumentando a fagocitose (PAREDES *et al*., 2007) e estimulando a produção de citocinas (ALVAREZ-GARCIA *et al.,* 2012). Injeções múltiplas diárias de melatonina na glândula pineal de ratos podem promover um aumento significativo no número de macrófagos (KAUR; LING, 1999). Além da expressão dos diferentes genes relacionados ao controle de relógio, a melatonina e os glicocorticóides regulam inúmeras atividades proteicas e expressões gênicas, que são reguladores críticos do relógio circadiano e do sistema imunológico (SILVER *et al*., 2012). Entre esses fatores, a melatonina aparece como um candidato-chave para a regulação circadiana durante a reprodução feminina. Além de ser um antioxidante potente, foi relatado o importante papel da melatonina na função folicular e do corpo lúteo, gravidez, puberdade e parto, indicando seu papel crucial na reprodução (BRZEZINSKI *et al.*, 1987).

A melatonina é rapidamente transferida da circulação materna para a fetal, fornecendo informações fotoperiódicas ao feto para a diferenciação tecidual e o metabolismo hormonal (OKATANI *et al*., 1998). Portanto, a interação entre o sistema imunológico e o relógio circadiano durante a gravidez é de vital importância para o crescimento e o desenvolvimento fetal. No entanto, essa interação envolve diversos fatores que ainda não foram completamente elucidados (MAN *et al*., 2017).

Estudos relacionados com perturbações do ritmo circadiano causados pela configuração de trabalho em turnos, apontaram que variações da relação entre o ciclo claro e o escuro durante o período gestacional podem refletir em alteração dos padrões de expressão gênica da prole, assim como danos metabólicos e comportamentais da mesma (ROMAN; KARLSSON, 2013; MENDEZ *et al*., 2016; VARCOE *et al*., 2016). A exposição constante à luz do 12º ao 18º dia de gestação também leva à alterações no metabolismo e expressão de alguns genes na fase adulta da prole, apontando que reflexos da alteração circadiana, não somente afetam o desenvolvimento intrauterino como podem refletir na fase adulta da prole, em roedores (SPICHIGER *et al*., 2015; VOICULESCU *et al*., 2016).

1.2 TESTÍCULO E EPIDÍDIMO

O sistema genital masculino é composto pelas gônadas (testículos), epidídimos, ductos deferentes, glândulas sexuais e órgão copulador (JUNQUEIRA; CARNEIRO, 2004). Nos túbulos seminíferos, acontece a espermatogênese e no interstício, a esteroidogênese; estas duas regiões (túbulos seminíferos e interstício) formam o testículo (RODRIGUEZ; FAVARETTO, 1999).

A região do interstício é formada por tecido conjuntivo, vasos (linfáticos e sanguíneos), tecido nervoso, células de defesa (macrófagos) e células de Leydig (responsáveis pela produção de hormônios andrógenos) (RUSSEL et al., 1990). Já o parênquima testicular é formado por uma série de túbulos seminíferos, que se organizam compartimentalizados em lóbulos de tecido conjuntivo nos humanos. Ao final de cada túbulo, há epitélio de transição (túbulos retos), que conectam cada túbulo seminífero a uma rede testicular de canais anastomosados (FOLEY, 2001). Em indivíduos que já atingiram a maturidade sexual, os túbulos seminíferos são organizados em camadas concêntricas de células de Sertoli (basais) e células germinativas.

As células germinativas são divididas em: espermatogônias, espermatócitos primários e secundários e espermátides, posteriormente dando origem aos espermatozoides (figura 1). A célula de Sertoli por sua vez, é uma célula somática com função de promover suporte estrutural e nutricional às células germinativas em formação, secretar fluidos e hormônios, realizar fagocitose e se organizar na forma de barreira hematotesticular (FOLEY, 2001).

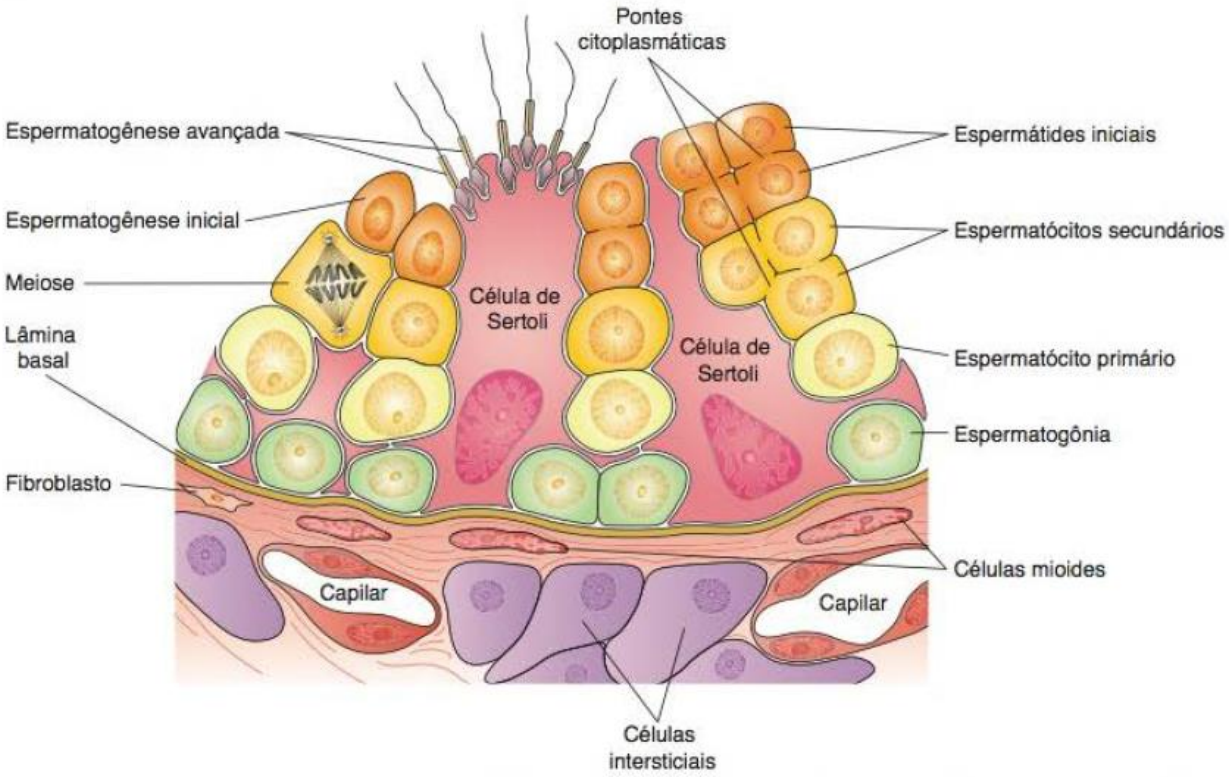

Figura 1 – Esquema de túbulo seminífero e da área intersticial. Os túbulos seminíferos são cercados por lâmina basal, células mioides e pelo tecido intersticial que contém células intersticiais (de Leydig), capilares sanguíneos e fibroblastos. Espermatogônias estão localizados na região basal do túbulo seminífero. Espermatócitos, 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 até o lúmen do túbulo, fazendo a ancoragem das células da linhagem germinativa.
Fonte: JUNQUEIRA; CARNEIRO, 2013.

O processo espermatogênico está sob o controle neuroendócrino do eixo hipotalâmico-hipofisário-gonadal e tem início a partir da puberdade, devido a um aumento na secreção de gonadotrofinas (FSH: hormônio folículo-estimulante; LH: hormônio luteinizante). O FSH age nas células de Sertoli estimulando suas funções sobre a espermatogênese, enquanto o LH estimula as células de Leydig a produzirem andrógenos (MAEDA *et al*., 2000; MARTY *et al*., 2003).

O processo de espermatogênese pode ser dividido em três fases distintas: mitótica ou proliferativa, meiótica e espermiogênica (CLERMONT, 1972; RUSSEL *et al.*, 1990). A fase mitótica é caracterizada pela proliferação das células tronco espermatogoniais com a finalidade de aumentar a população espermatogonial. Em determinado momento, grupos de espermatogônias tornam-se diferenciados 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 as espermátides (haploides). Durante a fase espermiogênica, as espermátides 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, finalmente, os espermatozoides (CLERMONT, 1972).

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 se unem para formar um ducto único e altamente enovelado, o epidídimo, que vai se ligar através de sua porção terminal ao ducto deferente (ROBAIRE; HINTON, 2015).

Nos roedores, o epidídimo é composto pelo segmento inicial, cabeça, corpo e cauda, enquanto nos humanos somente as regiões de cabeça, corpo e cauda compõe o órgão (ROBAIRE; HINTON, 2015). Em ratos, 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 os quais são: basais, principais, estreitas, halo, claras e apicais. Cada zona apresenta um microambiente bioquímico próprio, necessário para as etapas da maturação espermática (ROBAIRE; HINTON, 2015).

Os espermatozoides formados nos testículos são morfologicamente prontos, mas ainda são imóveis e incapazes de fertilizar um ovócito II (DACHEUX; DACHEUX, 2015). Assim, além do transporte até o ducto deferente para ser ejaculado, o epidídimo tem papel crucial sobre a maturação espermática. É neste órgão que acontece a aquisição 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 (DACHEUX; DACHEUX, 2015; ROBAIRE; HINTON, 2015).

1.3 ÚTERO E OVÁRIOS

Nas fêmeas, o sistema genital é formado por dois ovários, duas tubas uterinas, o útero, a vagina e a genitália externa. Sua função consiste em, além da produção de gametas femininos e hormônios sexuais, manter o ovócito fertilizado

durante seu desenvolvimento completo ao longo das fases embrionária e fetal até o nascimento (JUNQUEIRA; CARNEIRO, 2013).

Em humanos, o sistema genital feminino sofre modificações periódicas a partir da primeira menstruação (menarca). Estas modificações são cíclicas e ocorrem durante toda a idade reprodutiva da mulher e se encerra na menopausa, onde inicia-se uma lenta involução da fertilidade feminina. Todos estes períodos são regulados por mecanismos neuro-humorais (GARTNER; HIATT, 2003; JUNQUEIRA; CARNEIRO, 2013).

Os ovários estão localizados na região pélvica, caudalmente aos rins. O ovário se conecta ao ligamento largo do útero pelo mesovário, uma dobra especial do peritônio, responsável pela irrigação sanguínea desta glândula. O epitélio superficial que cobre o ovário, denominado epitélio germinativo, é uma modificação do peritônio e, apesar deste nome, este epitélio não dá origem às células germinativas (figura 2A; KOMÁREK *et al*., 2000).

Imediatamente abaixo do epitélio germinativo está localizada a túnica albugínea, que consiste em uma cápsula de tecido conjuntivo denso não modelado com pouca vascularização. O tecido ovariano é subdividido em córtex, onde estão presentes os folículos ovarianos, e medula, constituída principalmente por tecido conjuntivo frouxo altamente vascularizado (GARTNER; HIATT, 2003). Em mamíferos, o ovário é formado por duas populações celulares: as células somáticas e as células germinativas, que são produzidas durante a vida intratuterina. Após o nascimento, os ovócitos entram em uma pausa de divisão em diacinese, onde permanecem até o momento da puberdade (BIGGERS, 1975).

O ciclo ovulatório em mamíferos, que acontece a partir da puberdade, inclui múltiplos eventos: foliculogênese; ovulação; preparação do sistema genital para a fertilização e implantação; gestação (BOUÉ; BOUÉ; LAZAR, 1975).

No ovário estão presentes uma quantidade variável e limitada de folículos, dependendo da idade da mulher. O folículo ovariano é formado por um ovócito envolvido por uma ou mais camadas de células foliculares ou células da granulosa. Ao nascimento, o indivíduo geneticamente feminino apresenta no córtex ovariano apenas folículos primordiais, formados na vida fetal. Estes folículos são formados por um ovócito primário envolvido por uma única camada de células foliculares achatadas (JUNQUEIRA; CARNEIRO, 2013).

A partir da puberdade, momento marcado pela menarca nas mulheres, um pequeno grupo de folículos primordiais inicia um processo chamado crescimento folicular. Esse processo é estimulado pelo FSH e caracteriza-se por modificações foliculares e no ovócito. À medida que os folículos se desenvolvem, é observado um aumento no tamanho do ovócito e o número e forma das células foliculares que o circundam. Assim, a primeira etapa do desenvolvimento folicular é a modificação de folículo primordial em primário, que passa a apresentar uma ou mais camadas de células foliculares cúbicas ao redor do ovócito I (GARTNER; HIATT, 2003). O próximo estágio de desenvolvimento é a produção de líquido folicular pelas células da granulosa; este líquido se acumula entre as células, formando o antro. Quando os folículos apresentam antro, são denominados folículos secundários ou antrais. Nesta etapa do amadurecimento folicular, além das células da granulosa, os folículos apresentam duas camadas celulares denominadas tecas. A teca interna é formada por células produtoras de estrogênios, enquanto a teca externa tem estrutura semelhante ao estroma ovariano e não apresenta função hormonal (JUNQUEIRA; CARNEIRO, 2013).

Ainda sob influência do FSH, um dos folículos secundários torna-se dominante, e apresenta crescimento muito mais acentuado que os demais. Este, então, desenvolve-se até formar o folículo maduro ou de Graaf, que apresenta um aumento significativo no tamanho da cavidade folicular e do número de células da granulosa, além de possuir suas tecas mais espessas (figura 2B; GARTNER; HIATT, 2003; JUNQUEIRA; CARNEIRO, 2013).

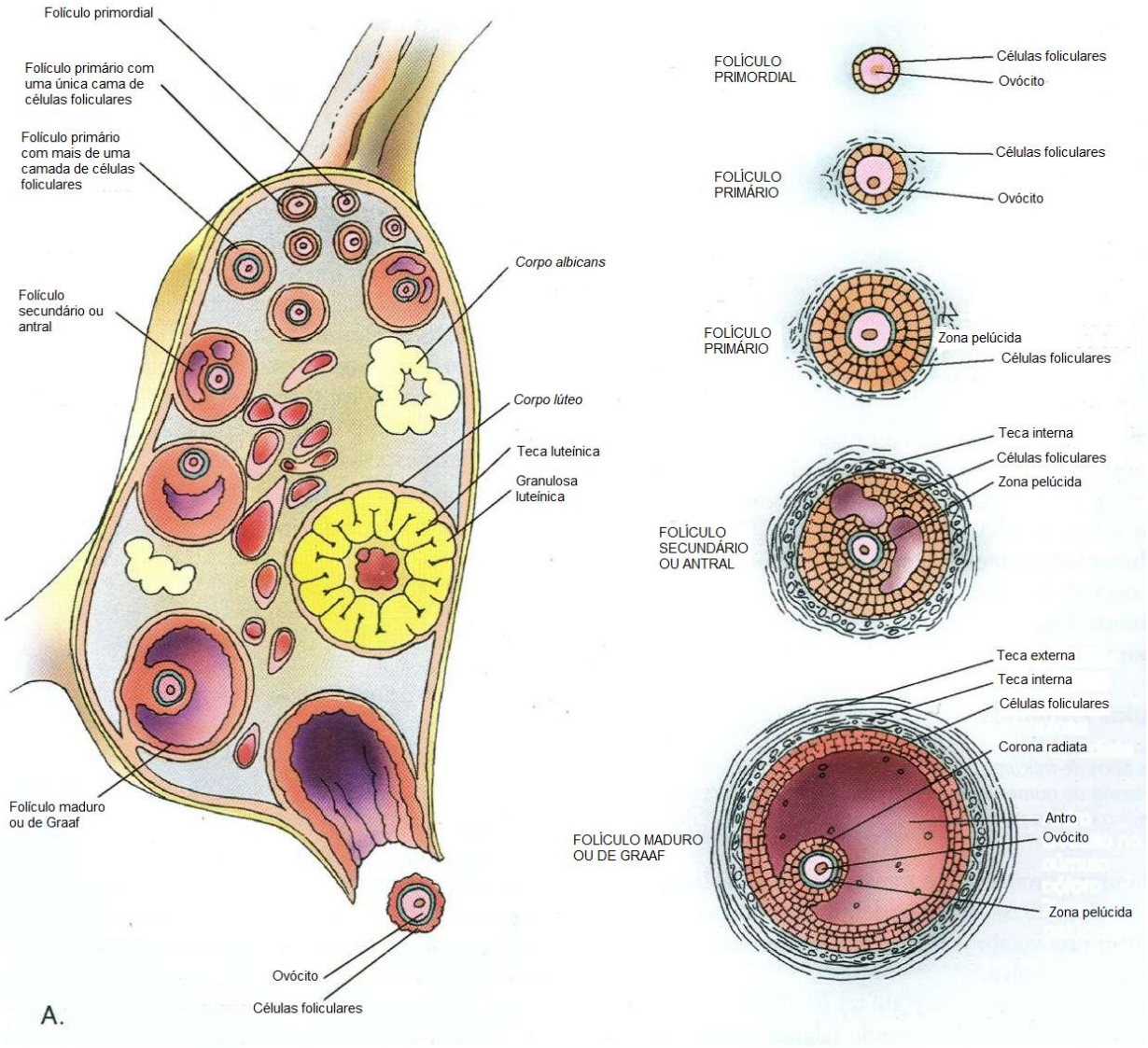

Figura 2 – (A) Esquema ilustrativo do ovário contendo os folículos e desenvolvimento. (B) Folículos ovarianos e suas estruturas.
Fonte: GARTNER; HIATT (2003).

A ovocitação, ou seja, liberação do ovócito II para posterior fertilização, é resultante de interações entre regiões hipotalâmicas, hipófise e ovário. O hipotálamo libera GnRH e como resposta, a hipófise é estimulada e os níveis de FSH e LH aumentam subitamente. Este súbito aumento hormonal leva ao desenvolvimento folicular e estimula o ovócito primário presente no folículo maduro a terminar a primeira divisão meiótica. Além disso, os hormônios hipofisários fornecem estímulos para a cascata de eventos ovarianos que resulta na ovocitação do ovócito secundário em metáfase da segunda divisão meiótica (BOUÉ; BOUÉ; LAZAR, 1975; SCHOENWOLF *et al*., 2015).

Após a ovocitação, as células foliculares remanescentes no ovário se diferenciam em corpo lúteo e iniciam a secreção de progesterona para manutenção de uma possível gestação. Os demais folículos que se desenvolveram, mas não ovocitaram, degeneram por um processo denominado atresia (KOMÁREK *et al*., 2000).

Em humanos, o útero tem a morfologia própria, na qual o corpo do útero é a porção dilatada, e a porção superior tem formato de cúpula (fundo do útero); a porção caudal é estreita e se abre na vagina (cérvix ou colo uterino). Esta morfologia é conhecida também como “forma de pera”. Em roedores, o útero é duplo, formado por dois tubos que se unem e abrem na vagina. O útero é um órgão muscular de parede espessa, que é formada por três camadas: 1) o perimétrio, caracterizado por uma camada delgada serosa; 2) o miométrio, uma espessa camada de músculo liso; 3) endométrio, que reveste internamente a luz uterina e é composto por um grande volume de vasos sanguíneos e glândulas, altamente responsivos à ação de hormônios sexuais (JUNQUEIRA; CARNEIRO, 2013).

As glândulas endometriais estão presentes em úteros de várias espécies, incluindo os roedores. Estas glândulas originam-se da invaginação do epitélio luminal em direção ao estroma uterino, formando um epitélio cuboide glandular (BRANHAM; SHEEHAN, 1985; OGASAWARA *et al*., 1983). Estas estruturas endometriais produzem e transportam secreções fundamentais para a implantação embrionária. Apesar da formação das glândulas acontecer de mandeira independente dos hormônios sexuais, a exposição inapropriada do sistema genital feminino imaturo a interferentes ambientais pode resultar em desregulação do desenvolvimento necessário para a fertilidade na idade adulta (BRANHAM; SHEEHAN, 1985).

1.4 Desenvolvimento intrauterino do sistema genital

A manifestação sexual do embrião em desenvolvimento começa com a determinação sexual genética que acontece com a fertilização (46, XX ou 46, XY em condições normais). O genótipo sexual direciona o desenvolvimento gonadal, que direcionará o desenvolvimento dos órgãos internos do sistema genital, bem como a genitália externa (SCHOENWOLF *et al*., 2015).

Apesar do sexo cromossômico ser definido no momento da fertilização, as características masculinas ou femininas são evidentes apenas na 7ª semana de

desenvolvimento embrionário. Até este momento, o sistema genital em ambos os sexos é semelhante; dessa forma, o período inicial do desenvolvimento genital é chamado de estágio indiferenciado do desenvolvimento sexual (MOORE; PERSAUD, 2008).

A formação das gônadas se inicia com a migração das células germinativas primordiais do saco vitelino para o mesoderma intermediário. Estas células se deslocam para a área adjacente ao epitélio celômico, localizado medial e ventralmente em relação aos rins mesonéfricos em desenvolvimento (figura 3A). Posteriormente, o epitélio celômico se prolifera e torna-se mais espesso; em conjunto com as células germinativas primordiais, estas estruturas são chamadas de cristas gonadais ou genitais (SCHOENWOLF *et al*., 2015; figura 3C).

As células derivadas do epitélio celômico originam as células somáticas de sustentação, durante a 6ª semana. Estas células envolvem as células germinativas presentes nas gônadas em formação e são responsáveis pela sustentação, sendo essenciais para o desenvolvimento das células germinativas (figura 3D). Com a diferenciação gonadal que acontece após a 6ª semana, estas células seguem destinos diferentes em machos e fêmeas (GARDINER; SWAIN, 2015; SCHOENWOLF *et al*., 2015).

Durante a 6ª semana, além da diferenciação das células somáticas, um novo par de ductos - os ductos paramesonéfricos ou de Müller - começa a se formar lateralmente aos ductos mesonéfricos em ambos os sexos (figura 3B). As extremidades caudais destes ductos crescem e se unem à uretra pélvica em desenvolvimento. As extremidades cranianas dos ductos paramesonéfricos formam aberturas em forma de funil para o celoma (MOORE; PERSAUD, 2008; SADLER, 2013).

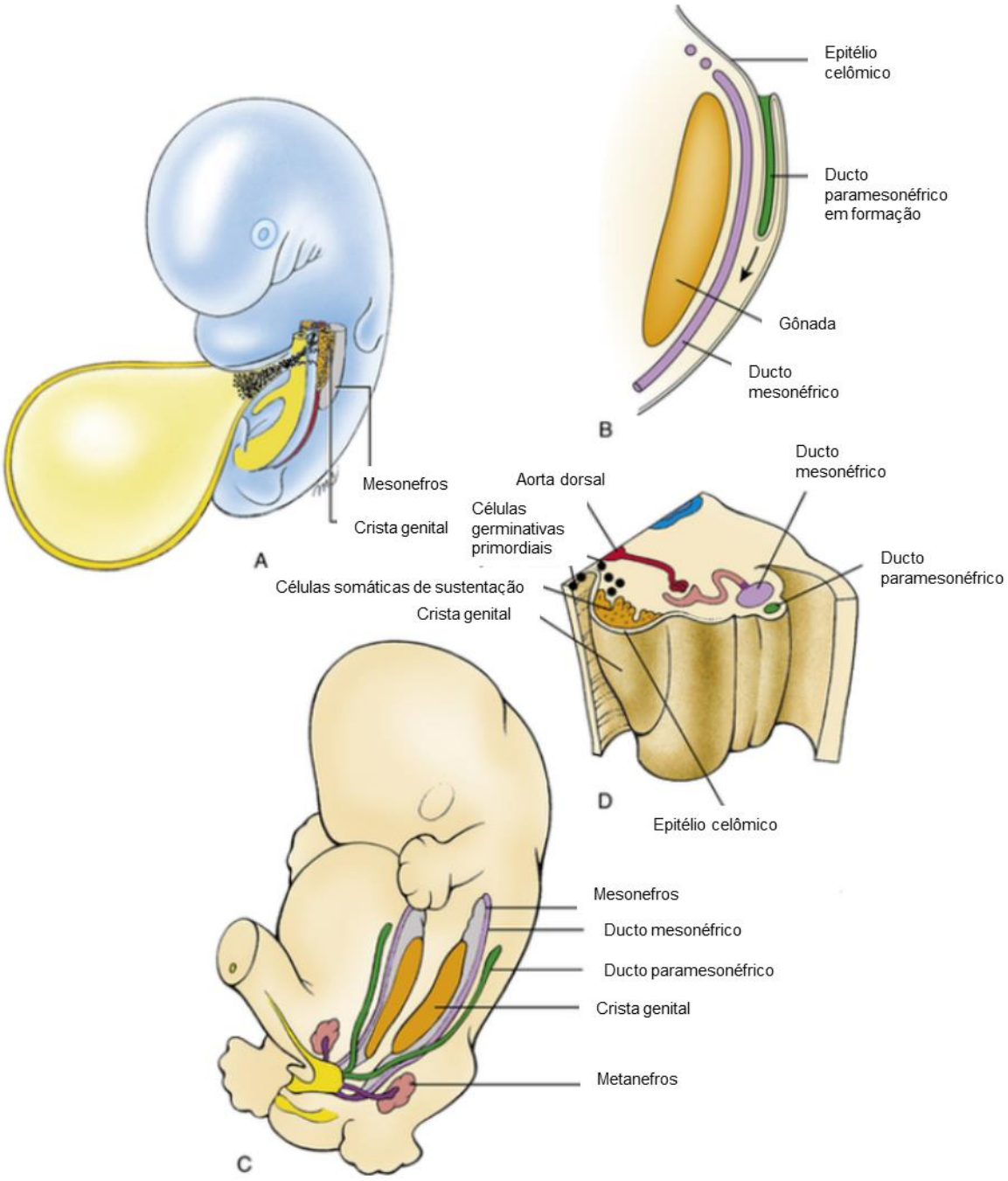

Figura 3 – (A) Esquema ilustrativo da migração das células germinativas primordiais; (B) da formação dos ductos paramesonéfricos; (C) da localização das cristas genitais e (D) da relação entre as células germinativas primordiais e as células somáticas de sustentação dentro da crista genital.

Fonte: SCHOENWOLF *et al*. (2015) com adaptações.

Ao final da 6ª semana, os sistemas genitais masculino e feminino são indistinguíveis externamente, mas apresentam diferenças celulares sutis. Em ambos os sexos, as células germinativas e as células somáticas de sustentação estão presentes nas gônadas em formação e os ductos mesonéfricos e paramesonéfricos

completos estão presentes. O período indiferenciado do sistema genital termina neste estágio. A partir da 7ª semana, o sistema genital segue vias de desenvolvimento diferentes em homens e mulheres, apesar dos órgãos se originarem dos mesmos precursores embrionários (MOORE; PERSAUD, 2008; SCHOENWOLF *et al*., 2015).

No sexo masculino, o gene SRY para o fator determinante do testículo (FDT) está presente no braço curto do cromossomo Y e direciona o desenvolvimento da gônada indiferenciada em testículo. O FDT induz os cordões sexuais presentes nas gônadas a se condensar e penetrar na medula do órgão, formando os cordões seminíferos. Estes cordões são formados por células germinativas (espermatogônias) e células somáticas (células de Sertoli). Concomitantemente, acontece o desenvolvimento de uma cápsula fibrosa e espessa, a túnica albugínea, que envolve a gônada em formação. Com o desenvolvimento desta cápsula, o testículo se separa do mesonefro – que se desenvolve para dar origem ao ducto epididimário. Parte dos cordões sexuais, além de originar os cordões seminíferos, originam os túbulos retos e a rede testicular (GARDINER; SWAIN, 2015; MOORE; PERSAUD, 2008).

Entre os cordões seminíferos, as células intersticiais em desenvolvimento originam as células de Leydig. Por volta da 8ª semana, estas células iniciam a secreção de androgênios – testosterona e androstenediona. Estes hormônios que induzirão a diferenciação da genitália externa e do ducto mesonéfrico em epidídimo. Além de androgênios, o testículo em formação secreta o hormônio anti-Mulleriano (AMH), produzido pelas células de Sertoli presentes nos cordões seminíferos. Este hormônio tem a função de inibir o desenvolvimento dos ductos paramesonéfricos, que no sexo feminino dará origem às tubas uterinas, útero e porção superior da vagina (MOORE; PERSAUD, 2008; SADLER, 2013).

Nos embriões do sexo feminino, o desenvolvimento das gônadas acontece de maneira mais lenta. Além dos cromossomos X, um gene autossômico também desempenha papel na organogênese ovariana. Em embriões, não é possível identificar o ovário até a 10ª semana de desenvolvimento. Por volta da 7ª semana, os cordões sexuais primários penetram na medula da gônada fetal e formam uma rede ovariana rudimentar, que posteriormente será degenerada. Em seguida, os cordões corticais se estendem do epitélio da superfície do ovário em desenvolvimento para dentro do mesênquima subjacente. As células germinativas

primordiais são incorporadas por estes cordões a medida que estes crescem em tamanho. Na 16ª semana, em média, os cordões sexuais se rompem e formam grupamentos isolados de células – os folículos primordiais. Estes folículos são constituídos de uma ovogônia, derivada de uma célula germinativa primordial, rodeada por uma única camada de células foliculares achatadas, derivadas do epitélio da superfície. Ainda na vida intrauterina, as ovogônias sofrem mitoses contínuas, produzindo milhares de folículos primordiais. Esta divisão mitótica não acontece no indivíduo geneticamente masculino devido à inibição deste processo pelas células de Sertoli (MOORE; PERSAUD, 2008; SCHOENWOLF *et al*., 2015).

O desenvolvimento sexual feminino não depende da presença de ovários ou hormônios. Na ausência de AMH, nos indivíduos geneticamente femininos, o ducto paramesonéfrico persiste, enquanto ocorre a degeneração do ducto mesonéfrico. Os ductos paramesonéfricos formam a maior parte do trato genital feminino – tubas uterinas desenvolvem-se a partir das partes craniais não fusionadas dos ductos paramesonéfricos; enquanto as porções caudais fundidas destes ductos formam o primórdio uterovaginal (MOORE; PERSAUD, 2008). Até a 7ª semana de desenvolvimento, as genitálias externas não apresentam diferenças significativas em ambos os sexos, e a diferenciação só é evidente a partir da 12ª semana.

Em indivíduos geneticamente masculinos, os androgênios secretados pelo testículo induzem o alongamento do tubérculo genital, formando o falo. As pregas uretrais direita e esquerda se fundem, formando a uretra peniana. A porção distal da uretra é formada por uma proliferação de células ectodérmicas, que por fim são origem ao óstio da uretra. As elevações genitais passam a ser denominadas elevações escrotais, que se movem caudalmente e, quando unidas, darão origem ao escroto. Nas mulheres, o tubérculo genital tem um alongamento discreto e dará origem ao clitóris. As pregas uretrais não fundem e darão origem aos pequenos lábios. As elevações genitais permanecem na posição lateral e desenvolvem-se, dando origem aos grandes lábios (figura 4; SADLER, 2013). A figura 5 resume os principais eventos do desenvolvimento pré-natal do sistema genital.

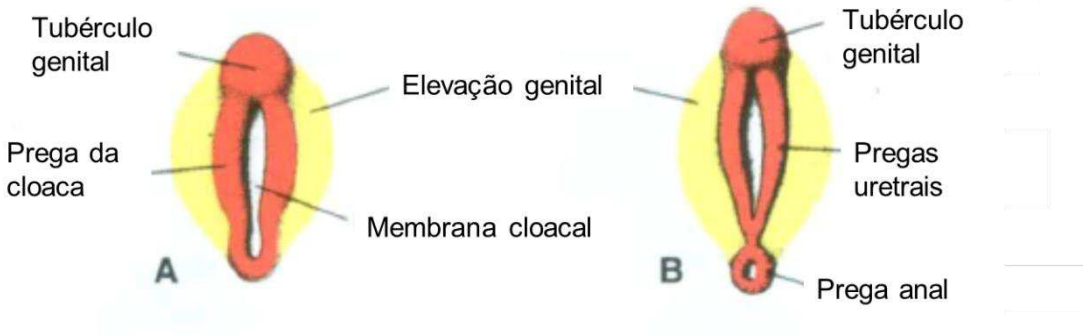

Figura 4 – estágios indiferenciados da genitália externa. (A) quatro semanas de desenvolvimento. (B) seis semanas de desenvolvimento.

Fonte: SADLER (2013).

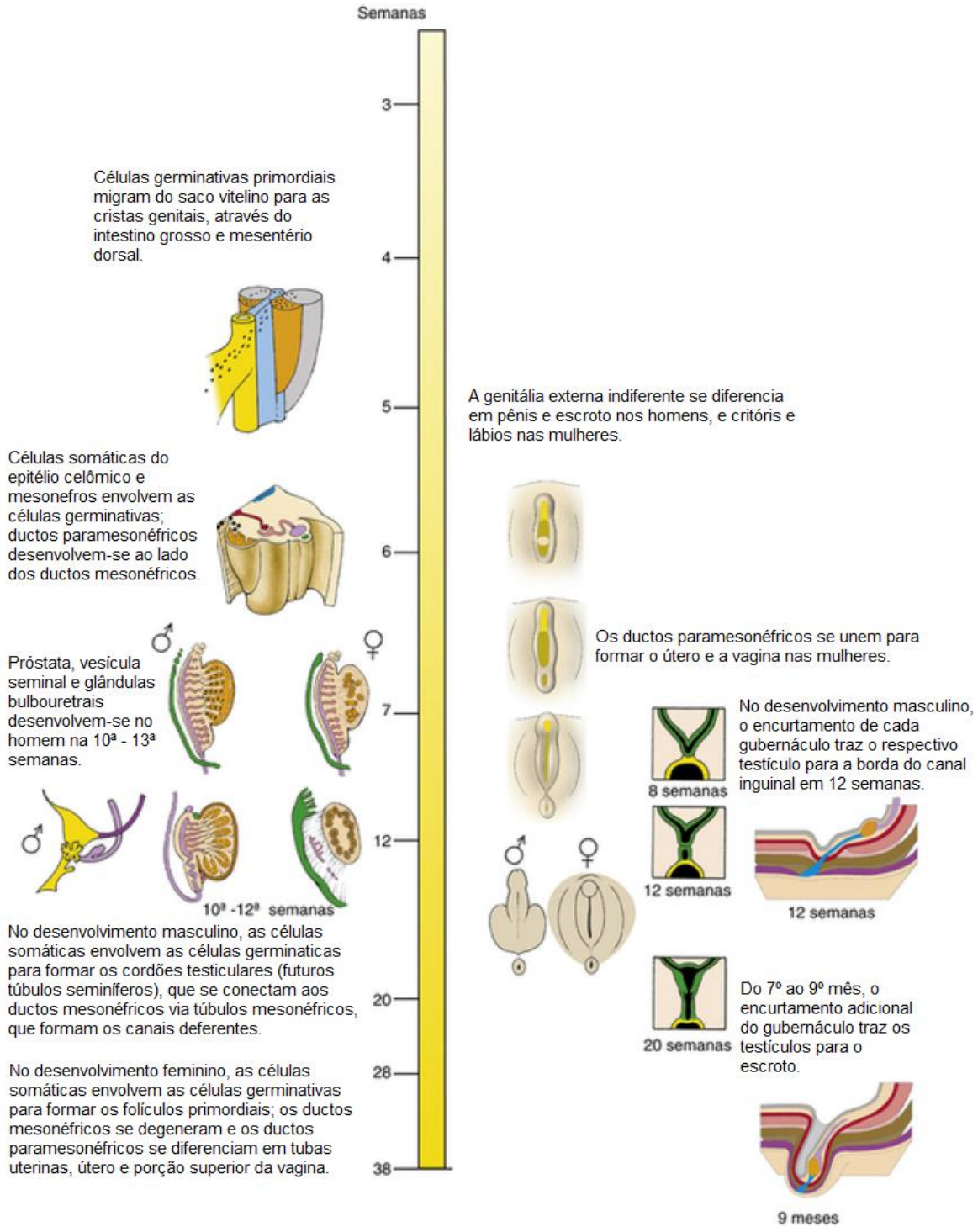

Figura 5 – Esquema representativo do desenvolvimento genital em ambos os sexos.

Fonte: SCHOENWOLF *et al*. (2015) com adaptações.

1.5 ORIGEM DESENVOLVIMENTISTAS DE SAÚDE E DOENÇA (DOHAD)

A saúde humana, muitas vezes é definida basicamente como ausência de doença, sendo determinada por vários fatores relacionados à dieta, meio ambiente e

economia, bem como o país de residência e realização educacional. Na interface de todas estas áreas destaca-se a relação entre fatores biológicos e socioeconômicos, que culminam em processos normais do desenvolvimento e, posteriormente, em uma condição saudável durante toda a vida de um ser humano (HOFFMAN; REYNOLDS; HARDY, 2017).

Com esta visão, um crescente número de evidências clínicas e estudos experimentais, tem relatado que adversidades e influências externas no início da vida são capazes de programar padrões de saúde-doença na vida adulta (BEYDOUN; SAFTLAS, 2008). Estas influências externas são relevantes principalmente na vida intrauterina, que é considerada uma janela crítica do desenvolvimento (ERIKSSON, 2010).

Um estudo pioneiro nesta área é o de Ravelli, Stein e Susser (1976), que estudaram uma população de 300.000 homens, filhos de mulheres expostas a um período de escassez alimentar. Este período ficou conhecido como fome holandesa, e aconteceu durante o cerco da Holanda pela Alemanha, na Segunda Guerra Mundial. Os autores observaram que, na vida adulta, esses indivíduos apresentavam padrões diferenciados de composição corporal dependendo do período de exposição à desnutrição materna. Quando os indivíduos sofreram exposição à desnutrição intrauterina no primeiro trimestre de gestação, a incidência de obesidade era significativamente maior. Contudo, quando a exposição ocorreu no último trimestre, a prole apresentava uma baixa incidência de obesidade.

Assim, nos últimos 30 anos, uma atenção substancial foi dada à influência de fatores externos durante os períodos críticos de desenvolvimento, e como estes interferentes influenciariam na saúde durante a idade adulta (HOFFMAN; REYNOLDS; HARDY, 2017). Inicialmente estes estudos faziam parte de uma área denominada “hipótese das origens fetais” ou “programação fetal”; esta denominação foi posteriormente modificada para as “origens desenvolvimentistas de saúde e doença” (DOHaD) para melhor refletir tanto o período gestacional quanto o pós-natal (SILVEIRA *et al*., 2007).

Atualmente os estudos são voltados a investigação da relação entre variações ambientais em momentos críticos do desenvolvimento, com fatores genéticos, epigenéticos e estocásticos; e quais desfechos metabólicos estas relações gerariam (SILVEIRA *et al*., 2007).

Os estudos envolvendo DOHaD e sistema genital ou fertilidade abordam principalmente a exposição intrauterina a agentes químicos ambientais com ação estrogênica ou antiandrogênica. Estes contaminantes são capazes de interferir na síntese de testosterona e consequentemente na diferenciação sexual, levando à disfunção testicular na vida adulta (CLARK; COCHRUM, 2007; DELBES; LEVACHER; HABERT, 2006). Em indivíduos geneticamente femininos, a exposição a substâncias desreguladoras endócrinas durante a vida fetal interrompe o desenvolvimento do sistema genital por meio da alteração na expressão gênica; esses efeitos têm consequências permanentes para a morfologia e função do trato reprodutivo tanto em roedores quanto em humanos (BAIRD; NEWBOLD, 2005; NEWBOLD, 2004).

Assim, a exposição a desreguladores endócrinos durante a gestação tem sido muito estudada nas linhas de DOHaD, dada a importância estrogênica e androgênica neste período (HO *et al*., 2017). Contudo, dados acerca da exposição intrauterina a outros influentes, como agentes estressores e o estilo de vida, ainda são escassos na literatura.

Neste sentido, é evidente o grande número de pessoas que realiza suas atividades, principalmente de trabalho, durante a noite. Esta inversão do ciclo claro e escuro resulta em uma produção alterada de melatonina e uma alteração no ciclo circadiano (TAIN; HUANG; HSU, 2017). O impacto destas alterações, em conjunto com a restrição de sono, pode aumentar o risco a alguns tipos de câncer, causar alterações no padrão metabólico – favorecendo a obesidade, prejudicar o sistema imune e gerar um estado pró-oxidativo (HAUS; SMOLENSKY, 2013).

Algumas evidências sugerem que o desbalanço de melatonina é responsável pelas alterações sistêmicas ocasionadas por modificações no ciclo circadiano. Durante a gestação, a melatonina tem papel crucial na manutenção da gestação e no desenvolvimento fetal (TAMURA *et al*., 2008; WADDELL *et al.*, 2012). Em condições normais, as concentrações plasmáticas de melatonina são elevadas durante a gestação, apresentando um pico significativo durante o parto; os valores voltam a níveis basais imediatamente após o parto (WADDELL *et al.*, 2012). Este aumento significativo ocorre pela produção do hormônio pela placenta, que também expressa receptores de melatonina e *CLOCK genes* (REITER *et al*., 2014). Assim, alterações na produção ou sinalização da melatonina pode prejudicar a função placentária. Além disso, a melatonina é capaz de ultrapassar a membrana

placentária e modificar o sistema circadiano do feto em desenvolvimento (TAIN; HUANG; HSU, 2017).

Estudos recentes sugerem que a melatonina possui propriedades epigenéticas, inibindo DNA metiltransferases ou histonas desacetilades (KORKMAZ; REITER, 2008; TAIN; HUANG; CHAN, 2014). Assim, alterações epigenéticas causadas pela alteração do ciclo circadiano durante o desenvolvimento intrauterino pode predispor a prole à uma variedade de distúrbios na vida adulta.

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2. OBJETIVOS

2.1 Objetivo geral

Avaliar se a exposição constante à luz durante a gestação altera o desenvolvimento intrauterino do sistema reprodutor na prole de ratos adultos.

2.2. Objetivos específicos

A. Avaliar os parâmetros espermáticos do ducto deferente: Motilidade e Morfologia.
B. Avaliar os parâmetros testiculares: peso do órgão, histopatologia, morfometria, dinâmica da espermatogênese, contagem espermática e produção diária de espermatozóides.
C. Avaliar os parametros epididimários: peso do órgão, histopatologia, estereologia, contagem espermática e transito espermático.
D. Avaliar parâmetros hormonais em ratos machos: Testosterona plasmática.
E. Avaliar parâmetros hormonais em ratas fêmeas: FSH, LH e progesterona plasmática.
F. Avaliar parâmetros ovarianos: peso do ovário e processo de foliculogenese.
G. Avaliar parâmetros úterinos: peso e morfometria.

3. CONCLUSÕES

A exposição constante à luz durante a gestação altera o ciclo circadiano materno, que atua de forma à induzir uma desregulação do ciclo circadiano fetal, desencadeando alterações no desenvolvimento morfológico dos órgãos reprodutores em na prole masculina de ratos adultos: Testículo e epidídimo e nos espermatozoides e alterações hormonais na prole feminina de ratas adultas.

3.1. Conclusões dos objetivos específicos

A. Foi observada alterações na morfologia espermática. Alterações na cabeça do espermatozóides foram mais frequentemente observadas no grupo LL (Luz-Luz). Não foi observada alterações na motilidade espermática.
B. Foram observadas alteração na morfologia dos túbulos seminéferos, tais como: vacuolização e redução no diâmetro dos túbulos seminíferos do grupo luz contínua. Não houve alteração na altura do epitélio.
C. Foram observado uma redução do peso epididimário, na contagem espermática na região da cabeça do epidídimo e redução do compartimento luminal e aumento do compartimento epitelial em ambas as regiões da cabeça e cauda epididimária no grupo LL em relação ao grupo LD (Luz-Escuro).
D. Foi observado uma redução nos níveis de testosterona plasmática no grupo LL.
E. Foi observado uma redução nos níveis de LH plasmático e um aumento nos níveis de Progesterona plasmáticas no grupo LL.
F. Não houve alterações na parâmetros ovarianos.
G. Não houve alterações na morfometria uterina. Porém, houve um aumento nas

glândulas úterinas no grupo LL em relação ao gupo LD.

- Artigo 1 –

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Extended light period in the maternal circadian cycle impairs the reproductive system of the rat male offspring

Running tittle: Impairment prenatal development by circadian cycle changes

Fernanda Mithie Ogo[1,2], Glaucia Eloisa Munhoz Lion Siervo[1,2], Ana Maria Praxedes[3], Katia Gama de Barros Machado[3], Suellen Ribeiro da Silva Scarton[1,2], Ana Tereza Guimarães Bitencourt[5], Alessandra Cecchini Armani[2], Andréa Name Colado Simão[4], Paulo Cesar de Freitas Mathias[3], Glaura Scantamburlo Alves Fernandes[1]

1 - Department of General Biology, Biological Sciences Center, State University of Londrina – UEL, Londrina, Paraná, Brazil
2 - Department of Pathological Sciences, Biological Sciences Center, State University of Londrina – UEL, Londrina, Paraná, Brazil
3 - Department of Biotechnology, Genetics and Cell Biology, State University of Maringá - UEM, Maringá, Paraná, Brazil.
4 - Department of Pathology, Clinical Analysis and Toxicology, Health Center, State University of Londrina – UEL, Londrina, Paraná, Brazil.
5 – Department of Biological and Health Sciences, State University of Western Parana, Cascavel, Paraná, Brazil.

***Corresponding author**
Dr. Glaura Scantamburlo Alves Fernandes
Department of General Biology
State University of Londrina (UEL)
Rodovia Celso Garcia Cid, Pr 445, Km 380, 86057-970
Londrina, PR, Brazil
Phone: +55 43 3371-4417
Email address: glaura@uel.br (G.S.A. Fernandes)

Abstract

Alterations in the circadian cycle are known to cause physiological disorders in the hypothalamic-pituitary-adrenal and the hypothalamic-pituitary-gonadal axes in adult individuals. Therefore, the present study aimed to evaluate whether exposure of pregnant rats to constant light can alter reproductive system development of male offspring. The dams were divided into a light-dark group (LD) of pregnant rats exposed to a light-dark photoperiod (12h/12h), and a light-light group (LL) of pregnant rats exposed to a photoperiod of constant light during the gestation period. After birth, offspring from both groups remained in the normal light-dark photoperiod until adulthood. The trunk blood was collected at PND90 to measure plasma testosterone levels, testes and epididymis for sperm count, oxidative stress and histopathological analyses, and the spermatozoa from the vas deferens, to perform the morphological and motility analyses. Results showed that a photoperiod of constant light caused a decrease in testosterone levels, epididymal weight and sperm count in the epididymis, seminiferous tubule diameter, Sertoli cell number and normal spermatozoa number. Histopathological damage was also observed in the testes, and stereological alterations, in the LL group. In conclusion, exposure to constant light during the gestational period impairs the reproductive system of male offspring in adulthood.

Key words: Circadian cycle, gestation, development, male reproductive system, adulthood.

Introduction

Clinical evidence and experimental studies have reported that the influence of environmental factors early in life is associated with alterations in the gene expression, and that these changes modify the programmed health-disease life pattern of adult individuals (Silveira et al., 2007; Beydoun; Saftlas, 2008). Studies involving the DOHaD (Developmental Origins of Health and Disease) concept are aimed at investigating the relationship among environmental variations occurring at critical moments of development, and during the development of diseases in adulthood (Silveira et al., 2007).

During development, the dynamic interaction among genome, epigenome, and stochastic and environmental factors contributes to the role of individual cells in the formation of functional organs. It also allows a critical balance to be maintained on a continuous basis between cell proliferation and death, and between cell regeneration and repair (Ho et al., 2017). Gestation, one of the three programming windows (gestation, lactation, and puberty), is considered a period of high vulnerability due to developmental events, such as germ cell specialization and migration, and morphogenesis of the gonads, which are structures of the reproductive tract and external genitalia (Ho et al., 2017). Moreover, prenatal stress exerts a negative impact on the development of the offspring throughout its childhood, and may also persist into adulthood (Beydoun; Saftlas, 2008; Eriksson, 2010). These critical periods of development should be considered not only as vulnerable stages, but also as cycles of opportunity, considering that an insult caused during this period can lead to permanent changes (Bouret, 2012).

In a circadian system, the light-dark cycle is the most reliable and efficient external signal that synchronizes the biological rhythms with the environment. In mammals, photopic information is perceived by specialized retinal photoreceptors, and is transmitted directly to the suprachiasmatic nucleus in the hypothalamus. Thus, the circadian information is transmitted to other organs through hormonal signals or the nervous system (Hastings, et al., 2003). The circadian rhythm is involved in several physiological processes, such as that of sleep/wake (Dijk; Duffy, 1999), regulation of body temperature (Van Someren, 2000), hormone secretion (Copinschi; Van Cauter, 1995), tissue repair (Janich et al., 2014) and cardiovascular functions (Cooke-Ariel, 1998). However, the fetus does not respond directly to the effects of light during development. Circadian information is transferred through the mother to the fetus (Reppert, Schwartz, 1983; Parraguez et al., 1996).

The effect of exposure to endocrine disrupters during gestation has been extensively studied under DOHaD lines, given the importance of estrogen and androgen in this period (Ho et al., 2017). However, information on intrauterine exposure to other stressors and on lifestyle is still unclear. In this scenario, the aim of the present study was to evaluate whether changes in the circadian cycle of pregnant rats exposed to constant light can alter the development of the reproductive system in the male offspring.

Material and Methods

Ethical approval

Experimental procedures were in accordance with the Experimental procedures were approved by the Ethics Committee on Animal Use of State University of Maringá (CEUA/UEM protocol number 4504080715).

Animals and experimental conditions

Rattus novergicus of the *Wistar* lineage (males and females, aged between 70 and 80 days of age) were obtained from the Sectorial Room of the Laboratory of Cellular Biology of Secretion, linked to the Department of Cell Biology and Genetics of the State University of Maringá. The animals were housed at the same place, into polypropylene cages (45 cm/30 cm/15 cm) under light controlled conditions with a 12-h light-dark cycle (07:00 a.m. to 07:00 p.m.) and a temperature of 22.0 ± 2°C. After one week of adaptation, rats were mated at a ratio of three females to each male. Pregnancy was confirmed by the presence of sperm in a vaginal smear, and pregnant dams were individually housed and separated into two groups: light-dark control group (LD) (group exposed to normal light and dark cycle - 12h/12h) and light-light group (LL) (group exposed to constant light - 24h).

For the control of the light and dark cycle, the pregnant rats were kept in a specific rack (Master-One®, Ribeirão Preto) for studies of circadian rhythms which controls the exposure to light. In the first compartment was the LD group with normal light/dark cycle, in the second compartment was the LL group that were kept at constant exposure to light (≅ 200 lux; mensuared by a luxmeter). The pregnant females (N = 8) were maintained at the specific rack during all the gestational period.

After birth, mothers and offspring were kept in normal lighting conditions (12h light/12h dark; light period from 07h a.m to 07h p.m.) cycle and controlled room

temperature (22 ± 2° C). The litter size was standardized to eight pups per female. At 21º postnatal day (PND) male offspring were kept in polypropylene cages (4 rats per cage). During all experimental period, the rats had free access to water and food (Nuvital®, Curitiba, Brazil).

Reproductive organs weight

At PND 90, male rats were anaesthetized (Thiopental, 150 mg / kg intraperitoneally) and euthanized by decapitation. Testis, epididymis, vas deferens and seminal vesicle (without the coagulating gland, full and empty of seminal fluid) were removed and their weights (absolute and relative to body weights) were determined. Testis and epididymis were used for sperm counts (n=7/group), histopathological analysis (n=5 rats per group) and oxidative stress analyses (n=10). Spermatozoa from the vas deferens were analyzed for morphological analysis and sperm motility (n=10/group).

Sperm number, daily sperm production per testis and sperm transit time through epididymis

Testicles decapsulated and epididymis were weighed and homogenized as described previously by Robb et al. (1978), with the adaptations described by Siervo et al., (2015). After dilution (1:10 for testis; 1:20 for each epididymal portion) of the homogenized, a small sample was transferred to Neubauer chamber and 4 fields were counted per animal sample) for counting homogenization- resistant spermatids (stage 19 of the spermatogenesis) in the testis, and spermatozoa in epididymis. To calculate the daily production of sperm (DSP), the concentration of spermatids per testis was divided by 6.1, which is the number of days in which mature spermatids are present in the seminiferous epithelium. To calculate sperm transit time through the epididymis, the number of sperm in each portion was divided by DSP.

Morphometric and histopathological analyses

The testicles and epididymis were removed, fixed in methacarn (Puchtler et al., 1970) and embedded in Paraplast (SIGMA Life Science). Histological sections (5 mm) were stained with hematoxylin–eosin (HE) and examined for general histopathological, morphometric and stereological analysis features as described by Favareto et al., (2011).

Histopathological analysis of the testis

One hundred random testicular cross-sections per rat were examined under Opton microscope at a magnification of 1000X. The seminiferous tubules were categorized as normal or abnormal. Abnormal tubules were identified as those with immature germ cells in the lumen, acidophilic cells and vacuolization (Siervo et al., 2015).

Nuclei of Sertoli cells were counted in 10 seminiferous tubules (3 non-consecutive testis cross-sections) per rat (n = 10 animals/group) at stage VII of spermatogenesis, under a light microscope (Opton), at 1000x magnification.

Morphometric analysis in the testis: Seminiferous tubule diameters and seminiferous epithelium height

Ten random testicular cross-sections were examined per rat in Stage IX of the seminiferous epithelium cycle. Seminiferous tubule diameters were measured using an Opton photomicroscope at a magnification of x 400 and BELview version 6.2.3.0 for Windows; BEL Engineering. The height of the seminiferous epithelium was measured using the same tubules and methodology described above. The mean of four measures of diameter and height was calculated for each seminiferous tubule and used for statistical analyses (Siervo et al., 2015).

Kinetics of spermatogenesis

One hundred random seminiferous tubular sections per rat were classified into one of four categories of the seminiferous epithelium cycles (Stages I–VI, VII–VIII, IX–XIII and XIV) according to Leblond and Clermont (1952), under a light microscope (Opton) at magnifications of x 100 and x 400.

Histopathological and stereological analysis in the epididymis

In the histopathological analysis, epididymal cross-sections (caput and cauda) were evaluated qualitatively using an Opton microscope (x 100 and x400 magnification). For stereological analysis, 10 random cross-sections per animal of caput (Region 2A) and cauda (Region 5A/B) epididymis (Miller and Killian 1987) were captured using a photomicroscope (Opton) and BELView version 6.2.3.0 (BEL Engineering) for Windows at a magnification of x 400 and analyzed. This analysis was performed using Weibel's multipurpose graticule with 168 points (Weibel 1963) to quantify the relative proportions of the epididymal components (epithelium, stroma and lumen).

Sperm morphology

The vas deferens was removed and washed internally with 1.0mL of 10% saline formol. Smears of histological slides were prepared from this solution and observed under a photomicroscope (Opton) at a magnification of x400. Two hundred spermatozoa were analyzed per rat. The morphological analysis was classified into three general categories: (1) normal morphology; (2) head abnormalities, that is spermatozoa without the characteristic curvature or in isolated form (i.e. no tail attached); and (3) tail abnormalities, namely broken tail, tail rolled into a spiral and isolated (i.e. no head attached). This evaluation was conducted as described previously (Siervo et al., 2015).

Sperm motility

To obtain sperm, left vas deferens were rinsing with 1.0 ml of modified HTF medium with gentamicin (Human Tubal Fluid, Irvine Scientific1), at 34°C –37°C. A warmed (34°C - 37°C) 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, in duplicate) under a light microscope (Motic) at x 100 magnification and was done by the same person throughout the study. Spermatozoa were classified as: mobile or immobile (Siervo et al., 2015).

Hormonal analysis

Blood plasma was obtained via centrifugation at 3000 g for 15 minutes at 4°C and stored at -20°C until assayed via immunoassay. The total testosterone present in the plasma was measured via chemiluminescence (2nd Generation Testosterone. Architect System, Abbott, Wiesbaden, Germany), according to the manufacturer's recommendations. The intra-assay coefficient of variation and minimum sensitivity of the assay were 4.6% and 0.15 nmol/L, respectively.

Biomarkers of Oxidative Stress

The testes and epididymis were separately homogenized in Tris-HCl buffer (pH 7.4) in a 1:20 ratio and centrifuged at 12,000 g for 10 min at 4ºC. The protein quantification of the samples was determined by the Bradford method, using bovine serum albumin as the standard (Bradford, 1976). This homogenate sample was diluted to 1 mg of protein.mL-1, and an analyses of oxidative stress were performed.

Lipoperoxidation (LPO) was measured with the aim of indirectly quantifying the peroxides that were produced. The result reflects the intensity of lipid peroxidation (Lushchak et al., 2009). The measurements were performed using the Thiobarbituric Acid Reactive Substances (TBARS) method at an absorbance of 535 nm (Buege and Aust, 1978) and with a comparison to a standard curve for malondialdehyde (MDA), which is the main by product of cellular lipid peroxidation. For the preparation of the sample, a 0.33 mg.mL-1 aliquot of the sample protein was added in 6.7% trichloroacetic acid (TCA) and in a final volume of 180 µL was vortexed, left in an ice bath for 5 min, and centrifuged for 5 min at 12,000 g at 4ºC. For the measurement of the thiobarbituric acid reactive substances (TBARS), the following substances were added to a microplate: 40 uL of the supernatant and different concentrations of MDA in triplicate, and a reaction medium containing 21.42 mM of thiobarbituric acid (TBA), 17.86 mM of NaOH (used for TBA solubilization), 0.73 M of TCA, 0.032 mM of butylated hydroxytoluene (BHT), and 3% ethanol (used for BHT solubilization) in PBS Buffer. The reading of the reaction was performed at 22ºC, after 60 min of incubation at 60ºC. The lipid peroxidation was estimated from the MDA curve, and the results were expressed as nmol of TBARS. mg of protein-1.

The activity of superoxide dismutase (SOD) was evaluated according method originally proposed by Crouch et al. (1981) with some modifications described below. The principle of this analysis was to quantify the complex formed between superoxide and nitro blue tetrazolium (NBT), which was measured at 560 nm during a 1hr period. An aliquot of 0.75 mg.mL-1 of protein in 25% ethanol was prepared in a total volume of 800 µL. The sample was centrifuged at 12,000 g at 4ºC for 20 minutes. In a 96 well microplate, the supernatant was pipetted in triplicate; a final volume of 200µL contained 0.1 mg of protein.mL-1, 0.09 mM of NBT, 0.015 mM of EDTA, 34.78 mM of hydroxylamine sulfate, and 79 mM of sodium carbonate buffer (pH 10.2), and the plate was read at 22ºC. The values of the activity of antioxidant enzymes were expressed as U. mg of protein-1. One unit of SOD activity was defined as the amount of enzyme that inhibits the oxidation reaction of NBT by 50% of the maximum inhibition.

The aim for the analysis of glutathione transferase (GST) is that the enzymatic activity of GST catalyzes the conjugation of GSH with the synthetic substrate CDNB, which produces a conjugate detected at 340 nm (Habig et al., 1976). During the assay, the enzymatic activity was proportional to the production rate of the conjugate compound. The assay was performed in triplicate in a microplate, and the final concentration of the

sample was 0.020 mg of protein.mL-1. The reaction medium produced final concentrations of 0.94 mM of CDNB and 0.94 mM of GSH. The reading was done at 22ºC. The molar extinction coefficient of the GSH/CDNB compound was 9.6 mM-1.cm-1, and the unit was expressed as mmol.min-1.mg of protein-1.

Glutathione reductase (GR) catalyzed the reduction of glutathione disulfide (GSSG) through the oxidation of NADPH; the decrease in absorbance was measured at 340nm (Sies et al., 1979). The assay was performed in duplicate in a microplate. The final concentrations within the reaction medium were 0.138 mM of NADPH, 3.81 mM of GSSG, and 3.75 mM of EDTA. The reaction was performed at 22ºC. The molar extinction coefficient of NADPH was 6.22 mM-1.cm-1. The unit was expressed as mmol.min-1.mg of protein-1.

Statistical analysis

All parameters were submitted to the Shapiro– Wilk test for normality and thus classified into parametric and nonparametric data. Parametric results were statistically analyzed by Unpaired *t* test. Nonparametric results were analyzed by the Mann-Whitney test. Differences were considered significant when $p < 0.05$. The statistical analyses were performed with GraphPad Prism (version 5.0 - GraphPad Software, La Jolla, California, USA) program.

Results

Reproductive organs weight

The weight of the reproductive organs is listed in Table 1. A reduction in epididymis weight was observed in the LL versus the LD group. In contrast, the weight of other organs remained unchanged between these two groups.

Sperm number, daily sperm production and sperm transit time through caput/corpus regions of epididymis

Exposure to continuous light during pregnancy did not change the sperm count, the sperm concentration or the daily sperm production of the testes of male offspring. Conversely, there was a decrease in the relative number of spermatozoa in the caput region of the epididymis of the LL group offspring, compared with the LD group.

However, no change was observed in the sperm count in the epididymal cauda or in the sperm transit time in the cauda region, in either experimental group (Table 2).

Sperm morphology and motility

Results of sperm morphology are presented in Table 3. A decrease in the number of spermatozoa with normal morphology was observed in group LL. Consequently, an increase in the number of abnormal sperm heads was noted in the group exposed to continuous light in relation to the control group ($P<0.0001$). The main abnormality observed in the spermatozoa was that their heads lacked the characteristic curvature. Continuous light exposure during the gestation period did not alter sperm motility (mobile sperm number: LD = 67.44 ± 3.31, LL = 63.88 ± 2.07, versus immobile sperm number: LD = 32.56 ± 3.31, LL = 36.13 ± 2.07; Mean ±SEM) in male offspring.

Histopathological analysis and spermatogenesis kinetics

Exposure to continuous light during gestation caused an increase in the number of seminiferous tubules with vacuolization in the male offspring ($P <0.04$) (Table 4 and Figure 1 (A and B). There was a significant reduction in the Sertoli cell number per seminiferous tubule at stage VII of the spermatogenesis cycle in the LL group (Table 4). The Leydig cells and the interstitial connective tissue seemed to be uniform in size and shape in both groups.

Analysis of the kinetics of spermatogenesis revealed no significant difference in the number of seminiferous tubules in the spermatogenesis stages between the groups (Mean±SEM): I-VI (LD = 36.8 ± 4.04, LL = 41.0 ± 2.19), VII-VIII (LD = 47.2 ± 4.09, LL = 42.2 ± 3.91), IX-XIII (LD = 9.60 ± 4.39, LL = 10.4 ± 8.90), and XIV (LD = 6.40 ± 1.07, LL = 5.60 ± 1.28).

Morphometric analysis of the testes

Exposure to continuous light during pregnancy caused a significant reduction in the diameter of the seminiferous tubule of the offspring in the LL versus the LD group ($P <0.02$). However, no significant difference was noted in the height of the seminiferous epithelium (Table 5).

Histopathological analysis and stereological analyses in the epididymis

The histopathological analysis revealed no cellular alterations in the epididymal tissue in either experimental group (Figure 1. C-D). Results of the stereological analyses are presented in Table 6. Specifically, a significant increase in the epithelial compartment was detected. This was followed by a decrease in the luminal compartment of the caput (2A region) and the compartment of the cauda epididymis (5A/B region) in the LL versus the LD group.

Hormonal analysis

Disrupting the circadian cycle caused a significant reduction in plasma testosterone levels in the male offspring in the LL versus the LD group (Figure 2.).

Biomarkers of Oxidative Stress

There was no significant difference between the experimental groups in relation to the lipid peroxidation (LPO) levels in the testis and epididymis. An increase in the enzymatic activity GPx (Glutathione Peroxidase) and GST (Glutathione s-Transferase) was observed in the testis of animals exposed to continuous light during the intrauterine period. However, the other SOD and GR enzymes remained unchanged in the testis. In the epididymis, continuous light caused a reduction in GR levels in relation to the LD group. The other SOD, GPx and GST enzymes remained unchanged (Table 7).

Discussion

Studies reveal how circadian alteration impairs fetal development; however, none of them have reported its relationship with the intrauterine development of the male reproductive system, and its effects in adulthood. The present study showed that exposure to continuous light during pregnancy impaired the reproductive system of the male offspring, evidenced by significant abnormal spermatozoa, vacuolization, a reduction in seminiferous tubule diameter, testosterone levels, epididymal weight, epididymal sperm concentration, and luminal compartment, and by an increase in the epithelial compartment. In addition, the damage caused by changes in the circadian rhythm can be associated with reduced testosterone levels, but not with oxidative stress.

Specific events in the intrauterine development of the male reproductive system are considered extremely important (Ho et al., 2017). The maternal circadian rhythm is involved in the programming of fetal and neonatal circadian clocks (Reiter et al., 2014). It can also influence the pineal-defining transcriptome, which is established prior to the

neonatal period (Hartley et al., 2015). Disorders of the circadian system in fetal life have been associated with long-term metabolic and behavioral consequences in the offspring (Cisternas et al., 2010; Voiculescu et al., 2015). The fetal circadian clock begins to develop in the second-half of pregnancy (Sladek et al., 2004). Epigenetics could be associated with prenatal stress and fetal programming, and could impact the offspring adversely throughout its childhood and persist during adulthood (Beydoun; Saftlas 2008; Eriksson, 2010). Thus, stress conditions during gestation can compromise testicular and epididymal functions in adult life.

In relation to the oxidative stress assay, previous studies have reported increased levels of lipoperoxidation, and reduced levels of antioxidant biomarkers in the brain, liver and kidneys of 3-month-old male rats exposed to continuous light for 26 days (Escribano et al., 2014). Verma et al. (2019) observed an increase in oxidative stress levels in the erythrocytes of male rats (150 ± 20g) exposed to continuous light for 10 days. However, there are no studies on how this deleterious event may affect the reproductive organs of male offspring undergoing circadian rhythm alteration during gestation. In the current study, non-alteration of the oxidative stress biomarkers may be associated with maintenance of sperm motility. It has been reported that oxidative stress impairs sperm motility, considering that the generation of high concentrations of ROS in the semen is associated with a decline in the energy metabolism of the sperm, in motility, in sperm viability and in DNA fragmentation (Baumber et al., 2002; Bilodeau et al., 2002). Alterations evidenced in the GST and GPx antioxidant enzymes in the testis and GR antioxidant enzymes in the epididymis indicate that the organism against oxidative damage was maintained, thus enabling protection of the cell membrane and attempting to maintain the redox homeostasis of the reproductive organs.

During the period of intrauterine development, the seminiferous cords are in formation. At puberty, spermatogenesis begins under hormonal influence, and these cords open in the seminiferous tubules for the production and passage of spermatozoa. Comparatively, in the present study, an alteration in the intrauterine circadian cycle affected the correct opening of these tubules, as evidenced by the reduction in the seminiferous tubule diameter.

In the present study, increased vacuolization in the seminiferous tubules was related to the activity and number of Sertoli cells, and to a reduction in testosterone levels. The main function of Sertoli cells is to maintain the seminiferous epithelium. According to Zambrano et al. (2014), Sertoli cells begin to differentiate between the 13^{th} to 14^{th} day

of gestation. In this period, an alteration in the circadian cycle affected Sertoli cell differentiation, leading to permanent changes. However, the sperm count in the testes remained unaltered.

Exposure to continuous light during the gestational period resulted in a change in sperm morphology, with an increase in sperm head changes. These results are associated with a reduction in the testosterone levels and an alteration in the spermiogenesis process. In this process, the nuclear and cytoplasmic compounds undergo a complex series of morphological, histochemical, and biochemical changes, ultimately ending with the reproduction of highly differentiated and specialized germ cells called spermatozoa (Guraya 1987).

The reduction in epididymal weight observed in the present study is related to the reduction in sperm concentration and luminal compartment in the caput region of the epididymis. A reduction in the luminal compartment allowed fewer sperm to pass through the epididymal duct, whereas sperm production in the testes remained unaltered. However, this reduction did not alter spermatozoa motility, indicating that the epididymal epithelial cells performed the function of sperm maturation correctly.

Light is a powerful element in circadian, neuroendocrine and neurobehavioral regulation, and has a profound influence on the health and well-being of all mammals, including laboratory animals (Dauchy et al., 2013). Many hormones and enzymes are essential for life, and are secreted following a circadian pattern, according to the photoperiod; testosterone is one such hormone. The organs of the male reproductive system develop as a result of hormonal action, like that of testosterone. In the present study, the faulty development of the morphology of male organs, such as seminiferous tubules and epididymal ducts, may be attributed to reduced testosterone levels. Despite this reduction, daily production and motility of spermatozoa was not affected. Thus, the reduction in testosterone caused morphological changes in the organs and spermatozoa, but not in the functioning of the spermatogenic cells, the seminiferous tubules, or the epididymal duct cells.

According to Gore et al. (2006), excessive exposure to glucocorticoids in adult male rats causes a reduction in GnRH (gonadotropin-releasing hormone) levels, ultimately decreasing LH (luteinizing hormone) levels, thereby acting indirectly on testosterone secretion and spermatogenesis. The alterations implicated in the male reproductive system by the excessive exposure of glucocorticoids are already well documented; however, the alterations caused during the development of the male

reproductive system are still unclear. Montano et al. (1991) have reported that an alteration in the light-dark cycle may increase corticosterone levels during pregnancy, and that corticosterone increases more rapidly in male offspring than in female offspring. Studies reveal that an enzyme called placental 11 β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) acts as a partial barrier to the passage of glucocorticoids from the mother to the fetus (Benediktsson et al., 1993). Some studies have reported that the manipulation of pregnant rats with ACTH (Stylianopoulou 1983), corticosterone, 11β-HSD2 inhibitors, and synthetic glucocorticoids increases fetal exposure to glucocorticoids by modifying the synthesis and secretion of adrenal glands and the fetal gonadal steroids. These act on the fetal hypothalamic-pituitary-adrenal and the hypothalamic-pituitary-gonadal axis during pregnancy (Harvey; Chevins, 1987).

Conclusion

In conclusion, the present study was able to demonstrate that exposure to constant light during the gestational period impairs the male reproductive system of the offspring in adulthood due a reduction in testosterone levels.

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The authors declare no conflicts of interest.

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663 **Legend of the figures**

664

665 **Figure 1. Histopathological analysis of seminiferous tubules of testes, caput and**
666 **cauda epididymis.** Photomicrograph of seminiferous tubules in testes (A and B), caput
667 epididymis (C and D) and cauda epididymis (E and F). Sections from LD group (A, C
668 and E) and LL group (B, D and F). (A) seminiferous tubules normal aspect, (B) Observed
669 vacuolization. (C, D, E and F) Epithelium, lumen and stroma normal aspect. Arrows in
670 (B) indicates vacuolization. Ep, Ephitelium It, interstitial tissue; L, lumen. Haematoxilin
671 and eosin stain. Magnification x100. LD (Light/Dark) group and LL (Light/Light) group.

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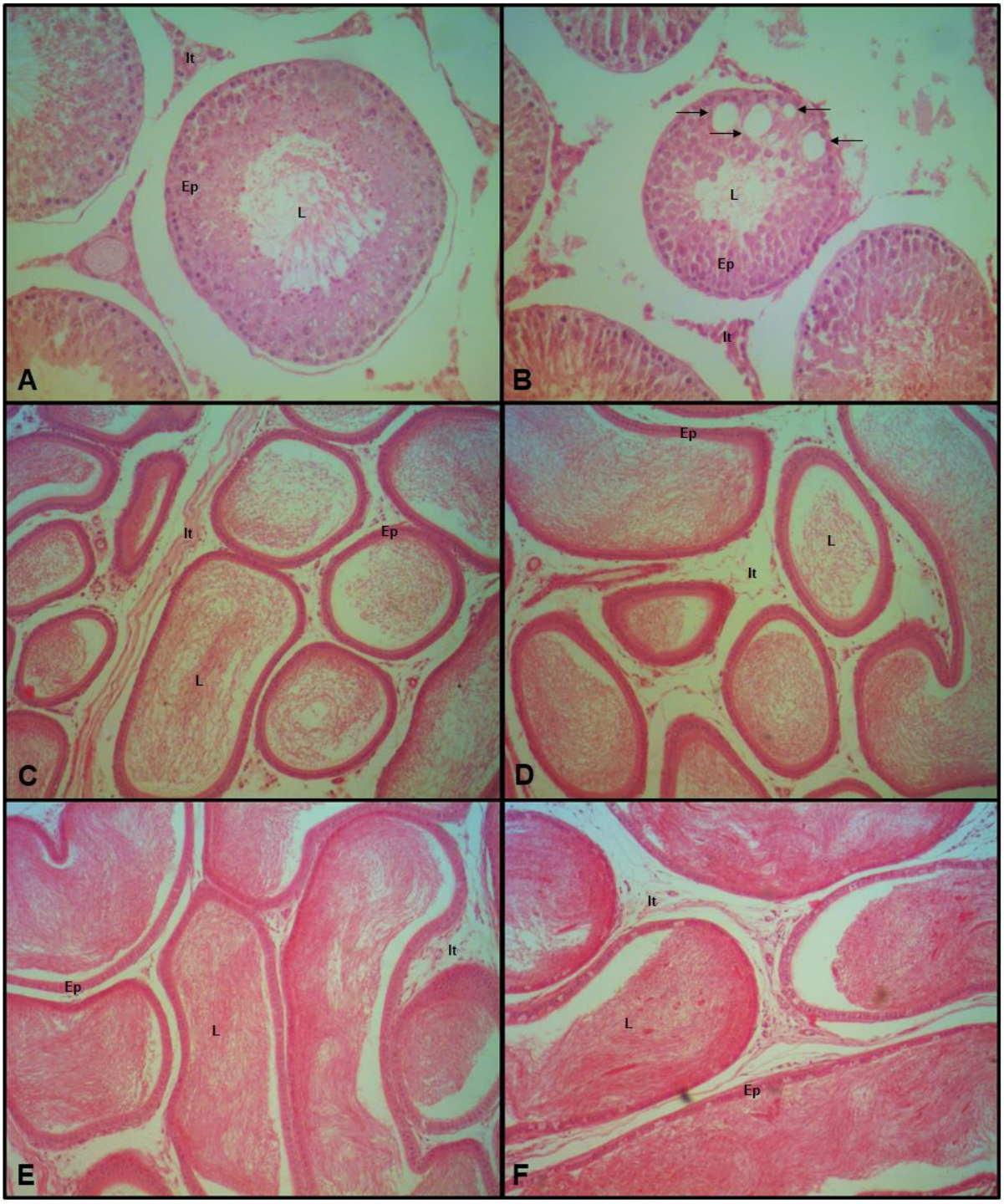
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Figure 2. Hormonal Analysis: testosterone plasma levels. Data are the mean ± SEM. *p > 0.05, Mann-Whitney test. LD (Light/Dark) group and LL (Light/Light) group.

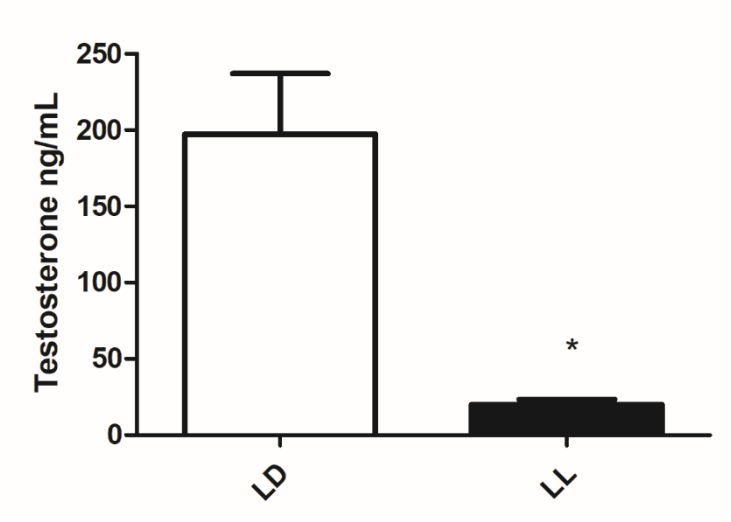

Table 1. Weight of reproductive organs

	LD (n = 10)	LL (n = 10)
Testis (g)	1.54 ± 0.03	10.44 ± 0.02
Testis (g/100g)	0.44 ± 0.01	0.42 ± 0.02
Epididymis (g)	0.51 ± 0.01	0.45 ± 0.02 *
Epididymis (g/100g)	0.14 ± 0.00	0.18 ± 0.03
Seminal vesicle (full) (g)	0.59 ± 0.08	0.85 ± 0.09
Seminal vesicle (full) (g/100g)	0.21 ± 0.02	0.25 ± 0.02
Seminal vesicle (empty)(g)	0.41 ± 0.06	0.43 ± 0.05
Seminal vesicle (empty)(g)	0.15 ± 0.02	0.13 ± 0.02
Vans deferens (g)	0.09 ± 0.01	0.08 ± 0.04
Vans deferens (g/100g)	0.02 ± 0.00	0.02 ± 0.00

Data are the mean ± SEM. Shapiro-Wilk normality test; $p > 0.05$. * indicate groups that differ statistically ($p < 0.05$, Unpaired *t*-test). LD (Light/Dark) group and LL (Light/Light) group.

740 **Table 2. Sperm Count**

	LD (n = 7)	LL (n = 7)
Sperm number in testis (x10^6)	56.51 ± 10.52	56.99 ± 3.03
Sperm number in testis (x10^6/g)	51.72 ± 6.77	49.68 ± 3.70
PDE	10.08 ± 1.72	9.34 ± 0.49
Sperm number in caput/corpus epididymal (x10^6)	20.68 ± 2.29	14.90 ± 2.85
Sperm number in cauda epididymal (x10^6)	22.39 ± 4.03	13.73 ± 2.28
Sperm concentration in caput/corpus epididymal (x10^6/g)	2.05 ± 0.04	2.00 ± 0.01 *
Sperm concentration in cauda epididymal (x10^6/g)	171.6 ± 24.3	114.0 ± 17.71
Transit time in caput/corpus epididymal (days)	3.39 ± 0.37	2.44 ± 0.46
Transit time in cauda epididymal (days)	3.67 ± 0.66	2.25 ± 0.37

741 Data are the mean ± SEM.Shapiro-Wilk normality test; $p > 0.05$.* indicate groups that differ
742 statistically ($p < 0.05$, Unpaired *t*-test). LD (Light/Dark) group and LL (Light/Light) group.
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Table 3. **Sperm morphology**

	LD (n = 10)	LL (n = 10)
Normal morphology sperm	157.5 [156.25 ± 160.75]	122.7 [112 ± 131.25] *
Abnormal head	38.0 [34.25 ± 41.25]	67.8 [60.75 ± 82.00] *
Abnormal tail	4.5 [2.00 ± 6.25]	9.44 [1.5 ± 19.00]

Data are the median [Q1 ± Q3]. Shapiro-Wilk normality test; $p < 0.05$. * indicate groups that differ statistically ($p < 0.05$, Mann-Whitney test). LD (Light/Dark) group and LL (Light/Light) group.

Table 4. Histopathological analysis of the testes

	LD (n = 5)	LL (n = 5)
Normal seminiferous tubules	99.2 ± 0.37	94.2 ± 2.08
- Immature germ cells in the lumen	0.20 ± 0.20	1.20 ± 0.58
- Vacuolization	0.20 ± 0.20	5.00 ± 1.58 *
Number of Sertoli cells	26.48 ± 0.38	14.66 ± 0.37 *

Data are the mean ± SEM. Shapiro-Wilk normality test; $p > 0.05$. * indicate groups that differ statistically ($p<0.05$, Unpaired *t*-test). LD (Light/Dark) group and LL (Light/Light) group.

Table 5. Morphometric analysis of the testis

	LD (n = 5)	LL (n = 5)
Seminiferous tubules diameter (µm)	271.00 ± 3.61	260.10 ± 3.37 *
Seminiferous epithelium height (µm)	78.56 ± 1.68	79.05 ± 1.25

Data are the mean ± SEM. Shapiro-Wilk normality test; $p > 0.05$. * indicate groups that differ statistically ($p < 0.05$, Unpaired *t*-test.). LD (Light/Dark) group and LL (Light/Light) group.

Table 6. Stereological analysis of the epididymis

	LD (n = 5)	LL (n = 5)
Caput (2A region)		
Epithelial	34.65 [29.00 ± 41.00]	39.75 [33.50 ± 45.50] *
Lumen	102.40 [93.25 ± 112.00]	93.22 [81.00 ± 104.50] *
Stroma	30.95 [23.25 ± 38.75]	35.02 [28.00 ± 42.50]
Cauda (5A/B region)		
Epithelial	19.89 [13.00 ± 26.00]	26.55 [20.00 ± 35.50] *
Lumen	120.30 [112.00 ± 130.00]	110.25 [90.50 ± 129.50] *
Stroma	27.83 [20.50 ± 32.50]	31.18 [18.00 ± 45.50]

Data are the Median [Q1 ± Q3]. Shapiro-Wilk normality test; $p < 0.05$. * indicate groups that differ statistically ($p < 0.05$, Mann-Whitney test.). LD (Light/Dark) group and LL (Light/Light) group.

Table 7. Biomarkers of oxidative stress in the testes and epididymis

	Testes		Epididymis	
	LD (n = 10)	**LL (n = 10)**	**LD (n = 10)**	**LL (n = 10)**
Glutathione Peroxidase (GPx)	95.65 ± 5.41	134.1 ± 15.1*	104.8 ± 3.01	95.99 ± 3.32
Glutathione Reductase (RD)	11.36 ± 0.87	12.33 ± 1.21	26.84 ± 3.56	15.29 ± 2.16*
Glutathione Transferase (GST)	123.5 ± 3.98	171.6 ± 16.19*	90.08 ± 7.59	72.99 ± 7.44
Superoxide Dismutase (SOD)	2.79 ± 0.44	2.14 ± 0.27	2.201 ± 0.52	2.29 ± 0.32
Lipoperoxidation (LPO)	1568 ± 358.9	2184 ± 73.7	253.2 ± 11.1	237.7 ± 6.46

Data are the mean ± SEM (Mm). Shapiro-Wilk normality test; $p > 0.05$. * indicate groups that differ statistically ($p < 0.05$, Unpaired *t*-test). LD (Light/Dark) group and LL (Light/Light) group.

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Increased the light period of the maternal circadian cycle impairs the development of the female reproductive system in adult offspring

Running tittle: Disruption of circadian cycle impairs prenatal development

Fernanda Mithie Ogo[1,2], Glaucia Eloisa Munhoz Lion Siervo[1,2], Ana Maria Praxedes[3], Henrique Rodrigues Vieira[3], Suellen Ribeiro da Silva Scarton[1,2], Ana Tereza Guimarães Bitencourt[5], Andréa Name Colado Simão[4], Marina Trevizan Guerra, Paulo Cesar de Freitas Mathias[3], Glaura Scantamburlo Alves Fernandes[1]*

1 - Department of General Biology, Biological Sciences Center, State University of Londrina – UEL, Londrina, Paraná, Brazil

2 - Department of Pathological Sciences, Biological Sciences Center, State University of Londrina – UEL, Londrina, Paraná, Brazil

3 - Laboratory of Secretion Cell Biology, Department of Biotechnology, Genetics and Cell Biology, State University of Maringá, Maringá, Brazil.

4 - Department of Pathology, Clinical Analysis and Toxicology, Health Center, State University of Londrina – UEL, Londrina, Paraná, Brazil

5 – Department of biological and health sciences, State University of Western Parana, Cascavel, Paraná, Brazil.

***Corresponding author**

Dr. Glaura Scantamburlo Alves Fernandes

Department of General Biology

State University of Londrina (UEL)

Rodovia Celso Garcia Cid, Pr 445, Km 380, 86057-970

Londrina, PR, Brazil

Phone: +55 43 33714417

E-mail address: glaura@uel.br (G.S.A. Fernandes)

Abstract

The circadian cycle plays numerous roles in the physiological processes of an organism, such us suppress or reproduce stimulation. The hypothalamic-pituitary gonadal axis receives and integrates environmental and physiological aspects of the circadian cycle and responds accordingly. This study aimed to evaluate whether circadian changes in rats pregnancy period may alter the development of the female reproductive system of the offspring. Wistar rats were divided: light-dark (LD) group, in which pregnant rats were exposed to a light-dark photoperiod during gestation. and light-light group (LL), in which pregnant rats were exposed to a photoperiod of constant light during gestation. After birth, pups remained in normal light-dark photoperiod until adulthood. At PND90, trunk blood was collected in the estrus phase for analyzing hormones in the plasma: FSH, LH and progesterone. The uterus was collected for morphometric and histological analyses and the ovaries for assessment of the number of follicles, and the corpora lutea. Exposure to continuous light during gestation interfered with the function of the hypothalamic-pituitary-gonadal axis in the offspring by reducing plasma LH and increasing plasma progesterone level, resulting in an increase in the number of uterine glands. The alteration in maternal circadian cycle was able to initiate alterations in the hormonal levels of offspring in adult life.

Key words: cycle circadian, female reproductive development, gestation

Introduction

Increasing clinical and experimental evidences in both humans and animals suggest that adversities and external factors at early stage of life are able to program health-disease patterns, which manifest later in adult life (Beydoun, Saftlas, 2008). These external factors are relevant mainly in intrauterine life, which is considered as a critical window of development (Eriksson, 2010). Developmental programming is considered as a specific challenge that occurs during the critical period of intrauterine development and alters the developmental trajectory with persistent effects on the progeny phenotype (Rabadan-Diehl, Nathanielsz, 2013). During intrauterine development of the female reproductive system, the migration of primordial germ cells to the genital crest occurs; this is followed by the proliferation of oogonia and cord formation, and finally, the oogonia undergo meiosis I (Zambrano et al., 2014).

Currently, the interference of various environmental stressors on the process of intrauterine development is being investigated. Among the various environmental stressors, disruptions to the circadian cycle have due to high exposure of the population to periods of artificial nocturnal lighting, transmeridian trips, and sleep disturbances (Evans, Anderson, 2018).

The circadian rhythm is involved in several physiological processes, including sleep / wake processes (Dijk, Duffy, 1999), body temperature regulation (Van Someren, 2000), hormone secretion (Copinschi, Van Cauter, 1995), tissue repair (Janich, Meng, Benitah, 2014), and cardiovascular function (Cooke-Ariel, 1998). Endogenous clocks exist in all organisms for the adjustment of physiology and behavior in anticipation of the occurrence of these daily changes. It receives and integrates different environmental and physiological factors, such as the circadian cycle and responds accordingly (Cristian, Moenter, 2010).

Female reproductive function is also controlled by circadian cycle (Cristian, Moenter, 2010). In adult animals, reproductive suppression or stimulation is considered to be regulated

by the hypothalamic-pituitary-gonadal axis. In addition, it is known that in the pre-ovulatory period, GnRH (Gonadotropin-Releasing Hormone) is released which stimulates the release and consequent increase of LH that is essential for ovulation to occur (Cristian, Moenter, 2010). The secretion of these hormones is regulated by internal and external factors such as the time of the day (Evans, Anderson, 2018). Studies have demonstrated that disruption of the circadian clock by shift work can result in infertility, menstrual dysregulation, and miscarriage (Stocker et al., 2014; Fernandez et al., 2016).

The functions of the circadian cycle in the control of some physiological processes are well documented; however, the influence of this cycle on the fetus is still obscure. The circadian clock is located within the suprachiasmatic nucleus of the hypothalamic region. , It contains about 20,000 neurons that act as "Pacemaker" (Dibner, Schibler, Albrecht, 2010). It is known that all tissues contain molecular clock genes that are expressed and controlled by transcriptional and translational feedback loops (Waddell et al., 2012). The humoral factors controlled by the circadian cycle including melatonin and glucocorticoids regulate numerous gene expressions and protein activities (Silver et al., 2012).

Melatonin plays an essential role in intrauterine development of the fetus. It passes through the maternal blood-brain barrier and goes into fetal circulation through the placenta, (Okatani et al., 1998). The melatonin receptors are disseminated in the fetus from the central tissue to the peripheral tissue throughout the period of fetal development. Melatonin is capable of reversing the rhythmic expression of fetal clock genes in response to maternal exposure to light (Cisternas et al., 2010). Man et al., (2017) reported that the maternal melatonin that crosses the placenta reduces the production of ROS (reactive oxygen species) and NO (Nitric Oxide) and increases antioxidant enzymes at the embryonic stage. In the fetal stage, maternal melatonin regulates the rhythm, promotes proliferation, differentiation, and neurodevelopment; while during childbirth, it regulates the time of labor. Considering the fact that the gestation period is

extremely vulnerable to environmental factors, any change in the circadian cycle can impair fetal development (Bouret, 2012).

Moreover, it is known that stress increases cortisol in pregnant women. The correlation between maternal and fetal cortisol may explain the origin of some prenatal diseases (Glover, 2015). Cortisol, being of lipophilic nature, passes easily through the placenta. However, there is a placental enzyme - 11β-hydroxysteroid dehydrogenase (11β-HDS2) which converts cortisol into inactive cortisone thereby protecting the fetus. However, any change that increases maternal cortisol level raises fetal glucocorticoid levels (Seckl, Holmes, 2007). According to O'Connor et al., (2002 and 2003) the increase in maternal cortisol may cause excess activity or dysregulation of the hypothalamic-pituitary-adrenal axis in offspring, resulting in changes in adulthood. Some studies indicated that excess cortisol can affect sexual maturation (Harvey, Chevins, 1987; Smith, Waddell, 2000), as well as decrease the fertility rate (Politch, Herrenkohl, 1979).

Varcoe et al., (2011) demonstrated that alteration in sex-dependent hyperleptinemia and hyperinsulinemia, increased adiposity, and decreased glucose tolerance and insulin sensitivity in offspring exposed to chronic phase shifts in their photoperiod (every 3-4 days) during intrauterine life. However, the effect of changes in circadian cycle on intrauterine development of the female reproductive system is still unclear. Therefore, the present study aimed to evaluate whether the circadian alterations caused in pregnant rats exposed to constant light throughout the period of gestation can alter the development of the reproductive system in their female offspring.

Material and Methods

Animals and experimental conditions

Rattus novergicus of the *Wistar* lineage (males and females, aged between 70 and 80 days of age) were obtained from the Sectorial Room of the Laboratory of Cellular Biology of Secretion, linked to the Department of Cell Biology and Genetics of the State University of Maringá. The animals were placed at the intersection at the Sectorial Room of the Laboratory of Cell Biology of the Secretion, linked to the Department of Biotechnology, Genetics and Cell Biology of the same institution. At birth the litters were kept with eight pups as standardized litter size. Weaning occurred at 21 days of age, keeping only female pups for evaluation. The rats were housed in polypropylene cages in number of 4 rats per cage, under light controlled conditions with a 12-h light-dark cycle (07:00 a.m. to 07:00 p.m.) and a temperature of 22.0 ± 2°C. with free access to water and (Nuvital®, Curitiba, Brazil) (After one week of adaptation, rats were mated at a ratio of three females to each male). Pregnancy was confirmed by the presence of sperm in a vaginal smear, and pregnant dams were individually housed and separated into two groups: light-dark control group (LD) (group exposed to normal light and dark cycle - 12h/12h) and light-light group (LL) (group exposed to constant light - 24h).

For the control of the light and dark cycle, the pregnant rats were kept in a specific rack (Master-One®, Ribeirão Preto) for studies of circadian rhythms which controls the exposure to light. In the first compartment were the LD group with normal light/dark cycle, in the second compartment were the LL group that were kept at constant exposure to light (≅ 200 lux; mensuared by a luxmeter). The pregnant females (N = 8) were maintained at the specific rack during all the gestational period.

After birth, mothers and offspring were kept in normal lighting conditions (12h light/12h dark; light period from 07:00 a.m to 07:00 p.m.) cycle and controlled room temperature (22 ± 2° C). At 21º postnatal day (PND) female offspring were kept in polypropylene cages (4 rats

per cage). During all experimental period, the rats had free access to water and food (Nuvital®, Curitiba, Brazil). Experimental procedures were approved by the Ethics Committee on Animal Use of State University of Maringá (CEUA/UEM protocol number 4504080715).

Reproductive organs weight

At the end of experimental period (PND 90), female offspring in the estrus phase from both experimental groups were anaesthetized with an anesthetic dose (Thiopental, 150 mg / kg intraperitoneally) according to the official standards and approval of the ethics committee (CEUA n°450408715). After decaptation, trunk blood was collected (between 08:00 and 11:30 hours) for determination of FSH, LH and progesterone concentrations (n = 7 rats per group). Uterus and ovaries were removed, and their weights were determined (n = 7 rats per group). Ovary was used for histopathological analysis - ovarian follicles and corpora lutea counting and oxidative stress analyses (n = 7 rats per group). Uterus was used for morphometric analysis to assess the height of endometrium and of luminal and glandular epithelium and counting the number of glands and oxidative stress analyses (n = 7 rats per group).

Hormonal Analysis

After decapitation, trunk blood was collected and allowed to clot at 4◦C for 30 min. Serum was collected after centrifugation (20 min at 2000 × g) and stored at −20◦C until analysis. Serum follicle-stimulating hormone (FSH), luteinizing hormone (LH) and progesterone concentrations were measured using a doubleantibody radioimmunoassay (RIA) kit (National Institute of Arthritis, Diabetes and Kidney Diseases [NIADDK], USA). Sample were performed to avoid intra-assay errors. Intra-assay errors for FSH, LH and progesterone were 2.8%, 3.4% and 4%, respectively.

185 ***Histological processing***

186 The histological evaluation was performed on reproductive organs: Uterus and right
187 ovary were fixed in methacarn (Puchtler et al. 1970) and embedded in Paraplast (SIGMA Life
188 Science). Histological sections (5 µm) were semi-serial with 10 interval cuts, stained with
189 hematoxylin–eosin (HE) and examined for general histopathological and morphometric.
190

191 ***Ovarian follicles and corpora lutea***

192 In the ovary, ovarian follicles and corpora lutea were counted. Follicles were classified
193 according to Borges et al. (2002) and Talsness et al. (2005). Primordial and primary follicles
194 were enumerated together; oocytes surrounded by a single layer of either squamous or cuboidal
195 epithelial cells were included. Follicles were classified as preantral when containing two to four
196 layers of granulosa cells with no antral space. Antral follicles were classified when there were
197 three or more layers of granulosa cells and a clearly defined antral space. Characteristics of
198 atretic follicles included pyknotic granulosa cells, disorganized granulosa cells, degenerating
199 oocyte, and detachment from the basement membrane.
200

201 ***Morphometric analysis in uterus: height of endometrium, myometrium, luminal and***
202 ***glandular epithelium and Count number of glands***

203 In the uterus, morphometric analyses were performed to assess the height of
204 endometrium and of luminal and glandular epithelium. For this, Option microscope at a
205 magnification of x 100 coupled to a digital camera and a PC with the software BELview version
206 6.2.3.0 for Windows; BEL Engineering were used. In each section, 5 different regions were
207 analyzed, resulting in a total of 15 measurements per animal by each parameter (Guerra et al.,
208 2010). The number of glands presented in each section was also recorded.
209

Biomarkers of Oxidative Stress

The uteurs and ovary were separately homogenized in Tris-HCl buffer (pH 7.4) in a 1:20 ratio and centrifuged at 12,000 g for 10 min at 4ºC. The protein quantification of the samples was determined by the Bradford method, using bovine serum albumin as the standard (Bradford, 1976). This homogenate sample was diluted to 1 mg of protein.mL-1, and an analyses of oxidative stress were performed.

Lipoperoxidation (LPO) was measured with the aim of indirectly quantifying the peroxides that were produced. The result reflects the intensity of lipid peroxidation (Lushchak et al., 2009). The measurements were performed using the Thiobarbituric Acid Reactive Substances (TBARS) method at an absorbance of 535 nm (Buege and Aust, 1978) and with a comparison to a standard curve for malondialdehyde (MDA), which is the main by product of cellular lipid peroxidation. For the preparation of the sample, a 0.33 mg.mL-1 aliquot of the sample protein was added in 6.7% trichloroacetic acid (TCA) and in a final volume of 180 µL was vortexed, left in an ice bath for 5 min, and centrifuged for 5 min at 12,000 g at 4ºC. For the measurement of the thiobarbituric acid reactive substances (TBARS), the following substances were added to a microplate: 40 uL of the supernatant and different concentrations of MDA in triplicate, and a reaction medium containing 21.42 mM of thiobarbituric acid (TBA), 17.86 mM of NaOH (used for TBA solubilization), 0.73 M of TCA, 0.032 mM of butylated hydroxytoluene (BHT), and 3% ethanol (used for BHT solubilization) in PBS Buffer. The reading of the reaction was performed at 22ºC, after 60 min of incubation at 60ºC. The lipid peroxidation was estimated from the MDA curve, and the results were expressed as nmol of TBARS. mg of protein-1.

The activity of superoxide dismutase (SOD) was evaluated according method originally proposed by Crouch et al. (1981) with some modifications described below. The principle of this analysis was to quantify the complex formed between superoxide and nitro

blue tetrazolium (NBT), which was measured at 560 nm during a 1hr period. An aliquot of 0.75 mg.mL-1 of protein in 25% ethanol was prepared in a total volume of 800 µL. The sample was centrifuged at 12,000 g at 4ºC for 20 minutes. In a 96 well microplate, the supernatant was pipetted in triplicate; a final volume of 200µL contained 0.1 mg of protein.mL-1, 0.09 mM of NBT, 0.015 mM of EDTA, 34.78 mM of hydroxylamine sulfate, and 79 mM of sodium carbonate buffer (pH 10.2), and the plate was read at 22ºC. The values of the activity of antioxidant enzymes were expressed as U. mg of protein-1. One unit of SOD activity was defined as the amount of enzyme that inhibits the oxidation reaction of NBT by 50% of the maximum inhibition.

The aim for the analysis of glutathione transferase (GST) is that the enzymatic activity of GST catalyzes the conjugation of GSH with the synthetic substrate CDNB, which produces a conjugate detected at 340 nm (Habig et al., 1976). During the assay, the enzymatic activity was proportional to the production rate of the conjugate compound. The assay was performed in triplicate in a microplate, and the final concentration of the sample was 0.020 mg of protein.mL-1. The reaction medium produced final concentrations of 0.94 mM of CDNB and 0.94 mM of GSH. The reading was done at 22ºC. The molar extinction coefficient of the GSH/CDNB compound was 9.6 mM-1.cm-1, and the unit was expressed as mmol.min-1.mg of protein-1.

Glutathione reductase (GR) catalyzed the reduction of glutathione disulfide (GSSG) through the oxidation of NADPH; the decrease in absorbance was measured at 340nm (Sies et al., 1979). The assay was performed in duplicate in a microplate. The final concentrations within the reaction medium were 0.138 mM of NADPH, 3.81 mM of GSSG, and 3.75 mM of EDTA. The reaction was performed at 22ºC. The molar extinction coefficient of NADPH was 6.22 mM-1.cm-1. The unit was expressed as mmol.min-1.mg of protein-1.

Statistical analysis

All parameters were submitted to the Shapiro– Wilk test for normality and thus classified into parametric and nonparametric data. Parametric results were statistically analyzed by Unpaired *t* test. Nonparametric results were analyzed by the Mann-Whitney test. Differences were considered significant when $p < 0.05$. The statistical analyses were performed with GraphPad Prism (version 5.0 - GraphPad Software, La Jolla, California, USA) program.

Results

Body Weight

The weight of the reproductive organs is given in Table 1. The weights of the uterus and the ovary of both experimental groups were not significantly different.

Hormonal Analysis

Exposure to continuous light during pregnancy (LL group) caused a decrease in the values of plasma LH ($P<0.03$) and an increase in the values of plasma progesterone ($P<0.04$) compared to LD group. In both groups of female offspring, the values of FSH maintained in the blood were equal (Figure 1).

Count of ovarian follicles and corpora lutea

The evaluation of the number of ovarian follicles: primary, pre-antral, antral, and atresic and the number of corpora lutea per area of the ovary in adulthood did not reveal significant changes in LL group compared to LD group (Table 2).

Morphometric analysis of the uterine parameters: height of endometrium, myometrium, luminal epithelium, and glandular epithelium and the evaluation of the number of uterine glands

Table 3 shows the evaluation of histopathology of the uterus. It was observed that the results of the morphometric parameters such as height of the endometrium, myometrium, luminal epithelium, and glandular epithelium were similar between the experimental groups. However, the LL group showed a significant increase in the number of uterine glands when compared to the LD group ($P< 0.01$) (Figure 2).

Biomarkers of Oxidative Stress

There was no significant difference between the experimental groups in relation to the lipid peroxidation (LPO), enzymatic activity GPx (Glutathione Peroxidase), GST (Glutathione s-Transferase), GR (Glutathione Reductase) and SOD (Superoxide dismutase) levels in the uterus (Table 4).

A reduction in the enzymatic activity GR (Glutathione Reductase) and GST (Glutathione s-Transferase) was observed in the ovarian of animals exposed to continuous light during the intrauterine period. However, the other GPx (Glutathione Peroxidase), and SOD (Superoxide dismutase) enzymes and lipid peroxidation (LPO) remained unchanged in the ovary (Table 4).

Discussion

The present study demonstrated that maternal exposure to continuous light during pregnancy reduces LH level but increases the level of progesterone, the number of glands and reduction in levels of antioxidant enzymes such as: glutathione reductase and glutathione s-transferase in the uterus in female offspring in adulthood.

It is well documented that circadian rhythm plays important roles in the physiological processes of an organism. However, its effect on intrauterine development of the female reproductive system is still obscure. It is known that circadian alteration can act on the clock genes that are present in the ovaries and the uterus during cell differentiation (He et al., 2007). Circadian alteration can cause maternal stress by increasing the amount of plasma glucocorticoids, which could interfere with the function of the hypothalamic-pituitary-adrenal and hypothalamic-pituitary-gonadal axes, causing alterations in hormonal production (O'Connor et al., 2002; O'Connor et al., 2003).

The period of intrauterine development is considered to be highly vulnerable to environmental changes, since important events occur in the process of development of the female reproductive system (Zambrano et al., 2014). It is also considered a cycle of opportunity, because certain insults to the reproductive organ in this period can lead to permanent changes (Bouret, 2012). According to Otha et al., (2008), during pregnancy, the fetus functions as an oscillator of the mother's body and uses maternal signals to adjust its fetal biological clock to the extra-uterine environment. The circadian rhythm regulates the behavior and some physiological processes. Some studies suggest that maternal circadian disturbance may induce long-term alterations in the offspring (Varcoe et al., 2011) and adversely impact pregnancy outcomes, growth, and metabolic health of the offspring (Varcoe et al., 2016).

The hypothalamus produces GnRH that acts on the pituitary gland and stimulates the synthesis of FSH and LH, which stimulate the ovary cells to produce estrogen and progesterone, which in turn control uterine functions in adulthood (Carson et al., 2000). The major uterine cell types respond differently to these two hormones produced by the ovary. In adult mouse, estrogen acts directly on the proliferation of uterine epithelial cells, while in the stromal cells, this process requires the action of both progesterone and estrogen (Pollard,1990). Progesterone is produced by the corpus luteum after ovulation and induces the proliferation of stromal cells.

In the present study, the increase in the serum progesterone level may be related to the increase in the number of uterine glands as progesterone acts on the endometrium. The glandular epithelium is the main source of uterine secretions which are necessary for establishment and maintenance of pregnancy (Gray et al., 2001). Studies by Jeong et al., (2010) demonstrated that Foxa2 gene is essential for the development of the uterine gland, and that gland reduction interferes with fertility in rats. In rodents, the uterine secretory products of the endometrial glands are necessary for uterine receptivity and fetal implantation (Gray et al., 2001).

The uterus is a highly dynamic tissue. It is only responsive and sensitive to a change in the classic hormonal signs. The most important part of the uterus is the endometrium, in which the drastic changes that happen throughout the estrus cycle occur. In rodents, hormones are important in the proliferation of endometrial stromal cells and decidualization (Dey et al., 2004). Nakamura et al., (2005) and Dolatshad et al., (2006) reported that clock genes were expressed rhythmically in the uterus of rats. The absence of the morphofunctional changes observed in this study may correlate with the weight of the uterus and ovary, which did not change. Thus, it is suggested that continuous light exposure was not able to act on the uterine clock genes and did not cause morphofunctional changes on the weight of the ovary and the uterus of the offspring. However, a reduction in the levels of Glutathione Reductase (GR) and Glutathione S-Transferase (GST) was observed in the uterus of females exposed to continuous light in the intrauterine period. This reduction in GR and GST levels did not lead to lipoperoxidation, since MDA levels were normal in both groups, promoting cell protection. The other antioxidant enzymes: Supeeroxide dismutase (SOD) and Glutathione Peroxidase (GPx) remained unchanged in both experimental groups.

The progesterone hormone is dominant in the luteal phase. The combination of estrogen and progesterone exerts negative feedback on the hypothalamus and adeno-hypophysis (Legan et al., 2015). In this present study, it is suggested that high production of progesterone, which

is evidence in the serum level, provides feedback to the hypothalamus and pituitary gland, thus inhibiting GnRH and consequently inhibits LH secretion.

During folliculogenesis, the granulosa cells of the ovary undergo proliferation and differentiation, which is regulated by follicle stimulating hormone (FSH) and other factors produced locally (Richards, 1994). This step is of great importance for ovulation to occur.Yoshikawa et al. (2009) reported that circadian ovarian clock can regulate the synthesis of steroid hormones in adult rats, where the light-dark cycles of animals were in advanced phase or delayed for 6 h. Although several previous studies have shown the presence and expression of clock genes in the ovarian follicles and in the corpus luteum (Fahrenkrug et al., 2006; Karman, Tischkau, 2006), the present study demonstrated that maternal exposure to continuous light throughout gestation did not triggered alteration in the lipoperoxidation (OLP) and levels of antioxidant enzymes, as well a changes in folliculogenesis in ovary and in the plasma FSH level. during the adult life of the female rats. Demonstrating that the balance of antioxidant enzymes helped in maintaining the levels of lipoperoxidation as well as in the homeostasis of ovarian functions.

Although, the presence of clock genes in the uterus and the ovary has been previously documented, in the present study, morphofunctional changes of the uterus or changes in folliculogenesis in the ovary were not observed. In conclusion, the alteration of the maternal circadian cycle triggered changes of the hormonal level, which in long-term, can lead to interruption of estrus cycle and consequently lead to infertility in adult female offspring.

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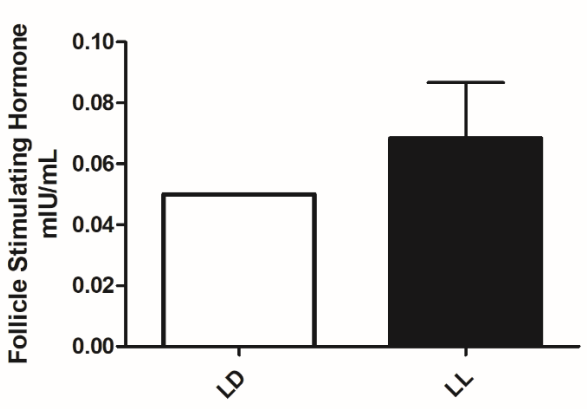
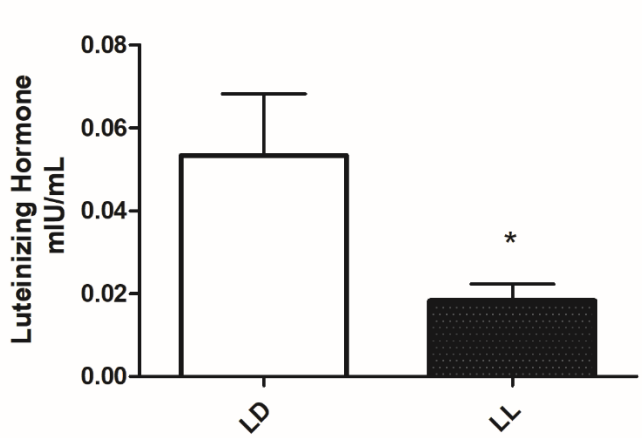
Figure 1 – Hormonal Analysis: FSH, LH and progesterone plasm levels. Values expressed as Mean ± SEM; Shapiro–Wilk test for normality ($p > 0.05$). Mann-Whitney test. *indicate groups that differ statistically ($p < 0.05$). LD (Light/Dark) group and LL (Light/Light) group.





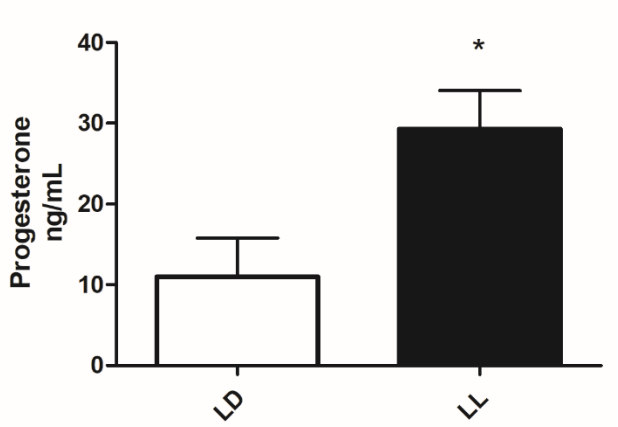


Figure 2 – Number of Glands in uterus. Values expressed as Mean ± SEM; Shapiro–Wilk test for normality; $p > 0.05$. Unpaired *t*-test; * indicate groups that differ statistically ($p < 0.05$). LD (Light/Dark) group and LL (Light/Light) group.

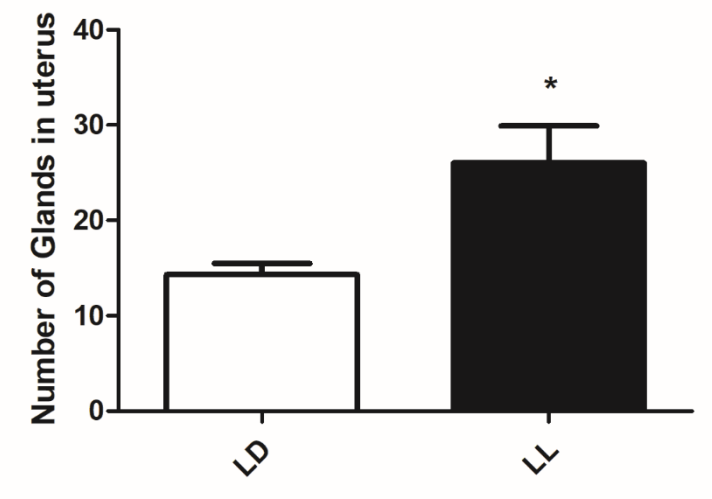


Figure 3 – Photomicrography in ovary and uterous. (A) Ovarian antral follicle in LD group; (B) Ovarian antral follicle in LL group; (C) Glands in uterous in LD group and (D) Glands in uterous in LL group. Arrow in (A) and (B) indicate antral follicle. Arrow in (C) and (D) indicate glands in uterous. It, interstitial tissue; L, lumen. Haematoxylin and eosin staining (original magnification x100). LD (Light/Dark) group and LL (Light/Light) group.

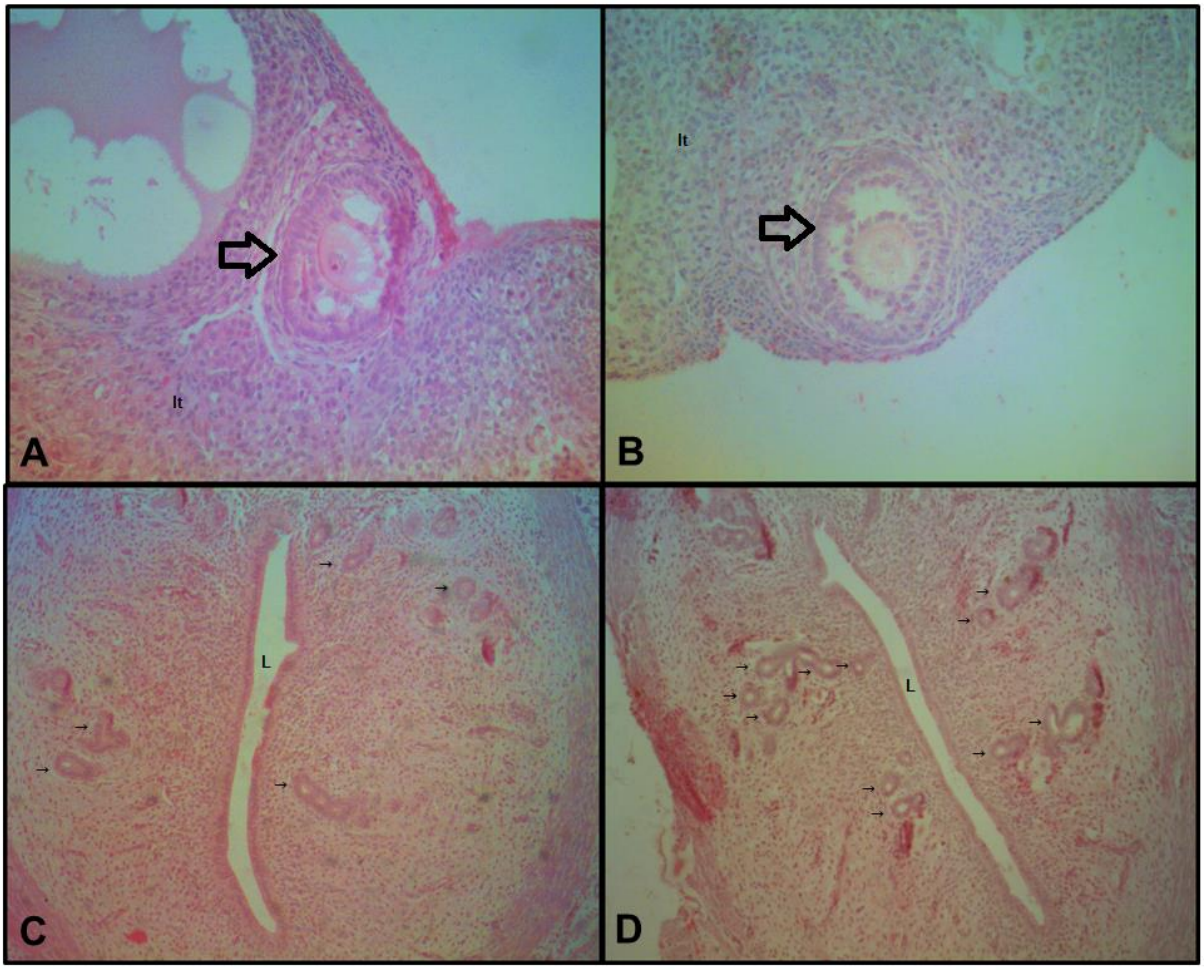


Table 1. Reproductive organs weight.

	LD (n = 7)	LL (n = 7)
Uterus (g)	0.25 ± 0.01	0.32 ± 0.02
Ovary (g)	0.05 ± 0.00	0.05 ± 0.00

Values expressed as mean ± SEM. Shapiro–Wilk test for normality ($p > 0.05$). * indicate groups that differ statistically. Unpaired *t*-test ($p < 0.05$). LD (Light/Dark) group and LL (Light/Light) group.

Table 2. Ovarian follicles and corpora lutea.

	LD (n = 7)	LL (n = 7)
Primary Follicle	4.71 ± 0.41	5.04 ± 0.52
Preantral Follicle	3.57 ± 0.90	3.66 ± 0.43
Antral follicle	5.04 ± 1.53	7.19 ± 0.97
Atretic follicle	3.38 ± 0.64	3.28 ± 0.57
Corpora lutea	5.85 ± 0.50	6.81 ± 1.16

Values expressed as mean ± SEM. Counted and classified ovarian follicles and corpora lutea. Shapiro–Wilk test for normality ($p > 0.05$). * indicate groups that differ statistically. Unpaired *t*-test ($p < 0.05$). LD (Light/Dark) group and LL (Light/Light) group.

Table 3. Morphometric analysis in uterus.

	LD (n = 7)	LL (n = 7)
Endometrium (µm)	455.4 ± 24.66	468.8 ± 26.43
Myometrium (µm)	94.64 ± 3.94	96.37 ± 3.57
Height of the luminal epithelium(µm)	20.10 ± 0.49	20.51 ± 0.40
Height of the grandular epithelium(µm)	16.00 ± 0.49	15.31 ± 0.50

Values expressed as Mean ± SEM. Shapiro–Wilk test for normality ($p > 0.05$).
* indicate groups that differ statistically. Unpaired *t*-test ($p < 0.05$). LD (Light/Dark) group and LL (Light/Light) group.

Table 4. Biomarkers oxidative stress in uterus and ovary.

	Uterus		Ovary	
	LD (n =7)	**LL (n =7)**	**LD (n =7)**	**LL (n =7)**
Glutationa Peroxidase (GPx)	192.3 ± 7.07	163.6 ± 12.82	134.9 ± 19.83	130.10 ± 18.85
Glutationa Reductase (RD)	32.80 ± 3.39	15.25 ± 2.95*	8.85 ± 1.33	9.80 ± 1.66
Glutationa s-transferase (GST)	22.86 ± 3.83	9.86 ± 1.43*	12.90 ± 2.78	6.88 ± 1.79
Superoxide Dismutase (SOD)	7.67 ± 2.93	9.60 ± 2.22	6.86 ± 1.53	4.71 ± 0.82
Lipoperoxidation (LPO)	14.71 ± 0.65	14.44 ± 0.58	17.31 ± 0.62	17.31 ± 0.62

Values expressed as Mean ± SEM. Shapiro–Wilk test for normality ($p > 0.05$). * indicate groups that differ statistically. Unpaired *t*-test ($p < 0.05$). LD (Light/Dark) group and LL (Light/Light) group.