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"DESFECHOS NOS DESENVOLVIMENTOS COGNITIVO, SOMÁTICO E REPRODUTIVO DA EXPOSIÇÃO PRÉ-NATAL E NEONATAL DE RATOS À BETAMETASONA"

THAMIRIS MOREIRA FIGUEIREDO
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Tese apresentada ao Programa de Pós-Graduação em Biologia Geral e Aplicada do Instituto de Biociências de Botucatu da Universidade Estadual de São Paulo (Unesp) como parte dos requisitos exigidos para a obtenção do título de Doutora em Biologia Geral e Aplicada, na área de Biologia Celular, Estrutural e Funcional.

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Epígrafe

"Depois da tempestade, as cores aparecem".

Paul David Hewson (Bono Vox)

Sumário

Resumo

A betametasona (BM) é o fármaco de escolha para a corticoterapia antenatal em casos de risco de parto prematuro por induzir a maturação pulmonar e garantir a sobrevivência fetal pós-natal. No entanto, estudos deste grupo de pesquisa com exposição de ratos à BM no período gestacional crítico para o desenvolvimento do sistema genital evidenciam programação fetal e alterações na função e desenvolvimento reprodutivos em F1. Nosso objetivo foi investigar os efeitos da BM sobre o desenvolvimento sexual em período que corresponde à janela de exposição em humanos, além de avaliar os efeitos da exposição pré-natal à BM com ênfase nos desenvolvimentos sexual, cognitivo e somático de machos e fêmeas F1 e F2. Para o experimento neonatal, ratos machos e fêmeas (n=10/grupo) foram expostos por via subcutânea à solução NaCl 0,9 % (Controle) ou 0,1 mg/Kg de BM nos dias pós-natal (DPN) 1, 2 e 3. Além disso, para a exposição pré-natal, ratas prenhes (n=10/grupo) foram expostas à solução NaCl 0,9% (Controle) ou 0,1 mg/Kg de BM por via intramuscular nos dias gestacionais 12, 13, 18 e 19. No DPN 90, os ratos machos e fêmeas (F1) expostos in utero foram acasalados com animais não tratados para obtenção de F2 (prole de ratos machos) e F2’ (prole de ratos fêmeas). A exposição neonatal de ratos à BM reduziu o ganho de peso corpóreo em machos e fêmeas durante o tratamento e a qualidade espermática em machos, além de ter causado desregulação do ciclo estral e redução na concentração de LH em fêmeas na maturidade sexual. Nos ratos expostos in utero, houve redução do ganho de peso em F0 e do peso da prole F1. Além disso, a instalação da puberdade estava atrasada e o ciclo estral desregulado em fêmeas F1, assim como a qualidade espermática e o peso testicular em machos F1 estavam reduzidos. Houve atraso dos desenvolvimentos cognitivo e somático em F1, F2 e F2’. Nas proles F2 e F2’, houve redução do peso no dia do desmame e aumento da distância ano-genital em fêmeas F2’ no DPN 01. Conclui-se que a exposição pré-natal à BM causou disfunções nos desenvolvimentos cognitivo e somático em F1, F2 e F2’ e ambos os períodos de exposição alteraram a qualidade espermática, desregularam o ciclo estral e causaram programação do desenvolvimento reprodutivo e restrição do crescimento em machos e fêmeas F1. Portanto a exposição de ratos à BM no período neonatal, embora tenha efeitos menos evidentes sobre o sistema genital masculino e feminino, confirma resultados observados anteriormente após exposição pré-natal. Apesar da reconhecida importância da corticoterapia antenatal humana, os achados do presente estudo sinalizam e estimulam novos estudos a fim de minimizar possíveis efeitos adversos colaterais pós-natais.

Abstract

Betamethasone (BM) is the drug of choice for antenatal corticosteroid therapy for women at risk of preterm delivery once it induces fetal lung maturation and enhances survival after birth. However, fetal programming and impaired reproductive function and development were reported by our group in F1 rats exposed to BM during the critical window of genital system development. Thus we aimed to investigate the effects of BM on sexual development in the neonatal period that corresponds to the exposure in humans and evaluate the effects of prenatal BM exposure, emphasizing the sexual, cognitive and somatic developments of male and female F1 and F2. Therefore male and female rats (n=10/group) were exposed subcutaneously to NaCl 0.9% solution (Control) or 0.1 mg/Kg BM at postnatal days (PND) 1, 2 and 3. For prenatal exposure, 20 pregnant rats (n=10/group) were exposed intramuscularly to NaCl 0.9% solution (Control) or 0.1 mg/kg BM at gestational days 12, 13, 18 and 19. At PND 90, F1 male and female rats exposed *in utero* were mated with untreated animals to obtain F2 (offspring of male rats) and F2' (offspring of female rats). Neonatal exposure to BM decreased body weight gain in male and female rats. Also, the estrous cycle was deregulated and LH level was decreased in female rats. The sperm concentration in the caput-corpus of the epididymis was decreased, while the sperm transit time was increased in male rats as well as the sperm concentration in the cauda of the epididymis. Prenatal exposure to BM reduced body weight gain of F0 female rats and at birth of F1 offspring, decreased body weight at weaning of F2 and increased anogenital distance of F2' female at PND01. Also, BM delayed puberty onset and deregulated the estrous cycle of F1 female rats. There were a decrease in sperm quality and testicular weight at sexual maturity of male rats. We also observed delayed physical and neurobehavioral development of F1, F2 and F2' male and female rats. Our results demonstrated that both neonatal and prenatal exposure to BM impaired body growth of male and female rats, impaired sperm quality and reproductive function and led to development programming, but only prenatal BM exposure led to dysfunctions in the cognitive and somatic developments in F1, F2 and F2'. Therefore, neonatal BM exposure corroborated results previously observed after prenatal exposure to this drug. Despite the recognized importance of human antenatal corticosteroid therapy, the findings of this study evidence and encourage further studies in order to minimize possible adverse postnatal effects.

Introdução

1. Introdução

1.1. O rato como modelo experimental

Roedores, como o rato Wistar (*Rattus novergicus albinus)*, são animais muito utilizados na pesquisa biomédica, principalmente em avaliações toxicológicas, devido ao seu tamanho reduzido, ótimo custo-benefício, facilidade de manipulação, além de possuírem fisiologia e desenvolvimento de doenças bastante semelhante aos humanos, ou seja, é um modelo experimental que, embora diferente, aproxima-se bastante da clínica humana (DUTTA; SENGUPTA, 2013, 2016).

Embora bastante semelhante à espécie humana, ratos e camundongos apresentam um período de vida bem menor, geralmente um roedor vive em torno de 4 anos, enquanto seres humanos vivem em média 80 anos. Além disso, foi estimado que um mês de vida em ratos adultos pode equivaler a 3 anos de vida de um ser humano. O período gestacional em ratos também é bastante diferente, dura cerca de 22 dias contra 280 dias gestacionais em humanos. Portanto, ao nascerem, ratos encontram-se em estágio de desenvolvimento correspondente ao que seriam os 150 dias de gestação e apenas no dia pós-natal (DPN) 12 considera-se que o desenvolvimento fetal está completo, sendo que este período corresponde ao último trimestre gestacional na espécie humana (QUINN, 2005; OJEDA; SKINNER, 2006).

Em relação ao desenvolvimento do sistema genital, sabe-se que roedores atingem a maturidade sexual mais cedo e não passam pelo período de quiescência testicular que dura até o final da juventude, como seres humanos (PICUT; ZIEJEWSKI; STANISLAUS, 2018). Além disso, roedores são reprodutivamente mais eficientes. O desenvolvimento sexual pós-natal e as janelas críticas de exposição em roedores e seres humanos são divididos nos seguintes períodos (PICUT; ZIEJEWSKI; STANISLAUS, 2018):

Período	Roedores	Seres humanos
Neonatal	DPN 0 -7	0 – 28 dias
Infância	DPN 8 – 20	28 dias – 2 anos
Juvenil	DPN 21 – 32	2 – 11 anos
Pré-puberdade	DPN 33 - 60	11 – 14 anos

No período pós-natal, ratos são desmamados por volta da 3ª semana de vida, possuem uma infância rápida, atingem a maturidade sexual até as 6 semanas de vida e se tornam adultos a partir da 8ª semana de vida, enquanto humanos desmamam após 6 meses de vida e entram na puberdade entre os 11-12 anos de idade. Portanto, todas essas diferenças entre

roedores e seres humanos devem ser levadas em consideração quando se pensa em realizar estudos comparativos (DUTTA; SENGUPTA, 2016; SENGUPTA, 2013).

1.2. Desenvolvimento do eixo hipotalamo-hipófise-adrenal (HHA)

O eixo HHA é composto pelo hipotálamo, hipófise e glândulas adrenais e é responsável pelo controle da resposta ao estresse, do metabolismo energético e das funções cardiovascular, imune, comportamentais e reprodutivas (JOSEPH; WHIRLEDGE, 2017). Uma vez estimulados, os neurônios do núcleo paraventricular no hipotálamo produzem e liberam o hormônio liberador de corticotrofina (CRH) no sistema porta-hipofisário que estimula a liberação de ACTH, a partir da pró-opielomelanocortina, pela adenohipófise. Então, o ACTH estimula a produção de cortisol (ou corticosterona em roedores) pela zona fasciculada do córtex das glândulas adrenais, que através de *feedback negativo* inibe a secreção de CRH e a transcrição de genes da pró-opielomelanocortina, reduzindo a atividade do eixo HHA (GRINO et al., 1995; AIRES, 2012).

A hipófise, glândula localizada na base do cérebro e no interior da sela turca, é dividida fisiologicamente em duas partes: hipófise anterior ou adenohipófise e hipófise posterior ou neurohipófise (RANG, 2016). Em ratos, aproximadamente no dia gestacional (DG) 13, células produtoras de ACTH são observadas na hipófise (NEMESKÉRI et al., 1988) e, são identificados neurônios produtores de CRH entre os DG 15,5 e 16,5 nas áreas laterais e no núcleo paraventricular hipotalâmicos. A produção de CRH tem início a partir do DG 16 e auxilia na diferenciação das células produtoras de ACTH, ao mesmo tempo, inicia-se a expressão de pró-opielomelanocortina, precursora do ACTH (DAIKOKU et al., 1984; GRINO et al., 1995). O CRH leva ao aumento na concentração de ACTH na hipófise em até 10 vezes entre os DG 17 e 20 (CHATELAIN; DUPOUY, 1981). Do DG 20 até o nascimento a produção de ACTH permanece constante e inalterada (MANOJLOVIĆ-STOJANOSKI et al., 2012).

As glândulas adrenais são estruturas pares que derivam da proliferação celular do mesoderma esplâncnico e estão localizadas na parte superior dos rins e subdivididas em medula e córtex, sendo que o córtex ainda é dividido em três zonas: fasciculada, reticulada e glomerulosa em indivíduos adultos (ALHEIRA; BRASIL, 2005; MANOJLOVIĆ-STOJANOSKI; NESTOROVIĆ; MILOŠEVIC, 2012; BUSADA; CIDLOWSKI, 2017). O ACTH age sobre o córtex das adrenais e estimula o crescimento, desenvolvimento, produção e liberação de glicocorticoides, mineralocorticoides e uma pequena quantidade de hormônios

esteroides, que são importantes durante o desenvolvimento pré-natal, pois fornecem substrato para produção de estrógenos pela placenta (NUSSDORFER; MAZZOCCHI; REBONATO, 1971; BUSADA; CIDLOWSKI, 2017).

A partir do DG 13, em ratos, as glândulas adrenais já produzem corticosterona, no entanto, só são responsivas ao estímulo de ACTH a partir do DG 16, coincidindo com o início da produção deste hormônio (MITANI et al., 1999). Então, próximo ao nascimento, mais precisamente a partir do DG 19, ocorre um pico na produção e liberação de glicocorticoides, que logo decresce a níveis basais (GRINO et al., 1995). Em seres humanos, as adrenais são identificadas entre a 3ª e 4ª semanas de gestação como um espessamento celular entre o mesentério e as cristas gonadais, porém apenas a partir da 8ª semana gestacional que é possível diferenciar as zonas na região do córtex e a produção de hormônios esteroides e glicocorticoides, assim como ocorre a ativação do eixo HHA e liberação de ACTH pela hipófise (MESIANO; JAFFE, 1997). Então, entre as semanas gestacionais 8 e 9 ocorre um pico na concentração de cortisol, que atinge o platô até a semana gestacional 14 e permanece desta forma até o período próximo ao nascimento (BUSADA; CIDLOWSKI, 2017).

1.3. Desenvolvimento do eixo hipotálamo-hipófise-gônada (HHG)

A fisiologia reprodutiva em mamíferos é regulada pelo hormônio hipotalâmico liberador de gonadotrofinas (GnRH) (WEN et al., 2010). Ao serem estimulados, os neurônios da área pré-óptica do hipotálamo produzem e liberam GnRH no sistema porta-hipofisário que, por sua vez, irá estimular a liberação de gonadotrofinas, os hormônios luteinizante (LH) e folículo estimulante (FSH) pela hipófise anterior e, então, estes hormônios irão estimular a gametogênese e esteroidogênese gonadal. O aumento na secreção de testosterona, estrógeno e progesterona inibem a liberação de GnRH, LH e FSH, através de *feedback* negativo e inibem a atividade do eixo HHG. Além disso, o hormônio inibidor de gonadotrofinas (GnIH), produzido pela núcleo dorsomedial do hipotálamo, inibe a liberação de GnRH e, portanto, o eixo HHG (GERAGHTY; KAUFER, 2015) (Figura 1).

Embora em roedores o desenvolvimento gonadal fetal seja independente de gonadotrofinas, a partir do DG 12, inicia-se a expressão de GnRH no hipotálamo e de seus receptores na hipófise e acredita-se que a presença de GnRH auxilia na diferenciação dos gonadotrófos hipofisários (OJEDA; SKINNER, 2006). No entanto, a concentração de GnRH permanece baixa até aproximadamente o DG 17, quando começa a aumentar e ocorre um pico próximo ao nascimento, que leva a síntese de LH imediatamente e de FSH após dois dias,

demonstrando que a síntese de FSH é dependente do início da produção de LH (OJEDA; SKINNER, 2006). Então, o início da síntese de gonadotrofinas, leva ao início da esteroidogênese gonadal.

Nos testículos de roedores por volta do DG 15, as células de Leydig fetais já expressam receptores para LH e produzem pequenas quantidades de andrógenos. No entanto, apenas por volta do DG 18, quando ocorre um aumento expressivo de receptores para LH nestas células, é que há um pico na concentração de testosterona que é essencial para o processo de diferenciação sexual cerebral e gonadal em ratos machos (WARREN et al., 1984; CORBIER; EDWARDS; ROFFI, 1992; OJEDA; SKINNER, 2006). Por outro lado, os receptores para FSH são identificados pela primeira vez nas células de Sertoli apenas a partir do DG 17,5, após o início da expressão de receptores para LH (PICUT; ZIEJEWSKI; STANISLAUS, 2018). Em fêmeas, só é possível identificar receptores para LH e FSH nos ovários a partir do DPN 4 ou 5, quando as gônadas se tornam responsivas às gonadotrofinas.

No entanto, embora o desenvolvimento gonadal fetal seja independente de gonadotrofinas, o estrógeno é indispensável para o desenvolvimento ovariano, manutenção dos folículos ovarianos e regulação do eixo reprodutivo (OJEDA; SKINNER, 2006). Então, devido à necessidade de esteroides para o desenvolvimento ovariano, a esteroidogênese em fêmeas inicia-se durante o período fetal e evidências sugerem que a noradrenalina e o polipeptídeo intestinal vasoativo estimulam esse processo a partir do AMP cíclico (DENEF et al., 1974; GEORGE; OJEDA, 1987; OJEDA; SKINNER, 2006).

Em seres humanos, o correto desenvolvimento gonadal e folicular é dependente de gonadotrofinas, que são secretadas a partir da 12ª semana gestacional, sendo que os picos nas concentrações de LH e FSH ocorrem por volta do 2º trimestre gestacional e é maior em fêmeas do que em machos (MANOJLOVIĆ-STOJANOSKI; NESTOROVIĆ; MILOŠEVIC, 2012).

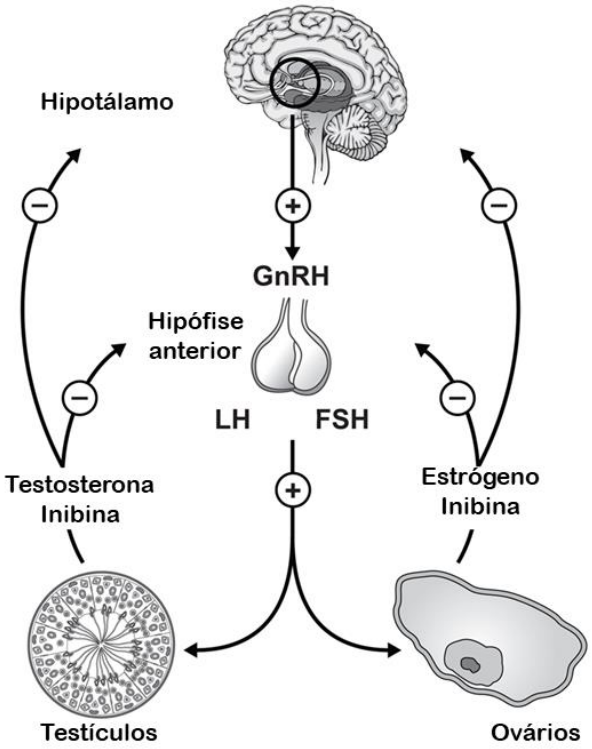

Figura 1. Esquema representativo do eixo hipotálamo-hipófise-gônada. Fonte: Adaptado de Whirledge e Cidlowski, 2010.

1.4. Determinação e diferenciação sexual

Inicialmente, as cristas gonadais são identificadas como dois espessamentos bipotenciais e indiferenciados do epitélio celômico localizados medialmente no mesonefro e que se diferenciaram do mesoderma intermediário pela influência dos fatores de transcrição *Pax 2* e *Pax 8*. Em mamíferos, a determinação sexual gonadal em testículos ou ovários ocorre pela presença ou ausência do gene SRY, respectivamente, que está localizado no braço curto do cromossomo Y em machos (LOVELL-BADGE; ROBERTSON, 1990; KOOPMAN et al., 1991; SCHMAHL et al., 2000).

Após a formação das cristas gonadais, entre os DG 9,5 e 11, as células germinativas primordiais, que derivam de células epiblásticas da linha primitiva e são precursoras dos gametas masculino e feminino, se proliferam e migram da parede posterior do saco vitelínico para a região das gônadas indiferenciadas. Então, após a migração das células germinativas primordiais, se o sexo cromossômico do animal for XY, ocorrerá a expressão de *Sry*, que levará à determinação sexual e à diferenciação gonadal em testículos. Caso o sexo cromossômico do indivíduo seja XX, as cristas gonadais se diferenciam em ovários (MCLAREN; SOUTHEE, 1997; YAO, 2003; CUPP; SKINNER, 2005). Já foi demonstrado que deleções ou inibição da expressão de *Sry* levam as gônadas XY a se diferenciarem em

ovários ou ovotestes, estrutura composta por tecido testicular e ovariano (SCHMAHL et al., 2000; ALBRECHT; EICHER, 2001).

Em seres humanos, as cristas gonadais são visualizadas apenas a partir da 4ª SG e se tornam diferenciadas apenas na 7ª semana gestacional. Então, durante este período, as células epiteliais das cristas gonadais se proliferam e formam cordões sexuais que irão se agregar com as células germinativas primordiais a partir da 6ª semana gestacional. Se o feto for XY, a proliferação continua até a 8ª semana gestacional e os testículos se formam. Se o feto for XX, os ovários contendo os folículos primordiais só são visualizados a partir da 16ª semana gestacional (GILBERT, 2003).

Assim como as gônadas no início do desenvolvimento, o cérebro também apresenta bipotencialidade. Com o aumento da produção de testosterona pelos testículos no período próximo ao parto ocorre, a diferenciação dos ductos mesonéfricos, genitália externa, além do dimorfismo sexual cerebral em ratos machos (SCHWARZ; MCCARTHY, 2008).

A diferenciação sexual cerebral é estimulada por hormônios esteroides, que atravessam livremente a barreira hematoencefálica, e ocorre em duas fases diferentes: organizacional e ativacional. A primeira inclui as etapas de masculinização, que é a organização de ume rede neuronal permissiva ao comportamento sexual típico masculino através da interação do estrógeno com receptores do tipo ERα em determinadas regiões cerebrais, como a área pré-óptica, e a defeminização, que é o processo de perda da capacidadede responder aos efeitos do estrógeno e progesterona na vida adulta. A presença de estrógenos induz a produção de prostaglandinas em áreas sexualmente dimórficas no cérebro e dessa forma promove mudanças morfofisiológicas que masculinizam o cérebro (AMATEAU; MCCARTHY, 2004). A fase ativacional ocorre na puberdade e durante toda vida adulta e compreende a exposição do animal aos hormônios esteroides, testosterona, em machos, e estrógeno e progesterona em fêmeas, que induzem o comportamento sexual e levam a manifestação dos caracteres sexuais secundários de acordo com o sexo cromossômico do animal.

Portanto, a expressão de hormônios esteroides durante o desenvolvimento, principalmente no período próximo ao nascimento em roedores, é essencial para definir o comportamento sexual em adultos (SCHWARZ; MCCARTHY, 2008; ROSENFELD, 2017; MCCARTHY; DE VRIES; FORGER, 2010).

1.5. Desenvolvimento do sistema genital masculino

Após a migração das células germinativas primordiais, a expressão de *Sry e Sox9* e a determinação sexual, aproximadamente no DG 12, as células mesenquimais somáticas do epitélio gonadal se proliferam e diferenciam em células de Sertoli que então se agregam com as células germinativas primordiais e com células que migraram dos mesonefros, que originam as células mióides peritubulares e endoteliais, dando início a formação dos cordões seminíferos. As células endoteliais formam a rede de vascularização dos testículos a partir do DG 12,5 e as células mióides peritubulares formam uma camada de células ao redor dos cordões seminíferos.

Então, por volta do DG 13, as células de Sertoli iniciam a produção e secreção do hormônio anti-Mülleriano (AMH), que promove a regressão dos ductos paramesonéfricos (CUPP; SKINNER, 2005). Além de produzirem AMH, também produzem substâncias que induzem a diferenciação de células de Leydig e prostaglandinas, que impedem que as células germinativas entrem em meiose (DOLCI; DE FELICI, 1990; MCLAREN; SOUTHEE, 1997; SHARPE et al., 2003; MANOJLOVIĆ-STOJANOSKI; NESTOROVIĆ; MILOŠEVIC, 2012). Em ratos, as células de Sertoli se proliferam com maior taxa entre os DG 18 e 21, coincidindo com o pico na concentração de testosterona (ORTH, 1982).

Aproximadamente no DG 12,5, as células de Leydig fetais se diferenciam e, no DG 14,5, a estereidogenese tem início com a produção de androstenediona e de ativina A, que controla a proliferação de células de Sertoli e o desenvolvimento dos cordões seminíferos (SVINGEN; KOOPMAN, 2013). No início do desenvolvimento, a androstenediona será convertida a testosterona pelas células de Sertoli devido a ausência de 17β-hidroxiesteroide desidrogenase nas células de Leydig fetais. A diferenciação de novas células de Leydig fetais depende tanto de substâncias produzidas pelas células de Sertoli quanto, produzidas pelas células de Leydig fetais já diferenciadas. Estas substâncias irão impedir a apoptose de células de Leydig fetais já diferenciadas (WEN; CHENG; LIU, 2016).

Após o nascimento, os gonócitos se diferenciam em espermatogônias e migram para base do epitélio, as células de Sertoli se proliferam até aproximadamente o DPN 18 e então amadurecem e formam a barreira Sertoli-Sertoli, que confere proteção imunologica aos túbulos seminíferos e ampara o processo espermatogênico que se inícia logo após o estabelecimento desta barreira. Além disso, as células de Leydig fetais regridem e dão lugar as células de Leydig progenitoras, que até a pré-puberdade se diferenciam em adultas e iniciam a estereidogenese (PICUT; ZIEJEWSKI; STANISLAUS, 2018).

Entre os DG 16,5 e 18,5, os ductos mesonéfricos, sob influência de andrógenos, se diferenciam em epidídimos, que então começam a passar pelo processo de enovelamento no sentido crânio-caudal. No período pós-natal, o epidídimo passa por três períodos de desenvolvimento: indiferenciado, de diferenciação e de expansão (HINTON et al., 2011; ARROTIA et al., 2012; ROBAIRE; HINTON, 2015). Ao nascimento, as diferentes regiões do epidídimo são visíveis e toda sua extensão está altamente enovelada, com exceção da região da cauda. Além disso, o epitélio caracteriza-se por colunar indiferenciado e é possível observar células em processo de mitose, sendo que até o DPN 15 ocorre um grande crescimento longitudinal deste órgão em ratos (HERMO; BARIN; ROBAIRE, 1992; ROBAIRE; HINTON, 2015).

A diferenciação histológica epididimária inicia-se com o surgimento das células halo apenas no início da infância, marcando o final do período indiferenciado. No período de diferenciação, as células colunares se diferenciam nos tipos adultos: principais, basais, narrow, halo e claras, e o epitélio passa a apresentar característica pseudoestratificada. Até o DPN 49, ou seja, o fim da puberdade, todas as células colunares já passaram pelo processo de diferenciação (HERMO; BARIN; ROBAIRE, 1992; ARROTIA et al., 2012; ROBAIRE; HINTON, 2015). A partir desse momento, já é possível visualizar espermatozoides na luz e o órgão continua crescendo longitudinalmente, caracterizando a fase de expansão (STAACK et al., 2003; ROBAIRE; HINTON, 2015).

Além de se diferenciarem em epidídimos, os ductos mesonéfricos originam os ductos deferentes e as glândulas seminais sob influência de andrógenos (ROBAIRE et al., 2006). O primórdio das glândulas seminais surge no DG 18 e forma-se como um divertículo que se alonga crânio-dorsalmente até o DG 21, quando então adquire o dobramento característico de adultos (PRICE, 1936). O processo de ramificação ocorre entre os DPN 5 e 15, após o DPN 30 o epitélio encontra-se diferenciado e no DPN 36 a glândula encontra-se funcional. Os ductulos eferentes, por sua vez, derivam dos túbulos mesonéfricos (ROBAIRE; HINTON, 2015).

O seio urogenital, uma estrutura derivada de endoderme, é um rudimento embrionário bipotente que em machos, devido a ação da testosterona, origina a próstata, uretra prostática, glândulas bulbouretrais e bexiga. Em ratos, o desenvolvimento prostático inicia-se com a formação de brotos epiteliais sólidos que emergem do seio urogenital e se projetam no mesênquima adjacente em resposta ao estimulo de andrógenos secretados pelos testículos a partir do DG 19 (CUNHA et al., 2004). Após o nascimento, as células epiteliais e basais se

diferenciam, os brotos alongam-se, adquirem um lúmen e até o final da puberdade encontram-se completamente ramificados (SUGIMURA; CUNHA; DONJACOUR, 1986; CUNHA et al., 2004). Embora a próstata esteja completamente desenvolvida até as 3 semanas de vida, apenas após a puberdade é que irá adquirir características adultas (PRICE, 1936).

Portanto, a determinação e a diferenciação sexual em gônadas XY dependem de três hormônios: AMH, que irá levar a regressão dos ductos paramesonéfricos, andrógenos, que promoverão o desenvolvimento dos derivados dos ductos mesonéfricos, a masculinização da genitália externa e o dimorfismo sexual cerebral, e o Insl3, que está envolvido com a descida dos testículos para o escroto (SHARPE et al., 2003; MANOJLOVIĆ-STOJANOSKI; NESTOROVIĆ; MILOŠEVIC, 2012).

1.6. Desenvolvimento do sistema genital feminino

Após a migração das células germinativas primordiais, entre os DG 11,5 e 12, a gônada XX, na ausência da expressão de *Sry*, expressa os fatores *Wnt4* e *Foxl2* e ácido retinoico que irão inibir a expressão de fatores ligados a masculinização e levarão a diferenciação do epitélio somático das gônadas em células da pré-granulosa. O primeiro sinal de início do desenvolvimento ovariano é o surgimento das regiões do córtex e da medula e de agrupamentos de células germinativas primordiais envoltas por células epiteliais escamosas, as células pré-granulosa, formando os cordões ovarianos (OJEDA; SKINNER, 2006).

Após o DG 12,5, em contraste com o que acontece no desenvolvimento das gônadas masculinas, o epitélio das cristas gonadais XX continua se proliferando e invagina-se. As células germinativas primordiais se agregam com células pré-granulosa, se diferenciam em ovogônias e, devido a citocinese incompleta durante a mitose, formam pontes intracitoplasmáticas entre si, originando os cistos ovarianos. Entre os DG 14,5 e 17,5, as ovogônias se diferenciam em ovócitos e passam e se proliferar por meiose. Enquanto isso, os cistos ovarianos são rompidos devido à ingressão de mais células da pré-granulosa, originando os ninhos ovarianos, contendo até dois ovócitos em seu interior. Então, os ovócitos entram em parada meiótica, que será reestabelecida apenas após o nascimento. Acredita-se que o ácido retinoico é o fator que leva as ovogônias em fêmeas a entrarem em meiose (OJEDA; SKINNER, 2006).

A presença da progesterona placentária impede que os ovócitos entrem em apoptose, porém, após o nascimento, este estímulo cessa e essas células entram em morte celular programada, formando os folículos primordiais. Após a formação dos folículos primordiais,

ocorre uma onda foliculogênica que leva ao desenvolvimento de folículos pré-antrais e antrais até o DPN 15. No entanto, eles entram em atresia e apenas ao início da puberdade é que ocorre a primeira ovocitação (LAFFAN et al., 2018). Depois de formados, os folículos primordiais representam todo o conjunto de gametas em um indivíduo do sexo feminino durante a vida reprodutiva, uma vez que as células germinativas em fêmeas só se proliferam durante o desenvolvimento embrionário, diferente do que ocorre em machos, que a proliferação é ao longo de toda vida (BRENNAN; CAPEL, 2004; PASK, 2016).

As células da granulosa, que são análogas das células de Sertoli e irão nutrir as células germinativas durante o desenvolvimento, e as células da teca envolvem o folículo primordial e são responsáveis pela esteroidogênese, ou seja, correspondem às células de Leydig. Elas fornecem os hormônios necessários para o crescimento e maturação folicular.

Os ductos paramesonéfricos formam-se após 37 dias de gestação, em seres humanos, e aproximadamente no DG 12 em camundongos. Futuramente, diferenciam-se em tubas uterinas, útero, colo uterino e na parte superior da vagina (PARKER; SCHIMMER, 2006). O seio urogenital irá originar a parte inferior da vagina, a uretra e a bexiga urinária (STAACK et al., 2003).

1.7. Desenvolvimento pós-natal inicial

Em mamíferos, a puberdade compreende o período de transição entre a infância e a vida adulta (ABREU; KAISER, 2016). Durante este período, a maturidade sexual, capacidade de fertilizar e caracteres sexuais secundários são adquiridos e tanto a genitália externa como as gônadas passam pelo processo de crescimento e maturação (HAN; LEE, 2013).

O início da puberdade, em roedores, é desencadeado por uma série de fatores que se iniciam no começo da vida do animal. Logo após o nascimento, ocorre aumento na pulsatilidade de GnRH que leva a maior na síntese e liberação de FSH e LH que dura até aproximadamente o DPN 12. No período pré-púbere, há novamente um aumento na pulsatilidade de GnRH que leva a maior liberação de LH e FSH e, consequentemente, de hormônios esteroides pelas gônadas, induzindo a maturação testicular e ovariana e a instalação da puberdade (OJEDA; SKINNER, 2006). A Kisspeptina é um dos fatores que estimula a síntese de GnRH e de gonadotrofinas e controla o mecanismo de *feedback* negativo e positivo dos hormônios esteroides, regulando o eixo HHG (HARTER et al., 2018).

Em fêmeas, a instalação da puberdade ocorre aproximadamente no DPN 30 e é evidenciada pela alteração no padrão de secreção de LH, que leva a maior secreção de

estrógeno e progesterona pelos ovários e, consequentemente, causa a queratinização e canalização do canal vaginal (abertura vaginal) e primeiro estro (VIDAL, 2017). O *feedback* positivo é o estímulo principal para o aumento pré-ovulatório na concentração de LH que induz o início da puberdade em fêmeas.

Em machos, ao nascimento, os testículos encontram-se na cavidade abdominal, então, por volta do DG 15, pelo estímulo do hormônio semelhante à insulina do tipo 3 ocorre a descida para o escroto. Por volta do DPN 45, em resposta a maior secreção de testosterona pelos testículos, ocorre a instalação da puberdade em roedores machos, evidenciada pelo crescimento acentuado testicular, pela separação do prepúcio da glande e da formação dos túbulos seminíferos (CLERMONT; PEREY, 1957; OJEDA; SKINNER, 2006). Microscopicamente é possível observar espermatozoides nos testículos a partir do DPN 40 e, aproximadamente 15 dias depois, é possível visualizar espermatozoides nos epidídimos (OJEDA; SKINNER, 2006).

1.8. Glicocorticoides e desenvolvimento intrauterino

Os glicocorticoides são hormônios esteroides sintetizados na zona fasciculada do córtex da adrenal a partir do estímulo de ACTH e liberados de acordo com o ritmo circadiano (DICKMEIS; WEGER; WEGER, 2013; WOOD; WALKER, 2015; BUSADA; CIDLOWSKI, 2017). Desempenham importante função no controle da homeostase corporal e no metabolismo de carboidratos, lipídeos e proteínas, garantindo a disponibilidade de glicose para o organismo e evitando estados hipoglicêmicos. Além disso, atuam como agentes imunossupressores e como gatilho durante o desenvolvimento intrauterino, promovendo o desenvolvimento morfofisiológico de órgãos e sistemas e a maturação de processos que são imprescindíveis para a sobrevivência após o nascimento, uma vez que inibem a proliferação e estimulam diferenciação celular (RHEN; CIDLOWSKI, 2005; AIRES, 2012; FOWDEN; FORHEAD, 2015; RANG, 2016; BUSADA; CIDLOWSKI, 2017).

Uma vez dentro da célula, os glicocorticoides se ligam aos receptores de glicocorticoides (GR) ou mineralocorticoides (MR), pertencentes à família de receptores nucleares, e induzem a hiperfosforilação e alterações na conformação molecular do receptor, que ligado ao hormônio forma um complexo que é translocado para o núcleo. Dentro do núcleo, esse complexo tem dois destinos: interagir diretamente com elementos do DNA responsivos ou não responsivos aos glicocorticoides estimulando ou inibindo a transcrição gênica, respectivamente (ALHEIRA; BRASIL, 2005; RHEN; CIDLOWSKI, 2005; AIRES,

2012). Grande parte dos glicocorticoides circulante está ligada à globulina transportadora de glicocorticoides, outra à albumina e uma pequena parte encontram-se na sua forma livre, que é a responsável pelas ações fisiológicas (RHEN; CIDLOWSKI, 2005; SPENCER; DEAK, 2017) (Figura 2).

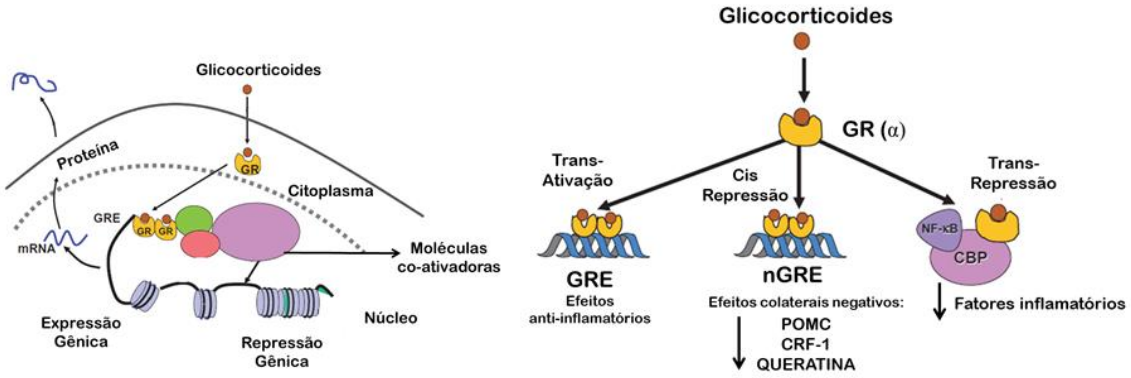

Figura 2. Mecanismo de Ação dos Glicocorticoides. Fonte: Adaptada de Barnes, 2010.

A atividade e biodisponibilidade dos glicocorticoides nos diferentes tecidos é regulada pelos tipos 1 e 2 da enzima 11β-hidroxiesteroide desidrogenase (11β-HSD), expressas em concentrações diferentes de acordo com o tipo de tecido, sendo que a primeira é expressa no fígado, tecido adiposo, músculos, ilhotas pancreáticas, cérebro, células imunes e nas gônadas, e a segunda, nos rins, placenta e tecidos fetais (CHAPMAN; HOLMES; SECKL, 2013). A 11β-HSD1 tem atividade de redutase, ou seja, converte a cortisona em cortisol. Já a 11β-HSD2 possui atividade de oxidase, ou seja, converte o cortisol em cortisona e seus metabólitos, inativando-o e permite que a aldosterona se ligue aos receptores do tipo MR nos tecidos (CHAPMAN; HOLMES; SECKL, 2013).

À medida que o parto se aproxima em roedores, a expressão de 11β-HSD2 na placenta diminui, assim como ocorre ativação do eixo HHA fetal, esses fatores em conjunto levam ao aumento na concentração de glicocorticoides endógenos no feto (FLAGEL et al., 2002; MURPHY; CLIFTON, 2003; HARRIS; SECKL, 2011; ROG-ZIELINSKA et al., 2013; FOWDEN; FORHEAD, 2015; AGNEW et al., 2018). Estudos em camundongos mostram um aumento na expressão de RNA mensageiro para GR ao fim da gestação e que logo em seguida, a concentração desses receptores volta a diminuir, o que corrobora a importância dos glicocorticoides no desenvolvimento fetal (KITRAKI; KITTAS; STYLIANOPOULOU, 1997).

Devido aos efeitos imunossupressor e anti-inflamatório dos glicocorticoides endógenos, foram desenvolvidos glicocorticoides sintéticos para o tratamento de uma série de

doenças inflamatórias, auto-imunes e alergias, além de serem amplamente utilizados na terapia de reposição hormonal e antenatal, como em casos de doença de Addison e em riscos de parto prematuro, respectivamente (RHEN; CIDLOWSKI, 2005; ZHANG et al., 2019). Os glicocorticoides sintéticos, como a betametasona (BM), dexametasona ou predinisolona, possuem mais especificidade e afinidade pelos receptores GR do que pelos MR, portanto, é possível separar o efeito glicocorticoide do mineralocorticoide e também minimizar os efeitos colaterais (RANG, 2016).

1.9. Terapia antenatal e parto prematuro

O parto prematuro é o nascimento de um bebê vivo antes de 37 semanas gestacionais completas e está associado à formação incompleta de órgãos, maior morbidade e mortalidade neonatal e infantil, doenças cardiometabólicas na vida adulta e alterações nos desenvolvimentos cognitivo e somático. Portanto, devido ao importante papel dos glicocorticoides no desenvolvimento fetal, mulheres em risco de parto prematuro são submetidas à corticoterapia antenatal com BM ou dexametasona, entre a 24ª e 34ª semanas gestacionais, para fornecer, de forma sintética, o gatilho para a maturação fetal final, principalmente dos pulmões, e diminuir os riscos de morbidade e mortalidade neonatal e infantil e de síndrome respiratória aguda (NIH, 1994; RAJU, 2006; LIU et al., 2012; BLENCOWE et al., 2013; AGNEW et al., 2018).

Tanto a BM como a dexametasona são equivalentes e 25 vezes mais potentes do que o cortisol e não são metabolizadas pela 11β-HSD2. No entanto, a primeira mostrou-se mais eficaz em induzir a maturação pulmonar fetal e possui menos efeitos adversos sobre o desenvolvimento neural de camundongos (JOBE; SOLL, 2004), sendo o medicamento de escolha para a terapia antenatal. A biodisponibilidade do fármaco no sangue fetal equivale a 30% da concentração plasmática materna (LIGGINS; HOWIE, 1972; HALLMAN, 2015). O fosfato de BM é rapidamente hidrolisado por fosfatases plasmáticas à sua forma livre e apresenta meia vida de 36 a 72 horas (HALLMAN, 2015). Portanto, mulheres em risco de parto prematuro recebem duas doses de BM na concentração de 12 mg por via intramuscular a cada 24 horas e na combinação de acetato e fosfato, o que proporciona maiores concentração plasmática e tempo de meia vida, aumentando os efeitos farmacológicos da droga (CROWLEY, 2007).

O estudo clínico pioneiro de Liggins e Howie com a corticoterapia antenatal com BM demonstrou redução nas taxas de síndrome respiratória aguda e de mortalidade neonatal

(LIGGINS; HOWIE, 1972). Mais tarde, uma metanálise também demonstrou que a corticoterapia em casos de risco de parto prematuro ajudava a reduzir hemorragias intraventriculares, enterocolite necrotizante e morte neonatal (CROWLEY, 2007). A corticoterapia antenatal, além de promover maior sobrevivência neonatal, reduz os custos na área da saúde com tratamentos de neonatos prematuros. Acredita-se que a exposição prematura aos glicocorticoides sintéticos leva ao afinamento da parede alveolar, aumentando a capacidade pulmonar para trocas gasosas e induz a produção de surfactantes (JOBE; IKEGAMI, 2000).

1.10. Superexposição aos GC, reprodução e programação fetal

Embora a corticoterapia antenatal tenha se mostrado bastante eficaz e benéfica, é necessário se atentar aos danos a longo prazo gerados pela exposição prematura aos glicocorticoides, uma vez que o desenvolvimento intrauterino é um período de intensa proliferação celular e sensível à ação de fatores químicos, físicos e biológicos que podem levar a alterações permanentes no desenvolvimento, predispondo o indivíduo a doenças cardiometabólicas na vida adulta (GILLMAN, 1995; ROSEBOOM; DE ROOIJ; PAINTER, 2006; MANOJLOVIĆ-STOJANOSKI; NESTOROVIĆ; MILOŠEVIC, 2012).

Durante a gestação em humanos, a enzima 11β-HSD2 é expressa em alta concentração na placenta e controla e o protege o feto da exposição exacerbada ou prematura aos glicocorticoides endógenos de origem materna. Sabe-se que o aumento na concentração de glicocorticoides endógenos de fonte materna, por restrição proteica ou estresse, ou sintéticos, em casos de corticoterapia antenatal, leva à diminuição da expressão de 11β-HSD2 na placenta, comprometendo não apenas o desenvolvimento fetal, mas também o crescimento e morfologia placentários (EDWARDS et al., 1996; SECKL, 1997; COOKE et al., 2004; WHIRLEDGE; CIDLOWSKI, 2010; FOWDEN; FORHEAD, 2015). Além disso, o eixo HHA fetal é bastante sensível a elevadas concentrações de glicocorticoides e sua desregulação está associada a distúrbios comportamentais, imunológicos, morfofuncionais e atrasos no desenvolvimento e crescimento somático (SECKL, 1997; WILLIAMS et al., 1999; MOISIADIS; MATTHEWS, 2014).

Sendo assim, alterações na expressão de 11β-HSD2 ou na atividade do eixo HHA materno expõem o feto a glicocorticoides de forma exagerada, o que sinaliza para um ambiente intrauterino adverso e, em consequência, leva a programação do desenvolvimento para garantir a sobrevivência após o nascimento. No entanto, a programação do

desenvolvimento está associada com restrição do crescimento intrauterino, baixo peso ao nascer e distúrbios psiquiátricos e comportamentais, aumentando a susceptibilidade de desenvolvimento de doenças na vida adulta, como diabetes e hipertensão (FOWDEN; FORHEAD, 2004; MANOJLOVIĆ-STOJANOSKI et al., 2012; BRAUN et al., 2013)

Sabe-se que os glicocorticoides são necessários para a função reprodutiva, porém em alta concentração desregulam o eixo HHG e causam distúrbios de fertilidade. Em situações estressoras, o eixo HHA é ativado e o cortisol liberado desencadeia a resposta de luta e fuga para disponibilizar energia para o organismo e garantir a sobrevivência e, da mesma forma, inativa vias fisiológicas dispensáveis à vida, sendo a reprodutiva uma delas. Em curto prazo esses efeitos podem ser revertidos, no entanto, em longo prazo os danos podem ser permanentes mesmo quando cessado o estímulo estressor (GERAGHTY; KAUFER, 2015). Os glicocorticoides interferem e modulam o eixo HHG por inibir a liberação do GnRH pelo hipotálamo, estimular a síntese de GnIH, inibir a síntese e liberação de gonadotrofinas pela hipófise, e de hormônios esteroides pelos testículos e ovários, impactando negativamente na gametogênese, diferenciação e comportamento sexual (MICHAEL; COOKE, 1994; DAVIES; NORMAN, 2002; GERAGHTY; KAUFER, 2015) (Figura 3).

Os glicocorticoides se ligam a receptores do tipo GR nas células germinativas, de Leydig e de Sertoli ou nos ovários e desencadeiam a maior apoptose celular e diminuem a estereidogenese e gametogênese. Além disso, os glicocorticoides inibem a estereidogenese através do silenciamento da expressão de proteína regulatória aguda estereidogênica, relacionada com a captação de colesterol para o processo de estereidogense (LIU et al., 2018).

PIFFER e colaboradores (2009) demonstraram que ratos expostos no período intrauterino à BM apresentaram diminuição na concentração de testosterona no período próximo ao parto, levando a diminuição deste hormônio na vida adulta e alteração na escolha de parceiro sexual. Estudos sugerem que a exposição intrauterina aos glicocorticoides sintéticos causa programação fetal e restrição do crescimento intrauterino (BORGES et al., 2016; DE BARROS et al., 2018), além de desregulação do eixo HHG, levando ao atraso do desenvolvimento reprodutivo inicial e redução da fertilidade em ratos machos e fêmeas. Além disso, em ratos machos, observaram-se alterações na qualidade espermática e nas morfofisiologias testicular e epididimária (PIFFER; PEREIRA, 2004; PIFFER et al., 2009; BORGES et al., 2017a, 2017c).

A exposição intrauterina aos glicocorticoides está relacionada com heranças transgeracionais, uma vez que a prole masculina F2 de ratos machos apresentou redução do

peso ao nascer, desregulação do eixo HHG, atraso no desenvolvimento sexual inicial, além de alterações na qualidade espermática, indicado pela diminuição da fertilidade (DRAKE et al., 2011; BORGES et al., 2017b). Ovelhas que foram expostas de maneira crônica a glicocorticoides sintéticos apresentaram redução na pulsatilidade de GnRH, no entanto a intensidade do efeito dependeu do tipo e duração do estímulo, além da presença de hormônios esteroides (OAKLEY et al., 2009). Além disso, estudos em ratos mostram que estresse por restrição combinado com administração de cortisol diminui a secreção de LH. No entanto, o estresse agudo está associado ao aumento na secreção de LH, prolactina e FSH, sugerindo ativação do eixo HHG (GERAGHTY; KAUFER, 2015).

Acredita-se que os glicocorticoides causem modificações epigenéticas, como metilação ou desmetilação, que são alterações na estrutura do DNA sem alterar a sequência nucleotídica e que modulam a expressão gênica. Essas alterações podem perpetuar e gerar danos ao longo das gerações subsequentes (MANOJLOVIĆ-STOJANOSKI; NESTOROVIĆ; MILOŠEVIC, 2012; FOWDEN; FORHEAD, 2015).

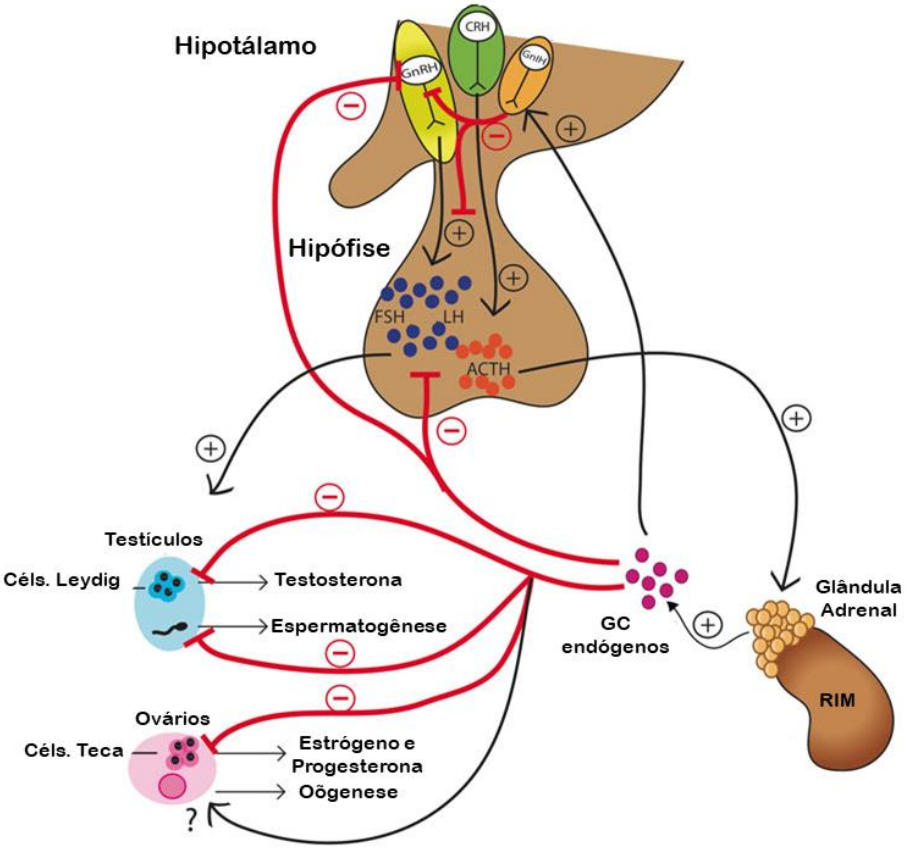

Figura 3. Representação dos eixos hipotálamo-hipófise-gônada e hipotálamo-hipófise-adrenal e da influência dos glicocorticoides. Fonte: Adaptado de Geraghty e Kaufer, 2015.

Justificativa

2. Justificativa do tema

Este estudo se justifica em virtude do uso de glicocorticoides por mulheres durante o último trimestre de gestação para acelerar o desenvolvimento e a maturação pulmonar fetal na eminência de parto prematuro. Além disso, nosso grupo de pesquisa demonstrou em trabalhos anteriores que a exposição de ratos machos e fêmeas à betametasona nos dias gestacionais 12, 13, 18 e 19, correspondentes à janela crítica de desenvolvimento do sistema genital e diferenciação sexual em ratos, tem efeitos deletérios no desenvolvimento sexual inicial e vida adulta, ocasionando desregulação do eixo hipotálamo-hipófise-gônada e diminuição da fertilidade. Desta maneira, é necessário estudo cuja exposição corresponda à terapia antenatal na clínica humana, o que em ratos equivale aos dias pós-natal 1, 2 e 3. Além disso, tendo em vista os resultados já obtidos pelo grupo de pesquisa com a exposição *in utero* a glicocorticoides e a necessidade de compreender os efeitos deste fármaco sobre os desenvolvimentos cognitivo e somático inicial, além de avaliar possíveis efeitos multigeracionais das alterações no sistema genital em F2, é pertinente a avaliação dos efeitos da exposição intrauterina à betametasona, seguindo os mesmos protocolos anteriores, nas proles masculina e feminina F1 e F2.

Objetivos

3. Objetivos gerais

O presente estudo teve como objetivos avaliar os efeitos da exposição neonatal de ratos à betametasona sobre desenvolvimento e fisiologia reprodutivos e avaliar os impactos da exposição pré-natal à betametasona, com ênfase em marcos dos desenvolvimentos reprodutivo, somático e cognitivo das proles F1 e F2, além de comparar ambos os períodos de exposição.

3.1. Objetivos específicos

- Mimetizar, com o tratamento neonatal, a terapia antenatal que ocorre em gestantes;
- Avaliar a influência da exposição pré-natal e neonatal à betametasona sobre o eixo hipotálamo-hipófise-ônadal e sobre os órgãos do sistema genital masculino e feminino;
- Avaliar os efeitos da exposição pré-natal e neonatal à betametasona no desenvolvimento sexual;
- Avaliar a influência da exposição pré-natal à betametasona sobre os desenvolvimentos cognitivo e somático inicial;
- Avaliar os efeitos da exposição pré-natal e neonatal à betametasona na qualidade espermática de ratos machos e na regularidade do ciclo estral em fêmeas;
- Comparar a exposição pré-natal e neonatal de ratos à betametasona;
- Determinar se há influência multigeracional da exposição *in utero* à betametasona no sistema genital masculino e feminino, a partir da observação da prole masculina e feminina F2.

Material e métodos

4. Material e métodos

Este estudo foi dividido em dois experimentos e tempos de exposição diferentes e os delineamentos experimentais estão ilustrados nas Figura 4 e Figura 5.

No experimento 01, avaliaram-se os efeitos da exposição neonatal à BM no sistema genital e no desenvolvimento sexual de ratos machos e fêmeas. No experimento 02, avaliaram-se os efeitos da exposição pré-natal à BM no sistema genital e nos desenvolvimentos cognitivo e somático inicial das gerações F1 e F2 masculina e feminina.

Ratos Wistar machos adultos (90 dias de idade, 300g) e fêmeas (70 dias de idade, 200g), provenientes do Centro Multidisciplinar para Investigação Biológica na Área da Ciência em Animais de Laboratório – CEMIB, Universidade Estadual de Campinas (UNICAMP), SP, foram mantidos no Biotério de Pequenos Mamíferos do Departamento de Morfologia do Instituto de Biociências da Unesp de Botucatu e também no Biotério do Centro de Assistência Toxicológica de Botucatu - CEATOX, em condições controladas de luminosidade (12 horas de luz/12 horas de escuro) e temperatura (média de 23ºC) para realizar acasalamentos para obtenção das ninhadas utilizadas no estudo. Os animais receberam água e ração para roedores à vontade.

Os acasalamentos foram realizados durante o período escuro do ciclo, colocando-se duas fêmeas na caixa do macho, e o dia gestacional um (DG 1) foi determinado pela presença de espermatozoides em esfregaços vaginais de fêmeas em estro, que após a confirmação da prenhez foram mantidas em gaiolas individuais até o nascimento espontâneo da prole no DG 22. Após o nascimento, o número de filhotes por ninhada foi reduzido para oito, visando sempre manter cinco filhotes do sexo masculino e três do sexo feminino, e ninhadas com número de filhotes inferior a oito foram eutanasiadas.

Os animais utilizados neste estudo foram submetidos à avaliação semanal do ganho de peso corpóreo e de sinais físico de estresse ou toxicidade até o fim do período experimental. Além disso, foram mantidos de acordo com os Princípios Éticos em Experimentação Animal, adotados pelo Colégio Brasileiro de Experimentação Animal. O projeto esteve sob protocolo número 923 (anexo 1), junto à Comissão de Ética em Experimentação Animal do Instituto de Biociências de Botucatu.

4.1. Experimento 1

Após o nascimento da prole, as fêmeas junto com os filhotes foram alocados em dois grupos experimentais (n=10 matrizes/grupo): controle (solução NaCl 0,9%) ou BM (Sigma®) a 0,1 mg/Kg e expostos por via subcutânea, nos DPN 1, 2 e 3, que correspondem às semanas

gestacionais 24 à 34 em humanos. A dose foi escolhida de acordo com a utilizada na terapia antenatal humana (İŞCAN et al., 2017).

Nos DPN 1, 4 e 21, os animais foram avaliados quanto à distância ano-genital (DAG) e, a partir do DPN 30, quanto à instalação da puberdade. As fêmeas foram avaliadas quanto à regularidade do ciclo estral a partir do DPN 60, por 15 dias consecutivos.

Tendo o intuito de observar os impactos da BM sobre o desenvolvimento sexual masculino da pré-puberdade até a idade adulta, um rato macho por ninhada (n=10/grupo experimental) foi eutanasiado para coleta de sangue para dosagens hormonais e de órgãos (testículos, epididídimos, ducto deferente, glândulas seminais, próstata, hipófise, pulmão, coração, fígado, rins e adrenais) para pesagem e avaliações histopatológicas (testículo e epidídimo) nos DPN 7, 14, 28, 45 e 112. Além de coleta de espermatozoides da cauda epididimária para avaliar motilidade e morfologia espermáticas. Os testículos e epidídimos direitos foram coletados para avaliação da contagem espermática, produção espermática diária e tempo de trânsito espermático.

Uma fêmea por ninahada foi eutanasiada no primeiro estro a partir do DPN 75 para coleta de sangue para dosagens hormonais e de órgãos (útero, ovários, hipófise, tireoide, pulmão, coração, fígado, rins e adrenais) para pesagem.

4.2. Experimento 2

Vinte ratas prenhes (F0) foram subdividas em dois grupos experimentais (n=10 matrizes/grupo experimental): controle (solução NaCl 0,9%) ou 0,1 mg/Kg de BM e foram expostas por via intramuscular nos DG 12, 13, 18 e 19. Essa dose foi baseada na utilizada em tratamento materno humano seguindo adaptações para roedores (SOUZA et al., 2001; PIFFER et al., 2009; BORGES et al., 2017a). Durante a gestação, F0 foi avaliada quanto ao ganho de peso gestacional.

As avaliações descritas a seguir foram realizadas em ratos machos e fêmeas F1 e F2. A partir do nascimento, os animais foram avaliados quanto ao desenvolvimento cognitivo e somático. Nos DPN 1 e 21, foi avaliada a DAG e, a partir do DPN 30, a instalação da puberdade. As fêmeas foram avaliadas quanto à regularidade do ciclo estral a partir do DPN 75, por 15 dias consecutivos e, a partir do DPN 90, uma fêmea em estro por ninhada foi eutanasiada para coleta de sangue para dosagens hormonais e de órgãos (útero, ovários, cérebro, hipófise, tireoide, pulmão, coração, fígado, rins e adrenais) para pesagem e avaliações histopatológicas (ovários e útero).

No DPN 120, um animal macho por ninhada foi eutanasiado para coleta de sangue para dosagens hormonais e órgãos (testículos, epididídimos, glândulas seminais, próstata, cérebro, hipófise, tireoide, pulmão, coração, fígado, rins e adrenais) para pesagem e avaliações histopatológicas (testículo e epidídimo). Além de coleta de espermatozoides da cauda epididimária para avaliar motilidade, morfologia, vitalidade espermáticas e atividade mitocondrial. Os testículos e epidídimos direitos foram coletados para avaliação da contagem espermática, produção espermática diária e tempo de trânsito espermático.

No DPN 90, um macho e uma fêmea por ninhada foram acasalados com animais não tratados e virgens para obtenção da geração F2 (prole de ratos machos) e F2’ (prole de ratos fêmeas).

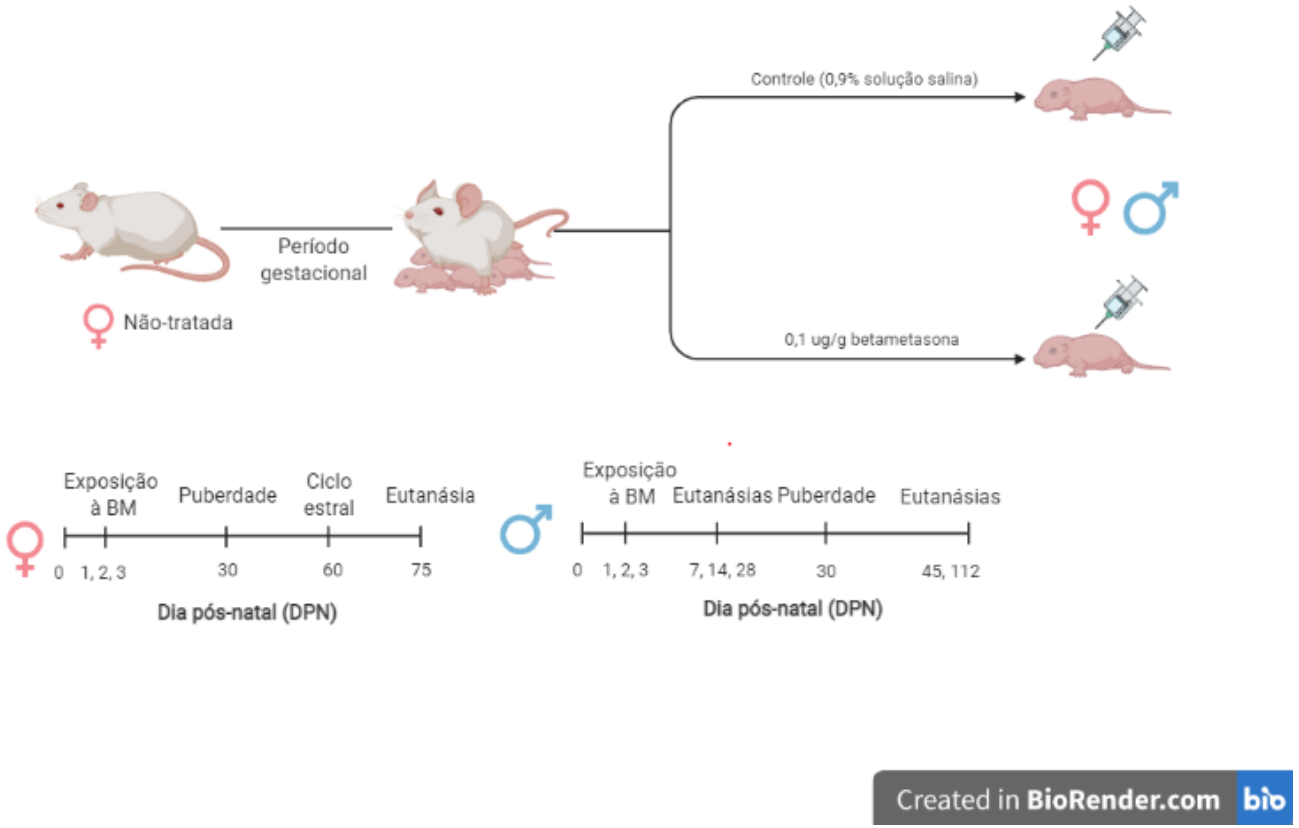

Figura 4. Delineamento experimental do experimento 1. BM: betametasona.

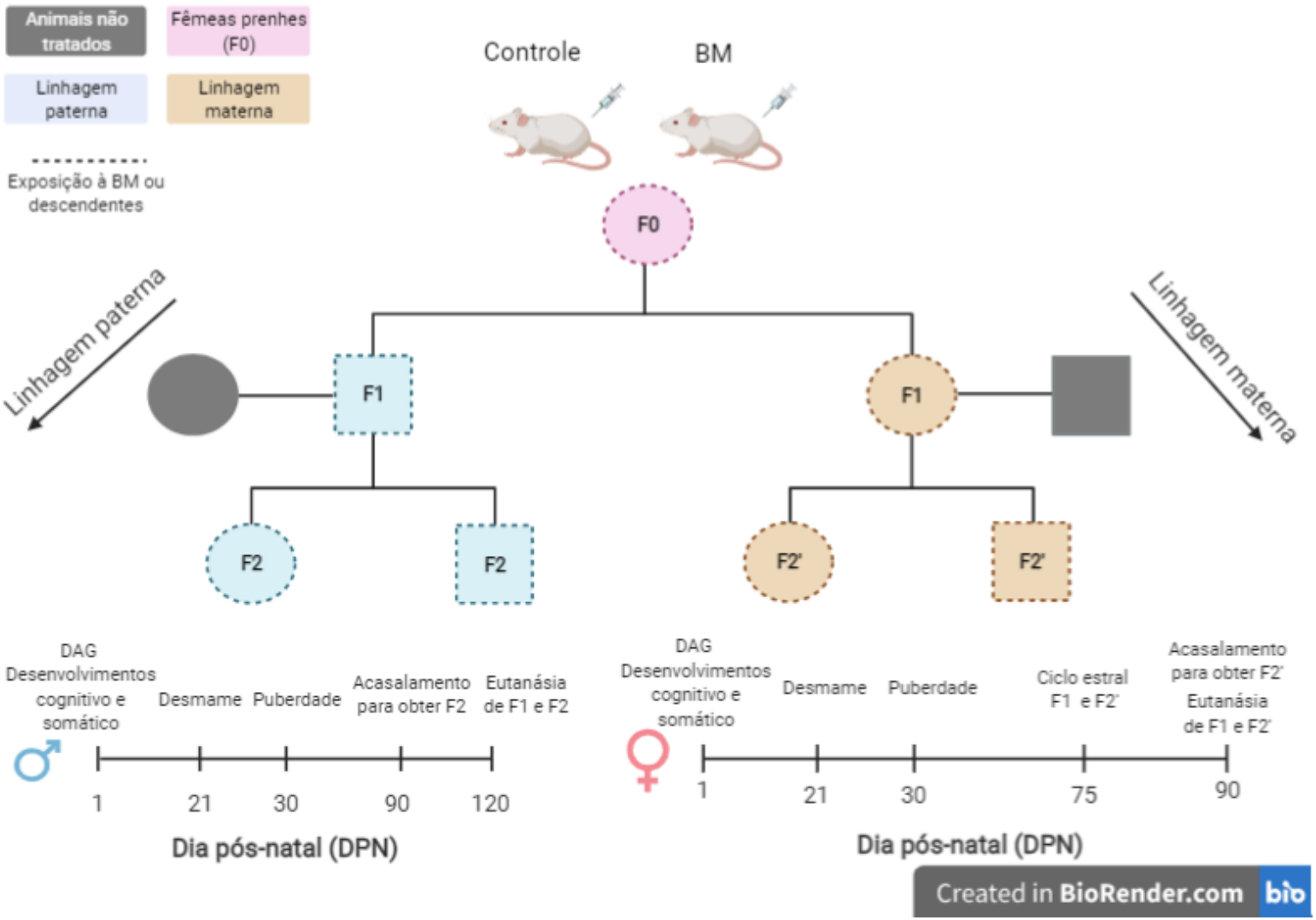

Figura 5. Desenho experimental do experimento 2. BM: betametasona 0,1 mg/Kg.

Capítulos

5. Capítulos

O presente trabalho resultou em dois manuscritos que foram elaborados e formatados de acordo com as regras da revista *Reproductive Toxicology* e estão apresentados a seguir.

5.1. Capítulo I: “Reproductive outcomes of neonatal exposure to betamethasone in male and female rats”.

REPRODUCTIVE OUTCOMES OF NEONATAL EXPOSURE TO BETAMETHASONE IN MALE AND FEMALE RATS

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ABSTRACT

Betamethasone (BM) is the drug of choice for antenatal corticosteroid therapy for women at risk of preterm delivery once it induces fetal lung maturation and enhances survival after birth. However, evidence of fetal programming and impaired reproductive development and function were reported by our group in rats exposed during the critical window of genital system development. Thus we aimed to investigate the effects of BM on sexual development of rats in a period that corresponds to antenatal corticosteroid therapy in humans. Male and female rats were exposed subcutaneously to 0.1 mg/Kg BM or to a NaCl 0.9% solution (Control) at postnatal days (PND) 1, 2, and 3. It was observed that neonatal exposure to BM decreased body weight and weight gain in male and female rats during treatment. The estrous cycle was deregulated and LH level was decreased in female rats. The sperm concentration in the caput-corpus of the epididymis was decreased, while the sperm transit time was increased in male rats, as well as the sperm concentration in the cauda of the epididymis. Our results demonstrated that neonatal exposure to BM impaired body growth of male and female rats, deregulated the estrous cycle of female rats and altered sperm quality of male rats. Therefore, BM exposure from PND 1 to 3 corroborated results previously observed after prenatal exposure to this drug. Despite the recognized importance of human antenatal corticosteroid therapy, the findings of this study evidence and encourage further studies in order to minimize possible adverse postnatal effects.

Key-words: betamethasone; sexual development; fetal programming; neonatal; corticosteroid therapy

1. BACKGROUND

Preterm delivery is the birth of an alive baby before 37 completed gestational weeks and leads to physical and neurobehavioral impairments. Towards term occurs a physiological rise in endogenous glucocorticoids levels that act as a maturational trigger that optimizes life after birth [1]. Thus, women at risk of preterm delivery are submitted to antenatal corticosteroid therapy, between gestational weeks 24 and 34, with betamethasone (BM) or dexamethasone to enhance fetal lung maturation and survival [2–4]. Due to some pharmacological advantages, BM is the drug of choice for antenatal corticosteroid therapy [5,6].

Antenatal corticosteroid therapy has beneficial effects, however early overexposure to endogenous or synthetic glucocorticoids impairs brain, neurobehavioral and physical development and reproductive functions, besides leading to intrauterine growth restriction and low birth weight. Thus glucocorticoids overexposure reprograms and deregulates the hypothalamic-pituitary-adrenal axis, leads to life-long physiological consequences and are associated to the developmental origins of diseases later in life [1,7–9]. In humans, children exposed prenatally to glucocorticoids present mental and behavioral disorders, besides insulin resistance [10,11].

It is also known that glucocorticoids play an important role in reproductive function, but at higher levels impair and program the hypothalamic-pituitary-gonadal (HPG) axis and lead to reproductive disorders and infertility [3,12]. Previous studies of our group demonstrated that rats exposed during the critical window of genital system development to synthetic glucocorticoids presented delayed sexual development, testicular and epididymal impairments, decreased testosterone levels, as well as fertility disorders and impaired sexual behavior [13–18]. Other studies demonstrate that female rats exposed *in utero* to BM presented delayed age of puberty onset, deregulation of the estrous cycle and uterine

morphophysiological impairment, as well as decreased fertility and gonadotropin levels. Glucocorticoids also regulate cell proliferation and organ development, and change the switch from tissue accretion to differentiation [19].

Although rodents are widely used in biomedical and toxicological studies due to some shared characteristics with humans, they have a shorter pregnancy and life span, better reproductive efficiency, and some differences regarding developmental events [20,21]. So, it is necessary to be aware to the differences when comparing results obtained in animal experimentation with humans.

Intrauterine glucocorticoids exposure in previous studies does not match the period of antenatal corticosteroid therapy in the human clinic, but rather corresponds to the critical window of genital system development. Therefore, this raises questions when comparing the effects observed in rats to humans. The study aimed to evaluate the effects of BM exposure during the period that corresponds to the antenatal corticosteroid therapy in humans on the reproductive development of male and female rats and on sperm parameters of male rats.

2. MATERIAL AND METHODS

2.1. Animals

Wistar male (90 days old, 300g) and female (70 days old, 200g) rats were obtained from the Multidisciplinary Center for Biological Investigation - CEMIB, State University of Campinas (UNICAMP) - SP, and maintained in the Small Mammal Biotherium at the Department of Structural and Functional Biology, sector of Morphology, Institute of Biosciences, Unesp, Botucatu, SP, Brazil, under light and temperature-controlled conditions (25º, 12/12h light/dark cycle) with food and water *ad libitum*. The animals were assessed regarding body weight, signs of general toxicity and stress weekly until the end of the experimental period.

The study was approved by the local ethics committee for the Use of Experimental Animal of the State University of São Paulo (protocol number 923 CEUA – IBB, Unesp) and according to the Guide for the Care and Use of laboratory Animals (National Institutes of Health – NIH). The animals were euthanized by decapitation following CO_2 asphyxiation.

2.2. Experimental design

Two nulliparous female estrus rats were mated with one naive male, during the dark period of the photoperiod. The gestational day (GD) 1 was determined by the presence of sperm in vaginal smears and from this day until weaning the female and their offspring were kept in individual cages and, after birth, randomly allocated into two experimental groups: control (NaCl 0.9% solution, n =10) and betamethasone (0.1 mg/Kg of betamethasone [BM] 21-phosphate disodium [Sigma-Aldrich, St. Louis, MO] diluted in NaCl 0.9% solution, n = 10). The puppies received a subcutaneous injection at postnatal day (PND) 1, 2 and 3, which correspond to the period of antenatal corticosteroid therapy in humans [24,23]. The dose of BM was chosen based on previous studies and on human use for antenatal corticosteroid therapy and modified to rodents [15,22,23]. After birth, at PND 1, the litters were reduced to 8

127 animals per dam (5 male and 3 female). At PND 21, the animals were weaned and allocated in
128 different cages (Figure 1).

129 **2.3. Anogenital distance (AGD)**

130 Since AGD can be influenced by exogenous factors besides the treatment, its measure
131 was normalized by the ratio between AGD and the cubic root of body weight to avoid biases
132 [25]. It was evaluated at PND 1, 4 and 21.

133 **2.4. Puberty onset**

134 Evaluated since PND 30, the physical sign of puberty onset in male rats was assessed
135 by the manual retraction of the prepuce [26]. In female rats, after the complete vaginal
136 opening, vaginal fluids were collected daily by inserting 10 µl of NaCl 0.9% solution in the
137 vagina and subsequently aspirating it. Then, vaginal fluids were placed on a slide and
138 analyzed under a light microscope (x200 magnification) to detect the first estrus by the
139 presence of cornified epithelial cells [27]. The animals were weighed on the day of puberty
140 onset.

141 **2.5. Estrous cyclicity**

142 Every morning over a period of 15 consecutive days (from PND 60 to 75) vaginal
143 fluids were collected and placed on a slide to evaluate the estrous cyclicity of each female rat
144 of the litter [27]. The samples were evaluated under a light microscope and the estrous cycle
145 phase was determined according to the predominance of cells as following described: estrus
146 (predominance of cornified epithelial cells), proestrus (predominance of nucleated epithelial
147 cells), metestrus (presence of nucleated and cornified epithelial cells and leukocytes) and
148 diestrus (predominance of leukocytes) [28]. The data obtained were used to estimate the
149 frequency of each phase of the cycle, the estrous cycle length, and the total number of cycles
150 during the evaluation period.

151 **2.6. Euthanasia, body and organ weights**

To evaluate the reproductive development of male rats, one animal per litter was euthanized at PND 7, 14, 28, 45, and 112. These days were chosen based on the testicular developmental and maturational events that occur during postnatal life [13,29,30]. One estrus female rat per litter was euthanized at PND 75. Soon after death, the blood was collected from the lower vena cava. Body weight was assessed weekly and just before the procedure.

To evaluate systemic and reproductive toxicity, the pituitary gland, heart, lungs, liver, kidney, and adrenal glands, *vas deferens*, prostate, and full and empty seminal vesicle were collected, weighed, and then discarded. The left testis and epididymis, uterus with fluid and ovaries were collected, carefully weighed and processed, sectioned in 5 µm and stained with hematoxylin and eosin (HE) for histopathological evaluation [31].

2.7. Testicular histopathological evaluations

An amount of 100 seminiferous tubules in each one of the ages mentioned were evaluated and classified according to toxicological guidelines as described: normal (presence of concentric and normally organized germ cell layers) or abnormal (presence of germ cells and cellular debris in the lumen, multinucleated formation, presence of acidophilic cells, few germ cells layers, vacuole formation or degeneration). Interstitial tissue and peritubular myoid cells were qualitative evaluated, as well as the general morphology of Leydig cells and the appearance of blood vessels [29]. At PND 14 and 28, it was also observed the presence of lumen.

At PND 28 and 45, 100 seminiferous tubules were evaluated regarding the maturational degree of the epithelium and classified according to the most mature cell as described by Leite et al. [32]. The number of each seminiferous tubules was multiplied by its degree and the values were summed and divided by 100, resulting in the average degree [33].

2.8. Sperm quality at sexual maturity

At PND 112, soon after euthanasia, sperm was collected from the cauda epididymis and each sample was diluted in 1mL of human tubal fluid (HTF) modified medium (Spectrun

178 901126) and supplemented with 1% bovine serum albumin (BSA) for evaluation of sperm
179 motility and morphology.

180 **2.8.1. Sperm motility**

181 A 10 µL aliquot was transferred to a Mackler's chamber and 100 spermatozoa were
182 classified under a phase-contrast microscope (200x magnification) as: type A (motile with fast
183 and progressive movement), type B (motile with non-progressive movement) and type C
184 (immotile). Sperm motility was expressed as the percentage of total sperm [34,35].

185 **2.8.2. Sperm morphology**

186 A 100 µL aliquot was diluted in 900 µL of buffered formaldehyde. Then, smears of
187 each animal were prepared on slides, air-dried and 200 spermatozoa were observed under a
188 phase-contrast microscope (400x magnification) and classified as: normal, sperm head
189 abnormalities (without characteristic curvature, pin-head or isolated form) and sperm tail
190 abnormalities (broken, isolated or rolled into a spiral) [36]. It was either evaluated the
191 presence of cytoplasmic droplets.

192 **2.8.3. Sperm counts in the testis and epididymis, daily sperm production, and**
193 **sperm transit time**

194 The right testis and the epididymis caput/corpus and cauda of adult male rats were
195 weighed, decapsulated, and frozen immediately after collection. Then at the day of the
196 analysis, were processed according to Leite et al. (2018) [35] to determine the number of
197 spermatid 19 and sperm in the testis and epididymis, respectively. Besides estimating the
198 daily sperm production (DSP) according to Robb et al. (1978) [37] and Ashby et al. (2003)
199 [38]. The sperm transit time throughout the epididymis was determined by dividing the
200 number of sperm in each part of the organ by the daily sperm production.

201 **2.9.Statistical analysis**

202 Data were checked for normal distribution using the Kolmogorov–Smirnov test. Data
203 are presented as mean ± standard error of the mean (S.E.M.) or median and interquartile

range. Student's t-test was used for comparison of parametric variables. Nonparametric data were compared using Mann-Whitney's test. Differences were considered significant when $p \leq 0.05$. Statistical analyses were performed using GraphPad Prism ® (version 5) (GraphPad Inc., San Diego, CA, USA).

3. RESULTS

Neonatal BM exposure led to a significant decrease of body weight of male rats at PND 3 and 4 and of body weight gain of male and female rats at PND 4. In other periods evaluated, there were no significant differences regarding body weight and body weight gain among the experimental groups, indicating a catch-up on growth (Figure 2).

Moreover, there were no significant differences regarding AGD at PND 1, 4, and 21 of male and female rats (Table 1).

Neonatal BM exposure did not change the age of puberty onset of male and female rats (Figure 3). The estrous cycle was evaluated from PND 60 to 75 and its length was significantly increased, while the total number of cycles was decreased in BM exposed female rats (Table 2).

At PND 28, the wet weight of the heart was decreased and at PND 45 the weights of the lungs and kidney of male rats exposed to BM were significantly increased (Table 3). There were no significant differences in the weight of these organs at PND 7, 14, and 112, as well as in the weights of testes, epididymis, ventral prostate, full and empty seminal vesicles, *vas deferens*, pituitary, liver and adrenal glands (data not shown). There was no significant difference in organ weights of female rats at PND 75 (data not shown).

It was observed a decrease in sperm number in the caput/corpus of the epididymis of male rats exposed to BM, as well as an increase in the sperm number in the cauda of the epididymis and in sperm transit time (Table 4). The animals exposed to BM did not present alterations in sperm motility and morphology (Figure 4 and Table 5).

It was also observed a decrease in LH levels at sexual maturity in female rats exposed to BM, but there were no significant differences in other hormones evaluated or in hormonal levels in male rats exposed to BM (Figure 5).

232 Data regarding testis histopathological evaluation at different PND are represented on
233 Table 6 and Figure 6. There was no significant difference between the experimental groups
234 for testis histopathology at different PND.
235

4. DISCUSSION

The neonatal period in rats, which extends from PND 0 to 7, is still quite sensitive to drugs and toxic agents [30]. Overexposure to glucocorticoids, such as BM, decreases cell proliferation and leads to impaired and delayed development [19,39]. Moreover, glucocorticoids exposure is associated to metabolic impairments, intrauterine growth restriction and decreased body weight gain [40–42]. Therefore, BM exposure during the neonatal period may lead to delayed development and body growth.

Glucocorticoids exposure increases the levels of the anorectic factor leptin and leads to reduced food intake and body weight gain [40,43,44]. Furthermore, insulin-like growth factor 1 (IGF-1) promotes body growth and cell metabolism, and epidermal growth factor (EGF) promotes development and cell proliferation are down-regulated due to glucocorticoids overexposure [45]. Although we did not evaluate leptin levels or IGF-1 and EGF expression, we believe that BM-reduced body weight is due to changes in these endpoints.

The perinatal period is important for sexual dimorphism in rodents, once there is a rise in testosterone levels that masculinizes and defeminizes the animal, thus leading to sexual differentiation of the brain, sexual accessory glands, and gonads. Therefore, exposure to drugs that leads to decreased testosterone levels during this period impairs sexual differentiation and development [46,47]. BM exposure decreased and changed the neonatal testosterone surge in male rats and disrupted sexual development and behavior [16]. In this study, BM exposure, although during the neonatal testosterone surge, did not led to impaired initial sexual development in male and female rats.

Once glucocorticoids exposure decrease cell proliferation, it is also associated to impaired development and physiology of organs, such as the heart and kidney, that last throughout life [48,49]. Therefore we believe that the weight changes of heart, lungs and

kidney in the animals exposed to BM are due to impaired development. Even though there was a catch-up on development of these organs, the physiology may be permanently impaired.

For normal reproductive function, optimal levels of glucocorticoids are required. Thus, overexposure to glucocorticoids inhibits the hypothalamic-pituitary-gonadal axis and the release of gonadotropins and steroid hormones, leading to impaired sexual development, reproductive dysfunction and infertility [12,50].

Puberty onset is a biomarker of exposure to toxic agents during pre and postnatal development and of HPG axis programming [51]. Puberty onset occurs due to a rise in GnRH release that will lead to testicular and ovarian maturation [52]. Glucocorticoids exposure delayed puberty onset in previous studies, which is related to deregulation of the HPG axis, once glucocorticoids inhibit GnRH, gonadotropins and steroid hormones release [14,43,53]. Although the day of vaginal opening, the physical landmark of puberty onset in female rats [52], was not altered in BM-exposed rats, the estrous cycle was deregulated. Impairments in the estrous cycle demonstrate deleterious effects of toxicants on the integrity of the HPG axis and fertility of female rats [54,55].

Sperm transit through the epididymis is important, once it is when sperm acquire the fertilization and motility capacity. Therefore decreased sperm transit time is related to poor sperm quality and fertility impairments [56,57]. Dexamethasone led to impairments in sperm morphology, motility and concentration, once glucocorticoids bind to glucocorticoids receptors in the initial segment and caput/corpus of the epididymis, and promote the expression of proapoptotic proteins and negatively impact sperm maturation process and lead to cell death [58,59]. We observed that exposure to BM decreased sperm concentration in the caput/corpus of the epididymis. As well, it increased sperm transit time, corroborating the increased sperm concentration in the cauda of the epididymis. So BM may have direct effects on epididymis, leading to poor sperm quality.

Spermatogenesis occurs within the seminiferous tubules under testosterone stimulus, and it is a complex and dynamic process that involves cell proliferation and differentiation. Glucocorticoids exposure are linked to cell death and decreased proliferation, thus it can impair spermatogenesis. Also, the testes are target organs of glucocorticoids, once this hormone binds to its receptors in Leydig, Sertoli and germ cells and promotes expression of proapoptotic proteins and inhibits steroidogenesis. Testicular histopathological evaluations are important to identify and classify the impact of drugs, such as BM, and their mechanisms of toxicity [60]. Although there were no significant changes regarding histopathological evaluation of the testis, glucocorticoids exposure has been already associated to impaired spermatogenesis and testicular morphophysiology [41]. We believe that BM exposure during PND 1 to 3 was not sufficient to promote testicular or spermatogenesis impacts, but directly acted at long-term in epididymis development, leading to the observed impairments in sperm concentration and sperm transit time.

Overexposure to glucocorticoids leads to inhibition of the HPG axis and, consequently, inhibits the release of GnRH and gonadal steroidogenesis [61], as it is demonstrated that prenatal exposure of rats to dexamethasone down-regulates acute steroidogenic regulatory protein and, therefore, decreases serum testosterone levels [59,62]. In this study, there was a decrease of LH levels in female rats at sexual maturity, which corroborates the estrous cycle deregulation and HPG axis deregulation and programming.

5. CONCLUSIONS

Although neonatal BM exposure did not have as obvious impacts as prenatal exposure, as previously described by our research group, it still impaired body growth of male and female rats, decreased sperm quality of male rats and deregulated the estrous cycle of female rats, suggesting HPG axis and developmental programming. Therefore confirmed partially the results previously observed after prenatal exposure to BM.

The differences between the experimental models used and the conditions of exposure to BM in the human clinic must be taken into account. In the study, newborn rats were treated directly with BM, while human fetuses are exposed indirectly via the maternal-fetal interface, since mothers receive the glucocorticoid. In addition, handling newborn animals may lead to stress. However, in addition to the minimum manipulation during the experimental period, it occurred for treated and control groups.

In addition, when comparing intrauterine and neonatal exposures of the offspring of rats to BM, it is noted that in the first, the glucocorticoid administered to the dams, besides being metabolized by the maternal organism, is distributed to the fetuses. The administration to newborn puppies is performed directly and individually, leading to greater exposure to the drug.

Thus, although the negative effects of neonatal BM exposure on the reproductive function and development of rats does not indicate discontinuing the use of glucocorticoids by pregnant women, they rather stimulate they rather stimulate further studies with the aim to minimize possible adverse effects.

6. CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

7. ACKNOWLEDGMENTS

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9. FIGURES AND TABLES

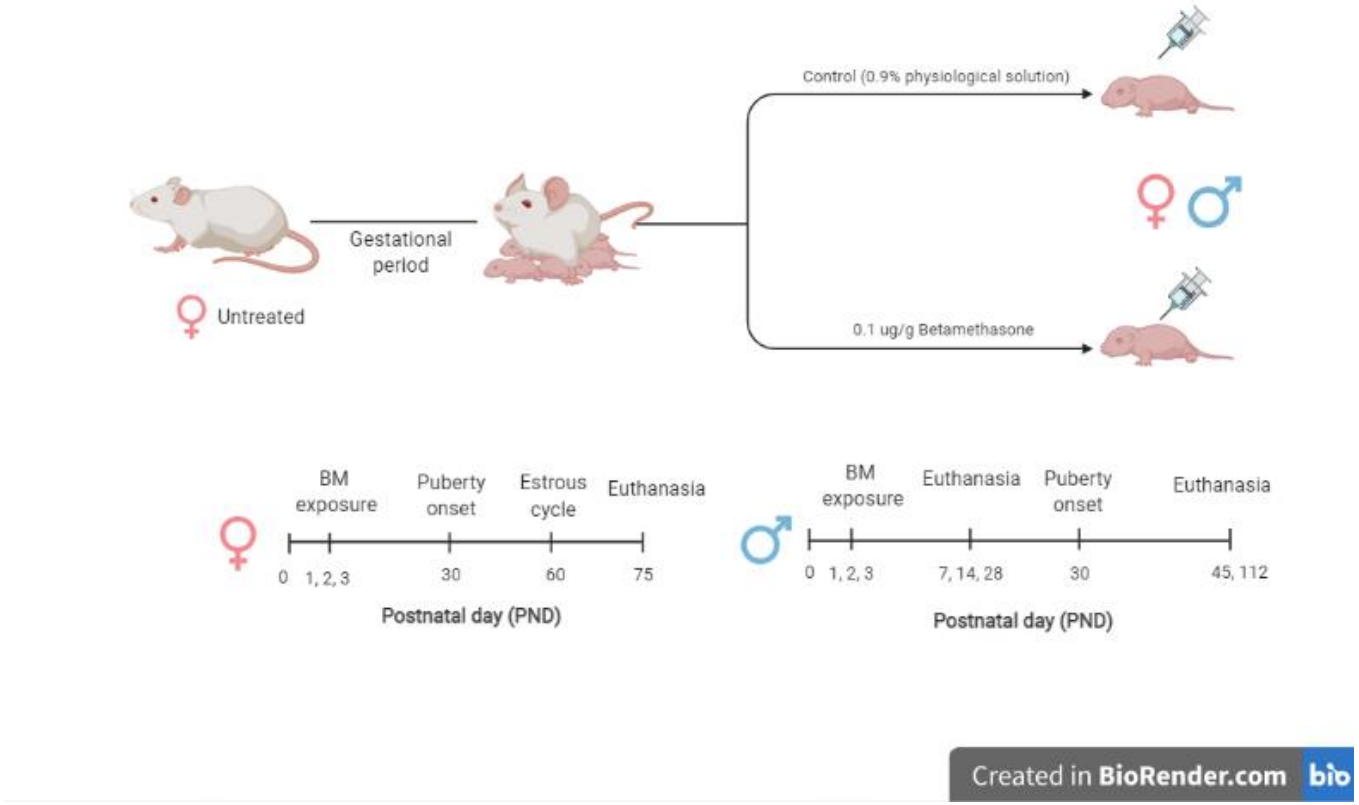

Figure 1. Experimental design. BM: betamethasone.

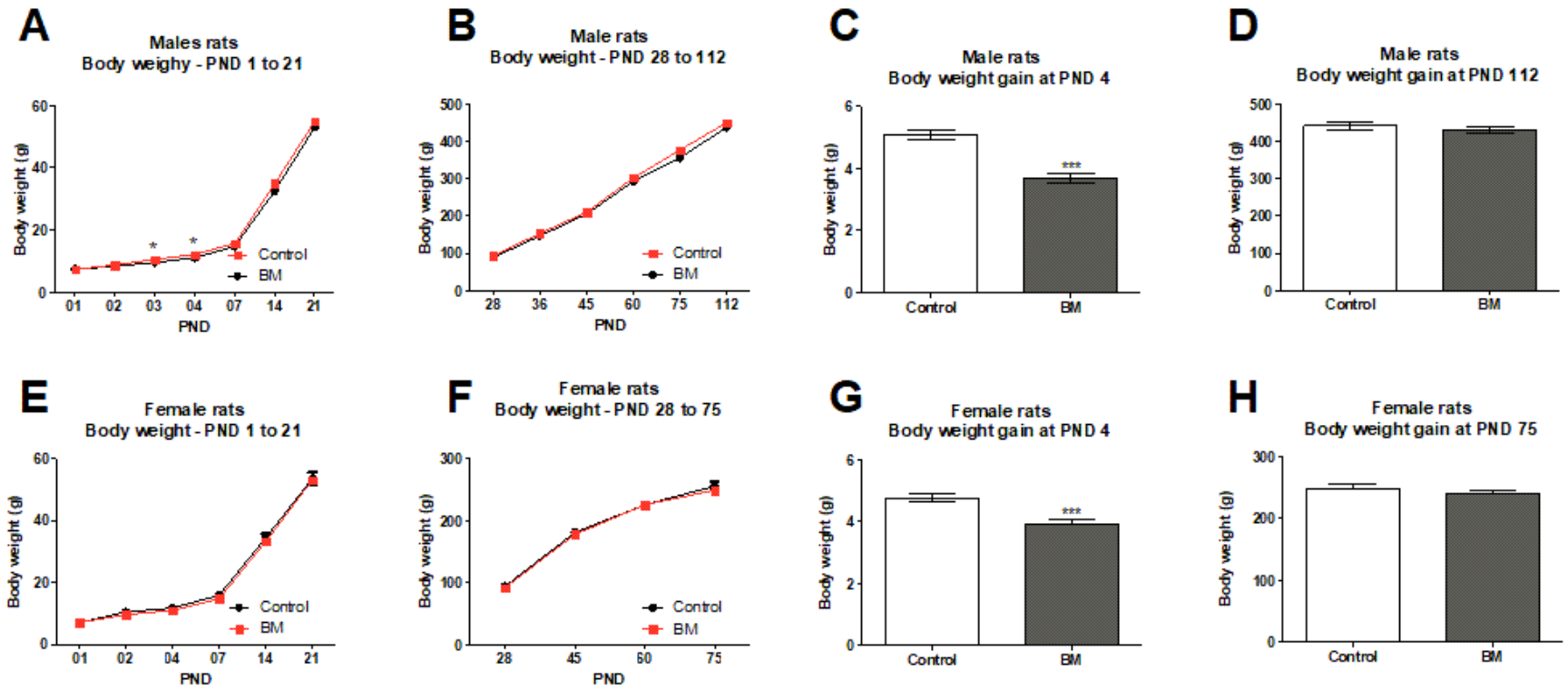

Figure 2. Body weight and body weight gain of male and female rats A to D: Body weight evolution from PND 1 to 21 and from PND 25 to 112 and body weight gain at PND 4 and 112 of male rats, respectively. E to H: Body weight evolution from PND 1 to 21 and from PND 25 to 75 and body weight gain at PND 4 and 75 of female rats, respectively. Values expressed as mean ± SEM. *p <0.05, ***p <0.001. Student's t test.

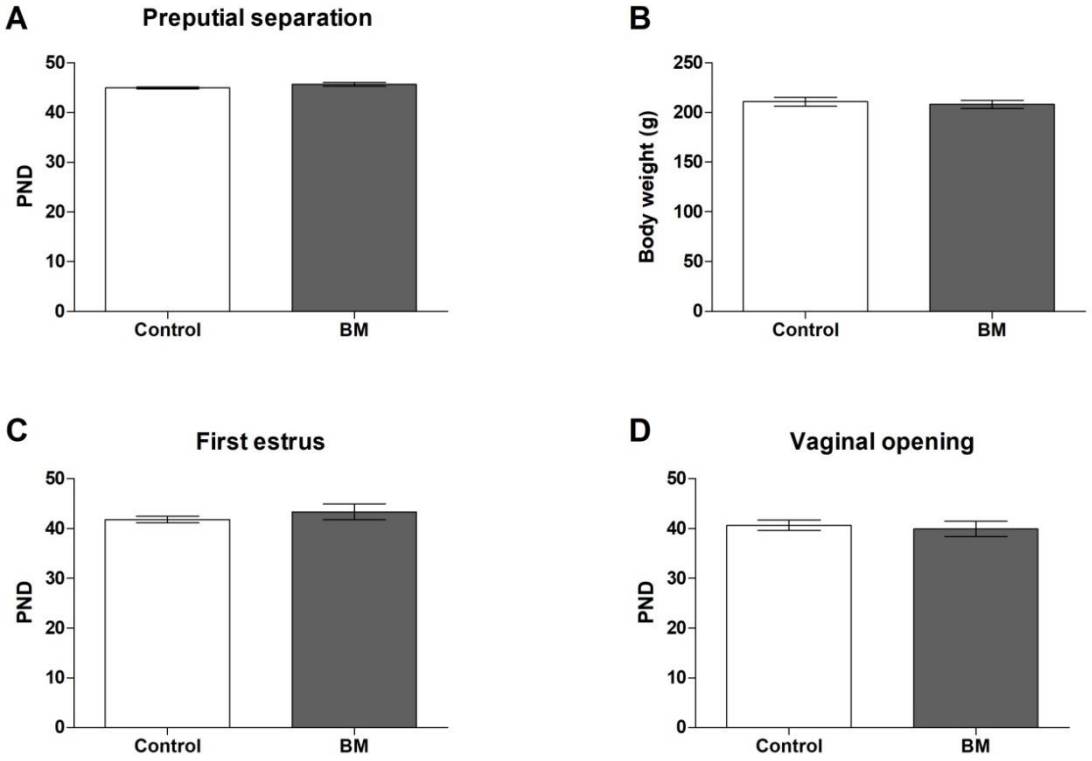

Figure 3. Puberty onset of male and female rats. A and B: Age of preputial separation and body weight of male rats. C and D: Age of vaginal opening and first estrus of female rats. Values expressed as mean ± SEM. $p>0.05$. Student's t test.

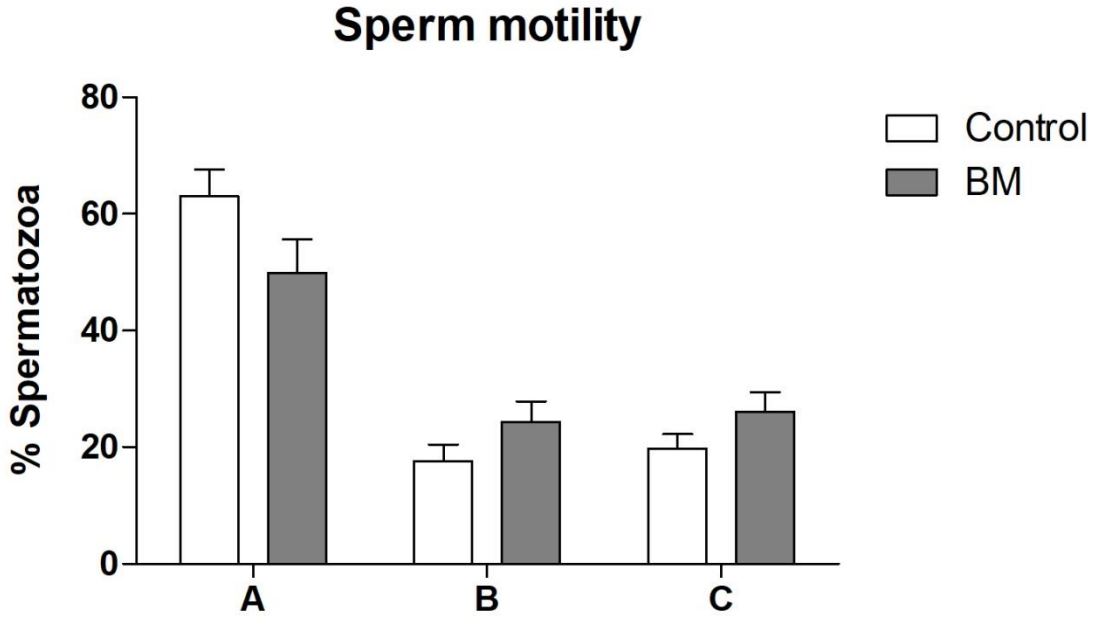

Figure 4. Sperm motility of male rats at PND 112. A: motile with fast and progressive movement, B: motile with non-progressive movement and C: immotile. Values expressed as median and interquartile range, Mann-Whitney's test. $p>0.05$.

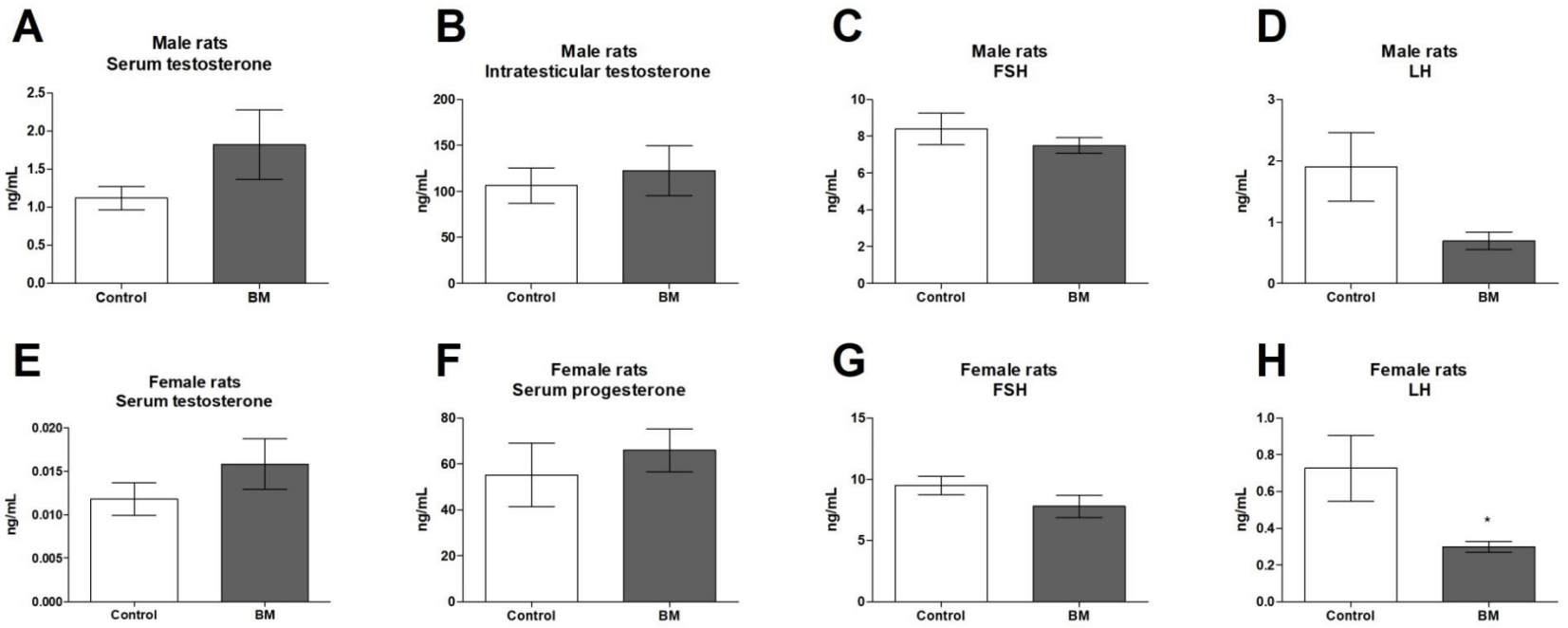

Figure 5. Hormone levels of male and female rats. A to D: Serum and intratesticular testosterone, FSH and LH levels of male rats. E to H: Serum testosterone and progesterone, FSH and LH of female rats. Values expressed as mean ± SEM. *$p<0.05$. Student's t test.

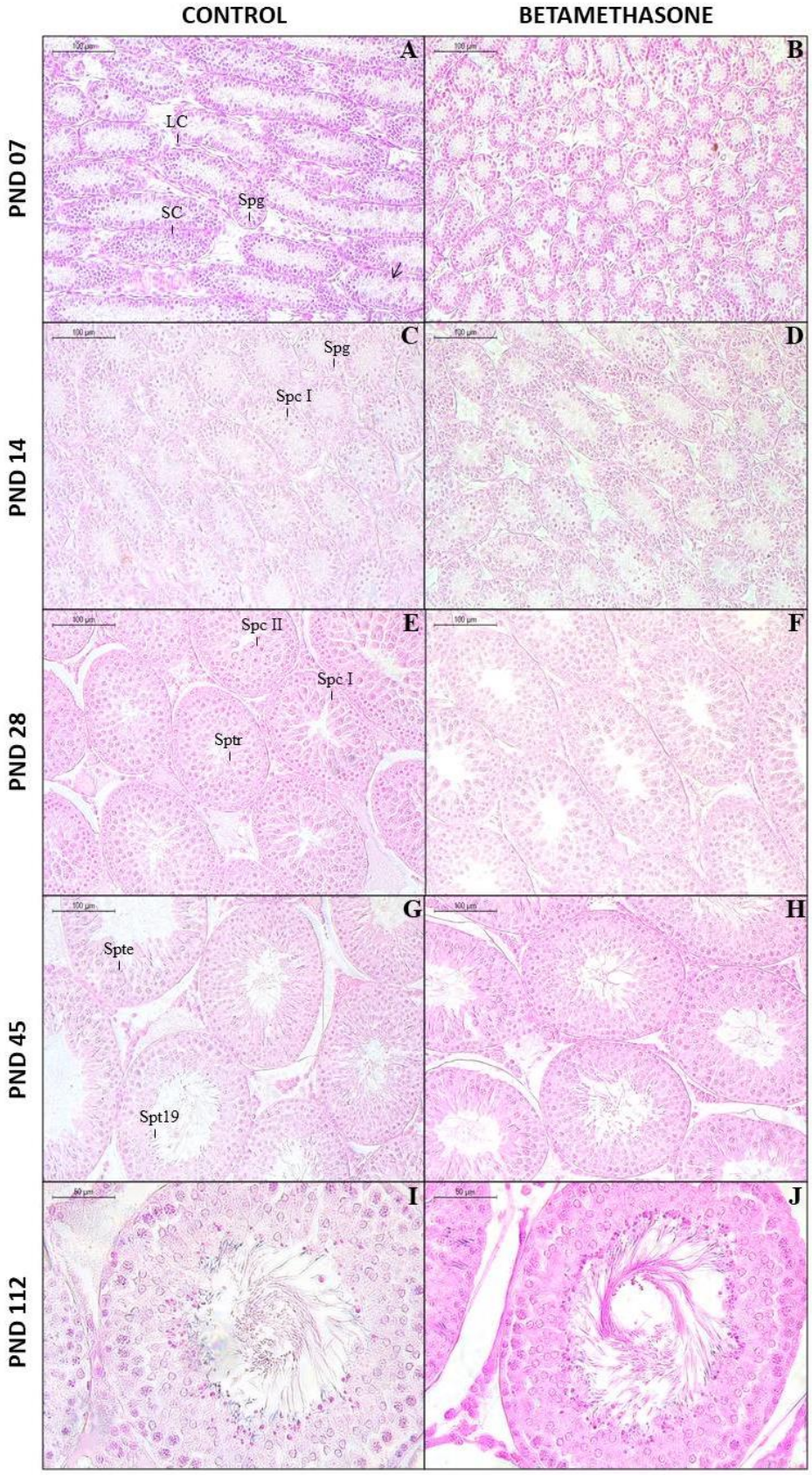

Figure 6. Seminiferous tubules of male rats exposed to BM in the neonatal period. A-B: PND 7, arrow: gonocyte; C-D: PND 14; E-F: PND 28; G-H: PND 45; I-J: PND 112. A-H: magnification of 200x; I-J: magnification of 400x. Spg: Spermatogonia, SpcI: Spermatocyte I; SpcII: Spermatocyte II; Sptr: Round Spermatids; Spte: Elongated Spermatids; Spt19: Spermatids 19, LC: Leydig cell, SC: Sertoli cell. Hematoxilin-eosin (HE).

Table 1. AGD of male and female rats.

AGD ($mm/g^{1/3}$)	Female (n=8/9)		Male (n=10)	
	Control	BM	Control	BM
PND 01	1.40 ± 0.11	1.36 ± 0.03	2.46 ± 0.07	2.56 ± 0.04
PND 04	1.41 ± 0.07	1.52 ± 0.08	2.57 ± 0.09	2.70 ± 0.08
PND 21	3.36 ± 0.12	3.16 ± 0.16	4.66 ± 0.08	4.74 ± 0.21

Values expressed as mean ± standard error of mean (SEM), Student's t test. $p>0.05$.

Table 2. Estrous cyclicity assessment of female rats from PND 60 to 75.

	Experimental groups (n=5/6)	
	Control	BM
[1]Estrous cycle length (days)	**5.36 ± 0.11**	**8.22 ± 0.80***
[1]Number of estrous cycles	**2.13 ± 0.08**	**1.44 ± 0.16****
[2]Estrus (%)	22.22 (21.11 – 25.56)	20 (19.44 – 28.33)
[2]Proestrus (%)	13.33 (13.33 – 20)	12.22 (8.88 – 21.11)
[2]Metestrus (%)	37.78 (34.44 – 37.78)	25.56 (15.00 – 33.70)
[2]Diestrus (%)	24.44 (20 – 34.44)	31.11 (27.41 – 37.78)

Values expressed as mean ± standard error of mean (SEM), Student's t test[1], or median and interquartile range, Mann-Whitney's test[2]. *$p<0.05$, **$p<0.01$.

Table 3. Body and organ weights of male rats at PND 7, 14, 28, 45 and 112.

	PND 28 (n=7)		**PND 45 (n=6)**	
	Control	**BM**	**Control**	**BM**
Body weight (g)	89.17 ± 1.71	84.57 ± 3.67	210.90 ± 5.39	209.60 ± 4.60
Heart (g)	**0.39 ± 0.01**	**0.35 ± 0.01 ***	-	-
Heart (mg/100g)	-	-	-	-
Lungs (g)	-	-	**1 ± 0.01**	**1.17 ± 0.05***
Lungs (g/100g)	-	-	**0.48 ± 0.01**	**0.56 ± 0.02***
Kidney (g)	-	-	**0.93 ± 0.02**	**0.98 ± 0.01***

Values expressed as mean ± standard error of mean (SEM), Student's t test[1]. *p<0.05.

Table 4. Sperm count, daily sperm production (DSP) and sperm transit time of male rats at PND 112.

	Experimental groups (n=8)	
	Control	**BM**
Testis		
Mature spermatid number ($x10^6$)	250.90 ± 4.43	240.30 ± 8.59
Mature spermatid number ($x10^6$/g)	164.90 ± 4.76	161.20 ± 5.46
Daily sperm production ($x10^6$/day)	41.13 ± 0.73	39.40 ± 1.41
Daily sperm production ($x10^6$/g/day)	27.03 ± 0.78	26.43 ± 0.90
Epididymis		
Sperm number in the caput/corpus ($x10^6$)	126.90 ± 10.58	105.00 ± 5.90
Sperm number in the caput/corpus ($x10^6$/g)	**371.10 ± 19.27**	**310.50 ± 13.47 ***
Sperm number in the cauda ($x10^6$)	**222 ± 14.35**	**271.70 ± 13.97 ***
Sperm number in the cauda ($x10^6$/g)	894.40 ± 50.63	988.90 ± 19.62
Sperm transit time in the caput/corpus (days)	3.02 ± 0.27	2.68 ± 0.13
Sperm transit time in the cauda (days)	**5.28 ± 0.42**	**6.97 ± 0.45 ***
Total sperm transit time (days)	**8.06 ± 0.46**	**9.64 ± 0.46 ***

Values expressed as mean ± standard error of mean (SEM), Student's t test. *$p<0.05$.

Table 5. Sperm morphology of male rats at PND 112.

(%)	Experimental groups (n=8)	
	Control	BM
Normal shaped sperm	99.25(98.50 - 99.88)	98.50(97 - 99)
Sperm head abnormalities	0(0 - 0.38)	0.50(0 - 0.88)
Sperm tail abnormalities	0.75(0.50 - 1.88)	1.50(1.50 - 3)
Sperm with cytoplasmic droplet	42.25(28.63 - 62.75)	49.25(32.63 - 69.88)

Values expressed as median and interquartile intervals, Mann-Whitney's test. $p>0.05$.

Table 6. Male rats exposed to BM in the neonatal period: Testicular histopathology and maturation degree of cells at PND 7, 14, 28, 45 and 112.

(%)	PND 07 (n=6)		PND 14 (n=6)		PND 28 (n=6)		PND 45 (n=6)		PND 112 (n=6)	
	Control	BM	Control	BM	Control	BM	Control	BM	Control	BM
Normal seminiferous tubules	82 (74- 85)	81 (73- 89)	76.50 (73-80)	68 (64-72)	82 (79 - 86)	79 (79- 86)	92 (91– 95)	98 (96– 99)	96 (93– 96)	86 (85– 94)
Acidophilic cells	18 (15- 26)	19 (11- 27)	23.50 (20- 27)	32 (28- 36)	16 (11- 20)	19 (10- 21)	3.00 (2– 5)	1 (1– 3)	2 (2– 7)	9 (6– 12)
Vacuole formation	-	-	-	-	2 (1- 3)	2 (0 - 4)	3 (1– 8)	1 (0 – 1)	2 (0 – 2)	1 (0 – 5)
Maturation degree of cells	-	-	-	-	1.39 (1.37 – 1.44)	1.42 (1.42 – 1.43)	4.37 (4.22 – 4.39)	4.22 (4.17 – 4.25)	-	-
Seminiferous tubules with lumen	-	-	-	-	92 (58- 98)	86 (85- 97)	-	-	-	-

Values expressed as median and interquartile intervals, Mann-Whitney's test. $p>0.05$.

5.2. Capítulo II: “Intrauterine betamethasone exposure impairs reproductive, physical and neurobehavioral development of F1 and F2 male and female rats”.

INTRAUTERINE BETAMETHASONE EXPOSURE IMPAIRS REPRODUCTIVE, PHYSICAL AND NEUROBEHAVIORAL DEVELOPMENT OF F1 AND F2 MALE AND FEMALE RATS

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ABSTRACT

Endogenous glucocorticoids act as a maturational trigger towards term that optimizes survival after birth, and betamethasone (BM) is prescribed to women at risk of preterm delivery to enhance fetal lung maturation and survival. Although antenatal corticosteroid therapy has beneficial effects, early overexposure to glucocorticoids impairs brain, neuroendocrine and reproductive functions and also leads to intrauterine growth restriction, indicating fetal programming and may have transgenerational effects. The study aimed to evaluate the impacts of prenatal BM exposure on physical, neurobehavioral, and reproductive initial developments of F1 male and female rats offspring, F2 (offspring of male rats) and F2' (offspring of female rats). Prenatal exposure to BM at gestational days 12, 13, 18 and 19 reduced body weight gain of F0 rats and at birth of F1 rats, decreased body weight at weaning of F2 and increased anogenital distance of F2' female. Also, BM delayed puberty onset and deregulated the estrous cycle of F1 female rats. There were a decrease in sperm quality and testicular weight at sexual maturity of male rats. We also observed delayed physical and neurobehavioral development of F1, F2 and F2' male and female rats. Thus, prenatal exposure to BM impaired physical and neurobehavioral developments of F1, F2 and F2', caused developmental programming and reproductive disorders in F1, such as decreased sperm quality and delayed puberty onset and deregulated estrous cycle in the female offspring. Hence, the detrimental effects of BM exposure during the critical window of rat genital system development raise question about the safety of antenatal corticosteroid therapy and stimulate further investigations to overcome possible adverse effects.

1. BACKGROUND

It is well known that glucocorticoids play important roles during intrauterine development, once they regulate maternal immune response to allow embryo implantation and act as a maturational trigger towards term that optimizes survival after birth (MOISIADIS; MATTHEWS, 2014a; WHIRLEDGE; CIDLOWSKI, 2017).

Currently, synthetic glucocorticoids, as betamethasone (BM) and dexamethasone, are widely prescribed to women who are at risk of preterm delivery to enhance fetal lung maturation and survival [4]. Even though antenatal corticosteroid therapy has beneficial effects, early overexposure to glucocorticoids might programs and deregulates the hypothalamic-pituitary-adrenal axis (HPA), impairs physical and neurobehavioral development and reproductive function, as well as leads to intrauterine growth restriction [1,5]. So, it is believed that glucocorticoids are related to diseases later life [6].

Besides that, prenatal glucocorticoids exposure is related to higher and lower fat mass index in girls and boys, respectively, while in rats is associated with lower birth weight, demonstrating developmental programming (PEREIRA et al., 2003; SECKL, 2004; VAN DIJK et al., 2012; DE BARROS et al., 2018). Also, early exposure to glucocorticoids leads to impaired development of the brain and decreased head circumference in newborns, hence leading to psychiatric diseases, increased sensitivity of the HPA axis to stress and impairments in cognitive function, once it causes degeneration of hippocampal neurons, dendritic atrophy and loss of synaptic connections [6,12].

Likewise glucocorticoids play an important role in reproductive function, but at excessive levels impair hypothalamic-pituitary-gonadal (HPG) axis via inhibition of the gonadotropin-releasing hormone (GnRH), gonadotropin and steroid hormones secretion or inhibition of gametogenesis, leading to reproductive disorders and infertility [4,13]. Previous studies of our research group have shown that male and female rats exposed to synthetic

glucocorticoids during the critical window of genital system development presented delayed puberty onset [11,14], impaired testicular [15] and epididymal morphophysiology, decreased sperm quality [16,17], deregulation of the estrous cycle and uterine morphophysiological impairment, and decreased gonadotropin and testosterone levels [10,18], as well as fertility disorders and impaired sexual behavior [19]. Still, glucocorticoids exposure increases proapoptotic protein expression in the testis which impairs the development of this organ and increases the index of Leydig and germ cells apoptosis [20,21].

In addition to impairments in cognitive and reproductive functions, glucocorticoids also promote developmental programming and transgenerational effects via epigenetic modifications, such as DNA methylation and histone acetylation, leading to alterations of gene expression, as observed in the female offspring of rats exposed during intrauterine development to synthetic glucocorticoids [22–24]. Also, the F3 generation of female rats exposed *in utero* to dexamethasone presented impairment in ovarian mitochondria and decreased estrogen levels and of factors related to steroidogenic process, besides early puberty onset and impairment of the estrous cycle [25]. Thus, the effects of glucocorticoids exposure evidence HPG axis deregulation and fetal programming [10,16,18].

Therefore, once prenatal exposure to BM impairs the reproductive function and fertility of F1 male and female rats and that glucocorticoids may negatively impact nervous system development, this study aimed to evaluate the impacts of prenatal BM exposure on the physical, cognitive and reproductive initial developments of F1 male and female offspring and the effects on paternal and maternal lineages on F2 male and female rats.

2. MATERIAL AND METHODS

2.1. Experimental design and animals

Adult naive male (90 days old, 300g) and female (70 days old, 200g) *Wistar* rats were mated during the dark period of the photoperiod to obtain the F1 male and female offspring. The gestational day (GD) 1 was determined by the presence of sperm in vaginal smears and the pregnant female rats (F0) were kept in individual cages and randomly allocated into control (NaCl 0.9% solution; n =10) and betamethasone (0.1 mg/Kg of betamethasone [BM] 21-phosphate disodium [Sigma-Aldrich, St. Louis, MO]; n = 10) groups. Both groups received an intramuscular injection at GD 12, 13, 18, and 19, which correspond to the critical window of genital system development [14,17]. The BM dose used, that was modified to rodents and was already used in previous studies of our research group, is based on the use for antenatal corticosteroid therapy in humans [10,16,26].

At postnatal day (PND) 1, the litters were reduced to 8 animals per dam (4 male and 4 female). At PND 21, the animals were weaned and male and female were allocated in different cages. At sexual maturity, F1 male were mated with untreated and nulliparous female to obtain the male and female F2 offspring. In the same manner, F1 female were mated with untreated and naive male to obtain F2' offspring (Figure 1). During the experimental procedures, the animals were monitored for general toxicity and stress signs due to the treatment, such as mortality and morbidity, body weight gain/loss, weakness, lethargy, presence of bristly hair, and abnormal behavior. Food and water were provided *ad libitum*. The rats were euthanized by decapitation following CO_2 asphyxiation.

The animals were obtained from the Multidisciplinary Center for Biological Investigation - CEMIB, State University of Campinas (UNICAMP) – SP, and kept in the Small Mammal Biotherium at the Department of Structural and Functional Biology, sector of Morphology, Institute of Biosciences, State University of São Paulo (Unesp), Botucatu, SP, Brazil, under light and temperature-controlled conditions (25º, 12/12h light/dark cycle).

The study was approved by the local ethics committee for the Use of Experimental Animal of the State University of São Paulo (protocol number 923 CEUA – IBB, Unesp) and according to the Guide for the Care and Use of laboratory Animals (National Institutes of Health – NIH).

2.2. Anogenital distance

Anogenital distance was measured at PND 01 and 21 and it was normalized by the ratio between anogenital distance and the cubic root of body weight, once it is influenced by exogenous factors besides the treatment [27].

2.3. Physical and neurobehavioral developmental landmarks

The 8 animals of the litter were assessed daily from birth until PND 21 and, before starting the evaluation, each animal was marked using a permanent pen to enable identification. Then, the animals were placed on a heat plate (37ºC) to keep the body temperature and the dams were placed in a cage with food and water *ad libitum*.

2.3.1. Physical development

The following developmental physical landmarks were evaluated according to Heyser et al. [28] and Coelho et al. [29]: pinnae detachment (usually occurs at PND 2), fur development (it was observed the appearance of a grayish hair), incisor eruption (usually occurs between PND 8 and 10 and its characterized by the incision of the upper and lower incisor through the gums), eye opening (it was observed the detachment of the upper and lower eyelid and the complete opening of both eyes), testes descent (it was determined by scrotum palpation and observed the presence of both testes) and nipple count.

2.3.2. Neurobehavioral development

The following neurobehavioral developmental landmarks were evaluated according to Heyser et al. [28] and Sandini et al. [30]: surface righting reflex (the puppies were placed on its back and had 30 s to turn to ventral position), negative geotaxis (the puppies were placed on a 40º inclined place with its head facing downwards and had 60 s to turn at least 90º

upwards), cliff avoidance (the puppies were placed on the edge of a platform with its nose and forepaws over the edge and had 60s to turn sideways), grasp reflex (a clip was used to stroke the forepaw of the puppies and the disappearance of this reflex was recorded).

2.4. Puberty onset

The separation of the foreskin of the glans is the physical sign of puberty onset in male rats and it was evaluated by the manual retraction of the prepuce [31]. In female rats vaginal opening and first estrus are the physical signs of puberty onset. To determine the day of first estrus, after vaginal opening, vaginal fluids were collected daily to detect the presence of cornified epithelial cells [32]. For this, 10 μL of NaCl 0.9% solution were inserted in the vagina and subsequently aspirated. Vaginal fluids were placed on a slide and analyzed under a light microscope (x200 magnification). Puberty onset was evaluated since PND 30 and once it occurred the animals were weighed.

2.5.Estrous cyclicity

From PND 75 to 90, every morning vaginal fluids were collected, placed on a slide and the samples were evaluated under a light microscope and the estrous cycle phase was determined according to the predominance of cells as described previously [32,33]. Then we estimated the frequency of each phase of the cycle, the estrous cycle length, and the total number of cycles during the evaluation period.

2.6. Euthanasia, body and organ weights

At PND 120, F1 and F2 male, and at the first estrus from PND 90, F1 and F2' female, were euthanized and thereafter the blood was collected from the lower vena cava. Body weight was assessed before the procedure.

To assess systemic and reproductive toxicity, the brain, pituitary gland, thyroid, heart, lungs, liver, kidney, adrenal glands, prostate, and full and empty seminal vesicle were collected, weighed, and then discarded. For histopathological evaluations, the left testis and epididymis, uterus with fluid, and ovaries were collected, carefully weighed, and then fixed in

Bouin's solution, processed, sectioned in 5 μm, mounted in a glass slide, stained with hematoxylin and eosin (HE) and evaluated under a light microscope [34] .

2.7. Ovarian and uterine histological evaluations

Three cross-sections of the ovaries and uterus per animal were randomly selected. Ovarian follicles were counted and classified according to Borgeest et al. [35], Talsness et al. [36] and Guerra et al. [37]. In the uterus, it were assessed the heights of perimetrium, myometrium, endometrium and luminal epithelium as previously described [38].

2.8. Testicular histopathological evaluations

An amount of 100 seminiferous tubules were classified as described: normal (presence of concentric and normally organized germ cell layers) or abnormal (presence of germ cells and cellular debris in the lumen, multinucleated formation, presence of acidophilic cells, few germ cells layers, vacuole formation or degeneration). Interstitial and peritubular myoid cells were qualitative evaluated, as well as the general morphology of Leydig cells and the blood vessels aspect [39].

2.9.Sperm quality at sexual maturity

Sperm was collected from the right cauda epididymis and diluted in 1 mL of human tubal fluid (HTF) modified medium (Spectrun 901126) and supplemented with 1% bovine serum albumin (BSA) for evaluation of sperm motility, morphology, mitochondrial activity, and vitality at PND 120.

2.9.1. Sperm motility

For sperm motility assessment, a 10 μL aliquot was transferred to a Mackler's chamber and 100 spermatozoa were analyzed under a phase-contrast microscope (200x magnification) and classified as: type A (motile with fast and progressive movement), type B (motile with non-progressive movement) and type C (immotile). Sperm motility was expressed as the percentage of total spermatozoa [40,39].

2.9.2. Sperm morphology

For sperm morphology evaluation an aliquot of 100 μL was added to 900 μL of buffered formaldehyde. On the day of the analysis sperm smears of each animal were prepared and 200 spermatozoa were observed under a phase-contrast microscope (400x magnification) and classified as: normal, sperm head abnormalities (without characteristic curvature, pin-head or isolated form) and sperm tail abnormalities (broken, isolated or rolled into a spiral) [41]. It was evaluated the presence of cytoplasmic droplets.

2.9.3. Sperm vitality

Samples for sperm vitality analysis were processed as described by Moskovtsev and Librach (44) and 100 spermatozoa were evaluated under a phase contrast-microscope (400x magnification) and classified as alive (not stained, indicating that the membrane is intact) and dead (stained in orange-red, indicating that the membrane is not intact and the stain passed through it). The data were expressed as a percentage of live and dead cells.

2.9.4. Mitochondrial activity

Samples for sperm mitochondrial activity analysis were processed as described by Hrudka [43] and Silva et al. [44] and 100 cells per animal were evaluated under a light microscope (200x magnification) and scored as type I (100% of the mid-piece stained, indicating complete mitochondrial activity), type II (50%-99% of the mid-piece stained, indicating some loss of mitochondrial activity) and type III (<50% of the mid-piece stained, indicating extensive mitochondrial activity loss) [45].

2.9.5. Sperm counts in the testis and epididymis, daily sperm production, and sperm transit time

The right testis and the epididymis caput/corpus and cauda were processed and analyzed as described by Borges et al. [17] to determine the number of spermatid 19 and sperm in the testis and epididymis, respectively, and to estimate the daily sperm production (DSP) according to Robb et al. [46] and Ashby et al. [47]. The sperm transit time throughout

239 the epididymis was determined by dividing the number of sperm in each part of the organ by
240 the DSP.

241 **2.10. Statistical analysis**

242 Data were checked for normal distribution using the Kolmogorov–Smirnov test. Data
243 are presented as mean ± standard error of mean (S.E.M.) or median and interquartile range.
244 Student's t-test was used to compare parametric variables. Nonparametric data were
245 compared using Mann-Whitney's test. Differences were considered significant when $p \leq 0.05$.
246 Statistical analyses were performed using the GraphPad Prism ® (version 5) (GraphPad Inc.,
247 San Diego, CA, USA).

248

3. RESULTS

Prenatal BM exposure led to a significant decrease of body weight gain of the dams (Figure 2), as well as of the body weight of F1 male at PND 1, 7, and 35 (Figure 3) and of F1 female at PND 1, 7 and 50 (Figure 4). The body weights of F2 (Figure 3) and F2' offspring (Figure 4) of male and female rats exposed to BM were significantly lower at PND 21. There were no significant differences between the groups regarding body weight gain of female and male rats at PND 90 and 120, respectively (Figures 3 and 4). Although the AGD of F1 and F2 male (Table 1) and F1 female was not altered (Table 2), the AGD of F2' female (Table 2) was significantly increased in the BM group when compared to the control group.

The developmental milestones were evaluated from PND 1 to 21 and BM exposure led to a significant delay in the age of incisor eruption, eye opening and negative geotaxis in F1 male rats, pinnae detachment and cliff avoidance in F2 male and female, and negative geotaxis in F2 female rats (Table 3). Also, led to a significant delay in the age of fur development, eye opening and incisor eruption in F1 female rats, fur development and pinnae detachment in F2' male and female and eye opening, negative geotaxis and surface righting in F2' female rats (Table 4).

BM exposure did not alter the day of preputial separation of F1 male rats, nevertheless, the body weight at puberty onset of F1 male was lower than the control group (Figure 5), but led to a delay in the vaginal opening of F1 female, but not in the age of first estrus (Figure 6). There were no significant differences in the age of puberty onset of F2 (Figure 5) and F2' (Figure 6) male and female. The estrous cycle was evaluated from PND 75 to 90 and its length was significantly increased while the frequency of proestrus was significantly decreased in F1 female exposed to BM, but not in F2' (Table 5).

The data regarding female rats organ weights are represented on Table 6. At PND 90, the wet weight of the brain of F1 female exposed to BM was increased while the wet weights

of adrenal and kidney were reduced in the same experimental group. At the same age, there was a reduction in the wet weight of the pituitary gland, as well as an increase in the weight of the uterus of F2' female. Other organs, such as thyroid, lungs, heart, liver, and ovaries were evaluated but there were no significant differences between the experimental groups (data not shown).

The data regarding male rats organ weights are represented on Table 7. At PND 120, the wet weights of the brain of F1 and F2 male of BM group were increased, but only F1 male had a significant increase in the weight of the seminal vesicles and a reduction in absolute testicular weight. Other organs, such as the pituitary gland, thyroid, lungs, heart, liver, kidneys, adrenal glands, prostate, and epididymis were evaluated but there were no significant differences among the groups (data not shown).

Data regarding sperm quality (morphology, mitochondrial activity, vitality, motility, and concentration) of F1 and F2 male rats are represented on Table 8 and 9 and Figure 7. At the same age, the sperm motility and mitochondrial activity of F1 male exposed to BM were decreased, but there were no significant differences in sperm morphology and concentration. There were no significant differences in sperm motility, morphology, mitochondrial activity, vitality, or concentration in F2 male rats.

There were no significant differences in histopathological evaluation of F1 and F2 male rats (Figure 8 and Table 10), as well as in histopathological evaluation of ovaries, follicles number and uterine morphometry of F1 and F2' female rats (data not shown).

4. DISCUSSION

Body and organ weights are biomarkers of drugs toxicity and decreased body weight gain and organ weights were associated to glucocorticoids exposure [48,15]. Moreover, decreased body weight gain is related to metabolic impairments, such as increased leptin levels, an anorectic factor synthesized by the adipocytes that sets food intake and energy expenditure [49–51]. Although we did not evaluated food intake, it was previously related to decreased body weight gain in pregnant rats exposed to BM [52,15]. Therefore, we believe that BM lower body weight gain might be associated with decreased food intake due to higher leptin levels.

Glucocorticoids exposure or stressful life events during pregnancy down-regulate glucose transporters in the placenta and insulin-like growth factor (IGF) expression, which impairs fetal development and body growth [53]. Furthermore, glucocorticoids act as a trigger that switches cell fate to differentiation at expense of cell proliferation, leading to fetal development [54]. However, early or overexposure to endogenous or synthetic glucocorticoids promotes delayed fetal development, intrauterine growth restriction and lower birth weight [52,25], as corroborated by us. Nevertheless, we also observed a catch-up on growth during development. This reduction in body weight is associated to fetal programming and have deleterious effects for an animal life-long [8,11].

Glucocorticoids exposure during intrauterine development is also related to HPA axis programming of F1 rats, leading to higher glucocorticoids exposure of F2 during development. It is believed that glucocorticoids transgenerational effects occur via epigenetic changes in the DNA [55]. We observed that male and female F2 and F2’ offspring presented decreased body weight with a catch-up on growth during development, indicating HPA axis programming and transgenerational inheritance.

The reproductive axis comprises the hypothalamus, pituitary and gonads and leads to the synthesis of steroid hormones that are important to brain and gonadal sexual differentiation and determination in the beginning of development [4]. At gestational days (GD) 18 and 19 and soon after birth there is a surge in testosterone levels that is necessary for brain sexual dimorphism, thus drugs or chemical compounds that alter testosterone levels have serious impacts on sexual development [19,56]. At adulthood, steroid hormones promote and support reproductive function and sexual behavior [57,58].

Anogenital distance, which is higher in male than in female rats, is a biomarker of androgen exposure: higher androgen levels lead to higher distance between the anus and genital papilla [59]. Nevertheless, exposure to glucocorticoids during fetal development is associated to higher anogenital distance in female rats, indicating a male phenotype [10]. Although the AGD was not altered in F1 female, it is increased in F2' female rats, so we believe that BM exposure led to developmental and hypothalamic-pituitary-gonadal (HPG) axis programming.

We also observed a delay in the physical development of the BM-exposed rats, as well as a decrease of adrenal and kidney weights of F1 female rats at sexual maturity. As previously discussed, glucocorticoids have deleterious effects on physical development and body growth once it cause metabolic impairments and lead to early cell differentiation. Moreover, early and overexposure to glucocorticoids during intrauterine development decreases the expression of EGF, which is related to organ and physical development, such as eyelid opening and incisor eruption, as well as body growth [60,61].

It was observed a reduction in the weight of the adrenal glands and kidney at sexual maturity of female rats exposed to BM in this study. The adrenal glands play a key role in the developmental origins of diseases in adulthood, regulate the HPA axis and synthesize steroid hormones that are important during pregnancy and fetal development [5]. In rats, exposure to

347 glucocorticoids in the prenatal period led to dysplasia and dysfunctions in the adrenal glands
348 and kidney, besides HPA axis deregulation [62–65].

349 Glucocorticoids also play an important role in normal brain development [66,67].
350 There is high expression of glucocorticoids receptors (GR) in the hippocampus, so this region
351 controls and inhibits de HPA axis. Nevertheless, high levels of endogenous or synthetic
352 glucocorticoids down-regulate the GR in the hippocampus and leads to neuronal degeneration
353 and cognitive, emotional, and behavioral disorders [52,68]. We observed that F1, F2 and F2'
354 male and female offspring presented delayed cognitive development, which can be explained
355 by the impact of BM on cell proliferation and neuronal degeneration. Also, we observed that
356 there was an increase in brain weight of F1 offspring and F2 male offspring, corroborating the
357 delayed cognitive development. This result may be related to a compensation mechanism.
358 Guinea-pigs exposed prenatally to synthetic glucocorticoids presented impairments in the
359 hippocampus for 3 different generations [24].

360 Steroids hormones are important to normal sexual development and reproductive
361 physiology. Glucocorticoids inhibit GnRH, FSH and LH release, thus impair steroidogenesis,
362 gametogenesis and sexual development [5]. Puberty onset requires a series of interrelated
363 events that begin in the infantile period and increase GnRH release, which will lead to a rise
364 in steroid hormone secretion and, thus, testicular and ovarian maturation [69]. Therefore, once
365 glucocorticoids impairs HPG axis, high as well as low levels of this hormone are linked to
366 delayed sexual development and puberty onset [44,11]. Our results showed a delay at the day
367 of puberty onset of female rats exposed to BM, corroborating previously studies [70,10], and
368 indicating HPG axis deregulation and programming and delayed sexual development.

369 Not only glucocorticoids, but also body mass and leptin levels are related to puberty
370 onset. The greater the body fat mass, the greater is leptin synthesis, which stimulates GnRH
371 release, and therefore leads to the onset of puberty[71,72]. Although, we observed that F1

male rats presented lower body weight at the day of puberty onset, there was not a delay of the day of preputial separation. Therefore, we believe that the lack of alterations on the day of puberty onset of F1 male rats is related to an overexpression of leptin, which is also associated to hypothalamic leptin resistance [73,74].

Evaluating the estrous cycle is important to infer about the integrity of the HPG axis and to identify the effects of toxicants on the fertility of rats [75,76]. Corroborating the puberty onset delay, we observed deregulation of the estrous cycle of F1 female rats, as also observed previously [18,10]. Even though we did not evaluated gonadotropin and steroid hormones levels, we believe that the HPG axis was deregulated as previously described, leading to decreased gonadotropin and steroid hormones levels [18,17,10].

BM exposure during fetal development delayed development and caused morphophysiological impairments in the testes of sheep, due to higher expression of proapoptotic proteins [21]. We observed that rats exposed during the critical window of genital system development presented a decrease in testicular weight, which may be related to impaired testicular development and cell proliferation [77]. The decreased testicular weight was previously associated to morphophysiological impairments, although we did not observed any histomorphometric alterations [16]. BM exposure also impaired seminal vesicles development, once the weight of this organ was increased.

Prenatal exposure to BM led to morphophysiological injuries in the epididymis and it was associated to poor sperm quality, such as sperm morphological impairment, decreased motility and concentration [17,16,78]. During the transit through the epididymis the spermatozoa acquire motility and fertilization capacity, thus increased sperm transit time can negatively impact motility and sperm concentration in the epididymis and decrease sperm quality [79,80]. We did not observed any changes in sperm transit time or concentration, but BM exposure led to poor sperm quality, associated with reduced sperm motility and

mitochondrial activity of F1, as previously reported [16,17,81]. In the initial segment and caput/corpus of the epididymis glucocorticoids modulate apoptosis via GR, so high levels of this hormone are associated to poor epididymal microenvironment and to impaired sperm maturation [82].

Previous studies demonstrated that glucocorticoids exposure during intrauterine development promoted histomorphometric alterations in the uterus [10], and impaired folliculogenesis [83], in F1 female rats. Nevertheless we did not observed alterations of uterine or ovary weights, as well as morphophysiological impairments in F1 female rats, but we observed increased uterine weight in F2' female rats, which may be related to HPA and HPG axis programming and deregulation.

During intrauterine development, the fetus is programmed under an adverse environment so that it can adapt and survive after birth [6]. However, fetal programming is associated with physiological changes, metabolic diseases in adulthood and transgenerational inheritance, as well as epigenetic changes that modify gene expression [1,23,84]. Although the molecular mechanisms that could elucidate fetal reprogramming and epigenetic inheritance have not been evaluated yet, it is believed that these factors are partly responsible for the changes observed by us in F1, F2 and F2'.

5. CONCLUSION

Therefore, the data obtained shows that gestational exposure to BM leads to toxic effects in F0, dysfunctions in the cognitive and somatic developments in F1, F2 and F2' as well as developmental programming of reproductive function in F1. Hence, the deleterious effects of BM exposure during the critical window of genital system development on the reproductive function and developmental landmarks. Hence, the detrimental effects of BM exposure during the critical window of rat genital system development raise question about the safety of antenatal corticosteroid therapy and stimulate further investigations to overcome possible adverse effects.

6. CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

7. ACKNOWLEDGMENTS

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9. FIGURES AND TABLES

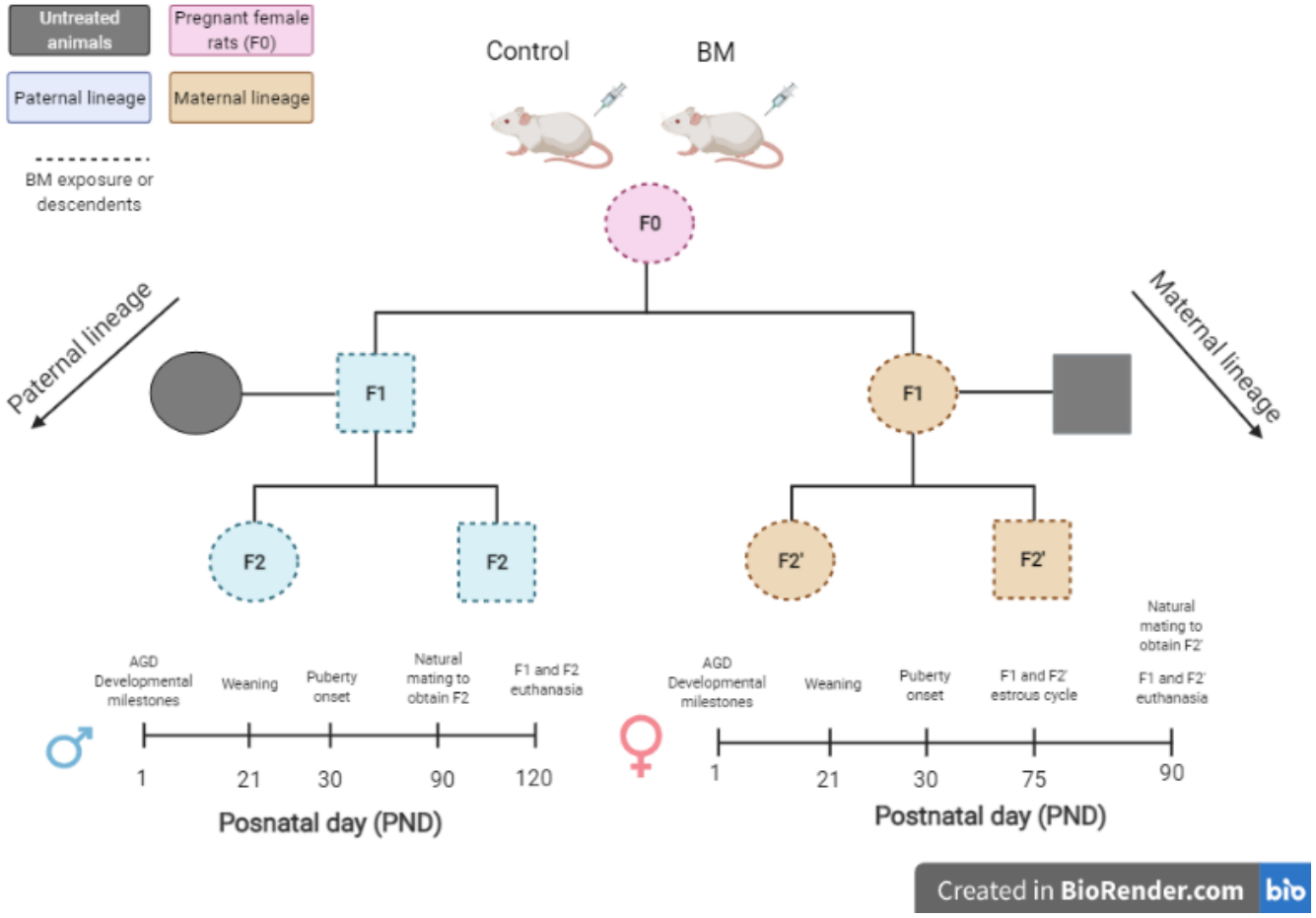

Figure 1. Experimental design.BM: betamethasone 0.1 mg/Kg.

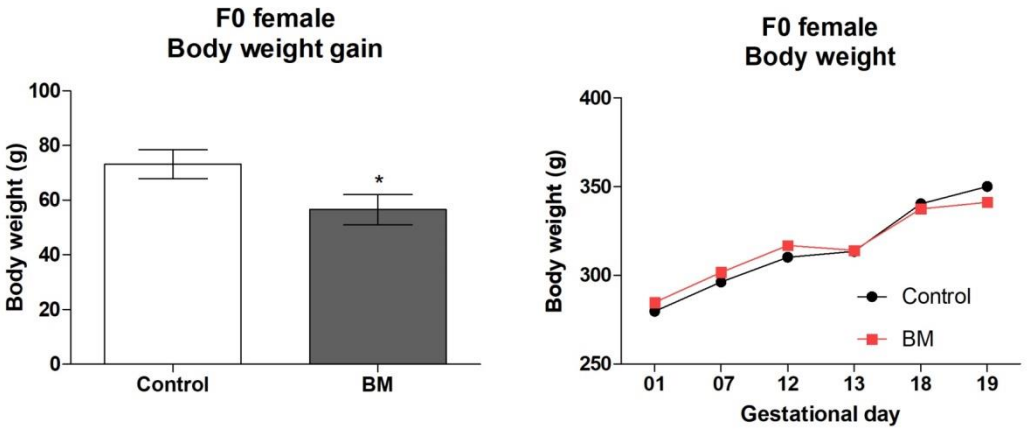

Figure 2. Body weight gain of F0 pregnant female rats. Values expressed as mean ± standard error of mean (SEM), Student's t test. *$p<0.05$.

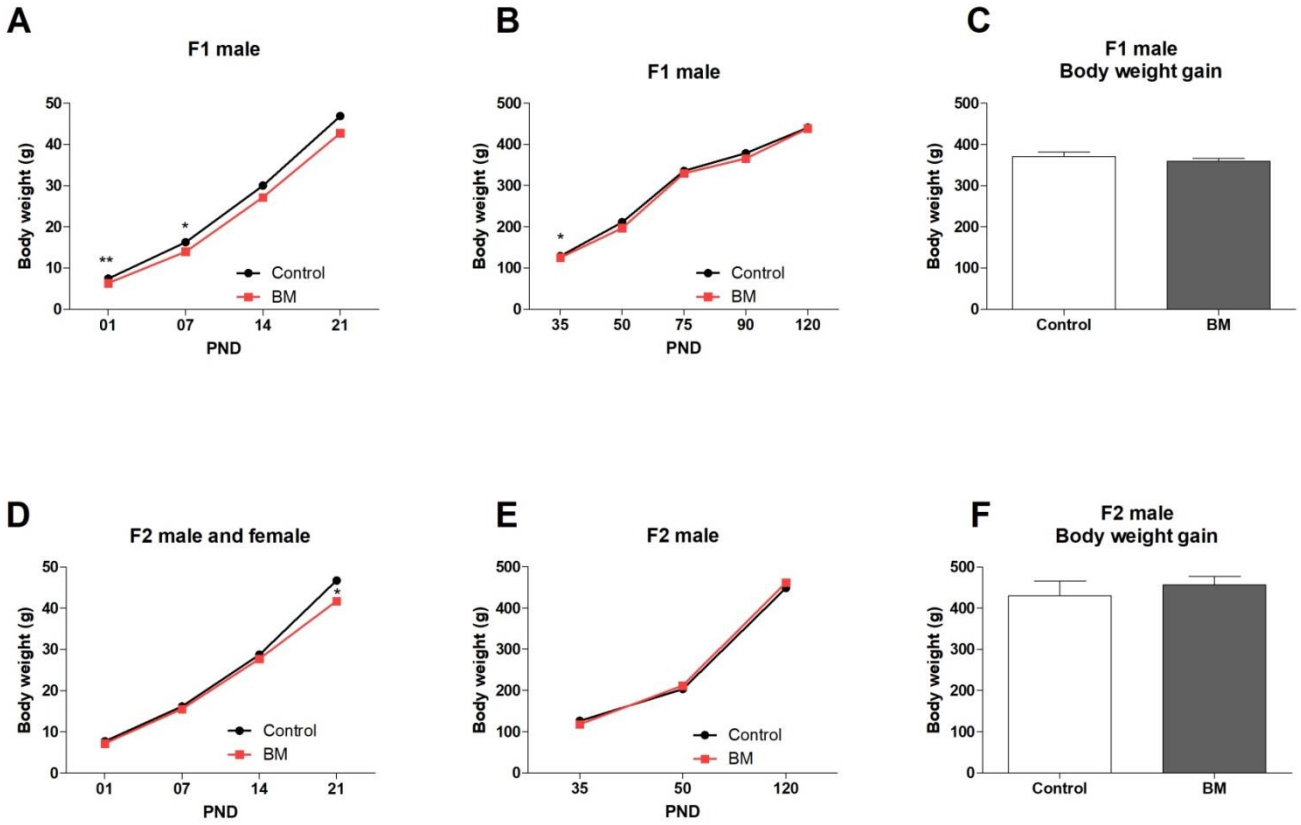

Figure 3. Body weight and body weight gain of F1 male and F2 male and female rats. A to C: Body weight evolution from PND 1 to 21 and from PND 35 to 120 and body weight gain at PND 120 of F1 male rats, respectively. D to F: Body weight evolution from PND 01 to 21 of F2 male and female and from PND 35 to 120 and body weight gain at PND 120 of F2 male rats, respectively. Values expressed as mean ± SEM. *p <0.05, **p <0.01. Student's t test.

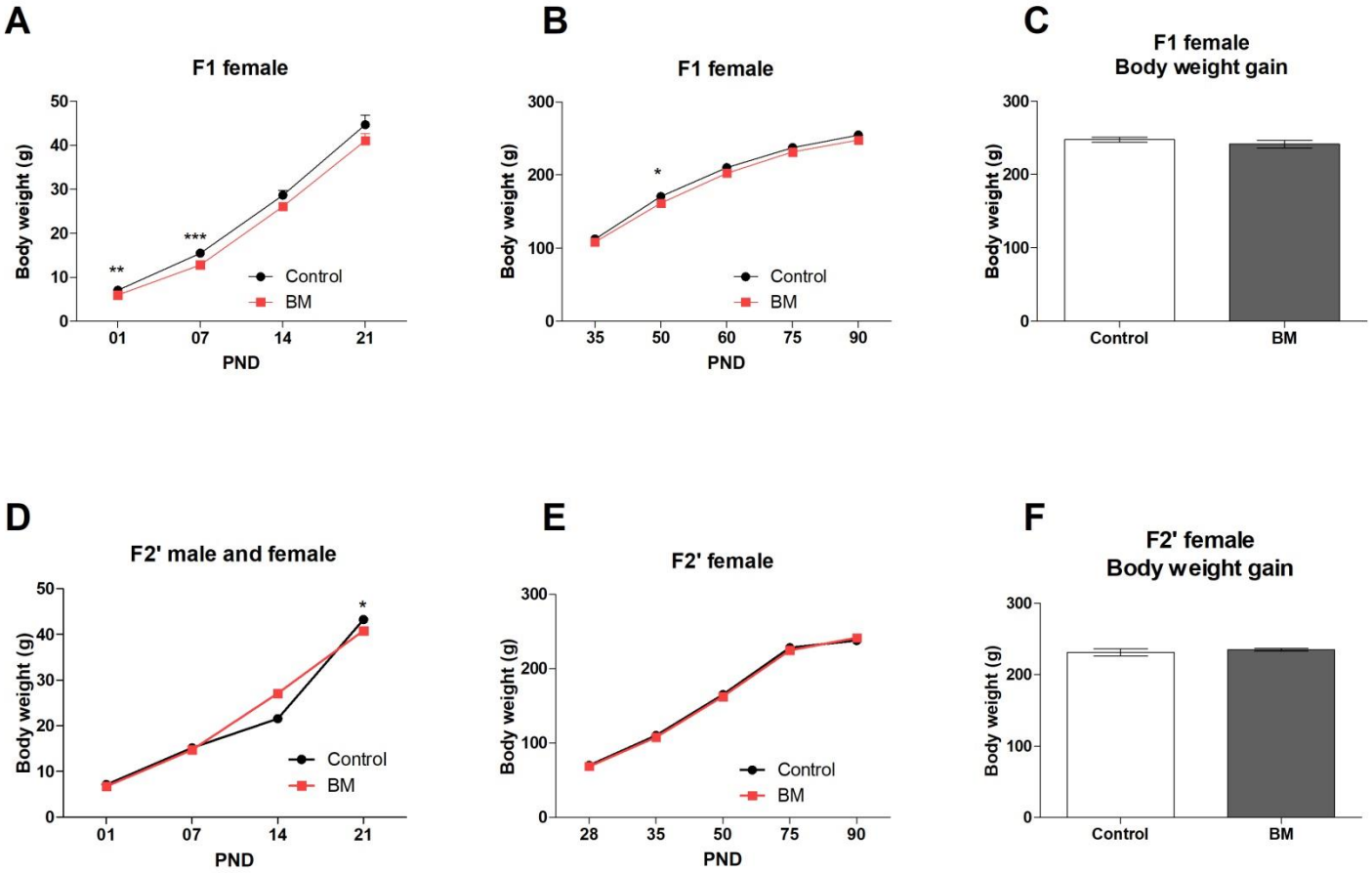

Figure 4. Body weight and body weight gain of F1 female and F2' male and female rats. A to C: Body weight evolution from PND 1 to 21 and from PND 35 to 90 and body weight gain at PND 90 of F1 female rats, respectively. D to F: Body weight evolution from PND 01 to 21 of F2' male and female and from PND 35 to 90 and body weight gain at PND 90 of F2' female rats, respectively. Values expressed as mean ± SEM. *p <0.05, **p <0.01, ***p<0.001. Student's t test.

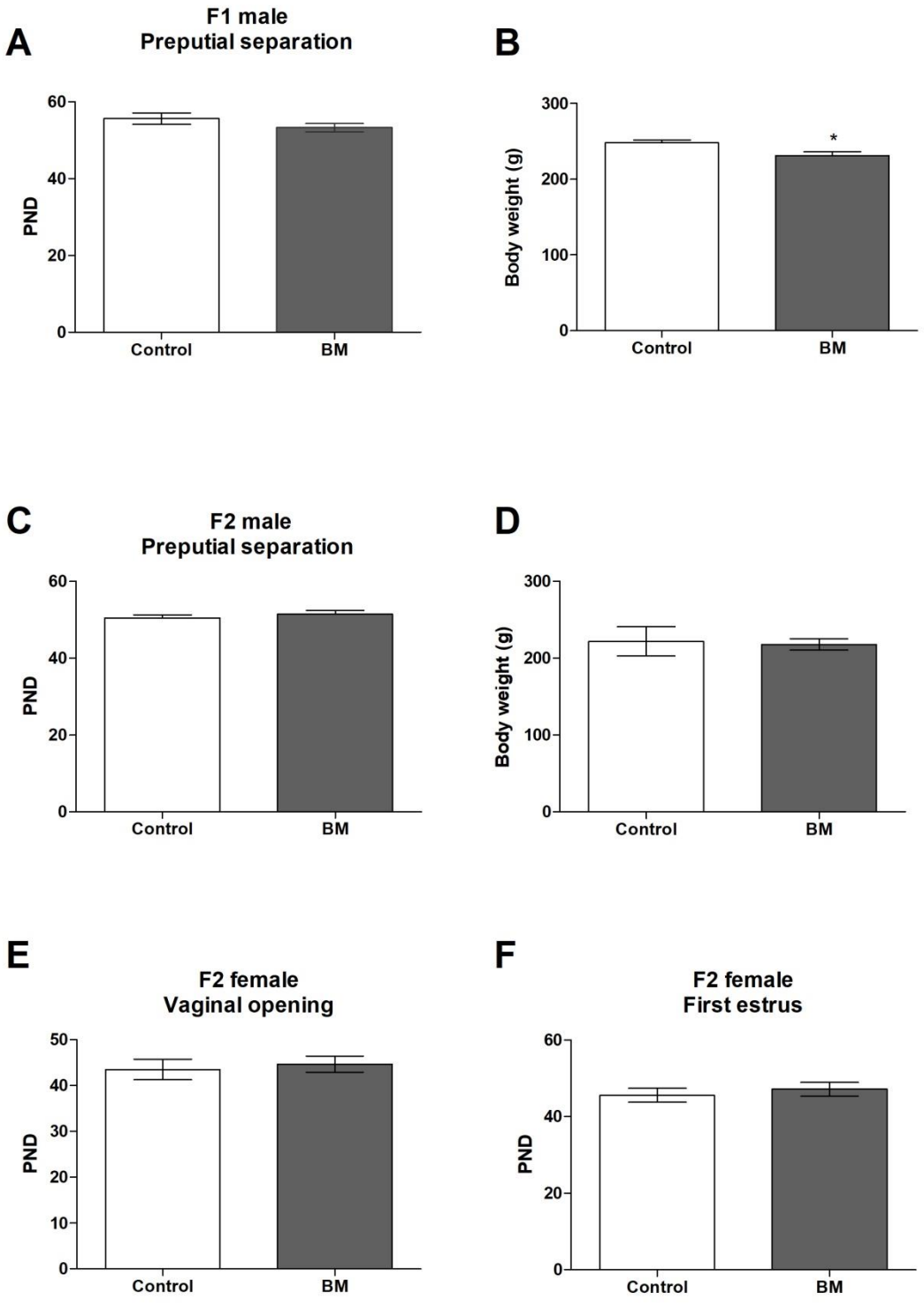

Figure 5. Puberty onset of F1 male and F2 male and female rats. A and B: Age of preputial separation and body weight of F1 male rats. C and D: Age of preputial separation and body weight of F2 male rats. E and F: Vaginal opening and first estrus of F2 female rats. Values expressed as mean ± SEM. *$p<0.05$. Student's t test.

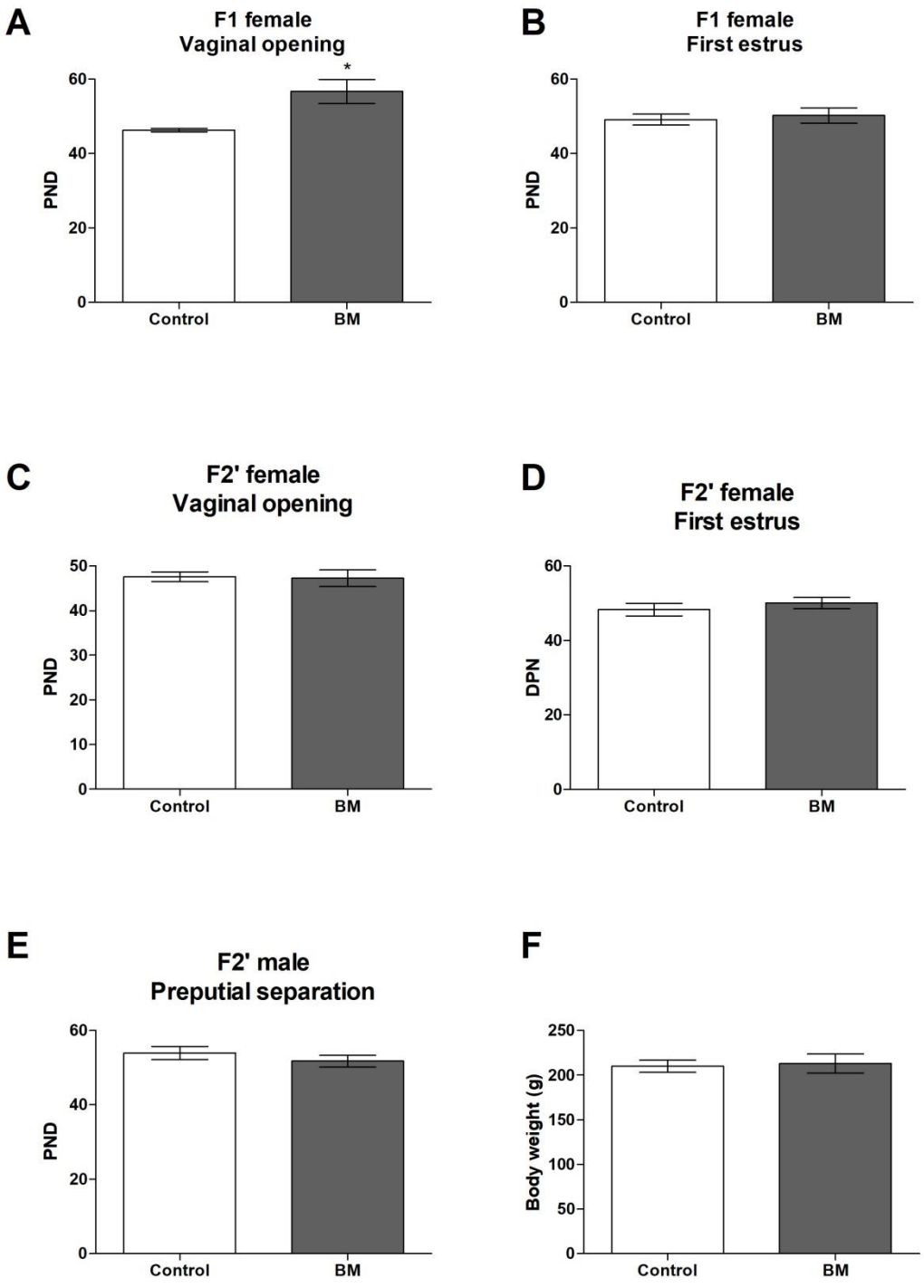

Figure 6. Puberty onset of F1 female and F2' male and female rats. A and B: Vaginal opening and first estrus of F1 female rats. C and D: Vaginal opening and first estrus of F2' female rats. E and F: Age of preputial separation and body weight of F2' male rats. Values expressed as mean ± SEM. *p<0.05. Student's t test.

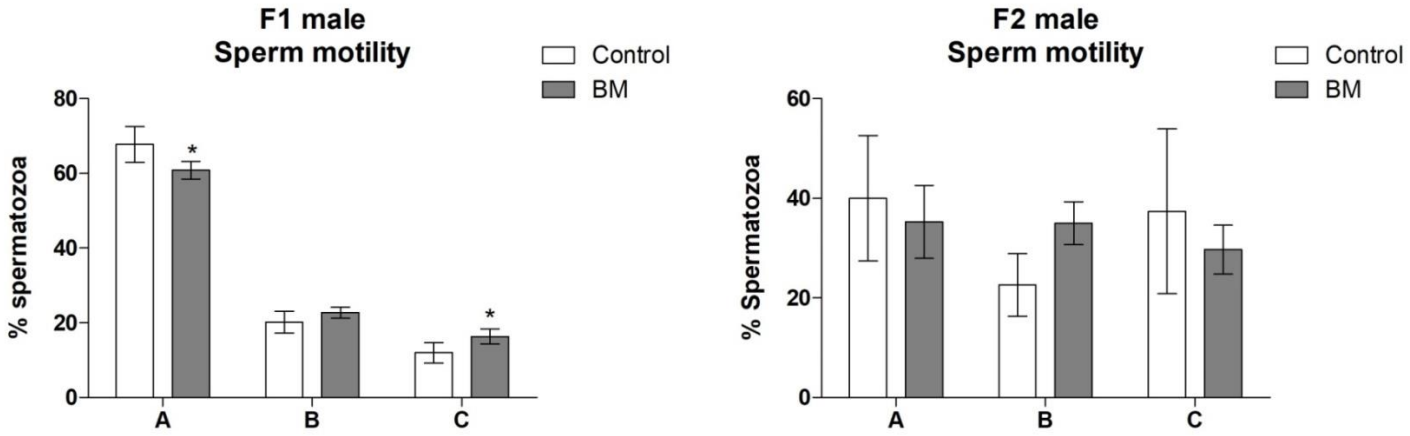

Figure 7. Sperm motility of F1 and F2 male rats at PND 120. A: motile with fast and progressive movement, B: motile with non-progressive movement and C: immotile. Values expressed as median and interquartile range, Mann-Whitney's test. *$p<0.05$.

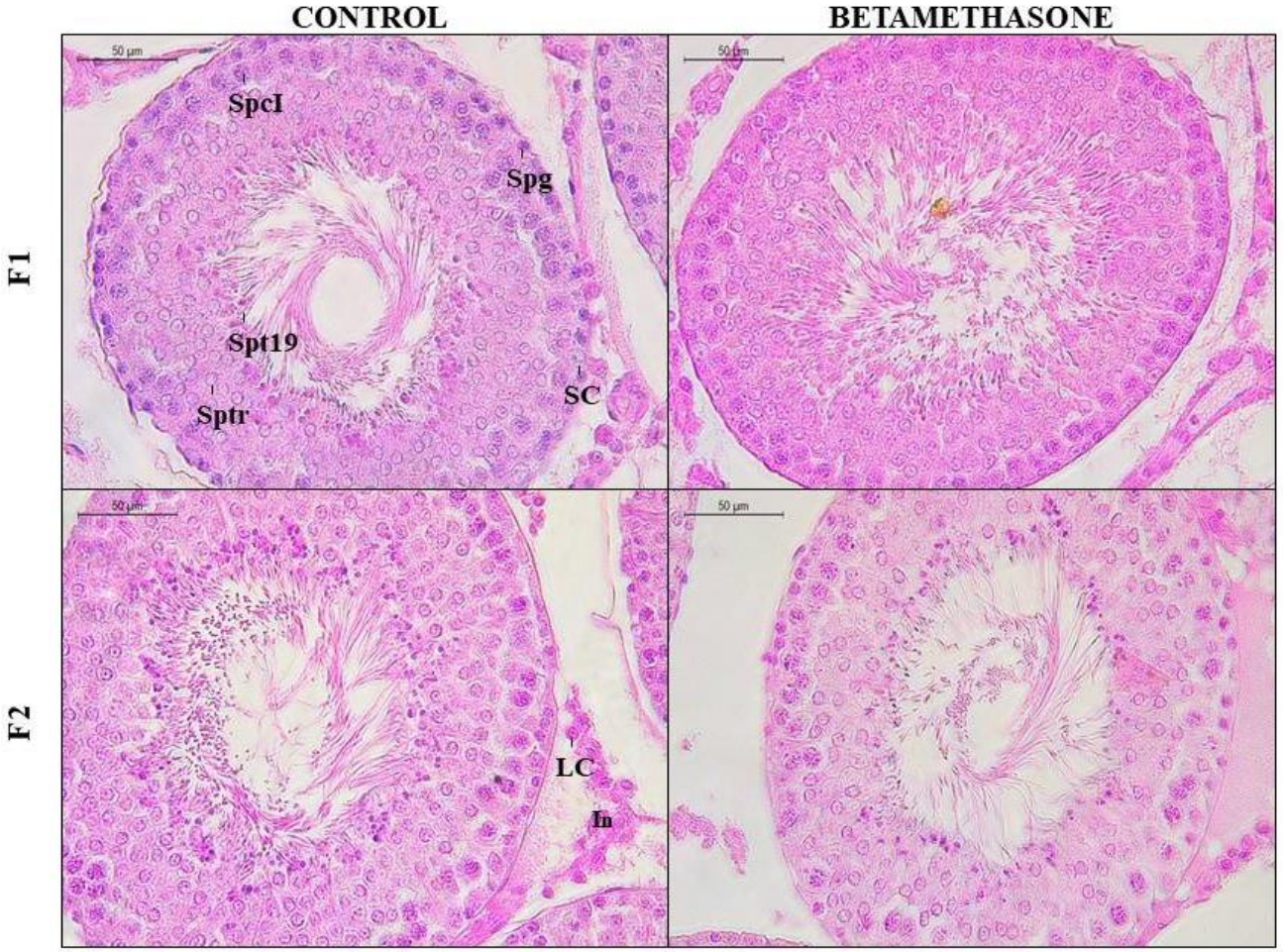

Figure 8. Histological aspect of seminiferous tubules from F1 and F2 male rats at PND 120. A – B: F1 and F2 control group, respectively. C – D: F1 and F2 BM group, respectively. Spg: Spermatogonia, SpcI: Spermatocyte I; Sptr: Round Spermatids; Spt19: Spermatids 19, In: Intersticium; LC: Leydig cell, SC: Sertoli cell.Hematoxilin-eosin (HE). Magnification: 400x.

Table 1. AGD of F1and F2 male rats and F2 female rats.

AGD (mm/g$^{1/3}$)	F1 male (n=9/8)		F2 male (n=5/6)		F2 female (n=5/6)	
	Control	BM	Control	BM	Control	BM
PND 01	2.32 ± 0.03	2.34 ± 0.11	2.33 ± 0.07	2.35 ± 0.05	1.25 ± 0.04	1.18 ± 0.03
PND 21	4.23 ± 0.08	4.10 ± 0.14	4.44 ± 0.08	4.14 ± 0.11	2.92 ± 0.09	2.85 ± 0.09

Values expressed as mean ± standard error of mean (SEM), Student's t test. $p>0.05$.

Table 2. AGD of F1 and F2' female rats and F2' male rats.

AGD (mm/g$^{1/3}$)	F1' female (n=9/7)		F2' female (n=6)		F2'male (n=6)	
	Control	BM	Control	BM	Control	BM
PND 01	1.22 ± 0.08	1.45 ± 0.09	**1.11 ± 0.03**	**1.28 ± 0.04 ****	2.26 ± 0.09	2.33 ± 0.05
PND 21	2.83 ± 0.05	2.95 ± 0.16	2.99 ± 0.11	2.77 ± 0.06	4.33 ± 0.21	4.18 ± 0.09

Values expressed as mean ± standard error of mean (SEM), Student's t test. $^{**}p<0.01$.

Table 3. Developmental landmarks of F1 and F2 male rats and F2 female rats.

	F1 male (n=8/9)		F2 male (n=5/7)		F2 female (n=5/7)	
	Control	**BM**	**Control**	**BM**	**Control**	**BM**
Physical landmarks						
Pinnae detachment	2.53 ± 0.22	2.72 ± 0.23	**2.60 ± 0.25**	**3.57 ± 0.20***	**2.65 ± 0.27**	**3.57 ± 0.20***
Fur development	8.22 ± 0.28	9.13 ± 0.44	9 ± 0.32	8.57 ± 0.37	9 ± 0.31	8.57 ± 0.37
Incisor eruption	**11.11 ± 0.22**	**12.34 ± 0.23***	11.55 ± 0.39	12 ± 0.37	11.55 ± 0.37	11.88 ± 0.38
Eye opening	**15.21 ± 0.23**	**16.28 ± 0.23****	15.30 ± 0.30	16.04 ± 0.28	15.20 ± 0.37	16.04 ± 0.28
Testes descent	16.67 ± 0.60	17.88 ± 0.73	16.50 ± 0.50	18 ± 0.41	12.32 ± 0.15	12.08 ± 0.06
Number of nipples	-	-	-	-	-	-
Neurobehavioral landmarks						
Surface righting	2.77 ± 0.26	2.99 ± 0.54	2.43 ± 0.19	2.57 ± 0.22	2.67 ± 0.28	2.29 ± 0.16
Negative geotaxis	**5.56 ± 0.45**	**7.48 ± 0.73***	4.35 ± 0.66	5.46 ± 0.43	**4.67 ± 0.71**	**6.56 ± 0.25***
Cliff avoidance	5.10 ± 0.66	6 ± 0.84	**5.03 ± 0.27**	**6.43 ± 0.30****	**4.97 ± 0.53**	**6.86 ± 0.37***
Grasp reflex	7.16 ± 0.37	7.73 ± 0.57	9.55 ± 0.60	9.64 ± 0.70	9.55 ± 0.60	9.64 ± 0.70

Values expressed as mean ± standard error of mean (SEM), Student's t test. *p<0.05. **p<0.01.

Table 4. Developmental landmarks of F1 and F2' female rats and F2' male rats.

	F1 female (n=9/7)		F2' female (n=6)		F2' male (n=6)	
	Control	**BM**	**Control**	**BM**	**Control**	**BM**
Physical landmarks						
Pinnae detachment	2.69 ± 0.23	2.95 ± 0.28	**3 ± 0**	**3.50 ± 0.22***	**3 ± 0.00**	**3.50 ± 0.19***
Fur development	**8.22 ± 0.28**	**9.43 ± 0.37***	**8.50 ± 0.22**	**9.17 ± 0.17***	**8.50 ± 0.22**	**9.17 ± 0.17***
Incisor eruption	**10.94±0.23**	**12.40 ± 0.33***	11.72 ± 0.30	12.03 ± 0.25	11.72 ± 0.34	12.08 ± 0.27
Eye opening	**15.39±0.28**	**16.57 ± 0.21***	**14.96 ± 0.34**	**16.25 ± 0.40***	15.21 ± 0.13	16.13 ± 0.42
Number of nipples	12.11±0.11	12.04 ± 0.04	12.10 ± 0.06	12.17 ± 0.08	-	-
Neurobehavioral landmarks						
Surface righting	3.07 ± 0.28	3.87 ± 0.28	**1.89 ± 0.20**	**2.93 ± 0.28 ***	2.06 ± 0.19	2.79 ± 0.36
Negative geotaxis	6.01 ± 0.51	7.65 ± 0.67	**4.04 ± 0.28**	**5.65 ± 0.36 ****	4.39 ± 0.35	4.54 ± 0.42
Cliff avoidance	5.71 ± 0.48	7.43 ± 1.31	5.33 ± 0.26	5.47 ± 0.26	5.25 ± 0.43	5.26 ± 0.41
Grasp reflex	7.72 ± 0.31	6.66 ± 0.59	9.99 ± 0.52	9.19 ± 0.26	9.76 ± 0.50	9.43 ± 0.13

Values expressed as mean ± standard error of mean (SEM), Student's t test. *p<0.05. **p<0.01.

Table 5. Estrous cyclicity assessment of F1 and F2' female rats from PND 75 to 90.

	F1 female (n=9/7)		**F2' female (n=5/6)**	
	Control	**BM**	**Control**	**BM**
[1]Estrous cycle length (days)	**4.61 ± 0.29**	**5.96 ± 0.44***	5.54 ± 0.53	4.83 ± 0.22
[1]Number of estrous cycles	2.41 ± 0.20	2.24 ± 0.23	2.53 ± 0.20	2.64 ± 0.09
[2]Estrus (%)	24.44 (21.11 – 28.89)	23.33 (18.33 – 26.67)	22.22 (17.78 – 26.67)	23.33 (21.67 – 26.11)
[2]Proestrus (%)	**20 (16.67 – 24.44)**	**15 (11.67 – 16.67) ***	22 (17.78 – 30)	21.11 (16.67 – 22.50)
[2]Metestrus (%)	31.11 (26.67 – 36.67)	27.78 (24.58 – 32.08)	26.67 (24.44 – 31.11)	28.89 (26.11 – 33.89)
[2]Diestrus (%)	20 (5.56 – 28.89)	31.67 (24.44 – 40)	28.89 (18.89 – 33.33)	26.11 (21.11 – 32.33)

Values expressed as mean ± standard error of mean (SEM), Student's t test[1], or median and interquartile range, Mann-Whitney's test[2]. *$p<0.05$.

Table 6. Body and organ weights of F1 and F2' female rats at PND 90.

	F1 female (n=8/6)		F2' female (n=5)	
	Control	**BM**	**Control**	**BM**
Body weight (g)	258.80 ± 4.10	248.70 ± 10.89	238.20 ± 4.80	241.50 ± 2.14
Uterus (mg)	514.80 ± 21.30	452.10 ± 41.66	**414.90 ± 16.35**	**684.70 ± 100.20***
Uterus (mg/100g BW)	200 ± 7.95	183.20 ± 18.53	**169.60 ± 8.22**	**282.00 ± 42.92***
Brain (g)	**1.88 ± 0.01**	**1.96 ± 0.03***	1.91 ± 0.02	1.84 ± 0.05
Brain (g/100g BW)	0.73 ± 0.01	0.79 ± 0.03	0.77 ± 0.02	0.75 ± 0.02
Pituitary (mg)	10.65 ± 0.92	13 ± 1.10	**10.80 ± 0.39**	**9.57 ± 0.25***
Pituitary (mg/100g BW)	4.10 ± 0.34	5.25 ± 0.44	4.43 ± 0.26	3.93 ± 0.12
Kidney (g)	**2.16 ± 0.05**	**1.94 ± 0.08***	1.93 ± 0.05	2 ± 0.07
Kidney (mg/100g BW)	833.90 ± 18.05	779.50 ± 20.90	784.50 ± 8.12	821 ± 28.67
Adrenal (mg)	**101.20 ± 2.47**	**87.80 ± 4.92***	87.26 ± 4.71	93.38 ± 5.53
Adrenal (mg/100g BW)	39.20 ± 1.22	35.46 ± 2.02	35.52 ± 1.42	38.28 ± 2.18

BW: Body weight. Values expressed as mean ± standard error of mean (SEM), Student's t test. *$p<0.05$.

Table 7. Body and organ weights of F1 and F2 male rats at PND 120.

	F1 male (n=8/9)		**F2 male (n=5/7)**	
	Control	**BM**	**Control**	**BM**
Body weight (g)	434.30 ± 16.22	440.50 ± 9.56	449 ± 19.60	461.70 ± 9.92
Testis (g)	1.95 ± 0.09	1.78 ± 0.02	1.74 ± 0.08	1.79 ± 0.05
Testis (mg/100g BW)	**444 ± 8.88**	**406.80 ± 9.51***	389.40 ± 22.21	387.30 ± 11.05
Full seminal vesicle (g)	**1.02 ± 0.06**	**1.19 ± 0.02***	0.99 ± 0.07	1.09 ± 0.06
Full seminal vesicle (mg/100g BW)	237.50 ± 15.21	269.70 ± 7.28	223.50 ± 21.42	235.50 ± 13.79
Empty seminal vesicle (g)	**0.37 ± 0.02**	**0.46 ± 0.02****	0.43 ± 0.03	0.43 ± 0.02
Empty seminal vesicle (mg/100g BW)	**85.87 ± 3.67**	**100.60 ± 6.12***	95.97 ± 5.89	94.19 ± 4.27
Brain (g)	**2.04 ± 0.02**	**2.10 ± 0.02***	**2.02 ± 0.03**	**2.09 ± 0.02***
Brain (g/100g BW)	0.47 ± 0.01	0.48 ± 0.01	0.45 ± 0.02	0.45 ± 0.01

BW: Body weight. Values expressed as mean ± standard error of mean (SEM), Student's t test. *$p<0.05$, **$p<0.01$.

Table 8. Sperm morphology, mitochondrial activity and vitality of F1 and F2 male rats at PND 120.

(%)	F1 male (n=8)		F2 male (n=5/7)	
	Control	**BM**	**Control**	**BM**
Sperm morphology				
Normal shaped sperm	97(94.75 – 98.00)	98(96.25 - 99.75)	95(95 – 98)	93.50(89 – 99)
Sperm head abnormalities	1(0 – 1)	0.00(0 - 1)	0 (0 – 0)	0(0 – 1.25)
Sperm tail abnormalities	2(1 - 5.25)	1.50(0.25 - 2.75)	3.50(2 – 5)	6.50(1.50 – 11)
Sperm with cytoplasmic droplet	59.50(46 – 64.50)	58 (48.50 – 67.50)	70(54.75 – 76.25)	59.00 (46 – 64)
Mitochondrial activity (%)	**Control (n=5)**	**BM (n=4)**	**Control (n=5)**	**BM (n=6)**
Type I	**92.50 (86.13 – 96.63)**	**59.25 (51 – 66.38)***	84.50 (82.13 – 95.50)	80.50 (72 – 91.50)
Type II	7.50 (4.75 – 27.25)	32.50 (27.13 – 43.88)	14 (4.25 – 17.38)	9.50 (2 – 19.50)
Type III	**0.75 (0 – 1.50)**	**7 (4.50 – 8.38)***	0.50 (0 – 1.75)	0 (0 – 0.50)
Sperm vitality	**Control**	**BM**	**Control (n=5)**	**BM (n=5)**
Live spermatozoa	D.N.A.	D.N.A.	82 (65.50- 86.50)	79 (76 – 90)
Dead spermatozoa	D.N.A.	D.N.A.	18 (13.50 – 34.50)	21 (10 – 24)

Values expressed as median and interquartile range, Mann-Whitney's test. *$p<0.05$. D.N.A.: Data not available.

Table 9. Sperm count, daily sperm production (DSP) and sperm transit time of F1 and F2 male rats at PND 120.

	F1 male (n=9/8)		F2 male (n=5/7)	
	Control	**BM**	**Control**	**BM**
Testis				
Mature spermatid number ($x10^6$)	225.20 ± 18.12	209.50 ± 10.07	234.20 ± 13.39	230 ± 9.44
Mature spermatid number ($x10^6$/g)	142.50 ± 6.83	138.50 ± 6.09	162.20 ± 17.12	151.70 ± 6.02
Daily sperm production ($x10^6$/day)	36.92 ± 2.97	34.34 ± 1.65	38.40 ± 2.20	37.70 ± 1.55
Daily sperm production ($x10^6$/g/day)	23.36 ± 1.12	22.70 ± 0.99	26.59 ± 2.81	24.86 ± 0.98
Epididymis				
Sperm number in the caput/corpus ($x10^6$)	122.10 ± 4.59	114.30 ± 5.05	104.30 ± 11.31	104.40 ± 2.94
Sperm number in the caput/corpus ($x10^6$/g)	360.20 ± 19.07	337.20 ± 8.71	334.30 ± 38.66	337.60 ± 5.67
Sperm number in the cauda ($x10^6$)	223.10 ± 24.41	201.10 ± 11.94	205.10 ± 14.87	203.40 ± 17.23
Sperm number in the cauda ($x10^6$/g)	813.30 ± 40.10	815.90 ± 25.89	869.80 ± 29.09	807,10 ± 57.96
Sperm transit time in the caput/corpus (days)	2.94 ± 0.15	3.37 ± 0.19	2.79 ± 0.42	2.80 ± 0.16
Sperm transit time in the cauda (days)	5.76 ± 0.39	5.86 ± 0.35	5.48 ± 0.66	5.51 ± 0.62
Total sperm transit time (days)	8.74 ± 0.34	9.27 ± 0.48	8.73 ± 0.33	9.27 ± 0.47

Values expressed as mean ± standard error of mean (SEM), Student's t test. $p>0.05$.

Table 10. Histophatological evaluation of F1 and F2 male rats at PND 120.

(%)	F1 male (n=7/6)		F2 male (n=5/7)	
	Control	**BM**	**Control**	**BM**
Normal tubules	89 (88 – 92)	92 (85.75 - 92)	92 (88 - 96.50)	93.50 (91.50 - 96)
Acidophilic cells	9 (8 - 12)	8 (5.75 - 13)	3 (2 - 9)	6 (3.50 - 7.25)
Vacuole formation	1 (0 - 2)	1.50 (0 - 2.25)	3 (1.50 – 4)	0.50 (0 – 2.25)

Values expressed as median and interquartile range, Mann-Whitney's test. $p>0.05$.

Conclusão

6. Conclusões

Através dos dados obtidos podemos concluir que a exposição pré-natal à BM, além de causar efeitos tóxicos em fêmeas da geração F0, levou a disfunções nos desenvolvimentos cognitivo e somático em F1, F2 e F2', além de programação do desenvolvimento reprodutivo e alteração da qualidade espermática em F1.

Embora a exposição neonatal não tenha causado impactos tão evidentes quanto à exposição pré-natal, como observado pelo grupo de pesquisa anteriormente, também foi capaz de diminuir a qualidade espermática de machos e desregular o ciclo estral em fêmeas, além de levar a alterações no peso, sugerindo programação do desenvolvimento.

Vale ressaltar que as diferenças entre os modelos experimentais utilizados e as condições de exposição à BM na clínica humana devem ser pontuadas. No estudo, os ratos recém-nascidos foram tratados diretamente com BM, enquanto que fetos humanos são expostos indiretamente via interface materno-fetal, uma vez que as mães recebem o glicocorticoide. Além disso, a manipulação de animais recém-nascidos pode levar a indução de estresse. No entanto, além da manipulação ter sido a mínima possível, ocorreu para ambos os grupos experimentais, tratado e controle.

Além disso, quando se compara as exposições intrauterina e neonatal da prole de ratos à BM, nota-se que na primeira o glicocorticoide administrado às matrizes, além de ser metabolizado pelo organismo gravídico, é distribuído entre os fetos. Já a administração aos filhotes recém-nascidos é realizada direta e individualmente, levando à maior exposição ao fármaco.

Portanto, a exposição de ratos à BM nos DPN 1 a 3, causa efeitos menos evidentes sobre o sistema genital masculino e confirma, em partes, resultados anteriormente observados após a exposição pré-natal. Além disso, demonstra alterações no desenvolvimento, evidenciando programação do desenvolvimento.

Apesar da reconhecida importância da corticoterapia antenatal humana, os achados do presente estudo sinalizam e estimulam novos estudos a fim de minimizar possíveis efeitos adversos colaterais pós-natais.

Resumo dos resultados

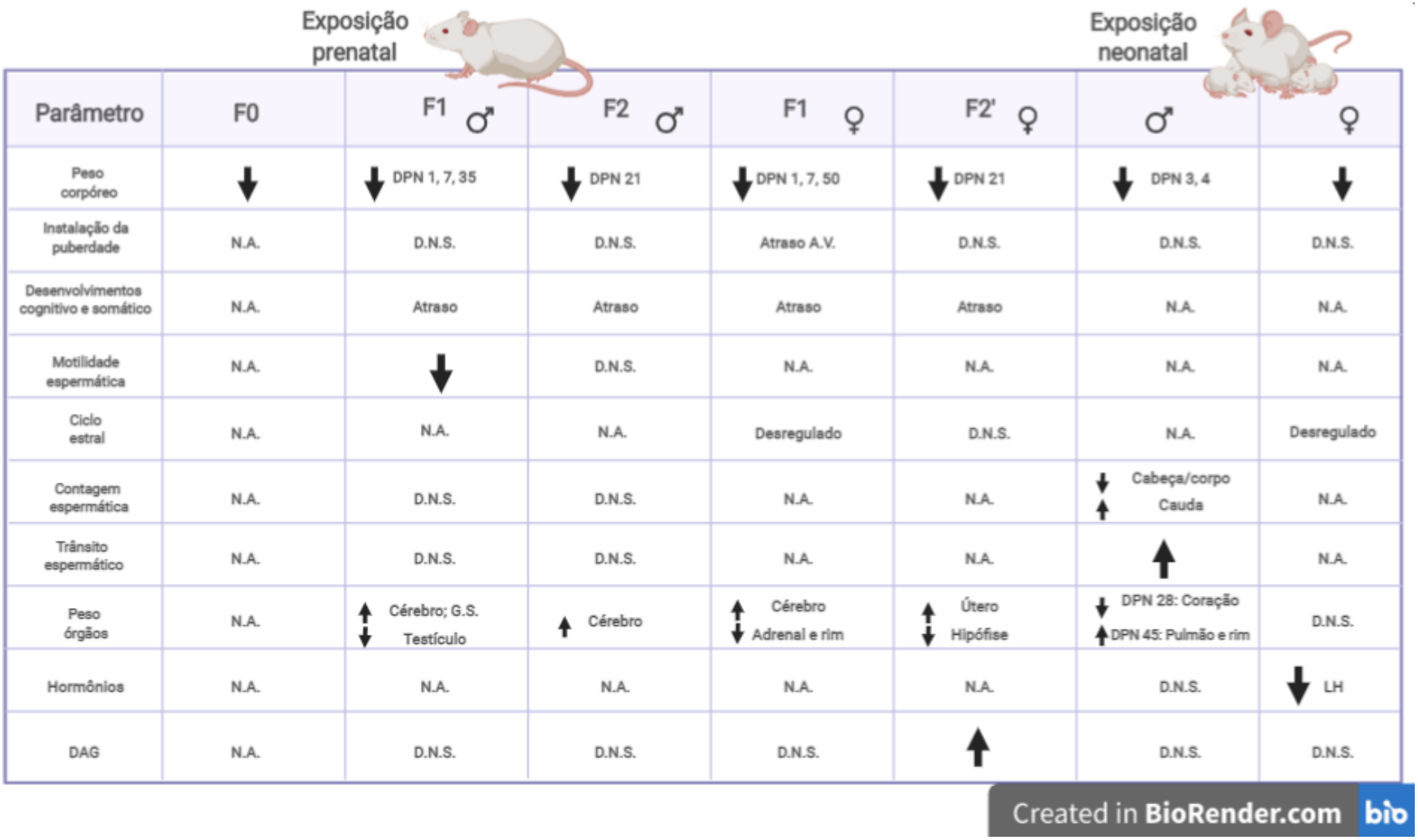

Figura 6. Resumo dos resultados dos experimentos 1 e 2. N.A.: Não avaliado; D.N.S.: Diferença não significativa; G.S.: Glândula seminal; Flecha para cima: aumento; Flecha para baixo: diminuição.

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Anexos

8. Comitê de Ética

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

Certificado

Certificamos que o projeto intitulado "Terapia perinatal com Betametasona, avaliação de parâmetros reprodutivos e transferência placentária: uma abordagem experimental em ratos", Protocolo nº **923-CEUA**, sob a responsabilidade de **Wilma De Grava Kempinas**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 9 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela ***COMISSÃO DE ÉTICA NO USO DE ANIMAIS*** (CEUA), nesta data.

Finalidade:	( ) Ensino	(x) Pesquisa Científica
Vigência do Projeto:	Início: 1/10/2016	Término: 31/07/2020
Espécie/linhagem:	*Rato Wistar*	
Nº de animais:	*370*	
Peso:	*350g*	Idade: 90 dias
Sexo:	*Machos (120) e fêmeas (250)*	
Origem	*CEMIB – Universidade de Campinas – Campinas/SP CNPJ: 49.607.336/0001-06*	

Botucatu, 09 de setembro de 2016.

Prof. Assoc. Wellerson Rodrigo Scarano
Coordenador da CEUA

Apêndices

9. Apêndices

9.1. Dados não apresentados no manuscrito I

Tabela 1. Pesos corpóreo final, absolutos e relativos de órgãos de ratas no DPN 75.

Parâmetros	Grupos Experimentais (n=7/8)	
	Controle	**BM**
Peso corpóreo final (g)	255,70 ± 6,73	249,10 ± 5,06
Útero (mg)	437,70 ± 11,50	461,60 ± 19,21
Útero (mg/100g PC)	171,50 ± 4,22	185,80 ± 8,81
Ovários (mg)	102,90 ± 9,64	107,10 ± 5,14
Ovários (mg/100g PC)	40,44 ± 4,02	43,11 ± 2,29
Hipófise (mg)	9,67 ± 0,44	9,53 ± 0,69
Hipófise (mg/100g PC)	3,80 ± 0,16	3,82± 0,25
Coração (g)	0,89 ± 0,06	0,82 ± 0,027
Coração (mg/100g PC)	347,30 ± 17,54	327,00 ± 6,19
Pulmão (g)	1,42 ± 0,07	1,25 ± 0,04
Pulmão (g/100g PC)	0,56 ± 0,03	0,50 ± 0,02
Fígado (g)	10,15 ± 0,31	10,19 ± 0,312
Fígado (g/100g PC)	3,97 ± 0,081	4,09 ± 0,07
Rim (g)	1,02 ± 0,03	0,98 ± 0,03
Rim (mg/100g PC)	398,10 ± 5,91	395,20 ± 8,54
Adrenal (mg)	42,90 ± 2,59	45,48 ± 1,69
Adrenal (mg/100g PC)	16,79 ± 0,97	18,27 ± 0,62

Valores expressos em média ± EPM. $p>0,05$. Teste t de Student. PC: Peso corpóreo.

Tabela 2. Pesos corpóreo final, absolutos e relativos de órgãos de ratos machos nos DPN 7, 14, 28, 45 e 112.

Parâmetros	DPN 7 (n=7)		DPN 14 (n=7)		DPN 28 (n=7)		DPN 45 (n=6)		DPN 112 (n=8)	
	Controle	BM	Controle	BM	Controle	BM	Controle	BM	Controle	BM
Peso corpóreo final (g)	17,13 ± 0,85	17,09 ± 0,68	33,93 ± 1,42	32,46 ± 1,13	89,17 ± 1,71	84,57 ± 3,67	210,90 ± 5,39	209,60 ± 4,60	450,60 ± 11,01	438,40 ± 10,01
Testículo + Epidídimo (mg)	21,40 ± 1,83	21,75 ± 1,21	65,09 ± 5,16	62,26 ± 2,80	-	-	-	-	-	-
Testículo + Epidídimo (mg/100g PC)	124,20 ± 7,06	127,30 ± 5,09	191,00 ± 11,58	192,30 ± 8,27	-	-	-	-	-	-
Testículo (g)	-	-	-	-	0,33 ± 0,01	0,31 ± 0,02	1,06 ± 0,02	1,09 ± 0,04	1,96 ± 0,06	1,81 ± 0,05
Testículo (mg/100g PC)	-	-	-	-	367,80 ± 13,50	366,80 ± 12,21	504,60 ± 11,25	522,50 ± 22,52	435,00 ± 11,00	413,60 ± 9,57
Epidídimo (mg)	-	-	-	-	36,35 ± 1,46	34,93 ± 2,72	119,60 ± 3,77	120,80 ± 4,20	585,30 ± 13,76	603,40 ± 14,98
Epidídimo (mg/100g PC)	-	-	-	-	40,59 ± 0,92	40,98 ± 1,78	56,72 ± 1,25	57,81 ± 2,60	130,1 ± 2,32	137,8 ± 2,95
Próstata (mg)	-	-	-	-	-	-	80,80 ± 6,14	83,55 ± 2,28	349,50 ± 23,23	324,9 ± 19,70
Próstata (mg/100g PC)	-	-	-	-	-	-	38,44 ± 3,13	39,89 ± 1,00	77,74 ± 5,19	74,35 ± 4,95
Glândula seminal (g)	-	-	-	-	-	-	0,05 ± 0,001	0,05 ± 0,003	1,07 ± 0,07	1,08 ± 0,048
Glândula seminal (mg/100g PC)	-	-	-	-	-	-	23,41 ± 0,81	25,07 ± 1,73	238,70 ± 15,51	244,90 ± 8,38
Glândula seminal sem fluido (mg)	-	-	-	-	-	-	-	-	407,1 ± 29,79	418,60 ± 13,82
Glândula seminal sem fluido (mg/100g PC)	-	-	-	-	-	-	-	-	90,22 ± 5,75	95,68 ± 3,21
Ducto deferente (mg)	-	-	-	-	-	-	-	-	90,16 ± 1,77	93,09 ± 2,39
Ducto deferente (mg/100g PC)	-	-	-	-	-	-	-	-	20,73 ± 0,57	21,29 ± 0,64
Hipófise (mg)	-	-	-	-	-	-	-	-	10,76 ± 0,45	11,10 ± 0,33
Hipófise (mg/100g PC)	-	-	-	-	-	-	-	-	2,39 ± 0,10	2,40 ± 0,143
Coração (g)	0,10 ± 0,01	0,10 ± 0,00	0,17 ± 0,01	0,18 ± 0,01	**0,39 ± 0,01**	**0,35 ± 0,01 ***	0,75 ± 0,01	0,72 ± 0,03	1,19 ± 0,01	1,12 ± 0,05
Coração (mg/100g PC)	598,20 ± 37,05	595,90 ± 24,35	504,40 ± 22,46	486,30 ± 20,93	439,10 ± 15,30	415,6 ± 14,26	354,60 ± 6,35	345,50 ± 9,71	262,00 ± 6,00	257,70 ± 11,00
Pulmão (g)	0,34 ± 0,02	0,34 ± 0,01	0,48 ± 0,03	0,52 ± 0,03	0,70 ± 0,03	0,63 ± 0,04	**1,00 ± 0,01**	**1,17 ± 0,05***	1,79 ± 0,09	1,91 ± 0,13
Pulmão (g/100g PC)	2,04,00 ± 0,09	1,97 ± 0,05	1,47 ± 0,11	1,61 ± 0,09	0,76 ± 0,02	0,79 ± 0,03	**0,48 ± 0,01**	**0,56 ± 0,02***	0,40 ± 0,02	0,44 ± 0,03
Fígado (g)	0,47 ± 0,03	0,45 ± 0,02	0,89 ± 0,02	0,89 ± 0,04	3,96 ± 0,13	3,98 ± 0,19	10,28 ± 0,24	10,09 ± 0,53	14,74 ± 0,32	14,31 ± 0,491
Fígado (g/100g PC)	2,74 ± 0,10	2,67 ± 0,08	2,78 ± 0,06	2,73 ± 0,100	4,70 ± 0,090	4,44 ± 0,11	4,88 ± 0,11	4,80 ± 0,17	3,28 ± 0,08	3,26 ± 0,08
Rim (g)	0,10 ± 0,01	0,11 ± 0,01	0,18 ± 0,01	0,16 ± 0,01	0,50 ± 0,01	0,46 ± 0,01	**0,93 ± 0,02**	**0,98 ± 0,01***	1,48 ± 0,027	1,46 ± 0,049
Rim (mg/100g PC)	595,90 ± 14,40	615,20 ± 12,24	551,30 ± 14,37	516,60 ± 9,31	556,10 ± 11,04	556,20 ± 9,31	441,90 ± 9,40	460,60 ± 1,83	329,50 ± 5,07	332,50 ± 6,59
Adrenal (mg)	2,06 ± 0,25	1,91 ± 0,20	5,07 ± 0,31	4,51 ± 0,56	12,21 ± 0,63	11,70 ± 0,62	20,58 ± 0,94	23,07 ± 1,97	30,96 ± 2,16	29,31 ± 1,90
Adrenal (mg/100g PC)	12,49 ± 1,52	11,24 ± 1,14	14,92 ± 0,60	13,92 ± 1,76	13,71 ± 0,71	13,97 ± 0,86	10,02 ± 0,56	10,99 ± 0,90	6,87 ± 0,46	6,74 ± 0,514

Valores expressos em média ± EPM. *p<0,05. Teste t de Student. PC: Peso Corpóreo.

9.2.Dados não apresentados no manuscrito II

Tabela 3. Pesos corpóreo, absolutos e relativos de órgãos de ratos machos F1 e F2 no DPN 120.

Parâmetros	Machos F1 (n=8/9)		Machos F2 (n=5/7)	
	Controle	BM	Controle	BM
Peso corpóreo (g)	434,30 ± 16,22	440,50 ± 9,56	449,00 ± 19,60	461,70 ± 9,92
Testículo (g)	1,95 ± 0,09	1,78 ± 0,02	1,74 ± 0,08	1,79 ± 0,05
Testículo (mg/100g PC)	**444,00 ± 8,88**	**406,80 ± 9,51***	389,40 ± 22,21	387,30 ± 11,05
Epidídimo (mg)	576,10 ± 19,73	585,10 ± 17,14	550,70 ± 27,25	561,90 ± 17,35
Epidídimo (mg/100 g PC)	136,50 ± 2,61	133,10 ± 4,15	122,60 ± 2,67	121,90 ± 4,18
Próstata ventral (mg)	327,10 ± 33,43	388,80 ± 22,38	374,50 ± 25,87	393,30 ± 42,16
Próstata ventral (mg/ 100g PC)	75,68 ± 7,95	88,24 ± 4,71	84,07 ± 6,87	85,63 ± 9,74
Gl. Seminal c/ fluido (g)	**1,02 ± 0,06**	**1,19 ± 0,02***	0,99 ± 0,07	1,09 ± 0,06
Gl. Seminal c/fluido (mg/100g PC)	237,50 ± 15,21	269,70 ± 7,28	223,50 ± 21,42	235,50 ± 13,79
Gl. Seminal sem fluido (g)	**0,37 ± 0,02**	**0,46 ± 0,02****	0,43 ± 0,03	0,43 ± 0,02
Gl. Seminal s/fluido (mg/100g PC)	**85,87 ± 3,67**	**100,60 ± 6,12***	95,97 ± 5,89	94,19 ± 4,27
Cérebro (g)	**2,04 ± 0,02**	**2,10 ± 0,02***	**2,02 ± 0,03**	**2,09 ± 0,02***
Cérebro (g/100g PC)	0,47 ± 0,01	0,48 ± 0,01	0,45 ± 0,02	0,45 ± 0,01
Hipófise (mg)	10,52 ± 0,47	10,69 ± 0,39	9,76 ± 0,53	10,03 ± 0,59
Hipófise (mg/100g PC)	2,42 ± 0,06	2,43 ± 0,09	2,18 ± 0,14	2,17 ± 0,13
Tireoide (mg)	20,32 ± 1,04	21,59 ± 1,63	20,34 ± 1,36	17,87 ± 1,18
Tireoide (mg/100g PC)	4,74 ± 0,31	4,94 ± 0,43	4,56 ± 0,33	3,87 ± 0,25
Pulmão (g)	1,93 ± 0,06	2,08 ± 0,09	2,11 ± 0,20	2,05 ± 0,12
Pulmão (g/100g PC)	0,45 ± 0,01	0,47 ± 0,02	0,47 ± 0,05	0,44 ± 0,02
Coração (g)	1,30 ± 0,04	1,27 ± 0,03	1,31 ± 0,05	1,32 ± 0,02
Coração (mg/100g PC)	295,30 ± 3,60	289,30 ± 5,80	293,60 ± 10,15	286,60 ± 3,53
Fígado (g)	16,94 ± 0,873	17,19 ± 0,37	14,91 ± 0,36	16,04 ± 0,70
Fígado (g/100 g PC)	3,87 ± 0,14	3,91 ± 0,07	3,33 ± 0,07	3,37 ± 0,06
Rins (g)	3,30 ± 0,13	3,25 ± 0,08	3,27 ± 0,11	3,30 ± 0,08
Rins (mg/100g PC)	739,10 ± 17,19	737,70 ± 14,00	730,50 ± 25,42	714,50 ± 17,24
Adrenais (mg)	70,19 ± 3,67	75,74 ± 5,66	73,24 ± 5,42	69,00 ± 3,67
Adrenais (mg/100g PC)	16,20 ± 0,70	17,15 ± 1,10	16,28 ± 0,87	14,90 ± 0,55

Valores expressos em média ± EPM. *p<0,05, **p<0,01. Teste t de Student. PC: Peso corpóreo.

Tabela 4. Pesos corpóreo, absolutos e relativos de órgãos de ratas F1 e F2' no DPN 90.

Parâmetros	Fêmeas F1 (n=8/6)		Fêmeas F2' (n=5)	
	Controle	BM	Controle	BM
Peso corpóreo (g)	258,80 ± 4,10	248,70 ± 10,89	238,20 ± 4,80	241,50 ± 2,14
Útero (mg)	514,80 ± 21,30	452,10 ± 41,66	**414,90 ± 16,35**	**684,70 ± 100,20***
Útero (mg/100g PC)	200,00 ± 7,95	183,20 ± 18,53	**169,60 ± 8,22**	**282,00 ± 42,92***
Ovários (mg)	118,90 ± 8,10	100,10 ± 5,33	110,60 ± 4,68	138,30 ± 9,20
Ovários (mg/100g PC)	46,12 ± 3,36	41,90 ± 1,93	45,05 ± 1,39	56,89 ± 4,08
Cérebro (g)	**1,88 ± 0,01**	**1,96 ± 0,03***	1,91 ± 0,02	1,84 ± 0,05
Cérebro (g/100g PC)	0,73 ± 0,01	0,79 ± 0,03	0,77 ± 0,02	0,75 ± 0,02
Hipófise (mg)	10,65 ± 0,92	13,00 ± 1,10	**10,80 ± 0,39**	**9,57 ± 0,25***
Hipófise (mg/100g PC)	4,10 ± 0,34	5,25 ± 0,44	4,43 ± 0,26	3,93 ± 0,12
Tireoide (mg)	19,35 ± 1,79	19,27 ± 1,81	12,40 ± 0,41	14,20 ± 0,93
Tireoide (mg/100g PC)	7,47, ± 0,70	7,88 ± 0,92	5,08 ± 0,28	5,82 ± 0,36
Coração (g)	0,90 ± 0,02	0,85 ± 0,03	0,85 ± 0,03	0,88 ± 0,04
Coração (mg/100g PC)	352,00 ± 9,69	343,90 ± 6,90	347,80 ± 10,60	361,50 ± 17,20
Pulmão (g)	1,95 ± 0,11	1,74 ± 0,18	1,49 ± 0,03	1,51 ± 0,09
Pulmão (g/100g PC)	0,76 ± 0,05	0,70 ± 0,07	0,61 ± 0,01	0,62 ± 0,03
Fígado (g)	9,98 ± 0,36	9,25 ± 0,47	8,65 ± 0,43	8,83 ± 0,14
Fígado (g/100 g PC)	3,86 ± 0,12	3,72 ± 0,10	3,52 ± 0,12	3,62 ± 0,04
Rins (g)	**2,16 ± 0,05**	**1,94 ± 0,08***	1,93 ± 0,05	2,00 ± 0,07
Rins (mg/100g PC)	833,90 ± 18,05	779,50 ± 20,90	784,50 ± 8,12	821,00 ± 28,67
Adrenais (mg)	**101,20 ± 2,47**	**87,80 ± 4,92***	87,26 ± 4,71	93,38 ± 5,53
Adrenais (mg/100g PC)	39,20 ± 1,22	35,46 ± 2,02	35,52 ± 1,42	38,28 ± 2,18

Valores expressos em média ± EPM. *p<0,05. Teste t de Student. PC: Peso corpóreo.

Tabela 5. Porcentagem de folículos ovarianos e morfometria uterina de ratas F1 e F2' no DPN 90.

Parâmetros	Fêmeas F1 (n=5)		Fêmeas F2' (n=5)	
	Controle	BM	Controle	BM
Porcentagem de folículos				
[1]Primordiais/primários (%)	19,31 (15,30 – 27,65)	22,17 (17,24 – 33,64)	41,76 (12,50 – 51,69)	24,55 (14,62 – 29,63)
[1]Pré-antrais (%)	9,05 (6,25 – 13,31)	10,04 (4,02 – 16,61)	8,99 (7,95 – 14,84)	8,96 (7,39 – 11,16)
[1]Antrais (%)	8,85 (5,78 – 14,58)	11,79 (8,10 – 13,83)	5,68 (0 – 10,99)	6,36 (5,81 – 10,71)
[1]Atrésicos (%)	4,51 (0,86 – 10,27)	6,33 (4,04 – 7,88)	4,49 (1,65 – 9,09)	5,66 (2,56 – 16,84)
[1]Corpos lúteos (%)	52,94 (42,58 – 68,02)	46,38 (43,86 – 55,26)	34,83 (30,77 – 64,77)	59,00 (33,11 – 65,86)
Morfometria uterina				
[2]Altura do perimétrio (µm)	22,93 ± 1,74	25,56 ± 1,72	23,99 ± 0,78	19,92 ± 1,96
[2]Altura do miométrio (µm)	415,70 ± 24,29	451,30 ± 26,23	446,80 ± 48,02	378,30 ± 32,50
[2]Altura do endométrio (µm)	559,00 ± 20,93	611,30 ± 42,64	509,10 ± 48,50	489,40 ± 34,09
[2]Altura do epitélio luminal (µm)	35,26 ± 2,03	37,18 ± 3,77	33,53 ± 2,63	29,80 ± 2,86

[1]Valores expressos em mediana seguidos pelo intervalo interquartílico. Teste de Mann-Whitney. [2] Valores expressos em média ± EPM. Teste t de Student. p>0,05.