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

PG-BGA

EFEITOS DA EXPOSIÇÃO INTRAUTERINA À BETAMETASONA SOBRE A REPRODUÇÃO DE RATOS *WISTAR*

CIBELE DOS SANTOS BORGES

Tese apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, para obtenção do título de Doutor no Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração Biologia Celular Estrutural e Funcional.

Orientadora: Profa. Dra. Wilma De Grava Kempinas

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Dedicatória

Este trabalho é dedicado à minha alma gêmea, minha mãe, ***Tereza Cristina dos Santos****, uma mulher maravilhosa, batalhadora, altruísta, única, que ao longo de toda sua vida sempre me colocou em primeiro plano, sucumbindo todos os seus sonhos para que eu sempre fosse feliz, mesmo que isso significasse ficarmos longe por tanto tempo. Ela foi a primeira a perceber o quanto esta vida me faz feliz e completa e por isso sempre me apoiou em todas as minhas escolhas. Mãe, esta etapa da minha vida também é sua. Mãe, eu te amo!*

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“Talvez não tenha conseguido fazer o melhor, mas lutei para que o melhor fosse feito. Não sou o que deveria ser, mas Graças a Deus, não sou o que era antes”. (Marthin Luther King)

Sumário

Resumo

A betametasona é o corticosteroide de escolha para o amadurecimento pulmonar fetal diminuindo a incidência de síndrome de angústia respiratória e mortalidade neonatal. Estudos realizados em parceria com o nosso laboratório demonstraram que a exposição pré-natal a este fármaco promoveu alterações nas concentrações de testosterona e parâmetros espermáticos da prole masculina de ratos. Recentemente, efeitos sobre o eixo hipotalâmico-hipofisário-adrenal foram observados por gerações. Assim, o objetivo do presente trabalho foi avaliar os efeitos nos parâmetros reprodutivos da prole feminina e masculina de ratos, provocados pela exposição *in utero* à betametasona. Para tanto, ratas *Wistar* prenhes foram alocadas em grupo controle (n=11) e tratado (n=13) com 0,1mg/kg de betametasona, via intramuscular, nos dias gestacionais 12, 13, 18 e 19. No experimento 1, foram avaliados: no dia pós-natal (DPN) 1 o peso inicial e a distância ano-genital da prole feminina, a partir do DPN 30, a instalação da puberdade, e a partir do DPN 70, a ciclicidade estral. No DPN 85, procedeu-se a eutanásia de uma fêmea em estro, e foram coletados uma alíquota de sangue para a determinação das concentrações hormonais e os órgãos reprodutores para pesagem. Ovário e útero foram fixados para análise histológica e morfométrica. No DPN 90 foi realizado comportamento sexual seguido de teste de fertilidade e no DPN 95 um segmento uterino de uma fêmea em estro por ninhada foi utilizado para a reatividade farmacológica. No experimento 2, foram avaliados: o consumo de ração e ganho de peso materno durante o tratamento, o peso inicial e a distância ano-genital da prole masculina no DPN 1, a instalação da puberdade a partir do DPN 30. No DPN 45 e no DPN110 procedeu-se a eutanásia de um animal de cada ninhada e foram coletados uma alíquota de sangue para a determinação das concentrações hormonais e os órgãos reprodutores para pesagem. O testículo foi fixado em Bouin e analisado para histologia, morfometria e imunohistoquímica para proteínas Cx43 e PCNA. No DPN 90, um animal de cada ninhada foi utilizado para a realização do teste de fertilidade natural. No experimento 3, dois machos da F1 foram separados e utilizados para a obtenção dos seguintes parâmetros: um animal de cada ninhada no DPN 90 foi utilizado para a realização do teste de comportamento sexual, e após 30 dias de recuperação procedeu-se a eutanásia dos animais e uma alíquota de sangue foi obtida para a determinação das concentrações hormonais e os órgãos reprodutores para pesagem. O testículo esquerdo teve a túnica albugínea removida e foi utilizado para a determinação da produção diária espermática. O epidídimo esquerdo foi utilizado para a determinação dos parâmetros espermáticos (contagem, motilidade e morfologia) assim como para o teste de fertilidade por meio da inseminação artificial *in utero.* O outro animal foi eutanasiado no DPN 120 e direcionado para os testes de reatividade farmacológica das glândulas sexuais acessórias. No experimento 4, um filhote macho de cada

ninhada da F1 foi utilizado para acasalar com fêmeas não tratadas para a obtenção da segunda geração (F2). A prole obtida da F2 foi avaliada quanto os mesmos parâmetros descritos nos experimentos 2 e 3. No experimento 5, os epidídimos direito dos animais tanto da F1 quanto da F2, no DPN 45 e 110, foram coletados e processados para histopatologia, imunomarcação para proteína Cx43 e p63 e estereologia, e o epidídimo esquerdo para reatividade farmacológica. A exposição *in utero* à betametasona promoveu uma redução no consumo de ração e no ganho de peso materno durante o tratamento. Na prole feminina foram observados: redução do peso da ninhada ao nascimento, atraso na instalação da puberdade, redução no número de estros, aumento do peso uterino, nas concentrações de LH, comprimento do ciclo estral, na contratilidade uterina e na área do miométrio. Em contrapartida, houve uma redução nas concentrações de FSH, no quociente de lordoses, na área endometrial e na fertilidade das ratas adultas. Na prole masculina da F1, foram observados redução do peso inicial ao nascimento, atraso na instalação da puberdade, redução das concentrações de testosterona no DPN 45, aumento do peso gonadal e alteração no padrão de organização das células de Sertoli e germinativas (DPN 45 e DPN 110). A produção diária espermática foi significativamente reduzida, assim como o diâmetro dos túbulos seminíferos, além da alteração na dinâmica da espermatogênese. No epidídimo foi observado um atraso na diferenciação das células do epitélio (DPN 45), redução da densidade volumétrica do epitélio, aumento no número de espermatozoides imóveis e malformados e alteração na expressão das proteínas Cx43 e p63. O teste de contratilidade das glândulas sexuais acessórias monstrou um aumento na atividade contrátil da vesícula seminal, cujo peso absoluto foi reduzido. Os testes de fertilidade revelaram redução no potencial fértil dos animais expostos à betametasona, tanto pelo acasalamento natural quanto pela inseminação artificial *in utero*. Na F2, ocorreu um padrão similar de alterações sobre o desenvolvimento sexual da prole masculina: redução do peso ao nascimento, atraso na instalação da puberdade e aumento no peso dos testículos. Além disso, foi observada redução no peso da vesícula seminal, assim como na sua atividade contrátil. As concentrações hormonais foram reduzidas para FSH e aumentadas para LH. Foi observada redução no volume das células de Leydig, redução na produção espermática, atraso na diferenciação celular do epitélio epididimário, alteração na expressão de Cx43, e redução no número de espermatozoides móveis e normais. Houve redução da frequência ejaculatória e de fertilidade. Diante do exposto, concluiu-se que a exposição de ratos à betametasona, em períodos críticos do desenvolvimento intrauterino promoveu uma reprogramação reprodutiva multigeracional. Do ponto de vista translacional estes resultados alertam para possíveis riscos reprodutivos da exposição pré-natal aos glicocorticoides na terapia antenatal.

Abstract

Betamethasone is the glucocorticoid of choice for fetal lung maturation, reducing the incidence of respiratory distress syndrome and neonatal mortality. Studies conducted in association with our laboratory have shown that prenatal exposure to this drug promoted changes in testosterone levels and sperm parameters of male rats offspring. Recently, effects on the hypothalamic-pituitary-adrenal axis were observed for generations. The aim of this study was to evaluatethe effects on reproductive parameters of male and female rats, caused by *in utero* exposure to betamethasone. Therefore, pregnant *Wistar* rats were divided into control group (n=11) and treated (n=13) with 0.1mg / kg betamethasone, intramuscularly, on gestational days 12, 13, 18 and 19. In the experiment 1, it was evaluated: the initial weight and the anogenital distance at PND 1, the puberty installation from PND 30, and from PND 70 as the estrus cyclicity. On PND 85, one female was euthanized in estrus and an aliquot of blood was collected for hormone levels and the reproductive organs were weighted. Ovary and uterus were fixed for histological and morphometric analysis. In DPN 90, sexual behavior was performed followed by fertility test, and in PND 95 an uterine segment from an animal per litter was used for pharmacological reactivity. In experiment 2, it was evaluated: the maternal food intake and weight gain during treatment, the body weight and anogenital distance of male offspring on PDN 1 and the puberty installation from PND 30. In DPN 45 and DPN110 an animal per litter was euthanized and an aliquot of blood was collected to hormone levels and the reproductive organs were weighted. The right testis was fixed in Bouin and processed: histology, morphometry and immunohistochemistry for PCNA and Cx43 protein. At PND 90 one animal from each litter was used to perform natural fertility test. In experiment 3, two males of F1 were used to obtain the following parameters: an animal from each litter on PND 90 was used to perform the sexual behavior test and after 30 days of recovery, it was euthanized and an aliquot of blood was obtained for hormone levels and the reproductive organs were weighted. The left testis had its tunica albuginea removed and used to determine daily sperm production. The left epididymis was used for the determination of sperm parameters (count, motility and morphology) as well as the fertility test by *in utero* artificial insemination. The other animal was euthanized in DPN 120 for pharmacological reactivity tests of male sex glands. In experiment 4, one male pup from F1 was used to mating with untreated females to obtain the second generation (F2). The offspring obtained from F2 was evaluated for the same parameters described in the experiments 2 and 3. In Experiment 5, the right epididymis from F1 and F2 animals, on PND 45 and PND 110 were processed for histopathology, immunostaining for Cx43 and p63 protein and stereology, and the left epididymis for pharmacology reactivity test. *In utero* exposure to betamethasone

promoted a reduction in food intake and in the weight gain of mothers during the treatment. In the female pups were observed: a reduction on weight at birth, delayed on puberty onset, reduction in estrus numbers, increase in uterine weight, LH levels, estrous cycle length, uterine contractility, and area of the myometrium. In contrast, there was a reduction in FSH levels, lordosis quotient, endometrial area and fertility. In F1 male offspring, there was a reduction in the initial weight of male offspring, delayed puberty onset, low testosterone levels in PND 45, a gonadal weight increase and alteration in the pattern of Sertoli and germ cells organization on PND 45 and 110. Reduction on daily sperm production, in the diameter of the seminiferous tubules and also an alteration in the dynamics of spermatogenesis. In the epididymis, there was a delay in epithelial cells differentiation (PND 45), reduction on epithelium density volume, an increase in the number of immobile and abnormal spermatozoa and alteration in the expression of Cx43 and p63. The seminal vesicle showed a decrease in its weight and an increase in contractility activity. Fertility tests revealed reduction in the fertility potential of animals exposed to betamethasone, either by natural mating and by *in utero* artificial insemination. In F2, there was a similar pattern of changes on the sexual development of male offspring, as well as in sperm quality, characterized by reduced birth weight, delay in puberty onset and increase in weight of the testicles. Furthermore, there was a reduction in weight of the seminal vesicle, as well as their contractility activity. Hormone levels showed reduced FSH and increased LH. It was observed decrease in Leydig cells volume, reduction on sperm production, delay in the epithelial cells differentiation, alteration in the expression of Cx43 and reduction in the number of normal and mobile spermatozoa. There was a reduction in the ejaculatory frequency and fertility potential. From the foregoing, it is concluded that betamethasone exposure, in critical periods of intrauterine development in rats, promoted a reproductive multigenerational reprogramming. From the translational perspective these results warn of possible adverse reproductive outcomes of prenatal exposure to glucocorticoids in the human antenatal therapy.

Introdução

1- DESENVOLVIMENTO INICIAL DO SISTEMA GENITAL

1.1- EIXO HIPOTALÂMICO-HIPOFISÁRIO-GONADAL

A fisiologia reprodutiva em mamíferos é regulada pelo eixo hipotalâmico- hipofisário-gonadal e depende da ação do hormônio liberador de gonadotrofina – GnRH (*gonadotropin releasing hormone*) sobre seus receptores específicos na região da hipófise anterior (Wen *et al.*, 2010). Uma vez estimulada, a hipófise anterior inicia a biossíntese e liberação de hormônio luteinizante – LH (luteinizing hormone) e hormônio folículo estimulante – FSH (*follicle stimulating hormone*) que são responsáveis pelo desenvolvimento e atividade das gônadas (Manojlović-Stojanoski *et al.*, 2012).

Em ratos, cuja gestação dura em torno de 21 dias, a partir do DG 12 pode-se perceber traços de GnRH em toda a região cerebral, entretanto, as concentrações deste hormônio permanecem baixos até o DG 16, e o aumento progressivo em sua concentração a partir do DG 17, coincide com o período final da gestação (Aubert *et al.*, 1985). O mesmo pode ser observado para a hipófise, que entre os dias DG 12 a 16 apresenta fracos sítios de ligação para o GnRH, e a partir do DG 17 sua afinidade para este hormônio aumenta progressivamente o que acarreta no aumento da secreção de LH pela hipófise anterior (Aubert *et al.*, 1985).

A expressão de receptores de LH é detectada a partir do DG 15,5 e permanece constante até o DG 17,5, sendo que após o DG 18,5 o aumento progressivo na expressão destes receptores coincide com o aumento da secreção deste hormônio (Warren *et al.*, 1984).

Já a expressão de receptores de FSH, inicia-se no DG 17,5, permanece baixa até DG 19,5 e a partir do DG 20,5 aumenta a sua expressão chegando ao máximo por volta do DG 21,5. Esse aumento na expressão coincide com o inicio da secreção de FSH pela hipófise anterior (Warren *et al.*, 1984). Interessante perceber que apenas após o aumento nas concentrações de LH, ocorre o aumento nas concentrações de FSH, pois é o LH que modula a expressão de FSH na hipófise, e não o GnRH (Wen *et al.*, 2010).

1.2- DESENVOLVIMENTO DA GÔNADA MASCULINA

O desenvolvimento da gônada masculina ocorre muito antes do aparecimento dos hormônios reprodutores e existem pelo menos 5 diferentes estágios na formação do testículo: desenvolvimento da crista gonadal, formação da gônada indiferenciada, determinação sexual, indução dos cordões testiculares e desenvolvimento funcional dos testículos (Cupp & Skinner, 2005).

Em ratos, a partir do DG 7,25 as células germinativas primordiais são especificadas no epiblasto do disco embrionário (Ginsburg *et al.,* 1990) e, por volta do DG 10 a 12, ocorre intensa proliferação e migração destas células para a crista gonadal urogenital, sendo possível identificá-las na superfície do mesônefro embrionário, que se origina a partir do mesoderma intermediário (Cupp & Skinner, 2005; Manojlović-Stojanoski *et al.,* 2012). No DG 13,5 a migração para a crista se encerra, no entanto, essas células continuam se proliferando (Cupp & Skinner, 2005).

Entre os dias DG 11,5 a 12,5 a gônada em formação apresenta bipotencialidade, ou seja, pode ser distinguida do mesônefro, mas ainda não é possível diferenciá-la em testículo e ovário. Após este período inicia-se a expressão de diferentes fatores de transcrição pelas células somáticas do epitélio celômico que promovem a diferenciação gonadal (Cupp & Skinner, 2005; Manojlović-Stojanoski *et al.,* 2012).

A expressão do gene SRY (*sex determining region of Y chromossome*), entre os DG 10,5 a 12, juntamente com a expressão de outros fatores de transcrição estão relacionados com a diferenciação das células de Sertoli, diferenciação sexual e a formação dos cordões testiculares (Capel *et al.,* 1999; Schmahl *et al.,* 2000; Albrecht & Eicher, 2001).

As células de Sertoli originam-se a partir das células epiteliais do celoma embrionário, sendo as primeiras a se diferenciarem, marcando o início do desenvolvimento testicular (Albrecht & Eicher, 2001; Bhandari *et al.,* 2012). Nos DG 11 a 11,5 essas células apresentam rápida proliferação e entre os DG 11,5 a 12,5 migram para o interior das gônadas (Cupp & Skinner, 2005; Manojlović-Stojanoski *et al.,* 2012) onde começam a sintetizar prostaglandina D_2 que exerce papel fundamental na própria diferenciação da células de Sertoli como também impedem o início da meiose nas células germinativas (Adams & McLaren, 2002).

A formação dos cordões seminíferos ocorre por volta do DG 12,5 e se completa por volta do DG 14, período no qual as células de Sertoli diminuem o ritmo de proliferação e se agregam com células germinativas formando os cordões testiculares (Cupp & Skinner, 2005). Logo em seguida, células do mesônefro tais como células endoteliais, peritubulares e células associadas a vascularização migram para estes cordões recém-formados (Buehr *et al.,* 1993).

Uma vez formados esses cordões desenvolvem-se funcionalmente devido a diferenciação e proliferação de determinados tipos celulares, incluindo as células de Leydig, que se originam das células do mesênquima gonadal (mesênquima do mesônefro), uma vez que, expressam a mesma proteína precursora das células esteroidogênicas adrenais (Yao & Barsoum, 2007; Wen *et al.*, 2016). Esta diferenciação que ocorre a partir do DG 14,5 a 15 corresponde ao mesmo período do início de sua atividade esteroidogênica (Yao & Barsoum,

2007). Por volta do DG 18 as células de Sertoli retomam sua atividade proliferativa o que coincide com o período próximo ao final da gestação (Levine *et al.*, 2000).

1.3 – DESENVOLVIMENTO DA GÔNADA FEMININA

O desenvolvimento dos ovários, em roedores, começam com as mesmas estruturas precursoras do desenvolvimento dos testículos, as cristas genitais, entre os dias DG 10 a DG 12 (Anderson *et al*., 2000; Parker & Schimmer, 2006; Windley & Wilhelm, 2015). À medida que as células germinativas alcançam as cristas genitais, começam a se proliferar mitoticamente, e como resultado de uma citocinese incompleta, elas desenvolvem pontes citoplasmáticas entre si e com as células somáticas da crista genital, formando os cordões sexuais (precursores dos folículos ovarianos) (Epifano *et al*., 2002; Grive & Freiman, 2015).

Inicialmente (DG 10 a 12,5) esta gônada em formação apresenta-se indiferenciada (Suzuki *et al*., 2015), e no caso do sexo feminino, as células somáticas da crista gonadal, pela ausência do gene SRY, permanecem indiferenciadas até aproximadamente o dia DG 13,5, quando as células germinativas começam a entrar em meiose (Yao *et al*., 2003; Bullejos e Koopman, 2004; Parker & Schimmer, 2006; Suzuki *et al*., 2015).

As células germinativas envoltas pelas células somáticas da crista urogenital, formando cistos, entram em meiose de forma sincronizada e em aproximadamente dois dias, por volta do DG 15,5 a maioria das células já se encontram estacionadas na prófase I da meiose (Miles *et al*., 2010; Windley & Wilhelm, 2015).

Em cada cisto, muitas células germinativas entrarão em processo apoptótico e a célula remanescente em prófase I (ovócito I) rodeada por uma única camada de células achatadas formará o futuro folículo primordial (Epifano e Dean, 2002; Windley & Wilhelm, 2015; Grive & Freiman, 2015). Durante este período, ocorre a diferenciação das células mesenquimais em células da teca, (Windley & Wilhelm, 2015) que iniciam sua atividade esteroidogênica, cujo pico de progesterona (DG 15,5) e estradiol (DG 17,5), desencadeiam a quebra dos cistos finalizando a formação dos folículos ovarianos (DG 17,5 até DPN 5) (Grive & Freiman, 2015; Rimon-Dahari *et al*., 2016).

1.4- DIFERENCIAÇÃO SEXUAL GONADAL

Durante a embriogênese, a diferenciação para o sexo masculino requer a secreção de três hormônios testiculares: hormônio anti-Muleriano (AMH), a testosterona e o fator de crescimento ligado a insulina 3 (insulin-like 3; INSL3) (Sharpe *et al*., 2003; Manojlović-Stojanoski *et al*., 2012). Por outro lado, a diferenciação para o sexo feminino, requer a

ausência da secreção destes hormônios, embora se saiba que este processo necessite da ativação de outros genes denominados pró-ovarianos e/ou antitesticulares, tais como Wnt4 e Dax1 (Schoenwolf *et al*., 2009).

O AMH é produzido pelas células de Sertoli fetais, induzindo a regressão dos ductos de Müller (Josso *et al.,* 2001); a testosterona, produzida pelas células de Leydig, promove o desenvolvimento de derivados do ducto de Wolff e a masculinização fetal (Corbier *et al*., 1992; Manojlović-Stojanoski *et al*., 2012) e o INSL3, também secretado pelas células de Leydig, é responsável pela descida do testículo para o escroto (Sharpe *et al.*, 2003).

O início da produção de testosterona ocorre concomitantemente com o início dos receptores de LH, a partir do DG 15,5 (Warren *et al.*, 1984). Entretanto, o processo de masculinização fetal hipotalâmica ocorre somente nos DG 18 e DG 19 e nas 2 primeiras horas após o nascimento (Gogan *et al.*, 1981) devido os dois importantes aumentos nas concentrações séricas e hipotalâmicas de testosterona secretada pelos testículos fetais (Corbier *et al*., 1992). Esses picos se correlacionam com a máxima produção de LH e expressão de seus receptores (Warren *et al.*, 1984). No homem, entre a 12ª e 20ª semana gestacional, as concentrações de testosterona no soro fetal são de 3 a 8 vezes maior do que na mulher (Manojlović-Stojanoski *et al*., 2012).

A ausência de testosterona e a ausência pré-natal do hormônio AMH, no sexo feminino, garantirá a diferenciação normal das estruturas do sistema genital interno feminino, útero e vagina (Durlinger *et al*., 2002).

1.5 - DESENVOLVIMENTO DOS DERIVADOS DOS DUCTOS MESONÉFRICOS E PARAMESONÉFRICOS

A origem da maioria dos outros órgãos reprodutores, tanto do sistema genital feminino (tubas uterinas, útero e parte superior da vagina) quanto do sistema genital masculino (epidídimo, ducto deferente e vesícula seminal) dá-se pela diferenciação dos ductos paramesonéfricos e mesonéfricos, respectivamente (Robaire *et al.,* 2006). A próstata, por outro lado, deriva do endoderma do seio urogenital, oriundo da parte terminal do intestino posterior (Staack *et al*., 2003; Cunha *et al*., 2004).

O desenvolvimento dos ductos mesonéfricos ocorre por meio da condensação do mesoderma intermediário, por volta dos DG 8,5 e DG 9,5, formando primeiramente um cordão de células epiteliais que expressam várias proteínas, principalmente Bmp4 e Pax2 (Robaire *et al.,* 2006). Rapidamente este cordão adquire uma luz, por cavitação, e em seguida ocorre seu alongamento, pelo rearranjo celular, proliferação, mudança conformacional das

células e transformação epitélio-mesenquimal, estendendo este ducto ao longo do embrião (Hinton *et al*., 2011; Robaire *et al.*, 2006).

Após seu alongamento, este ducto induz células mesênquimais subjacentes a formarem os túbulos mesonéfricos (Robaire *et al.,* 2006) e logo em seguida, por invaginação do epitélio celômico, ocorre a formação dos ductos paramesonéfricos, por volta do DG 14. (ductos de Muller). O desenvolvimento dos túbulos mesonéfricos é importante, pois estão relacionados ao desenvolvimento dos ductos eferentes e possivelmente a região do segmento inicial do epidídimo (Rodriguez *et al.,* 2002; Robaire *et al.,* 2006). Por outro lado, o ducto mesonéfrico mediado pela ação de testosterona produzida pelas células de Leydig por volta do DG 15 garantirá a formação do epidídimo, assim como do ducto deferente e da vesícula seminal (Robaire *et al*., 2006).

As células epiteliais e mesenquimais dos ductos mesonéfricos começam a expressar receptores androgênicos a partir do DG15, período que coincide com o inicio da secreção de testosterona pelas células de Leydig (You & Sar, 1998). Este hormônio irá atuar como fator de crescimento sobre os ductos de Wolff, promovendo sua diferenciação em ductos reprodutores masculinos, dentre eles o epidídimo (Rodriguez *et al.,* 2002). Além disso, por volta do DG 12, as células do ducto mesonéfrico já iniciam a formação do que será a futura barreira hemato-epididimária, uma vez que neste período ocorre o aparecimento das primeiras junções oclusivas (Suzuki & Nagano, 1978).

Ao mesmo tempo, a diferenciação das células de sustentação em células de Sertoli entre os dias DG 10,5 a DG 12, faz com que estas sintetizem o hormônio AMH a partir do DG 11,5 que irá atuar sobre os ductos paramesonéfricos, formado por volta do DG 14 (Durlinger *et al*., 2002), promovendo sua regressão por volta dos dias DG 14,5 a DG 15,5 (Tsuji *et al.,* 1992; Roberts *et al.,* 1999; Rodriguez *et al.,* 2002). Durante este mesmo período de desenvolvimento (DG 15,5) ocorre o inicio do enovelamento dos ductos epididimários, que irá finalizar apenas após o nascimento (Murashima *et al.,* 2015).

Vale ressaltar que a ausência de testosterona, no sexo feminino, promoverá a regressão dos ductos mesonéfricos, e a ausência pré-natal do hormônio AMH, garantirá a diferenciação normal das estruturas do sistema genital interno feminino (útero e vagina) a partir dos ductos paramesonéfricos (Durlinger *et al*., 2002).

Ao nascimento, o epidídimo se encontra diferenciado do seu antecessor, o ducto de Wolff, entretanto apenas as regiões iniciais (segmento inicial ao corpo) apresentam-se enoveladas, a região caudal continua em processo de enovelamento (Rodriguez *et al.*, 2002).

Hermo *et al*. (1992) e Orgebin-Crist *et al.* (1978) demonstraram que o desenvolvimento pós- natal do epidídimo também é muito importante, e é marcado por influência tanto de testosterona quanto de fatores vindos do fluido testicular. Existem três estágios de desenvolvimento epididimário pós-natal (Figura 2): período indiferenciado (DPN 1 ao DPN 15), período de diferenciação (DPN 16 ao DPN 44) e período de expansão (DPN 44 a idade adulta) (Sun & Flickinger, 1979; Marty *et al.,* 2003).

Durante o estágio indiferenciado do epidídimo, seu epitélio apresenta apenas um tipo celular, caracterizado por células colunares contendo inúmeras figuras mitóticas. No período de diferenciação celular, estas células colunares se direcionam, formando as células halo, a partir do DPN 14, estreitas (DPN 15), principais (DPN 28), basais (DPN 28) e claras (DPN 36). Por volta do DPN 49 todas as células do epitélio epididimário encontram-se diferenciadas (Hermo *et al.*, 1992; Marty *et al.,* 2003; Robaire *et al.*, 2006). Por fim, o período de expansão é marcado pelo inicio do aparecimento de espermatozoides na luz de todo o epidídimo (Robaire *et al.*, 2006) que por volta do DPN 91 atinge a sua máxima concentração (Marty *et al.*, 2003).

Portanto, o epidídimo é um órgão do aparelho genital masculino presente na maioria dos mamíferos, formado por um único ducto altamente enovelado que liga os ductos eferentes ao ducto deferente (Rodriguez, 2002; Hermo & Robaire, 2002; Robaire *et al*., 2006), dividido em segmento inicial, cabeça, corpo e cauda (Bedford, 1965; Hermo *et al.*, 1992; Kempinas & Klinefelter, 2014; Domeniconi *et al.*, 2016) e funcionalmente é responsável pelo processo de maturação espermática (Hinton *et al.*, 1995; Kempinas & Klinefelter, 2010; Hinton *et al.*, 2011), além de desempenhar um importante papel sobre o transporte, proteção, estocagem e concentração dos espermatozoides vindos dos testículos (Cuasnicú *et al.*, 2002; Robaire *et al.*, 2006; Fernandes *et al*., 2008; Kempinas & Klinefelter, 2010).

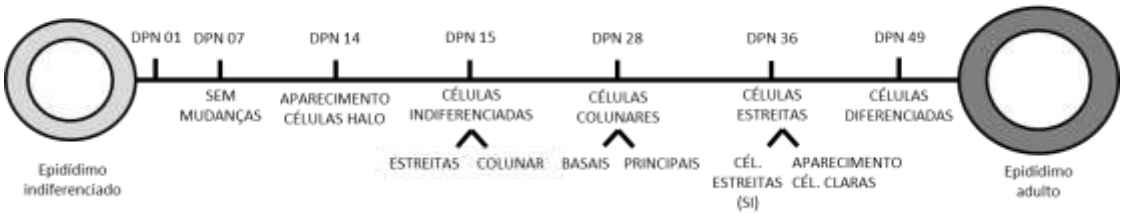


Figura 1: desenho esquemático do desenvolvimento epididimário pós-natal. DPN: dia pós-natal, SI: segmento inicial (adaptado de Hermo & Robaire, 2002).

A vesícula seminal, órgão andrógeno-dependente (Kierszenbaum, 2008), consiste em um ducto único, dilatado e enovelado, revestido por um epitélio pseudoestratificado altamente pregueado (Hayward *et al.*, 1996; Risbridger & Taylor, 2006).

Sua formação se inicia após o inicio da secreção de testosterona, no DG 15, com uma dilatação da região mais caudal do ducto mesonéfrico próximo a uretra (Flickinger, 1970; Hayward *et al.*, 1996). Entretanto, rudimentos da vesícula seminal apenas são detectados após o DG 18 (Flickinger, 1970; Risbridger & Taylor, 2006) na forma de um simples par de divertículos que cresce da região dorsal do ducto mesonéfrico circundado por mesênquima (Sugimura *et al.*, 1986; Price, 1936).

Vale ressaltar que o crescimento destes divertículos se dá por influencia de hormônios testosterona, uma vez que células do mesênquima expressam receptores androgênicos a partir do DG 15 e por estrógenos (Flickinger, 1970; Risbridger & Taylor, 2006). Este par de divertículos continua seu crescimento até por volta do DG 21,5 quando sua região mais cranial dobra ventralmente formando a flexura característica da vesícula seminal adulta (Price, 1936).

Durante o desenvolvimento da vesícula seminal, aumenta-se a complexidade de seu epitélio, assim como dos níveis de dobramentos, seu estroma se diferencia em músculo liso, e se separa do epitélio glandular por uma lâmina própria (Marker *et al.*, 2003).

Após o nascimento, a vesícula seminal continua seu crescimento de forma lenta e por volta do DPN 36, atinge sua etapa final de desenvolvimento, se tornando uma verdadeira glândula de secreção (Price, 1936).

A próstata, outro órgão andrógeno-dependente, é a maior glândula sexual acessória masculina, localizada na pelve, inferiormente à bexiga (Kierszenbaum, 2008). Apresentando-se como um conjunto de glândulas que entram na uretra em diferentes pontos, a próstata de roedores, como mencionado anteriormente, deriva do endoderma do seio urogenital (Staack *et al.*, 2003; Cunha *et al.*, 2004).

O seio urogenital pode ser visto em roedores a partir do DG 14, contudo apenas após o DG 16 que ele pode ser identificado como uma estrutura separada da bexiga em formação (Shaw *et al.*, 2008). No DG 19, ocorre a formação de cordões sólidos do epitélio urogenital que crescem bilateralmente a partir do seio urogenital em direção ao mesênquima subjacente (Hayward *et al.*, 1996; Timms *et al.*, 1994). Entre os dias DG 19 a DG 20 ocorre um rápido desenvolvimento das regiões ventral, dorsal e laterais da próstata (Timms *et al.*, 1994). Todo este processo se dá na presença hormônios androgênicos vindos do testículo e estimulando os receptores androgênicos presentes no mesênquima desde DG 16 (Thompson *et al.*, 1986;

Cunha *et al.*, 2004; Ricke *et al.*, 2007; Shaw *et al.*, 2008). Além disso, estrógenos presentes neste período também influenciam o desenvolvimento prostático, assim como fatores de crescimento vindos do fluido testicular (Prins *et al*., 2006; Ricke *et al*., 2007).

O desenvolvimento da próstata em roedores, entretanto, termina apenas na puberdade, com o final da morfogênese dos ramos prostáticos (Kinbara *et al*., 1996; Sugimura *et al.*, 1986). Durante os primeiros 15 dias após o nascimento, tem-se o inicio do desenvolvimento dos ramos prostáticos (Hayashi *et al.*, 1991). Esta glândula pode ser dividida em três regiões de acordo com o seu posicionamento em relação aos demais órgãos pélvicos: um par de lobos dorsolaterais, um par de lobos ventrais e um par de lobos anteriores ou glândula de coagulação que estão associadas às vesículas seminais (Abbott *et al.*, 2003) que se desenvolvem, mesmo na presença de baixas concentrações de testosterona (Risbridger & Taylor, 2006). Entretanto, por ser um órgão andrógeno-dependente e muito sensível à ação de compostos anti-androgênicos, a próstata se tornou um indicador preciso sobre a influência de qualquer composto que possa interferir no controle do eixo hipotalâmico-hipofisário-gonadal (Klinefelter & Hess, 1998).

A Figura 2 esquematiza todo o processo de desenvolvimento do sistema genital, tanto masculino, quanto feminino, demonstrando a importância do controle hormonal e do período crítico de desenvolvimento intrauterino sobre a diferenciação de cada órgão reprodutor.

1.6- DIFERENCIAÇÃO SEXUAL HIPOTALÂMICA

O cérebro no início do desenvolvimento também se encontra indiferenciado, e o aumento nas concentrações de testosterona no final da gestação ocorre ao mesmo tempo que este órgão é particularmente sensível a exposição hormonal, caracterizando um período crítico de diferenciação sexual hipotalâmica (Schwarz & McCarthy, 2008).

Para o desenvolvimento normal do cérebro de um macho, devem ocorrer 2 processos distintos: masculinização e defeminização. Na ausência destes processos, acredita-se que o cérebro se desenvolva com padrão feminino. A masculinização é a organização do sistema nervoso de modo a torná-lo permissivo a expressão de comportamento sexual masculino enquanto que a defeminização é a perda da capacidade de responder aos efeitos do estradiol e progesterona que induzem um comportamento sexual feminino (Schwarz & McCarthy, 2008). Assim, ambos os processos devem ocorrer ao mesmo tempo, e em roedores, por volta dos DG 18 e 19, e nas 2 primeiras horas após o nascimento, para que tanto o cérebro quanto a gônada sejam diferenciadas para o sexo masculino (Schwarz & McCarthy, 2008).

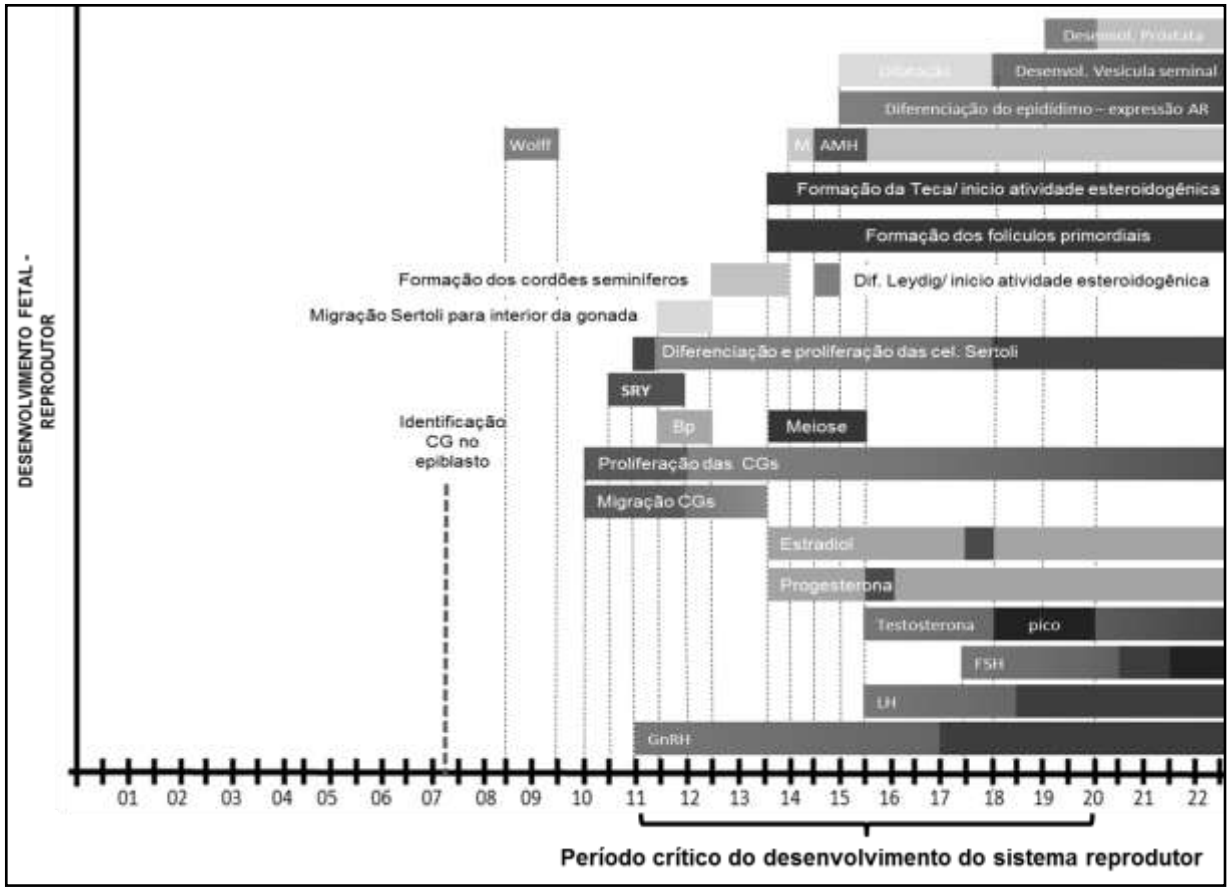


Figura 2: Desenho esquemático do desenvolvimento do sistema genital masculino e feminino de ratos, com base nos dias gestacionais, mostrando o período crítico do desenvolvimento dos órgãos reprodutores, assim como do eixo hipotalâmico-hipofisário-gonadal, expresso por meio dos hormônios sexuais.

Estudos recentes têm demonstrado que o processo de feminização do hipotálamo não é um processo totalmente passivo. Foi observado que geneticamente o cérebro apresenta genes que promoveriam sua masculinização, entretanto estes se encontram silenciados via metilação, mediado pela ativação de receptores esteroidais nucleares (Nugent *et al*., 2015). Assim, a exposição destes receptores diretamente ao estradiol ou a tratamentos farmacológicos que diminuem o grau de metilação global, como o realizado pela zebularina (um análogo da citidina) promovem masculinização em fêmeas (Nugent *et al.,* 2015).

Além disso, Juraska *et al.* (2013) mostraram que o processo de diferenciação sexual hipotalâmica não é restrito ao final do período pré-natal, mas também pode ser influenciado pela ação hormonal, tanto de testosterona para o sexo masculino, como de estradiol e progesterona para o sexo feminino, durante todo o desenvolvimento sexual inicial.

Os desrreguladores endócrinos, compostos que desempenham uma atividade anti-androgênica, anti-estrogênica ou estrogênica, como a flutamida, o bisfenol A e o dietilbestrol, assim como fármacos que agem diretamente sobre o sistema neuroendócrino, como a metildopa, e os glicocorticoides, como a betametasona, se administrados durante períodos críticos de desenvolvimento intrauterino, como por exemplo durante o período que compreende a diferenciação sexual, podem desencadear uma série de alterações tanto sobre o eixo hipotalâmico-hipofisário-gonadal, como no desenvolvimento do sistema reprodutor (Pereira & Piffer, 2005; Gusmán & Zambrano, 2007; Piffer *et al.,* 2009a/b; Svechnikov *et al.,* 2014; Resnikov *et al.,* 2015). Portanto, os efeitos sobre o eixo hipotalâmico-hipofisário-gonadal dependem do estágio de desenvolvimento intrauterino, da janela de exposição, bem como da dose utilizada durante o tratamento (Resnikov *et al.*, 2015).

2 – CORTICOTERAPIA ANTENATAL E SEUS EFEITOS SOBRE A REPROGRAMAÇÃO FETAL COM ÊNFASE NO SISTEMA GENITAL

2.1- GLICOCORTICOIDES E DESENVOLVIMENTO FETAL

Durante o desenvolvimento, a síntese de glicocorticoides pelas glândulas adrenais fetais inicia-se a partir do décimo terceiro dia gestacional (DG) em ratos, e na oitava semana de gestação em humanos, demonstrando que os hormônios endógenos desempenham papel crucial no crescimento e desenvolvimento de tecidos fetais (Manojlović-Stojanoski *et al.*, 2012).

Os glicocorticoides atuam sobre o metabolismo de carboidratos, proteínas e lipídeos (Rang *et al.,* 2004). O seu mecanismo de ação (Figura 3), de modo geral, envolve interações entre estes corticoides e receptores intracelulares. Após penetrarem nas células, os glicocorticoides ligam-se a receptores específicos GRα e GRβ (*glucocorticoids receptors*) no citoplasma, tornando-os ativos em razão da exposição do domínio de ligação do DNA. Este complexo glicocorticoide-receptor forma dímeros que migram para o núcleo e se ligam ao DNA, desencadeando uma resposta de inibição ou de indução da transcrição de genes específicos (Rang *et al.,* 2004; Alheira & Brasil, 2005).

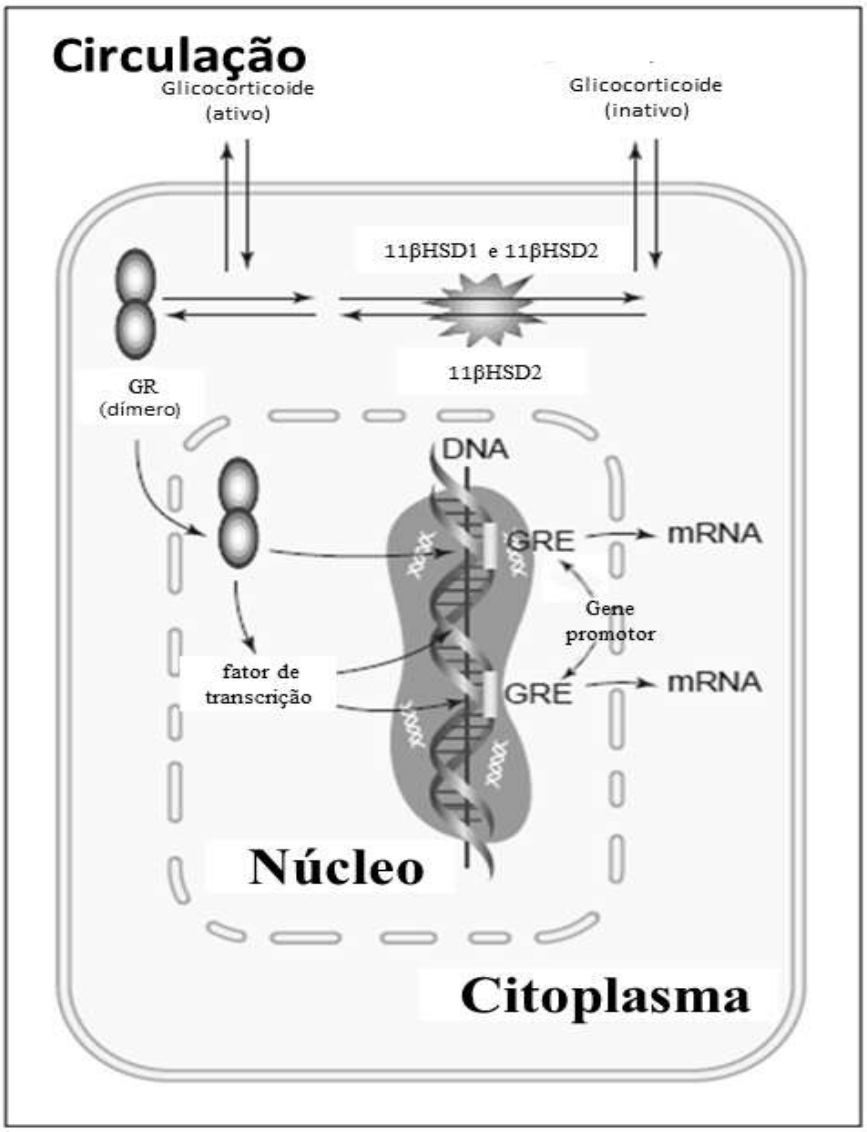


Figura 3: Desenho esquemático do mecanismo de ação dos glicocorticoides nas células, adaptado de Bertram & Hanson (2002).

Além disso, os glicocorticoides também podem atuar diretamente sobre as membranas celulares, interagindo com receptores do tipo $GABA_B$ (receptores acoplados a proteína G) e desencadeando um bloqueio na condução de íons Ca^{2+} (Wolkowitz & Reus, 1999; Alheira & Brasil, 2005).

Vale ressaltar que, as concentrações de glicocorticoides fetais são muito menores que o materno, devido a presença da enzima placentária 11β-hidroxiesteroide desidrogenase tipo 2 (11-HSD2) que degrada estes hormônios em metabólitos inativos (Figura 4). Entretanto, aproximadamente 20% das moléculas de glicocorticoides endógenas ainda conseguem atravessar a placenta, ou grande parte das moléculas de glicocorticoides sintéticos, como

dexametasona ou betametasona, pois apresentam baixa afinidade para esta enzima, podendo influenciar o ambiente intrauterino (Seckl, 1997).

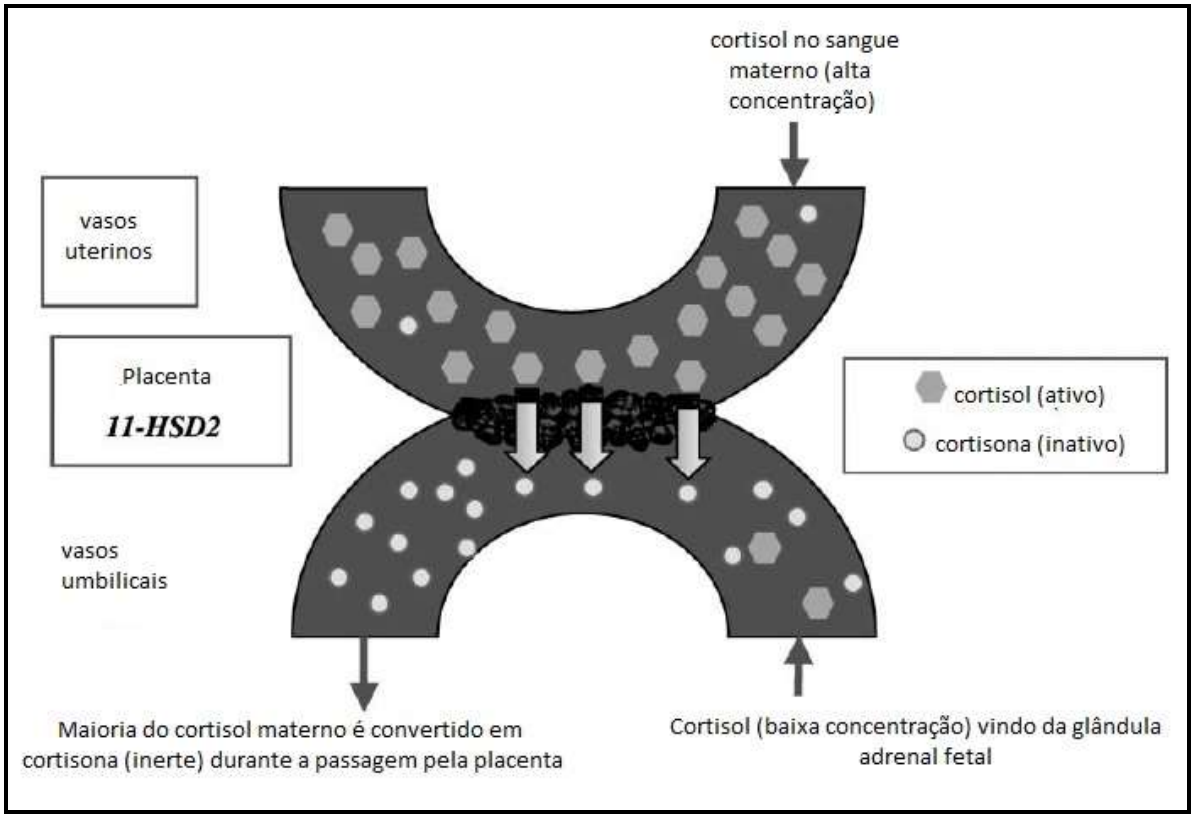


Figura 4: Desenho esquemático da ação da enzima 11-HSD2 na placenta sobre o cortisol materno, adaptado Seckl (2004).

Sabe-se também que no último terço da gestação, ocorre um aumento nas concentrações de glicocorticoides fetais, devido principalmente ao aumento na taxa de transferência placentária do cortisol materno (Manojlović-Stojanoski *et al.*, 2012). Este aumento nas concentrações fetais de glicocorticoides ocorre graças à diminuição da expressão da enzima placentária 11-HSD2 (Seckl, 2004). Uma vez aumentado, os glicocorticoides irão proporcionar uma série de modificações em diversos tecidos e órgãos essenciais para a sobrevida fetal. Estas modificações denominadas “preparação ao nascimento” incluem a maturação pulmonar fetal e produção de surfactante (Manojlović-Stojanoski *et al.*, 2012).

Órgãos como a tireoide, rins, cérebro e hipófise também são dependentes do aumento das concentrações de glicocorticoides endógenos no final da gestação, entretanto, um aumento ou redução fora do período esperado pode desencadear alterações funcionais graves nestes órgãos caracterizando uma possível reprogramação fetal (Moisiadis & Matthews, 2014).

2.2- UTILIZAÇÃO DOS GLICOCORTICOIDES SINTÉTICOS DURANTE O DESENVOLVIMENTO INTRAUTERINO

Atualmente, cerca de 5 a 13% das gestações em todo o mundo apresentam complicações relacionadas ao nascimento prematuro, sendo este a causa mais importante correlacionada a morbidade e mortalidade neonatal (Nijman *et al.*, 2016). Em situações de parto prematuro, principalmente em períodos críticos e finais da gestação, as concentrações endógenos de glicocorticoides ainda encontram-se inferiores aos necessários para a promoção do crescimento e desenvolvimento pulmonar fetal (Fowden & Forhead, 2004). Portanto, a utilização de glicocorticoides, principalmente a betametasona, no que se denomina terapia antenatal com glicocorticoides ou corticoterapia antenatal, se faz necessária.

A corticoterapia antenatal é portanto, o nome dado ao tratamento de mulheres grávidas com risco de parto prematuro, entre a 24ª a 34ª semana de gestação (Jobe & Soll, 2004) e, a betametasona é o glicocorticoide sintético de escolha nesses tratamentos, uma vez que, promove a maturação pulmonar fetal diminuindo a incidência da síndrome de angústia respiratória, mortalidade e morbidade neonatal (Sadiq & Devaskar, 1984; Crowley, 1995; Samtini *et al.*, 2005; Piffer *et al.*, 2009a).

Em roedores, tem sido demonstrado que a corticoteria antenatal ocorre entre os dias gestacionais 12 a 19 (Pereira *et al.*, 2003; Pereira & Piffer, 2005; Piffer *et al.*, 2009a/b; Drake *et al.*, 2011). Durante este período do desenvolvimento intrauterino, sabe-se que modificações importantes no desenvolvimento fetal, tanto masculino quanto feminino estão ocorrendo, tornando-os vulneráveis a qualquer perturbação, tais como exposição alterada a hormônios e a glicocorticoides (Barker *et al.*, 1998; Manojlović-Stojanoski *et al.*, 2012).

2.3- EFEITOS GERAIS DA CORTICOTERAPIA ANTENATAL: REPROGRAMAÇÃO FETAL

O tratamento antenatal com glicocorticoides, especificamente a betametasona, embora eficiente com relação à maturação pulmonar (Jobb & Soll, 2003) demonstrou promover alterações sobre a função cerebral e neuroendócrina assim como supressão da adrenal materna e fetal em diferentes espécies (Matthews, 2000; Souza *et al.*, 2001).

Foi observado que estes hormônios possuem papel chave na reprogramação intrauterina, induzindo mudanças permanentes nos sistemas fisiológicos por meio de alterações nas concentrações hormonais, expressão celular de receptores, enzimas, canais iônicos, transportadores e várias proteínas constituintes dos tecidos fetais, além de alterar fatores de crescimento e as vias de sinalização celular (Fowden & Forhead, 2004).

Os glicocorticoides interferem diretamente nas concentrações hormonais, devido ao fato de alterar a produção e secreção de vários hormônios, tais como estrógeno, insulina, gastrina, neuropepitídeo Y, angiotensina II, T3, noradrenalina e adrenalina, tanto pela placenta quanto pelas glândulas fetais. Além disso, os glicocorticoides ainda regulam a quantidade de receptores hormonais e a atividade de várias enzimas envolvidas na ativação e inativação destes hormônios nos tecidos fetais (Manojlović-Stojanoski *et al*., 2012).

Deve-se entender que a atividade biológica dos glicocorticoides é determinada pela fração biologicamente ativa, ou seja, pela fração livre encontrada no plasma, já que as ligadas a proteínas plasmáticas tornam-se inativas. Normalmente a concentração livre de glicocorticoides aumenta à medida que a concentração administrada aumenta (Gayrard *et al.*, 1996). Assim, a potência dos glicocorticoides em um sistema biológico depende de sua afinidade com os seus receptores, e no caso da betametasona é 25 vezes maior que o cortisol (Buttgereit *et al.*, 2000). Além disso, enquanto os glicocorticoides endógenos apresentam meia-vida biológica de 8-12 horas, os glicocorticoides sintéticos dexametasona e betametasona variam entre 36 a 54 horas (Manojlović-Stojanoski *et al.*, 2012) o que pode sinalizar seus possíveis efeitos sobre a reprogramação fetal.

Bi *et al*. (2014) observaram que o aumento nos níveis de estresse oxidativo em rins da prole de ovelhas expostas durante os DG 80 e 81 à betametasona devido a alterações nas concentrações de angiotensina II. Além disso, sabe-se que a exposição à betametasona durante a vida intrauterina interfere diretamente no crescimento fetal, incluindo redução na expressão de fatores de crescimento ligados a insulina (IGF-2; Manojlović-Stojanoski *et al*., 2012).

Entre os efeitos observados após a exposição pré-natal a um glicocorticoide, destaca-se a redução do peso ao nascimento, primeiro indício de reprogramação fetal (Braun *et al*., 2015), assim como o aumento do risco de doenças cardiovasculares, metabólicas e psicológicas (Drake *et al*., 2011). Nos últimos anos, estudos tentam demonstrar a influência destes tratamentos com uma abordagem intergeracional, salientando o impacto desta terapia durante toda uma descendência (Crudo *et al*., 2012; Moisiadis & Matthews, 2014).

Drake *et al.* (2011), seguindo modelo de reprogramação multigeracional, mostraram alterações nos pesos dos fetos de ratas expostas ao glicocorticoide dexametasona durante o período intrauterino (DG 15 ao 20), tanto na primeira quanto na segunda geração. Além disso, Drake *et al*. (2004) demonstraram que a exposição *in utero* a dexametasona afetou além do peso dos filhotes, a expressão de fatores de crescimento ligados à insulina e de proteínas no fígado tanto na primeira quanto na segunda geração.

Iqbal *et al.* (2012) por sua vez, constataram que a exposição a betametasona no período pré-natal de porquinhos da índia (DG 40/41, 50/51 e 60/61) levaram a alterações transgeracionais no eixo hipotalâmico-hipofisário-adrenal. Esses efeitos foram demonstrados pela redução na resposta ao estresse e deficiência de sinalização da hipófise para a glândula adrenal.

Crudo *et al*. (2012) observaram, na prole de porquinhos da índia tratadas durante o período intrauterino (DG 40/41 e 50/51), alterações no padrão de metilação de vários genes e, essas alterações foram transmitidas para a geração seguinte, demonstrando que a terapia antenatal pode afetar tanto a primeira geração quanto a geração seguinte.

Portanto, fica claro o impacto da exposição de glicocorticoides na reprogramação fetal, uma vez que promove alterações permanentes sobre vários sistemas fisiológicos tanto da F1 quanto da próxima geração (Fowden & Forhead, 2004; Drake *et al.*, 2011; Iqbal *et al.*, 2012). Contudo, há poucos estudos sobre a influência direta da exposição pré-natal à glicocorticoides no sistema genital, tanto masculino quanto feminino, visto que a corticoterapia antenatal, principalmente em roedores, ocorre exatamente durante o estabelecimento do eixo hipotalâmico-hipofisário-gonadal, o desenvolvimento das gônadas e dos demais órgãos reprodutores (Pereira *et al.*, 2003; Pereira & Piffer, 2005; Piffer *et al.*, 2009a/b).

2.4 – IMPACTO DA CORTICOTERIAPIA SOBRE O DESENVOLVIMENTO DO SISTEMA GENITAL

Foi observado que o aumento na concentração de glicocorticoides durante a terapia antenatal interfere diretamente no controle do eixo hipotalâmico-hipofisário-gonadal. Primeiramente, o aumento de glicocorticoides acarreta em uma diminuição na liberação do GnRH e, consequentemente, diminuição da secreção hipofisária de gonadotrofinas, tanto FSH quanto LH (Sapolsky *et al*, 2000; Alheira & Brasil, 2005; Gore *et al*., 2006).

Além disso, o aumento nas concentrações de glicocorticoides interfere diretamente na concentração de testosterona sérica, uma vez que inibe a atividade enzimática de biossíntese deste hormônio, levando a diminuição das taxas de secreção de testosterona pelas células de Leydig (Ward & Weisz, 1984; Page *et al.*, 2001; Hardy *et al.*, 2005).

Como dito anteriormente, a secreção de testosterona, principalmente nos períodos finais de desenvolvimento fetal, é fundamental para os processos de masculinização e defeminização do hipotálamo. Pereira *et al.* (2003a) observaram que a exposição intrauterina a hidrocortisona nos dias gestacionais 18 e 19 interfere nestes dois processos e, Pereira &

Piffer (2005) atestaram uma incompleta masculinização do hipotálamo seguido de danos sobre a contratilidade da vesícula seminal na puberdade ou na vida adulta de ratos expostos *in utero* a hidrocortisona (DG 17, 18 e 19). Além disso, Yazawa *et al*. (2000) observaram indução de apoptose das células germinativas em ratos após tratamento durante 7 dias consecutivos com dexametasona na idade adulta.

Piffer *et al.* (2009b), em trabalho realizado em colaboração com a nossa equipe, seguindo modelo experimental de exposição intrauterina a betametasona durante os dias gestacionais 12, 13, 18 e 19, observaram alterações nas concentrações de testosterona sérico e na produção espermática da prole masculina durante a fase adulta, assim como mudança na preferência sexual. Estes achados demonstram os danos da exposição a glicocorticoides sobre o desenvolvimento normal do processo de masculinização do hipotálamo de roedores.

Pedrana *et al*. (2008) observaram que a exposição a betametasona *in utero* (DG 104 e/ou 111) afetou diretamente o desenvolvimento dos testículos de ovelhas, diminuindo o tamanho dos cordões testiculares e do espaço intersticial, o que sugere impacto sobre as células de Leydig, uma vez que estas expressam receptores para glicocorticoides.

Outro ponto que merece atenção é o controle que os esteroides exercem sobre a contratilidade e excitabilidade dos órgãos sexuais acessórios, sendo que Pereira & Piffer (2005) observaram que a exposição intrauterina de ratos ao glicocorticoide hidrocortisona (DG 17, 18 e 19) promoveu alterações sobre a resposta contrátil da vesícula seminal destes animais.

Portanto, reconhece-se o impacto da exposição a glicocorticoides, principalmente a betametasona, sobre o desenvolvimento do sistema genital, embora estudos adicionais devam ser conduzidos com o objetivo de clarificar, com base nos períodos de exposição, como cada órgão reprodutor pode ser reprogramado ao longo do desenvolvimento por este fármaco, considerando principalmente os períodos críticos de organogênese do sistema genital.

Justificativa

Sabendo-se do grande emprego dos glicocorticoides em clinica humana e animal, especialmente a betametasona, na corticoterapia antenatal e que a exposição intrauterina a este hormônio em períodos críticos do desenvolvimento de ratos promoveu alterações reprodutivas na prole masculina, justifica-se a realização de um estudo experimental abrangente sobre a influência da reprogramação fetal promovida pela betametasona em parâmetros reprodutivos de ratos machos e fêmeas.

Objetivos

OBJETIVO GERAL

O presente trabalho teve por objetivo avaliar os efeitos nos parâmetros reprodutivos da prole feminina e masculina de ratos, provocados pela exposição *in utero* à betametasona.

OBJETIVOS ESPECÍFICOS

- Investigar os efeitos tóxicos da betametasona sobre as ratas prenhes durante o período de tratamento (F0);
- Obter parâmetros iniciais de desenvolvimento sexual que possam caracterizar uma possível reprogramação fetal induzida pelo tratamento com a betametasona;
- Avaliar a morfofisiologia dos órgãos reprodutores, com ênfase nas gônadas masculinas e femininas;
- Investigar a atividade contrátil dos órgãos reprodutores frente a exposição à neurotransmissores adrenérgicos e/ou colinérgicos;
- Avaliar o comportamento sexual da prole femina (F1) e masculina (F1 e F2);
- Avaliar os efeitos da betametasona sobre a qualidade espermática dos ratos machos tanto da F1 quanto da F2, e sobre a ciclicidade estral das fêmeas da F1;
- Avaliar os efeitos da betametasona sobre a fertilidade, de machos e fêmeas da F1 quanto de machos da F2;
- Avaliar a influência multigeracional sobre a exposição *in utero* à betametasona no sistema genital masculino.

Desenho experimental

Ratos adultos (n=12, 90 dias de idade, pesando aproximadamente 300 ± 50g) e fêmeas adultas (n=24, 70 dias de idade, pesando aproximadamente 200 ± 50g) da linhagem *Wistar*, provenientes do CEMIB da Unicamp, foram mantidos no Biotério de Pequenos Mamíferos do Departamento de Morfologia do Instituto de Biociências da UNESP de Botucatu, em condições controladas de luminosidade (12 horas de luz/ 12 horas de escuro) e temperatura (média de 23*C). Os animais receberam água e ração para roedores à vontade. Os animais utilizados neste experimento foram mantidos de acordo com os Princípios Éticos em Experimentação Animal, adotados pela Sociedade Brasileira de Ciência em Animais de Laboratório sob protocolo número 451 junto à Comissão de Ética em Experimentação Animal do Instituto de Biociências de Botucatu.

Os acasalamentos foram realizados durante o período escuro do ciclo, colocando-se duas fêmeas na caixa do macho, e o dia um de prenhez (DG1) foi determinado pela presença de espermatozoides em esfregaços vaginais de fêmeas em estro sendo posteriormente mantidas em gaiolas individuais.

O estudo foi realizado de acordo com delineamento experimental descrito a seguir (Fig.2). As fêmeas prenhes, previamente pesadas, foram alocadas em dois grupos experimentais: controle (n=11) e tratado (n=13) com 0,1mg/kg de betametasona (21-fosfato dissódico de betametasona diluído em veículo; Sigma-Aldrich, St Louis, MO). Essa dose foi escolhida com base na dose utilizada em tratamento materno seguindo adaptações para roedores (Piffer *et al*., 2009a, b). O período de tratamento que compreende os DG 12, 13, 18 e 19 foram escolhidos com base na corticoterapia materna adaptada por Souza *et al*. (2001) e que provocou alterações na diferenciação sexual hipotalâmica na descendência masculina em ratos (Pereira *et al*., 2003; Pereira & Piffer, 2005; Piffer *et al*., 2009a/b). As fêmeas do grupo tratado receberam as quatro doses de betametasona, via intramuscular, diluída em solução fisiológica (veículo) enquanto as fêmeas do grupo controle receberam apenas o veículo nos mesmos dias gestacionais. Após o nascimento, o número de filhotes por ninhada foi reduzido para oito, sendo 4 filhotes do sexo masculino e 4 filhotes do sexo feminino.

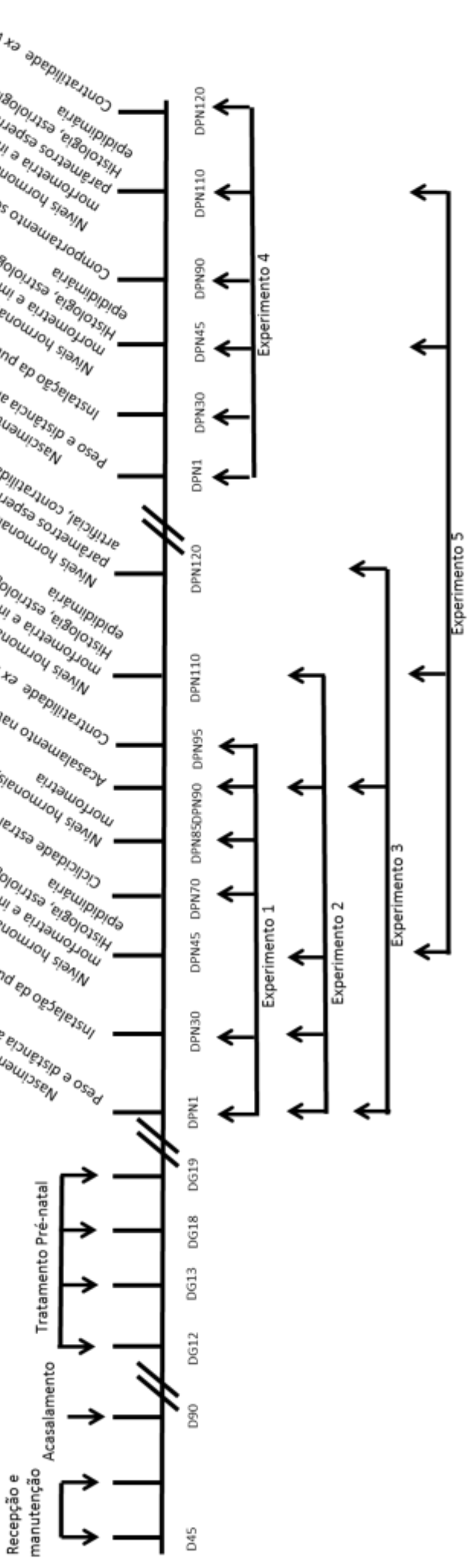


Figura 2: Delineamento experimental com suas respectivas etapas e técnicas utilizadas. D (dia), DG (dia gestacional) e DPN (dia pós-natal). As setas indicam os dias em que as análises foram realizadas. Setas sobre o mesmo dia indicam avaliações concomitantes entre os experimentos.

O estudo foi dividido em cinco experimentos (que correspondem aos capítulos subsequentes). No primeiro experimento, a prole feminina (F1) foi mantida para a avaliação do desenvolvimento sexual inicial, ciclicidade estral, histopatologia ovariana, morfometria e contratilidade uterina, comportamento sexual e fertilidade. No segundo experimento, as ratas prenhes tratadas com betametasona ou veículo foram monitoradas quanto a ingestão de alimentos e ganho de peso, e a prole masculina (F1) foi mantida para avaliação dos efeitos causados durante o desenvolvimento sexual inicial, morfogênese testicular e fertilidade. No terceiro experimento, foram avaliados o impacto sobre o comportamento sexual, qualidade espermática e fertilidade dos machos da F1 na idade adulta. No quarto experimento, os filhotes machos (F2) provenientes do acasalamento da geração F1 com fêmeas não tratadas foram submetidos aos mesmos protocolos e procedimentos utilizados na avaliação da geração F1 masculina, a fim de verificar os possíveis efeitos multigeracionais da betametasona nas funções reprodutivas, qualidade espermática e fertilidade. No quinto e último experimento, os epidídimos provenientes da prole masculina, tanto da geração F1 quanto da geração F2, em ambas as idades (púberes e adultos) foram utilizados para avaliação dos efeitos causados na morfogênese epididimária e seu possível impacto multigeracional.

Experimento 1

1- DPN 01: No DPN 01, foi realizada a redução de ninhada para 8 filhotes (4 machos e 4 fêmeas) e foram obtidos o peso ao nascimento e a distância anogenital da ninhada feminina.

2- DPN 13: No DPN 13, foi realizada a contagem de mamilos na ninhada feminina.

3- DPN30: A partir dos 30 dias de idade, as fêmeas de cada ninhada (F1) foram avaliados quanto à abertura vaginal, sinal físico externo da instalação da puberdade, e primeiro estro.

4- DPN70: A partir do DPN 70, durante 15 dias, foram realizados o monitoramento da ciclicidade estral de cada ninhada.

5- DPN85: Aos 85 dias de idade, uma fêmea em estro foi eutanasiada por decapitação, após narcose induzida em câmara de CO2, amostras de soro foram coletadas para dosagens

hormonais, foram pesados os órgãos reprodutores, rim, adrenal e hipófise, e os ovários e útero foram fixados para histologia e morfometria, respectivamente.

6- DPN95: uma fêmea em estro de cada ninhada (F1) foi destinada ao teste de comportamento sexual seguido de fertilidade natural.

7- DPN90: uma fêmea em estro de cada ninhada foi eutanasiada por decapitação e um segmento de aproximadamente 2 mm da região mediana do corno uterino foi utilizado para registro da tensão desenvolvida.

Experimento 2

1- Período gestacional: A partir da constatação de prenhez até o DG21, o ganho de peso materno e o consumo de ração foram monitorados.

2- DPN 01: No DPN 01, foi realizada a redução de ninhada para 8 filhotes (4 machos e 4 fêmeas) e foram obtidos o peso ao nascimento e a distância anogenital dos filhotes machos.

3- DPN30: A partir dos 30 dias de idade, os machos de cada ninhada (F1) foram avaliados quanto à separação prepucial, sinal físico externo da instalação da puberdade.

4- DPN45: Aos 45 dias de idade, um macho de cada ninhada (F1) foi morto por decapitação, após narcose induzida em câmara de CO2, com o objetivo de avaliar os seguintes parâmetros do sistema genital masculino: foi realizada a coleta do sangue dos vasos cervicais rompidos para posteriores dosagens hormonais, testículo, epidídimo e glândulas anexas foram pesados, e os testículos direitos foram destinados a análise histopatológica, morfométrica e de imunohistoquímica para proteínas Cx43 e PCNA.

5- DPN90: Aos 90 dias de idade, um macho de cada ninhada (F1) foram destinados ao teste de fertilidade natural.

6- DPN110: Aos 110 dias de idade, um macho de cada ninhada (F1) foi morto por decapitação, após narcose induzida em câmara de CO2, e destinados as mesmas análises descritas na DPN 45.

Experimento 3

1- DPN 01: No DPN 01, foi realizada a pesagem dos filhotes machos utilizados neste experimento, assim como foi obtida a distância anogenital.

2- DPN90: Um macho de cada ninhada (F1) foi submetido à avaliação de comportamento sexual. Este macho foi mantido isoladamente por 30 dias para a recuperação total de suas reservas espermáticas, e posteriormente utilizado para a avaliação dos seguintes parâmetros do sistema genital masculino: coleta de sangue dos vasos cervicais para posteriores dosagens hormonais; peso de órgãos reprodutores, da cauda do epidídimo esquerdo foi retirada uma amostra de espermatozoides utilizada na técnica de inseminação artificial *in utero*, motilidade e morfologia espermática, e então esta cauda foi congelada a -20ºC para determinação do número de espermatozoides e tempo de trânsito espermático, juntamente com a região da cabeça/corpo do epidídimo esquerdo; o testículo esquerdo de cada rato foi retirado e congelado a –20^{o}C para posterior determinação do número de células germinativas.

3- DPN120: Aos 120 dias de idade, um macho de cada ninhada (n=5/grupo) foi sacrificado por decapitação e destinado aos ensaios de reatividade farmacológica *ex vivo* das glândulas sexuais acessórias.

Experimento 4

Nestes experimentos, foram realizados os mesmos procedimentos e análises descritas nos experimentos 1 e 2, com a finalidade de verificar os possíveis efeitos multigeracionais nas funções reprodutivas, qualidade espermática e fertilidade masculina dos ratos da segunda geração (F2) provenientes do acasalamento da geração F1.

Experimento 5

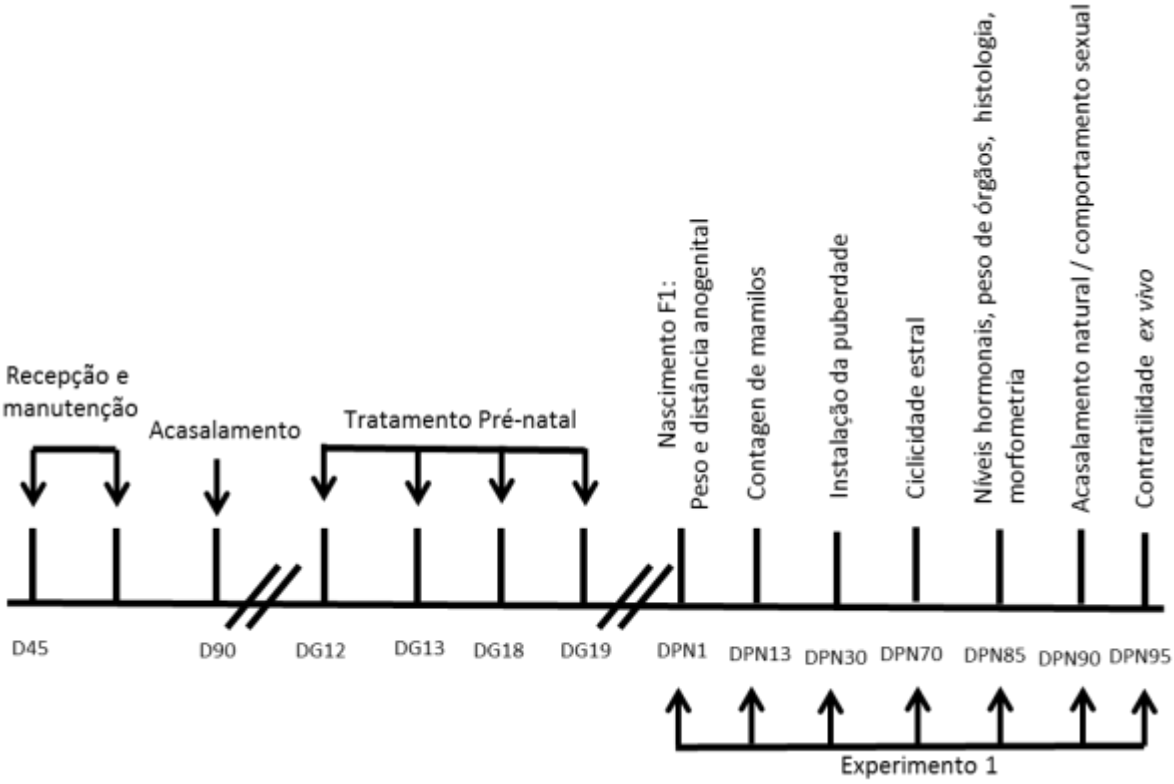
1- DPN45: Aos 45 dias de idade, o epidídimo de um macho de cada ninhada (F1 e F2) morto por decapitação, após narcose induzida em câmara de CO_2 (experimento 1 e 3) foi coletado, fixado em Bouin e processado para análises histopatológica e de imunohistoquímica para proteínas Cx43 e p63.

2- DPN110: Aos 110 dias de idade, o epidídimo direito de um macho de cada ninhada (F1 e F2) morto por decapitação, após narcose induzida em câmara de CO_2, também do experimento 1 e 3, foi destinado as análises histopatológicas, estereológicas e de imunohistoquímica para proteínas Cx43 e p63. O epidídimo esquerdo proveniente do mesmo animal, foi utilizado para a realização do ensaio de reatividade farmacológica do ducto epididimário.

Capítulo 1

Este trabalho deu origem ao manuscrito “REPRODUCTIVE DISORDERS IN FEMALE RATS AFTER PRENATAL EXPOSURE TO BETAMETHASONE” que está aceito para publicação no periódico “Journal of Applied Toxicology” (fator de impacto: 2,72), http://onlinelibrary.wiley.com/wol1/doi/10.1002/ jat.3457/abstract.

Neste trabalho foram demonstrados os efeitos promovidos pela exposição intrauterina à betametasona sobre o desenvolvimento da prole feminina, seguindo o desenho experimental abaixo, com ênfase na ciclicidade estral, morfofisiologia uterina e fertilidade.



O tratamento resultou na redução do peso da ninhada ao nascimento, atraso na instalação da puberdade, redução no número de estros, aumento do peso uterino, nas concentrações de LH, prolongamento do ciclo estral, na contratilidade uterina e na área do miométrio. Em contrapartida, houve uma redução nas concentrações de FSH, no número de lordoses feitas durante o comportamento sexual, na área do endométrio culminando na redução da fertilidade destas fêmeas, expressa pelo aumento no número de reabsorções fetais.

REPRODUCTIVE DISORDERS IN FEMALE RATS AFTER PRENATAL EXPOSURE TO BETAMETHASONE

Running title: Prenatal betamethasone exposure and female reproductive disorders

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ABSTRACT

Betamethasone is the drug of choice for antenatal treatment, promoting fetal lung maturation and decreasing mortality. Previous studies in rats reported male programming and alteration in sperm parameters and sexual behavior following intrauterine betamethasone exposure. The impact on the female reproductive development is not known. In this study rat female offspring was assessed for sexual development, morphophysiology of the reproductive tract and fertility after maternal exposure to 0.1 mg/kg of betamethasone or vehicle on gestational days 12, 13, 18 and 19. The treatment promoted reduction of litter weight on postnatal day 1, morphological masculinization in females, delay in the age of puberty onset, reduction in estrus number, increase in estrous cycle length and also increase in LH serum levels and uterus weight. The females from betamethasone group showed an increase of myometrial uterine area and decrease in endometrial uterine area. These animals also performed less lordosis during the sexual behavior test and showed impaired reproductive performance. The uterus showed higher contraction in the treated group as shown by a pharmacological assay. In conclusion, prenatal betamethasone exposure promoted, in rats, female masculinization, altered sexual development and reproductive parameters.

Additional keywords: betamethasone, female rat, reproductive disorders, sexual development, estrous cycle, sexual behavior, reproductive performance.

INTRODUCTION

During intrauterine life several alterations occur to promote the correct fetal development, and one of the most important events is the increase of endogenous fetal glucocorticoids (Barker, 1998; Fowden & Forhead, 1998; Moisiadis & Matthews, 2014). This increase provides a critical developmental trigger, one that is essential for normal maturation of the fetal lung and developmental switching in many other organ systems, including the thyroid, kidney, brain and pituitary (Fowden & Forhead, 1998; Moisiadis & Matthews, 2014). Pregnant woman at risk of preterm delivery needs to be treated with exogenous glucocorticoids to promote the same results as in the normal intrauterine conditions for fetal development (Gyamfi-Bannerman et al, 2016; Lindsley et al, 2015).

Betamethasone, a synthetic glucocorticoid, is considered the glucocorticoid of choice for this antenatal treatment (Alexander et al, 2016; Lindsley et al, 2015) being used for pregnant women between 24 and 34 weeks of gestation (Jobe & Soll, 2004; Rayburn et al, 1997; Wapner, 2013), promoting fetal lung maturation and thus reducing the incidence of respiratory distress syndrome, neonatal mortality and morbidity (Gyamfi-Bannerman et al, 2016; Lindsley et al, 2015). However, the prenatal exposure to betamethasone demonstrated several systemic long-term deleterious effects on offspring in different experimental protocols (Alexander et al, 2016; Belanoff et al, 2001; Bertram & Hanson, 2002; Pascual et al, 2014; Su et al, 2015).

During critical period of *in utero* sexual development of rodents, which corresponds to gestational days (GD) 12 to 20, important morphological and functional alterations occur in the fetus, including sex differentiation (Corbier et al, 1992; Gogan et al, 1981; Ward, 1972; Yun et al, 2016). It is known that rats sexual differentiation is dependent on the hormonal status during the last period of gestation (Wolf et al, 2002). Then, programmed rats exposed to betamethasone during intrauterine development (GD 12, 13, 18 and 19) shows a reduction in the testosterone levels and decrease in sperm quality, resulting in feminization of the male offspring (Piffer et al 2009a; Piffer et al, 2009b). However, on female reproductive system there is no information demonstrating any impact of intrauterine treatment with betamethasone. Thus, this study aimed to evaluate the effects of prenatal exposure to betamethasone on the initial sexual development and, at adulthood, morphophysiology of the female reproductive tract, fertility and reproductive performance.

MATERIALS AND METHODS

Animals and Treatment

Male (90 days old/ 300 – 350 g) and female (90 days old/ 225 – 230 g) Wistar rats were obtained from the Multidisciplinary Center for Biological Investigation, State University of Campinas and maintained under controlled conditions (25°C, 30% air humidity, 12/ 12-h light/dark cycle) with food and water available *ad libitum*. Two nulliparous female rats were mated with one male, during the dark cycle of the photoperiod. The detection of sperm in the vaginal smear of rats in estrus was considered as gestational day 1 (GD 1). Pregnant and lactating rats were maintained in individual cages. The experimental procedures used in this study were approved by the local Ethics Committee for the Use of Experimental Animals of the University of São Paulo State (protocol number 451-CEEA) in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health). All euthanasia was performed by decapitation following CO_2 asphyxiation.

Experimental groups

Pregnant female rats were randomly assigned to two experimental groups (n=11-13/group): control, treated with saline (vehicle) alone and treated (0.1 mg/kg; Betamethasone 21-phosphate disodium diluted in vehicle; Sigma-Aldrich, St Louis, MO). The dosing paradigm used in the present work has been previously adopted (Borges et al, 2016; Borges et al, 2017; Piffer et al, 2009a; Piffer et al, 2009b; Souza et al, 2001) and takes into account the beginning of the critical window of rat reproductive tract development (Crain et al, 2008; Davis et al, 1996; McIntyre et al, 2002; Wilson et al,2004) and fetus brain sexual differentiation (Pereira et al, 2003; Pereira et al,2005; Piffer et al, 2009a; Piffer et al, 2009b). Rats received an intramuscular injection of vehicle or betamethasone on days 12, 13, 18 and 19 of pregnancy. After birth, on post-natal day 1 (PND 1), the number of pups per litter was reduced, randomized and redistributed in order to have 8 pups (with 4 or 3 females) per lactating female. The offspring were weaned at PND 21 and housed in separate cages (n=3 females per cage). For each set of experiments, one female rat from each litter was sampled in order to prevent potential variation due to litter effects.

Anogenital Distance and Number of Nipples/areola

Female offspring were weighed and anogenital distance (AGD, the distance from the anus to the genital tubercle) was measured at PND 1 in four/three females per litter and the

mean was taken per litter and used to calculate the group mean (n = 11-13 per group) using Vernier calipers. AGD was normalized against the cube root of body weight (Gallavan et al, 1999).

On PND 13, the number of areolas was recorded. Observations were scored based on the presence or absence of a nipple bud or a discoloration of the skin surrounding the nipple (Mylchreest et al, 2000). This data was also expressed as litter mean (the mean from four/three females per litter was taken and used to calculate the group mean).

External Signs of Puberty Onset

On PND 30, all females were evaluated daily for vaginal opening (VO). The day of full opening of the vaginal orifice was recorded. On the day of VO, female rats were weighed and daily vaginal fluid was collected as described by Marcondes et al (2002), to detect the day of first estrus (predominance of cornified epithelial cells). Ten microliters of 0.9% saline was instilled into the vagina and subsequently aspirated. Vaginal fluids were placed in a slide and analyzed under a light microscope (Leica MicroStar IV) at 200×magnification. Data was expressed as litter mean (the mean from three females per litter was taken and used to calculate the group mean).

Estrous Cycle

The estrous cycle was assessed on the basis of vaginal smears collected every morning over a period of 15 days (PND 70 to 85) in the same animals that VO was recorded. The samples were observed under a light microscope and estrous cycle phases classified as diestrus, proestrus, estrus and metestrus. A proestrus phase consists of a predominance of nucleated epithelial cells while estrus consists of anucleated cornified cells. A metestrus phase consists of the same proportion among leukocytes, cornified, and nucleated epithelial cells and the diestrus phase primarily consists of a predominance of leukocytes (Marcondes et al, 2002). The estrous cycle duration was calculated as the number of days between one estrus phase to the next and the number of estrous cycle during the assay.

Collection and Analysis of Organs

On PND 85, one female rat from each litter was euthanized by CO_2 inhalation followed by decapitation in estrus phase, for collection of blood samples from cervical vessels and determination of the weight of organs including pituitary, liver, kidneys, adrenals, uterus

(with fluid) and ovaries. For histological evaluation of uterus and ovaries, they were fixed in Bouin's solution, dehydrated in ethanol, embedded in Paraplast® and sectioned in 5 μm (three sections per animal, each section were collected in a distance of 10 more sections), and stained with hematoxylin and eosin (H&E). In each ovary, ovarian follicles and corpora lutea were counted. Follicles were classified according to Guerra et al (2010). Primordial and primary follicles were enumerated together; oocytes surrounded by a single layer of either squamous or cuboidal epithelial cells were included. Follicles were classified as pre-antral when containing 2-4 layers of granulosa cells with no antral space. Antral follicles were classified when containing three or more layers of granulosa cells and a defined antral space. Characteristics of atretic follicles included pyknotic granulosa cells, disorganized granulosa cells, degenerating oocyte and detachment from the basement membrane. The numbers of follicles were expressed in percentage of total follicles observed. In the uterus, three sections of the right uterine horns per animal were used to measure the following parameters in Panoramic Viewer Software (3DHistech Ltd): total uterine area (μm^2), myometrial, endometrial and luminal epithelium areas (μm^2), thickness and width index. The areas and perimeters of each region were measured by tracing around the images of the sections with a cursor, and the values were used to calculate the thickness, as described by Mosquete et al (2007), with adaptations. Endometrial and luminal epithelium width index were measured by deriving the width from their respective areas.

Serum hormone levels

Blood samples were allowed to coagulate and the serum obtained by centrifugation (2400 rpm) for 20 min at 4°C and stored at -20°C to determine the concentration of serum follicle-stimulating hormone (FSH), luteinizing hormone (LH), estrogen and progesterone from one female per litter. LH and FSH were measured using specific kits supplied by the National Institute of Arthritis, Diabetes and Kidney Diseases (NIADDK). Progesterone hormone levels determined by the technique of double antibody radioimmunoassay using specified kits of ImmuChemTM Double Antibody supplied by MP Biomedicals, LLC Diagnostic Division Orangeburg , NY. Estradiol levels were measured by ELISA using specific kits of DRG® Estradiol ELISA (EIA- 2693) supplied by DRG International, Inc., USA. All samples were measured within the same assay to avoid the 1 inter-assay errors. Intra-assay variabilities were 3.4% for LH, 2.8% for FSH, and 4% for estradiol and progesterone.

Sexual behavior

During the first estrus after PND 95, one female rat per litter from different experimental groups (n=10/group) were used for the fertility test. Rats were maintained under controlled temperature conditions on an inverted 12-hr light/dark cycle, for at least 7 days, with food and water *ad libitum*. For the evaluation of female sexual behavior, sexually experienced males were allowed to perform 10 mounts on the female and the presence of lordosis was recorded. Results were expressed as the lordosis quotient (LQ, number of lordosis/10 mounts×100) (Beach, 1976). All females were used only once.

Fertility Test and Reproductive Performance

This analysis was performed by natural mating. After the end of sexual behavior test, the same female were maintained with the same male for additional 4 hours. At the end of this period, rats were separated and vaginal smears collected and sperm detection determined to assess GD 01. On GD 21 females were killed by decapitation. After collection of uterus and ovaries the numbers of corpora lutea, implants, resorptions, live fetuses, and dead fetuses were determined. From these results the following parameters were calculated: gestation rate: number of pregnant females/number of inseminated females ×100; fertility potential (efficiency of implantation): implantation sites/corpora lutea ×100; rate of pre-implantation loss: [number of corpora lutea – number of implantations/number of corpora lutea] ×100; rate of post-implantation loss: [number of implantations – number of live fetuses]/number of implantations ×100; and sex ratio: number of male fetuses/number of female fetuses ×100.

Pharmacological Reactivity of Isolated Uterus

Five females per experimental group (one per litter) were weighed and euthanized at PND 90 by decapitation. A ring of median region of the uterus with approximately 2 mm of height were prepared for the *in vitro* tension recording as described below. Tissues were mounted in 10 ml organ baths in a modified Tyrode's solution at 30ºC under 1g resting tension and allowed a 30 minutes equilibration period. After the resting period, the tissues were repeatedly challenged with 80 mM KCl every 30 minutes until two reproducible contractions were obtained. After that, 100 µl of norepinephrine 10^{-4} M (an adrenergic neurotransmitter) was added in the bath and 5 min of tension developed was recorded. The tissue was washed 4 times and 30 minutes later new recorded was done with 100 µl of carbachol 10^{-4} M (a muscarinic neurotransmitter).

Statistical Analysis

Data are presented as mean ± standard error of mean (SEM) or median and interquartile range. A Student T-test was used for comparison of parametric variables. Non-parametric data were compared using a Mann-Whitney test. Differences were considered significant when $p \leq 0.05$. Statistical analyses were performed using the GraphPad InStat software (version 5).

RESULTS

Betamethasone group (BM) or vehicle did not show visible signs of toxicity during gestational treatment. On PND 1 the offspring from betamethasone group had significantly reduced body weight compared to the control (C) group (BM = 6.80 $\pm$ 0.11g and C = 7.55 $\pm$ 0.13g, $p \leq 0.001$; Figure 1A), and there was a significant increase in the relative AGD (BM = 1.22 $\pm$ 0.02 and C = 1.15 $\pm$ 0.02, $p \leq 0.05$; Figure 1B). At PND 13 evaluation of number of nipples also revealed an increase in this parameter on betamethasone-exposed group (BM = 12.25 and C = 12, $p \leq 0.05$; Figure 1C).

The assessment of the external signs of puberty onset, VO (BM = 39.66 $\pm$ 1.31 days and C = 34.67 $\pm$ 0.40 days, $p \leq 0.01$; Figure 2A) and first estrus (BM = 42.73 $\pm$ 1.23 days and C = 35.31 $\pm$ 0.48 days, $p \leq 0.0001$; Figure 2B) demonstrated that betamethasone treatment interfered in both parameters. Moreover, 22% of the females from betamethasone exposed mothers showed incomplete vaginal opening by PND 70 (Figure 2C). In the same manner the estrous cycle regularity evaluated from PND 70 to PND 85 also showed that the cycle length was significantly increased and number of cycles significantly reduced during the period of evaluation of betamethasone group (Table 1).

At PND 85 there were no significant differences in body weight and absolute weight of most organs. However, animals exposed during the intrauterine life to betamethasone showed a significant increase in the absolute uterus weight (Table 2). Regarding hormonal analysis, we observed a significant increase only in LH levels (BM = 1.49 $\pm$ 0.51ng/mL and C = 0.56 $\pm$ 0.12ng/mL, $p \leq 0.05$; Figure 3) of treated group.

A light microscopy analyses of ovary and uterus was carried in order to evaluate the reproductive organs histology and reveal no morphological changes related to treatment (data not shown). The percentage of ovarian follicles and corpora lutea were similar between the groups (Figure 4A). However, the uterine evaluation revealed a decreased endometrium area (BM = 2.62 $\pm$ 0.07mm^2 and C = 2.88 $\pm$ 0.64 mm^2, $p \leq 0.05$; Figure 4B) and simultaneously an increased myometrium area in the betamethasone group (BM = 3.69 $\pm$ 0.08 mm^2 and C = 3.22 $\pm$ 0.14 mm^2, $p \leq 0.05$; Figure 4B).

At PND 85 sexual behavior was assessed, followed by fertility evaluation. The frequency of lordosis was significantly reduced in the betamethasone group when compared with the control group (Table 3). Although fertility potential remained unchanged, betamethasone group presented an increase in post-implantation loss and in number of

resorptions (Table 3). Furthermore, a reduction on fetus weight in the betamethasone group was also observed.

Assays for norepinephrine and carbachol allowed evaluation of uterine developed tension at PND 90. In this evaluation, a significant increase of tension during 5 minutes was observed in the betamethasone group only for carbachol (BM = 96.64 $\pm$ 15.26mN and C = 53.92 $\pm$ 10.38mN, $p \leq 0.05$; Figure 5).

DISCUSSION

This work is part of a comprehensive study we are performing concerning the effects of prenatal betamethasone exposure on the female and male offspring of rats. In males, we reported delay in puberty onset, testicular dysfunction and altered sperm quality and fertility, and part of these injuries were seen in the next generation (Borges et al, 2016; Borges et al, 2017; Borges et al, submitted manuscript). In females, as shown in the present work, the main findings were delayed sexual maturation, alteration in estrous cycle and LH levels, deleterious effects on fertility and uterus morphology and physiology.

Sexual differentiation of rodent brain is dependent upon hormonal exposure during a critical periods beginning in late gestation and ending in early neonatal life (Davis et al, 1996). Proper sexual differentiation of the offspring depends upon an increase in testosterone levels for males and absence of this hormone for females (Schwarz & McCarthy, 2008; Ward, 1972). It is known that female masculinization can be promoted after *in utero* exposure to several compounds, such as androgens, stressors or glucocorticoids (Gandelman & Rosenthal, 1981; Wolf et al, 2002). Reduction in birth weight, nipples count and increase in AGD indicate partial male phenotype in females (Gandelman & Rosenthal, 1981; Guerra et al, 2014; Wilson et al, 2002; Wolf et al, 2002). In our study masculinization was confirmed by the larger relative AGD at PND 1 in the betamethasone female pups compared to controls. However, in this same females the number of nipples increased, contradicting the literature assumption that female virilization promotes reduction on nipples count (Gandelman & Rosenthal, 1981; Wolf et al, 2002). We postulate that the alteration in the number of nipples indicates possible hormonal dysfunction caused by prenatal betamethasone exposure. Lower lordosis quotient, observed in the adult females from treated group, demonstrated the long-term impact of virilization on sexual behavior. These findings are in agreement with those from Piffer et al (2009b), which demonstrated the occurrence of an altered partner preference and sexual behavior in the male offspring exposed prenatally to betamethasone.

Fetal programming occurs when the fetus is submitted to an adverse prenatal environment that permanently programs its physiology and increases the risk of developing disorders in adulthood (Barker & Thornburg, 2013; Habbout et al, 2013; Seckl & Holmes, 2007). Although molecular mechanism was not explored, herein, the reduction in birth weight of females at PND 1 and compromised puberty onset, could be effects of a possible fetal programming after prenatal betamethasone exposure (Braun et al, 2015; Drake et al, 2011; Moisiadis & Matthews, 2014). The establishment of puberty in female rats results from a

cascade of events that leads to ovarian maturation (Andrews &Ojeda, 1981). After that, estradiol blood levels increase, in addition to vaginal opening and first estrus occurrence, considered the external indicators of puberty installation (U.S. Environmental Protection Agency [EPA], 1996). The delayed pubertal onset detected in the betamethasone group may be due to a dysfunction of the hypothalamus–pituitary–gonadal (HPG) axis (Zambrano et al, 2013). Interestingly, Smith et al (2000) and Ristic et al (2008) observed that dexamethasone *in utero* exposure also delayed puberty onset in female offspring.

Monitoring estrous cyclicity, which is under HPG axis, is a way of evaluate the female capacity of reproduction and the impact on reproductive function (Shaikh et al, 1971).It is also known that stress and glucocorticoids exposure can affect estrous cyclicity (Everett et al, 1989; Hardy et al, 2005). In the present work female prenatal exposure to betamethasone promoted disruption in estrous cyclicity, shown by the increase in estrous cycle length and decrease in the number of cycles. The increase in LH levels with not impact on estradiol levels reinforce the dysfunction on HPG axis, possibly affecting female reproductive capacity (Camille Melon & Maguire, 2015; Risma et al, 1995).

Follicular formation in the fetal ovary and activation of follicles during the postnatal period are potential targets of hormonal alterations (Crain et al, 2008). Ovarian histological analysis demonstrated no marked morphological alterations after betamethasone treatment. The uterus, on the other hand, showed a significant difference between the areas of myometrium and endometrium in the treated group, that also showed an increase of post-implantation loss and resorptions in the reproductive performance assessment, without alteration in fertility potential. It is possible that this result can be correlated with a lower endometrium tissue area, which might have compromised embryo implantation, placental and fetus development, leading to decreased fetus weight (Ingaramo et al, 2006; Jauniaux et al, 2006) as observed in the present work and also an increase of myometric tissue area, promoting more tension inside the uterus and exacerbated contractility activity of this organ.

Uterine rhythmic contractions are essential for the reproductive functions in all phases of the cycle (Dodds et al, 2015). The transport of eggs and sperm, the proper placement of the embryo for implantation, the maintenance of the fetus during its development and the expulsion of fetus or debris are organized by these contractions and, any increase in this rhythmus can promote uterine abnormal function and be related to infertility and implantation failure (Dodds et al, 2015). Thus, in the present work, the increased post-implantation losses by the number of resorptions observed in the betamethasone group could be correlated to the increased contractility activity of the uterus to muscarinic neurotransmitters. Moreover, the

increase in myometrial area, as discussed above, might lead to more vigorous uterine contractions, contributing, at least in part, to the increased of post-implantation losses. In fact, we can not determine, with the evaluations performed in the present study, the exact cause of the increase in the number of resorptions in the betamethasone group, since gamete DNA damage can also impair the embryo/fetal development (Codrington et al, 2004; Kedia-Mokashi et al, 2011). In fact, in a previous work we reported sperm DNA damage in rats exposed to betamethasone using the same experimental paradigm (Borges et al, submitted manuscript). Further studies are needed to clarify the molecular mechanisms involved.

In the present work, the organ weights did not change after intrauterine exposure to betamethasone, indicating possible absence of general toxicity (Clegg et al, 2001). However, there was a significant increase in uterus weight, although the total area of the organ remained unchanged. We speculate, but it remains to be elucidated, that the increase in weight may be due to, at least in part, to an increase in luminal uterus fluid.

We recognize strengths and weaknesses in our work. First, it reports new data on the effects of gestational exposure to betamethasone on critical windows of reproductive and sexual development of female rat. Additionally, it reinforces the reproductive disorders exerted by betamethasone after prenatal exposure, since similar effects were seen in the male and female rat offspring. However, our dosing regimen in the rat covers a wider developmental time span than in humans, since it covers both the critical period of reproductive organ development (GD 12, 13) and the period of reproductive organ and brain sexual maturation (GD 18, 19), whereas weeks 24-34 in humans receiving betamethasone only cover the period of sexual maturation and myometrium and endometrium differentiation (İşcan et al, 2017; Konishi et al, 1984; Gray et al, 2001). Even so, important findings are presented which warrant further investigations.

In conclusion, prenatal betamethasone treatment promoted, in this experimental model, female reproductive disorders, compromising initial sexual development and reproductive performance at adulthood. Taking into consideration these experimental results and recognizing the importance of antenatal betamethasone therapy, further studies are encouraged to investigate possible late adverse reproductive outcomes in women exposed to betamethasone *in utero*.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

FIGURE LEGEND

Figure 1: Body weight **(A)**, relative anogenital distance **(B)** and number of nipples **(C)** of female offspring from the betamethasone and control groups (n=10 litters/group). Values are expressed as mean ± SEM. * $p \leq 0.05$ and *** $p \leq 0.001$ (Student´s t-test for A and B, Mann-Whitney test for C).

Figure 2: Day of vaginal open **(A)** and first estrus **(B)** of female offspring from betamethasone and control groups (n=10 litters/group). Values are expressed as mean ± SEM. ** $p \leq 0.01$ and *** $p \leq 0.001$ (Student´s t-test). **(C)** Representative picture of one betamethasone-exposed female rat at PND 75 presenting incomplete vaginal opening.

Figure 3: Hormonal serum levels from the female offspring of betamethasone and control groups in PND 85. Values are expressed as mean ± SEM. * $p \leq 0.05$ (Student's t-test).

Figure 4: (A) Percentage of primordial and primary follicles (PP), pre-antral follicles (PA), antral follicles (AN), atretic follicles (AT) and corpora lutea (CL) and **(B)** Area of epithelial (Ep), endometrial (En), myometrial (Mio) and perimetrial (P) uterus from female offspring from betamethasone and control group (n=7/group). Values are expressed as mean ± SEM. * $p \leq 0.05$ (Student's t-test).

Figure 5: (A) Tension developed in 5 min in the presence of norepinephrine and carbachol in female offspring of betamethasone and control groups (n=5/group). Values are expressed as mean ± SEM. * $p \leq 0.05$ (Student's t-test). **(B)** Representative curve of tension developed in the presence of norepinephrine (NE) and carbachol (Cch) during 5 min.

1 **TABLES**

Table 1 - Analysis of the estrous cycle (15 consecutive days) from female offspring exposed to betamethasone or vehicle during the gestational period†.

Parameters	Control (n=10)	Betamethasone (n=10)
Proestrus (days)	3.409 ± 0.148	3.808 ± 0.230
Estrus (days)	3.545 ± 0.207	3.077 ± 0.225
Metestrus (days)	3.545 ± 0.196	4.115 ± 0.267
Diestrus (days)	4.455 ± 0.333	4.038 ± 0.406
Number of estrous cycle	2.500 ± 0.117	2.154 ± 0.104*
Estrous cycle length	4.955 ± 0.275	6.116 ± 0.431*

Values expressed as mean $\pm$ SEM. (Student's t- test). * $p \leq 0.05$.

† Data was expressed as litter mean (the mean from three females per litter was taken and used to calculate the group mean).

2

3

1

Table 2 - Body and organ weights at PND 85 in the females in estrus of betamethasone and control groups.

Parameters	Control (n=09)	Betamethasone (n=09)
Final body weight (g)	266.20 ± 3.69	256.70 ± 4.93
Organ weights		
Ovaries (mg)	104.70 ± 5.71	122.10 ± 9.16
Uterus (mg)	450.10 ± 18.99	816.50 ± 158.50*
Kidneys (g)	1.89 ± 0.04	2.12 ± 0.26
Adrenals (mg)	93.96 ± 2.92	91.63 ± 3.02
Liver (g)	9.20 ± 0.24	9.08 ± 0.30
Pituitary (mg)	11.03 ± 0.72	12.83 ± 1.56

Values expressed as mean $\pm$ SEM. (Student's t- test). *$p \leq 0.05$.

2

3

1

Table 3 – Reproductive performance of the females of control and betamethasone groups.

Parameters	Control (n=10)	Betamethasone (n=10)
[1] Lordosis quotient (%)	100.00 (90.00 – 100.00)	85.00 (70.00 – 100.00)*
Pregnancy rate (%)	70	80
Fertility potential (%)	100.00 (61.54 – 100.00)	93.33 (82.86 – 100.00)
[1] Pre-implantation loss (%)	0.00 (0.00 - 38.43)	6.67 (0.00 – 17.14)
[1] Post-implantation loss (%)	0.00 (0.00 – 7.14)	8.33 (3.13 – 38.75)*
[2] Body weight of dams (g)	343.40 ± 20.01	345.30 ± 9.07
[2] Uterus weight with fetuses (g)	51.06 ± 6.99	57.44 ± 3.52
[2] Corpora lutea number	13.14 ± 0.34	14.67 ± 0.83
[2] Implantation number	11.00 ± 1.43	12.78 ± 1.32
[1] Resorption number	0.00 (0.00 – 1.00)	1.00 (0.50 – 4.50)*
[2] Number of fetuses	11.83 ± 0.94	9.75 ± 1.56
[2] Fetus weight (g)	2.99 ± 0.18	2.69 ± 0.06*
[2] Placental weight (g)	0.50 ± 0.02	0.46 ± 0.01

[1] Values expressed as median and interquartile intervals. (Mann-Whitney test). [2] Values expressed as mean ± SEM. (Student's t- test). *$p \leq 0.05$.

2

3

1

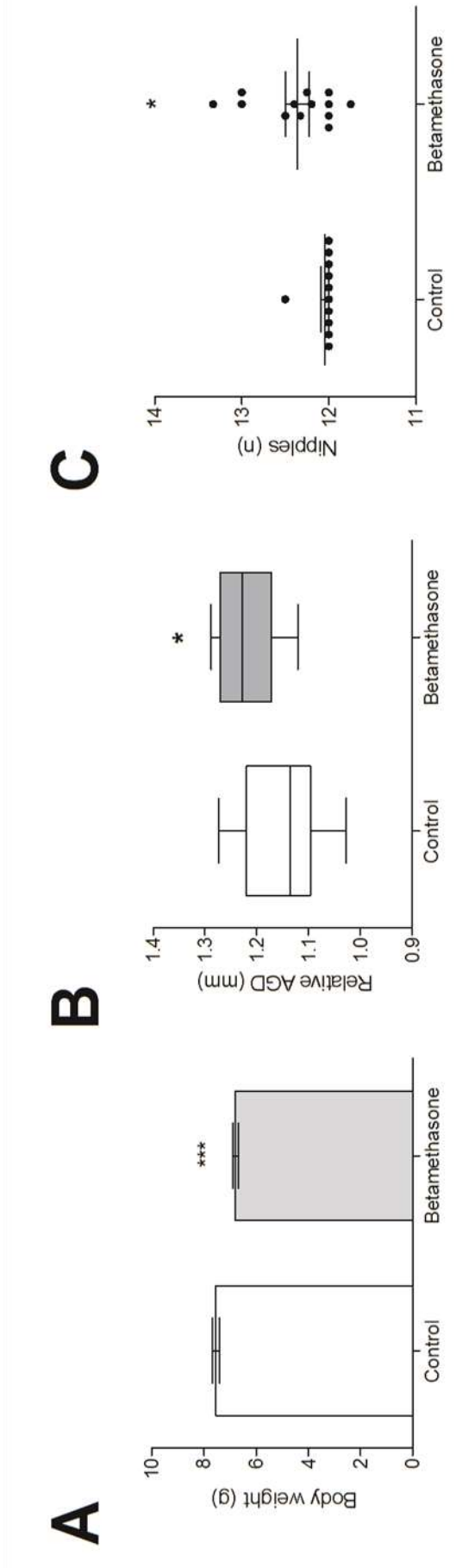

A
Body weight (g)
10
8
6
4
2
0
***
Control
Betamethasone
B
Relative AGD (mm)
1.4
1.3
1.2
1.1
1.0
0.9
*
Control
Betamethasone
C
Nipples (n)
14
13
12
11
*
Control
Betamethasone

Figure 1

Figure 2

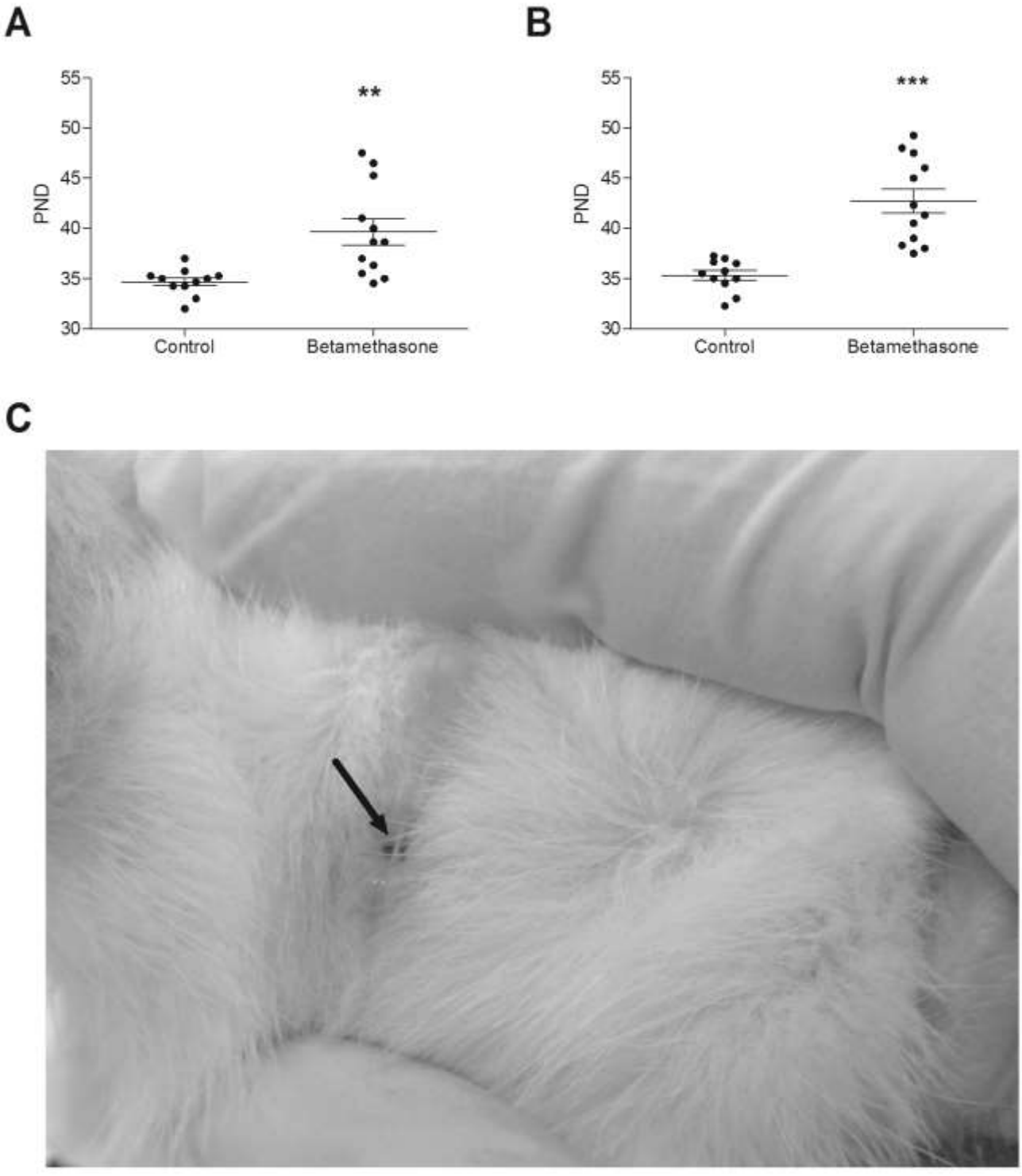
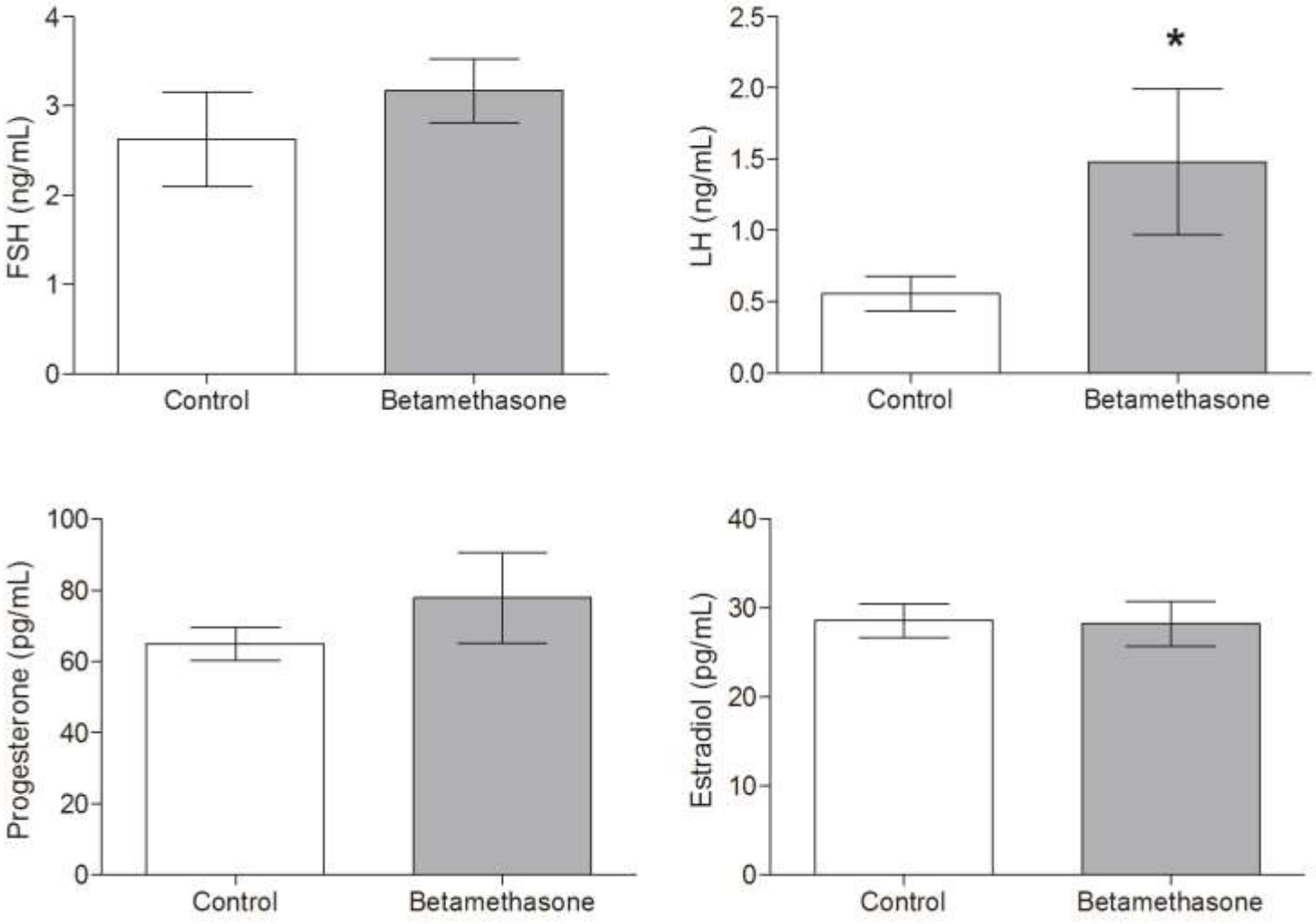


Figure 3


FSH (ng/mL)
4
3
2
1
0
Control
Betamethasone
LH (ng/mL)
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
Betamethasone
Progesterone (pg/mL)
100
80
60
40
20
0
Control
Betamethasone
Estradiol (pg/mL)
40
30
20
10
0
Control
Betamethasone

1

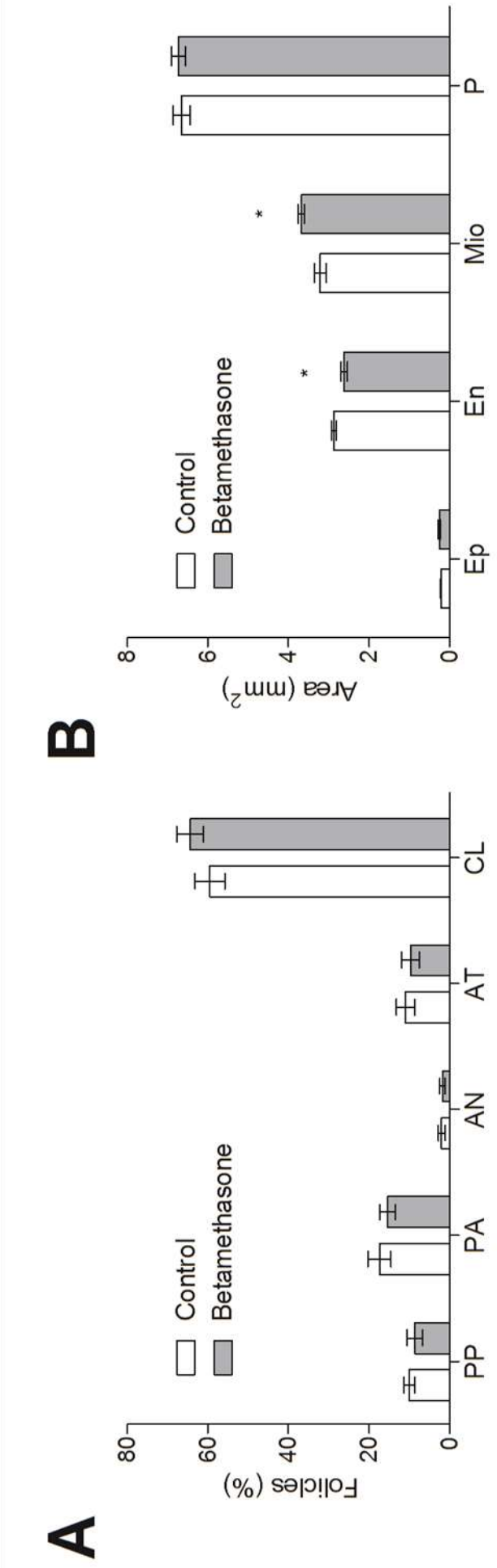

A
Follicles (%)
0
20
40
60
80
Control
Betamethasone
PP
PA
AN
AT
CL
B
Area (mm²)
0
2
4
6
8
Control
Betamethasone
Ep
En
Mio
P
*
*

Figure 4

1
2

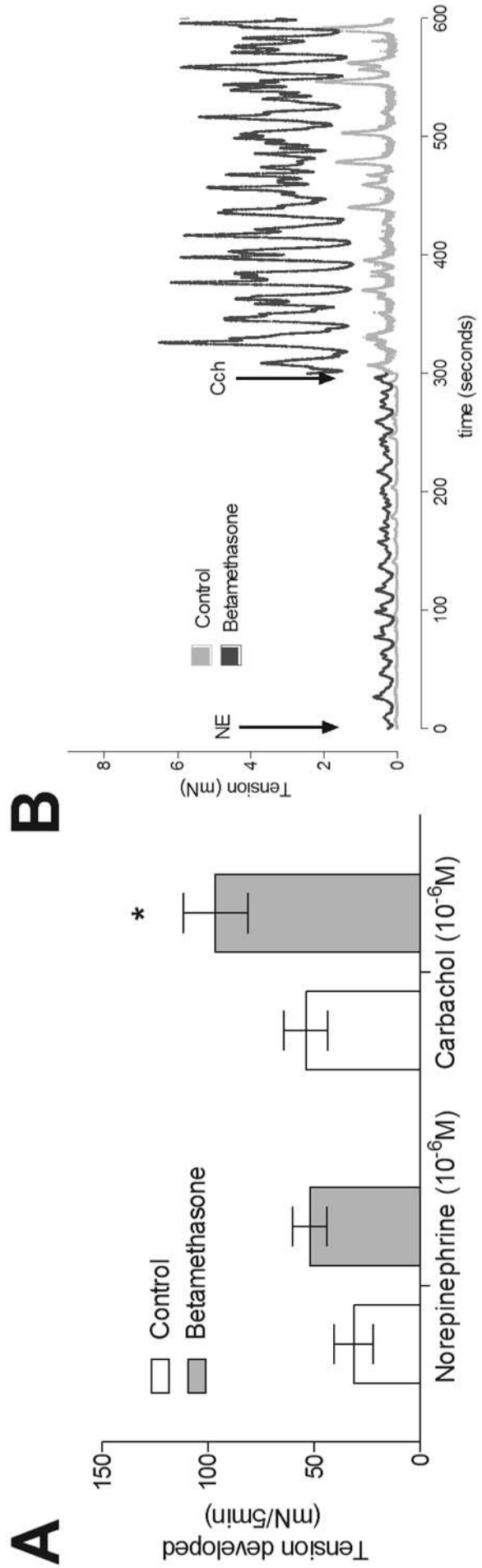

A
Tension developed (mN/5min)
150
100
50
0
Control
Betamethasone
*
Norepinephrine (10^{-6}M)
Carbachol (10^{-6}M)
B
Tension (mN)
8
6
4
2
0
NE
Cch
Control
Betamethasone
0
100
200
300
400
500
600
time (seconds)

Figure 5

SUPPORTING INFORMATION

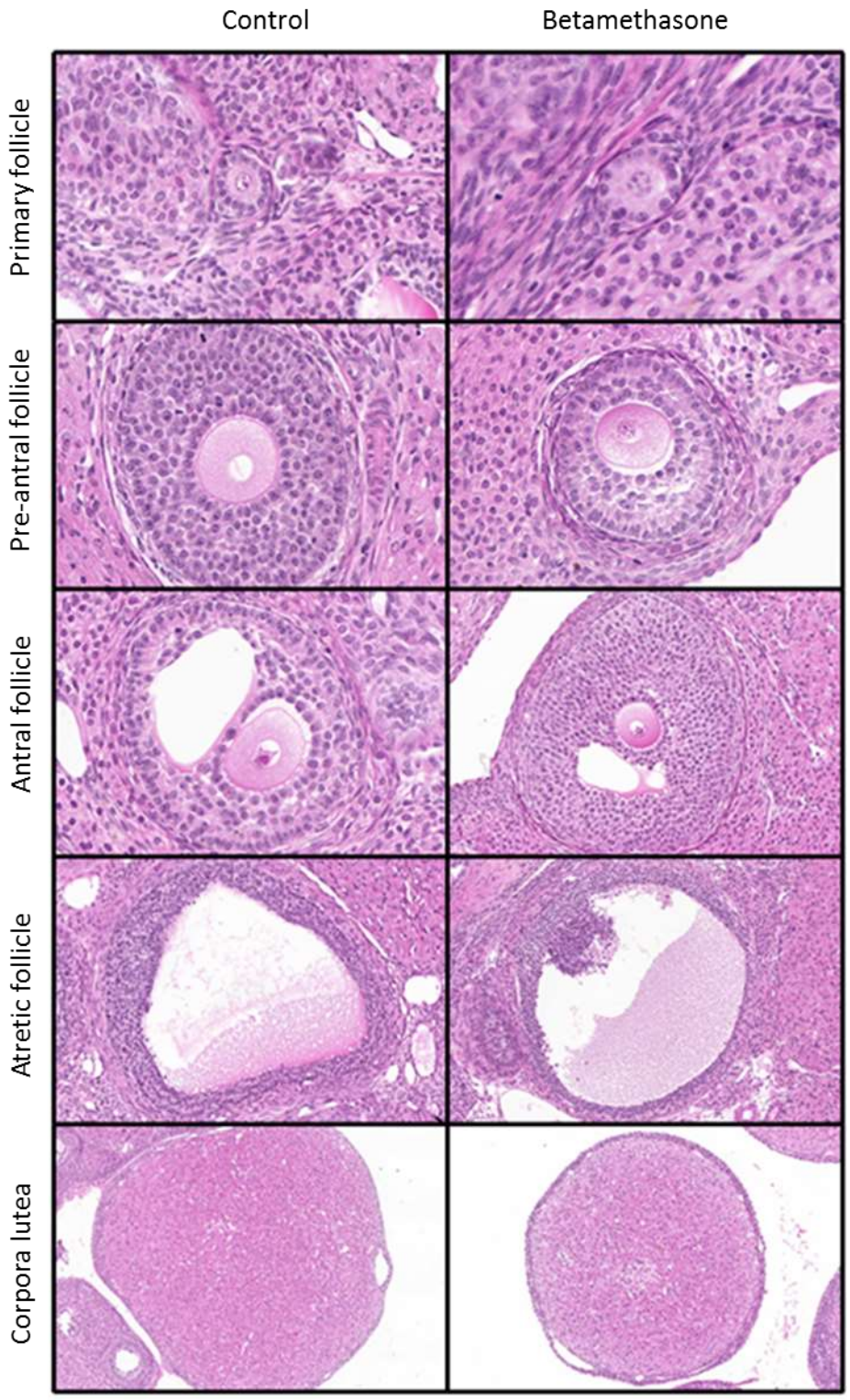
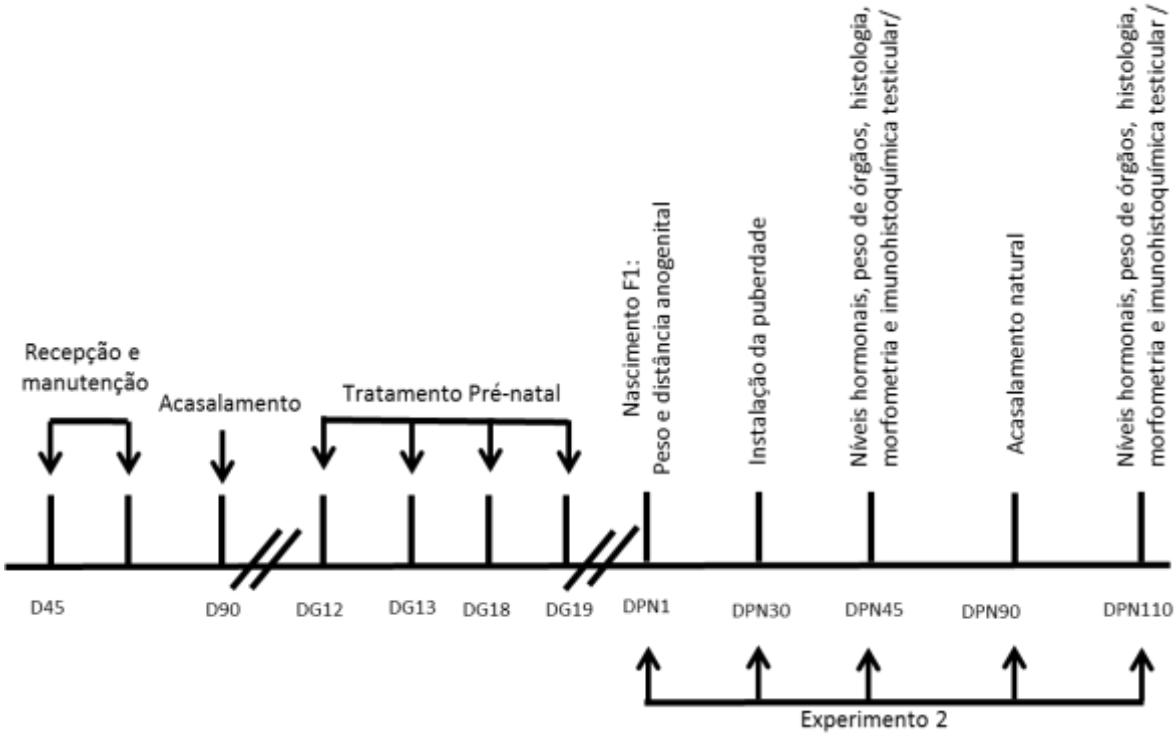


Figure S1: Histological sections of ovarian follicles of female rats from *in utero* betamethasone exposre and control group.

Capítulo 2

Este trabalho deu origem ao artigo “ALTERATIONS IN MALE RATS FOLLOWING *IN UTERO* EXPOSURE TO BETAMETHASONE SUGGESTS CHANGES IN REPRODUCTIVE PROGRAMMING” que foi publicado no periódico “Reproductive Toxicology” (fator de impacto: 2,85), http://dx.doi.org/10.1016/j.reprotox .2016.05.021.

Neste trabalho foi demonstrado parte dos efeitos promovidos pela exposição intrauterina à betametasona durante o período crítico de desenvolvimento masculino, com ênfase sobre os parâmetros iniciais do desenvolvimento sexual, histologia testicular e fertilidade dos ratos machos da primeira geração (F1) como desenho experimental abaixo:



Em suma, o tratamento resultou na redução do peso da ninhada ao nascimento, atraso na instalação da puberdade, aumento do peso dos testículos, diminuição do peso da vesícula seminal, das concentrações de testosterona e na fertilidade dos animais do grupo betametasona. Além disso, nos testículos, foi observado um padrão alterado de distribuição das células germinativas e de Sertoli, na qual encontravam-se em direção ao lúmen.

ALTERATIONS IN MALE RATS FOLLOWING *IN UTERO* EXPOSURE TO BETAMETHASONE SUGGESTS CHANGES IN REPRODUCTIVE PROGRAMMING

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ABSTRACT

Antenatal betamethasone is used for accelerating fetal lung maturation for women at risk of preterm birth. Altered sperm parameters were reported in adult rats after intrauterine exposure to betamethasone. In this study, male rat offspring were assessed for reproductive development after dam exposure to betamethasone (0.1mg/kg) or vehicle on Days 12, 13, 18 and 19 of pregnancy. The treatment resulted in reduction in the offspring body weight, delay in preputial separation, decreased seminal vesicle weight, testosterone levels and fertility, and increased testicular weight. In the testis, morphologically abnormal seminiferous tubules were observed, characterized by an irregular cell distribution with Sertoli cell that were displaced towards the tubular lumen. These cells expressed both Connexin 43 (Cx43) and Proliferative Nuclear Cell Antigen (PCNA). In conclusion, intrauterine betamethasone treatment appears to promote reproductive programming and impairment of rat sexual development and fertility due to, at least in part, unusual testicular disorders.

Additional keywords: betamethasone, reproductive programming, sexual development, testis, Sertoli cell, fertility.

1. INTRODUCTION

Betamethasone is used for pregnant women at risk for preterm birth, between 24 and 34 weeks of pregnancy [1-5]. In laboratory animals, studies have reported that this treatment may cause changes in brain and neuroendocrine functions, suppression of maternal and fetal adrenal function, as well as problems with male fertility [6-12].

During the treatment period, betamethasone promotes changes in intrauterine development that can impact fetal development resulting in persistent effects on the phenotype of the offspring [13, 14]. In addition, fetal programming is most likely to result in permanent structural and physiological alterations, including risk of cardiovascular, metabolic and psychological diseases [4, 13].

Glucocorticoid administration to adult animals can decrease serum levels of testosterone [15, 16]. This decrease in testosterone appears to result from the inhibition of steroidogenic enzymes in Leydig cells [15-17].

During the second phase of intrauterine development, specifically between days 11-13 and 18-19 of gestation, several changes in the morphology of the testis occur, e.g. an increase in Sertoli cell and germ cell proliferation, as well as an increase in testosterone levels [18-21]. Previous studies using this model reported alteration in semen quality of males exposed *in utero* to betamethasone [22]. The present study investigated the sexual development and fertility of male rats whose mothers were exposed to betamethasone during this period, with emphasis on the testicular development.

2. MATERIALS AND METHODS

2.1 - Animals and Treatment.

Male (90 days old/300–350 g) and female (90 days old/225–230 g) Wistar rats were obtained from the Multidisciplinary Center for Biological Investigation, State University of Campinas and maintained under controlled conditions (25°C, 30% air humidity, 12/12-h light/dark cycle) with food and water available ad libitum. Two nulliparous female rats were mated with one male, during the dark cycle of the photoperiod. The detection of sperm in the vaginal smear of rats in estrus was considered as gestational day 1 (GD1). Pregnant and lactating rats were maintained in individual cages. The experimental procedures used in this study were approved by the local Ethics Committee for the Use of Experimental Animals of the University of São Paulo State (protocol number 451-CEEA) in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health). All surgical procedures were performed under ketamine-xylazine anesthesia and euthanasia was performed by decapitation following CO_2 asphyxiation.

2.2 - Experimental groups

Pregnant female rats were randomly allocated into two experimental groups: control group (n=11; treated with saline (vehicle) alone) and treated group (n=13; 0.1 mg/kg; Betamethasone 21-phosphate disodium diluted in vehicle; Sigma-Aldrich, St Louis, MO). This dose was selected based on the dose used in clinic for maternal treatment and modified to rodents [9, 10]. Rats received an intramuscular injection of vehicle or betamethasone on days 12 and 13 (period of germ cell and Sertoli cell proliferation), 18 and 19 (peak testosterone levels) of pregnancy. The injection protocol was based on the maternal corticosteroid therapy adapted by Souza et al. [11], which takes into account differences in the sexual differentiation in male fetuses [9, 10, 23, 24]. During the treatment, the body weight and food consumption was monitored in pregnant females.

After birth, on post-natal day 1 (PND1), the number of pups per litter was randomized and redistributed in order to have 8 pups (4 males and 4 females) per lactating female. Rats were weaned at PND21 and housed in separate cages (n= 4 males per cage). Male rats (n=2-3) from each litter were used in order to prevent potential variation due to litter effects, as described below.

2.3 - External Examination at Puberty

Rats pups were weighed and anogenital distance measured at PND1 in four male rats per litter (n = 11-13 per group). The onset of puberty was determined by manual retraction of the prepuce [25].

2.4 - Organ Weights

On PND 45 (n= 10/group) and PND110 (n=5/ group), rats were weighed and euthanized. After euthanization, blood was collected (9:00 and 11:30 AM) and the right testis, epididymis, seminal vesicle (full and empty, without the coagulating gland), and ventral prostate were dissected and weighed. The left testes were fixed for histology and immunohistochemistry.

2.5 - Serum Hormonal Levels

Blood samples were allowed to coagulate and the serum obtained by centrifugation (2400 rpm) for 20 min at 4°C and stored at -20°C. Serum testosterone, FSH and LH levels were determined by radioimmunoassay. Testosterone levels were measured using the Coat-A-Count® assay (Diagnostics Products Corporation, Los Angeles, USA) while LH and FSH were measured using specific kits supplied by the National Institute of Arthritis, Diabetes and Kidney Diseases (NIADDK). All samples were measured within the same assay to avoid the inter-assay errors. Intra-assay variabilities were 3.4% for LH, 2.8% for FSH, and 4% for testosterone.

2.6 - Histological Analyses

Left testes were fixed by immersion in Bouin's fixative, dehydrated, and embedded in Paraplast Plus® (P3683, Sigma-Aldrich). Tissues were sectioned (5µm), mounted on a glass slide and stained with hematoxylin and eosin (HE) to evaluate testicular morphology. Sections destined for immunohistochemistry were mounted on silanized glass slides and stored at room temperature. Histopathology was analyzed in a blind assay using a Zeiss microscope (model Scope A1-Axio) mounted with a digital camera and analysis software (ZeissAxio Vision, version 4.7.2).

Three non-serial testicular sections of 50 µm apart were taken for each animal in order to evaluate 100 seminiferous tubules. Seminiferous tubules were classified as being either

normal or abnormal. The interstitium and peritubular myoid cells were also examined, as were the general morphology of the Leydig cells and the appearance of blood vessels [26].

The diameter of the seminiferous tubules and thickness of germinal epithelium was evaluated in 10 sections of seminiferous tubules per animal (n= 5 per group) in tubules at stage IX of spermatogenesis. This was done using a Leica DMLB microscope with a magnification of 200X and analyzed with the Leica Q-Win software (version 3).

In order to evaluate the degree of maturation of the seminiferous epithelium, 100 cross-sections of seminiferous tubules per animal (n = 5 per group) were randomly evaluated by assigning values according to the abundance of mature germ cells in the epithelium [27]. The following scale was used: degree 1 (I): young spermatids with rounded nucleus (stages 1–8 of the cycle of the seminiferous epithelium); degree 2 (II): spermatids in maturation phase, with ovoid or elongated nucleus (stages 9–14); degree 3 (III): spermatids in maturation phase, with elongated nucleus (stages 15–18); degree 4 (IV): mature spermatids (stage 19) in small numbers; degree 5 (V): mature spermatids (stage 19) in average numbers; degree 6 (VI): mature spermatids (stage 19) in high abundance. Each symbol means the numbers of tubules in this degree. The “average degree” was determined using the following mathematical formula: Average degree = [(I x 1) + (II x 2) + (III x 3) + (IV x 4) + (V x 5) + (VI x 6)]/100.

In order to evaluate the dynamics of spermatogenesis, the relative frequency of stages was estimated: I-VI (two generations of spermatids), VII-VIII (mature spermatids), IX-XIII (only one generation of spermatids), XIV (secondary spermatocyte) in 100 cross-sections of seminiferous tubules per animal. The relative frequency of stages estimates the rate or duration of spermatogenic process [28].

Furthermore, the number of Sertoli cell nuclei and the volume of Leydig cells also determined. Sertoli cell nuclei were determined in histological sections in 20 seminiferous tubules per rat testis in stage VII of spermatogenesis. The nuclear volume of Leydig cells was measured by randomly selecting 50 circular or elliptical cells, their diameters (D) were measured and the volume was obtained using the formula: $V = [D \times \pi\}/6$

2.7 - Immunohistochemistry for Cx43 and PCNA in the testis

Histological sections (n=5 animals per group) were deparaffinized and hydrated in graded ethanol and washed in TBST (Tris-Buffered Saline, 0.1% Tween-20). Sections were then subjected to antigen retrieval in citrate buffer (0.01 M, pH 6.0) and heated in a microwave. Slides were washed in ddH_2O, followed by hydrogen peroxide (3%) for 15 min,

tap water (5 min), and then incubated in blocking solution (TBST, 5% BSA) for 1 hr at 37^{o}C in a humidified chamber. Sections were then incubated with primary antisera. Sections were incubated overnight with anti-Cx43 (0.625 µg/ml, C-6219; Sigma-Aldrich); or with anti-PCNA (4 µg/ml, SC-56/ PC-10; Santa Cruz Biotechnology). Sections were then washed 3 times in TBST and incubated with the appropriate secondary antibody for 1 hr at 37°C (for Cx43: goat anti-rabbit HRP (1 µg/ml, C# 6721;Abcam); for PCNA: goat anti-mouse HRP (1.6 µg/ml SC- 2005; Santa Cruz Biotechnology)). Sections were subsequently washed with TBST and incubated with diaminobenzidine (0.5 mg/ml, Sigma-Aldrich) for 15 min, rinsed in tap water and counterstained with methylene blue for 5 min. Negative control sections did not receive primary antibody.

2.8 - Fertility Evaluation after Natural Mating

At PND90, one male rat per litter (n=11-13/ group) was placed in a cage alone for 5 min prior to the introduction of a sexually receptive female (70 days old) and the paired animals were kept together for 3 hrs after the first ejaculation. Females were removed and vaginal smears were collected to confirm whether or not insemination occurred. Females naturally inseminated by rats from the control and treated groups were euthanized 20 days after mating to assess male fertility. A median laparotomy was done and the uterus and ovaries were collected. The numbers of corpora lutea, implants, resorptions, as well as the number of live and dead fetuses was determined. From these results the following parameters were calculated: Pregnancy rate or index: (number of females achieving a pregnancy/number of potentially fertile females that were mated) x 100; fertility potential (efficiency of implantation): (implantation sites/corpora lutea) × 100; rate of pre-implantation loss: [(number of corpora lutea - number of implantations)/number of corpora lutea] × 100; and rate of post-implantation loss: [(number of implantations - number of live fetuses)/number of implantations] × 100.

2.9 - Statistical Analysis

Data are presented as mean ± standard error of mean (SEM) or median and interquartile range. A Student T-test was used for comparison of parametric variables. Non-parametric data were compared using a Mann-Whitney test. Differences were considered significant when $p \leq 0.05$. Statistical analyses were performed using the GraphPad InStat software (version 5).

3. RESULTS

Prenatal betamethasone treatment resulted in a significant reduction in food intake (Figure 1A) and weight gain of the dams (Figure 1B) as well as in the weight of the male offspring at PND 1 (Figure 1C). However, the relative anogenital distance was similar between control and betamethasone-treated group (2.23 ± 0.04 for control vs 2.30 ± 0.02 for betamethasone group). After weaning, the differences in body weight between groups did not persist (data not shown).

Preputial separation, which is the first sign of puberty, was delayed in the betamethasone offspring (Figure 1D). These animals had lower levels of serum testosterone levels at PND45. However, at PND110 there were no differences in testosterone levels. Interestingly, there was a significant increase in LH levels which extended into adulthood (Figure 2).

Testicular weights were significantly higher in the betamethasone group at both PND45 and 110. Furthermore, ventral prostate weights were also significantly higher, but only at PND110. In contrast, seminal vesicle weights were significantly lower at PND45, when testosterone levels were lower, but not at PND110 (Table 1).

The degree of maturation of the seminiferous epithelium at PND 45 was significantly different between the treated and control group, thus, the betamethasone group showed a delay in the maturation of the germ cells (Table 2). In the same way, the spermatogenesis showed a significant decrease in the percentage of seminiferous tubules in VII-VIII stages (Table 2).

Morphometric analysis of testis indicated a decrease in the diameter of the seminiferous tubules in both ages (Table 2). Even though the height of epithelium was smaller in the treated group, this effect was not statistically significant (PND45, $p = 0.06$; PND110, $p = 0.17$). The number of the Sertoli cell nuclei in stage VII of spermatogenesis and the volume of Leydig cells were similar between the groups (Table 2).

Testicular histopathology indicated that approximately 20% of tubules from betamethasone-treated rats showed abnormalities at both ages (Table 2). Of those, 6% of tubules in PND45 and 14% in PND110 displayed Sertoli cell nuclei migration towards the lumen of the tubule, while this abnormality was noted in two tubules of a single control rat in PND110 (Figure 3). It is important to note that in these seminiferous tubules, spermatogenesis is active and there are no apoptotic cells observed (Figure 3D and 3H). The mis-localized germ and Sertoli cell nuclei seem to maintain the same organization as the normal tubule, and

the cellular organization of Sertoli and germ cells appears to be maintained in the displaced, or invaginated, Sertoli cells.

In the seminiferous tubules of control animals, Sertoli cells were identified at the basal region of the seminiferous tubule, and the blood-testis barrier appeared morphologically intact. Cx43 was localized to the base of the tubules between adjacent Sertoli cell nuclei in the area of the blood-testis barrier. In the betamethasone group, Cx43 immunostaining was less intense; however, in the tubules in which Sertoli cell nuclei migration was altered, Cx43 immunostaining remained localized between displaced Sertoli cells (Figure 4). In the seminiferous tubules from control rats, PCNA was localized to nuclei of spermatogonia located near the base of the seminiferous tubule. Likewise, PCNA remained localized to the nuclei of spermatogonia in betamethasone-treated rats in which Sertoli cells and spermatogonia had migrated towards the lumen of the tubule (Figure 5). This indicated that not only were the Sertoli cells mis-localized, but that the germ cells remained associated with the displaced Sertoli cells.

There was a significant reduction in the fertility potential, as well as an increase in pre-implantation loss (Table 3). Interestingly, body weights of fetuses from treated males were significantly lower as compared to control animals, suggesting a potential trans-generational effect (Table 3).

4. DISCUSSION

At the end of pregnancy, an increase in plasma cortisol levels is essential for the final development and differentiation of fetal organs, especially the lungs [29]. These final stages of maturation can be induced with the use of synthetic glucocorticoids, as in the case of preterm delivery, thereby reducing neonatal mortality [30]. Betamethasone, a potent synthetic glucocorticoid, is generally the drug of choice for this antenatal therapy [4]. However, animal studies have shown that fetal exposure to synthetic glucocorticoids promotes an increased incidence of behavioral, metabolic and endocrine issues in the long-term [8, 12, 29]. Previous studies in rats have shown that betamethasone treatment can result in decreased testosterone levels and altered sperm parameters of male offspring [9, 10]. Our results demonstrated that betamethasone treatment during the sensitive window of development caused a delay in the onset of puberty in male rat offspring, possibly resulting from testicular alterations, which persisted into adulthood and which negatively impacted male fertility.

Preputial separation was delayed in rats exposed to betamethasone, suggesting a delay in the onset of puberty [31]. In these animals, a delay in the maturation degree of germ cells in PND45 and a significant decrease in serum testosterone levels were also observed. Glucocorticoid exposure during intrauterine development at gestational days 12-19 interferes directly with circulating testosterone levels and this change may extend throughout development [9, 10, 15-18]. Thus, our present data confirm the impact of betamethasone on testosterone levels until puberty and its impact on spermatogenesis. However, testosterone levels in the treated rats recovered to control levels by adulthood. These results parallel Leydig cell volumes, which are similar to controls at this age. In contrast, Piffer et al [9] reported that testosterone levels in betamethasone-treated rats were significantly lower through adulthood. The reason for these differences is unknown, but the increase in LH levels in the present data may have promoted a recovery in testosterone levels.

The significant reduction in litter weights observed in the betamethasone group may have contributed to the delayed onset of puberty , since lower birth weight, one of the first signs of altered fetal programming [32], also observed after betamethasone treatment, is correlated to the initiation of puberty [9, 10, 33]. However, anogenital distance, often used as an indicator of feminization [34], was not significantly different between control and treated rats.

In addition to the decreased offspring weight, there was also a decrease in the body weight of mothers during betamethasone-treatment. This reduction appears to be, at least in part, associated with decreased food intake. Franko et al. [35] and Woods [36] observed

similar results in rats treated with glucocorticoids. The seminal vesicles and ventral prostate are androgen-dependent organs, and their weight generally reflect changes in bioavailable androgens [24, 37-39]. While the weights of seminal vesicles appear to be correlated with circulating levels of androgens, betamethasone appears to exert a specific effect on the ventral prostate that is independent of circulating androgen levels. This is reflected by the significantly higher ventral prostate weight observed in treated adults when androgen levels are similar to those in control rats. While androgens regulate prostate weights, toxicants such as tributyltin have been reported to alter prostate weights independent of circulating androgens [40]. Indeed, Zhao et al. [41] demonstrated androgen-independent growth of prostate cells mediated by glucocorticoids. Thus, our data would suggest that *in utero* betamethasone can exert an effect on the development of the prostate resulting in increased ventral prostate weight in adults. Additional studies will be necessary to understand the mechanisms responsible for this.

Betamethasone-treatment also resulted in an increase in testis weight at both PND45 and 110. Testicular histology in the betamethasone group showed a different pattern of Sertoli and germ cell distribution. Our data indicate that 14% of the seminiferous tubules displayed disrupted germ cell organization and distribution with abnormal migration of Sertoli and germ cells toward the lumen of the seminiferous tubule in adult animals as well as in 6% of the tubules of pubertal animals. Sasso-Cerri and Cerri [42] reported that the antihistamine cimetidine, which exerts anti-androgenic effects, altered the organization of seminiferous tubules, and it is possible to observe the Sertoli cell nuclei and germ cells localizing toward the lumen of the tubule. In the present study, a similar pattern was observed in which betamethasone resulted in mis-localized Sertoli cells.

In order to determine whether or not the Sertoli cells that appear to migrate towards the lumen of the seminiferous tubules maintained their interaction with adjacent cells, immunolocalization of the gap junction protein Cx43 was performed. Cx43 has been shown to be expressed between adjacent Sertoli cell nuclei in the testis and is necessary for Sertoli cell nuclei and germ cell migration during development and spermatogenesis in the adult [43-47]. Cx43 has also been implicated in the organization of the blood-testis barrier and intercellular communication between Sertoli cell nuclei and between Sertoli and germ cells [44, 45, 48]. In the present study, Cx43 immunostaining appeared to be less intense in betamethasone group. Alterations in the expression of Cx43 in the testis have been shown to result in alteration in spermatogenesis and increased germ cell apoptosis [47, 49]. In tubules in which the Sertoli cell nuclei appear to show invagination towards the lumen, there is less intense Cx43 staining,

and this appeared to remain localized to gap junctions between adjacent Sertoli cell nuclei. These results would suggest that, at least in part, the treatment with betamethasone impacts normal expression of Cx43, which may lead to problems in spermatogenesis.

PCNA immunolocalization was done to determine the proliferative capacity of the cells [50, 51]. In the testis, the staining was primarily associated with spermatogonia with no detectable immunostaining in spermatids [52]. PCNA-positive spermatogonia were distributed around the tubules in both controls and betamethasone-treated animals. PCNA immunostaining was also observed in cells present at the base of the displaced Sertoli cell nuclei, suggesting that these remained associated with Sertoli cell nuclei. This would support the notion that the Sertoli cells maintained many of their normal function, including the interaction with developing germ cells, despite the loss of interaction with the basement membrane. However, in these tubules, the spermatogenesis seems to be compromised, since there is no lumen in these seminiferous tubules.

Morphometric analysis of testes revealed that the tubular diameters of the seminiferous tubules at both ages were significantly reduced in the betamethasone-group. Tubular diameter is a sensitive indicator of spermatogenic capacity of several species of animals [53, 54]. It is possible that the betamethasone treatment reduced sperm production. Piffer et al [9] demonstrated a similar reduction in tubule diameter in rats treated with betamethasone. Furthermore, the reduction in the frequency of stage VII-VIII tubules at PND110, may be correlated to the diameter of the tubules, since these are in the final stages of the spermatogenesis [54]. Thus, the histological alterations observed appears to be promoted by a restriction in the size of the tubule which forces the invagination of the cells, as there appears to be no changes in the number of Sertoli cells in stage VII of spermatogenesis.

In order to determine whether or not the changes observed in this study altered fertility of the male rats, animals were mated. Our results indicated that the fertility potential of betamethasone-treated rats was significantly reduced and that this could result from fewer sperm being ejaculated or from a reduction in sperm quality, as previously observed by Borges et al [22]. Piffer et al., [9] also reported that betamethasone-treatment resulted in increased post-implantation loss. These observations indicate that betamethasone-induced alterations in the testis alter sperm production and quality, leading to decreased fertility and decreased production of viable offspring. Considering that rodents have a higher fertility potential than humans [55], our results suggest that betamethasone may have a greater impact on human reproduction.

In conclusion, the administration of betamethasone during a critical period of pregnancy in rats promotes reproductive re-programming, leading to impairment of sexual development and fertility due to, at least in part, an unusual testicular disorder. These results raise concern for children exposed to betamethasone antenatally.

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7. CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

8. FIGURE LEGEND

Figure 1 – **Pregnant weight evolution and Preputial separation.** (A) Food intake of females during the treatment with betamethasone or vehicle. (B) Changes in body weight of pregnant females in the betamethasone treated group and control group. (C) Body weight of the control and betamethasone-treated group on PND 01. (D) Complete preputial separation day of control and betamethasone treated group litters. Values are expressed as mean ± SEM. * $p < 0.05$ (Student t-test, A and C).Values are expressed as median and interquartile ranges (first and third) * $p < 0.05$ (Mann-Whitney test, B and D).

Figure 2 – **Hormonal levels.** Hormonal levels in the male offspring of treated and control group in (A) PND45 and (B) PND110. Values are expressed as mean ± SEM. * $p < 0.05$ (Student's t-test).

Figure 3 – Testis histology at PND45 and PND110. Histological aspect of the testis from animals on PDN45 (n=5/group) and PDN110 (n=5/group): (a, c) control group from PND45, (b, d) betamethasone group from PND45, (e, g) control group from PND110 and (f, h) betamethasone group from PND110. GC: germ cells; L: lumen; In: interstitial tissue. Observe the presence of 19 stage spermatids (arrow) and spermatocytes and spermatogonial cells in the middle of the tubule (asterisk) Final magnification: (e, f) 100x (a, b) 200x and (c, d,g,h) 400x.

Figure 4 - Immunostaining for Cx43 in testis at PND45 and PND110. Immunostaining for Cx43 in testis from animals on PND45 and PND110: (a, c) control group from PND45, (e) control group from PND110, (b, d) betamethasone group from PND45 and (f, g, h) betamethasone group from PND110. GC: germ cells; L: lumen; In: interstitial tissue. Observe typical immunostaining in normal tubules (a, c, e, f) and in tubules with a peculiar disruption (b, d, g, h) atypical Cx43 staining was observed around the germ cells in the displaced layer of cells (arrow), that is, towards the center, rather than only along the margin of the tubules. In addition, the normal Cx43 staining between Sertoli cell nuclei and marginal germ cells was faint or absent (b). Final magnification: (a, b, e, f, g) 160x and (c, d, h) 640x.

Figure 5 - Immunostaining for PCNA in testis at PND45 and PND110. Immunostaining for PCNA in testis from animals on PND45 (n=5/group) and PND110 (n=5/group): (a, b) control group from PND45, (e, f) control group from PND110, (c, d) betamethasone group

from PND45 and (g, h) betamethasone group from PND110. GC: germ cells; L: lumen; In: interstitial tissue. Observe typical immunostaining in normal tubules and in tubules with a peculiar disruption (c, d, g, h) atypical PCNA staining was observed in germ cells in the displaced layer of cells (arrow). Final magnification: (a, c, e, g) 160x and (b, d, f, h) 640x.

Table 1 - Reproductive parameters

Parameters	Experimental groups			
	PND45		PND110	
	Control (n=10)	Betamethasone (n=10)	Control (n=05)	Betamethasone (n=05)
Final body (g)	224.30 ± 2.76	225.20 ± 3.33	450.20 ± 11.37	432.30 ± 14.15
Testis (g)	1.13 ± 0.023	1.19 ± 0.017*	1.75 ± 0.05	1.87 ± 0.01*
Epididymis (mg)	120.70 ± 3.36	130.70 ± 5.69	539.10 ± 22.79	564.60 ± 14.06
Ventral prostate (mg)	89.02 ± 3.90	97.36 ± 6.07	359.20 ± 15.00	426.30 ± 12.13**
Seminal vesicle (mg)	83.36 ± 4.048	69.78 ± 3.93*	1289.00 ± 48.52	1117.00 ± 102.70
Testis (g/100g)	0.51 ± 0.01	0.53 ± 0.02*	0.39 ± 0.01	0.43 ± 0.01*
Epididymis (mg/100g)	54.11 ± 1.58	57.94 ± 2.13	120.10 ± 6.17	131.30 ± 5.84
Ventral prostate (mg/100g)	40.03 ± 2.09	43.30 ± 2.76	79.85 ± 3.29	99.15 ± 4.96**
Seminal vesicle (mg/100g)	37.48 ± 2.06	31.24 ± 1.69*	287.30 ± 13.68	260.40 ± 27.09

Values expressed as mean ± SEM. T-Student test. *$p \leq 0.05$; **$p \leq 0.01$

1

Table 2 – Histology and morphometric assay (n=05/group)

Parameters	Control	Betamethasone
[1]Normal tubules PND45 (%)	95.00 (90.00 -96.00)	83.00 (81.50 – 88.00)*
[2]Closed tubules PND45 (%)	0.0	6.0***
[1]Normal tubules PND110 (%)	95.00 (94.50 – 98.50)	80.00 (77.50 – 91.00)**
[2]Closed tubules PND110 (%)	0.8	14.0***
[3] Degree of maturation cells (1-5)	3.16 (2.95 – 3.23)	2.70 (2.64 – 2.86)**
[3] Dynamics of spermatogenesis		
I – VI (%)	32.00 (28.00 – 36.00)	37.00 (35.00 – 39.00)
VII – VIII (%)	29.00 (27.00 – 32.50)	19.00 (12.00 – 22.50)**
IX – XIII (%)	33.00 (29.50 – 35.50)	36.00 (34.00 – 41.00)
XIV (%)	6.00 (4.50 – 8.00)	8.00 (7.00 – 10.00)
[3]Tubular diameter PND45 (µm)	265.10 ± 8.34	241.10 ± 5.72*
[3]Epithelium high PND45 (µm)	80.93 ± 2.56	75.10 ± 1.11
[3]Tubular diameter PND110 (µm)	279.40 ± 12.18	253.70 ± 3.54*
[3]Epithelium high DP110 (µm)	90.12 ± 5.76	83.41 ± 1.14
[1]Sertoli cell nuclei number PND110 (n)	16.46 $\pm$ 0.84	16.82 $\pm$ 1.41
[1]Leydig cells volume PND110 (μm^3)	122.20 $\pm$ 16.40	98.91 $\pm$ 6.92

[1]Values expressed as median and interquartile intervals (Mann-Whitney test). [2] Values are expressed as a percentage (Chi-Square test). [3] Values expressed as mean $\pm$ SEM (T-Student test). *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

2

1

Table 3 - Fertility after *natural mating*.

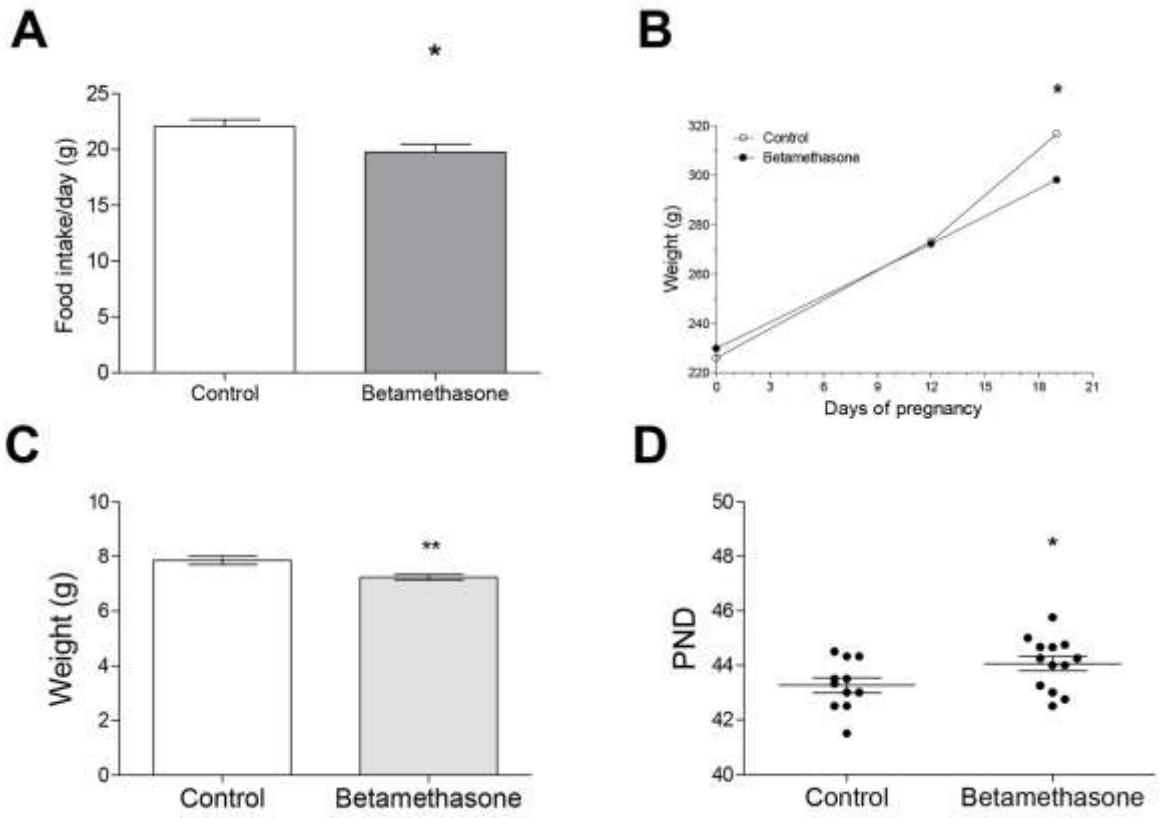
Parameters	Control (n=11)	Betamethasone (n=11)
Pregnancy rate (%)	90.90	76.92
[1] Fertility potential (%)	92.58 (85.00 – 100.00)	78.57 (72.50 – 81.53)*
[1] Pre- implantation loss (%)	0.00 (7.42 – 15.00)	21.43 (8.17 – 27.50)*
[1] Post- implantation loss (%)	8.33 (7.69 – 17.50)	8.39 (0.00 – 11.36)
[2] Body weight of dams (g)	336.90±8.55	338.00±10.78
[2] Uterus weight with fetuses (g)	52.72±3.85	48.05±2.38
[2] Corpora lutea number	12.70±0.72	14.80±1.03
[2] Implantation number	11.60±0.82	11.50±0.43
[2] Resorption number	1.20±0.25	1.00±0.30
[2] Fetus weight (g)	3.12±0.08	2.62±0.19*
[2] Placental weight (g)	0.50±0.02	0.47±0.01

[1] Values expressed as median and interquartile intervals (Mann- Whitney test). [2]Values expressed as mean ± SEM. (Student's t- test). *p< 0.05

2

3

Figure 1


A
Food intake/day (g)
25
20
15
10
5
0
*
Control
Betamethasone
B
Weight (g)
320
300
280
260
240
220
Control
Betamethasone
0
3
6
9
12
15
18
21
Days of pregnancy
*
C
Weight (g)
10
8
6
4
2
0
**
Control
Betamethasone
D
PND
50
48
46
44
42
40
*
Control
Betamethasone

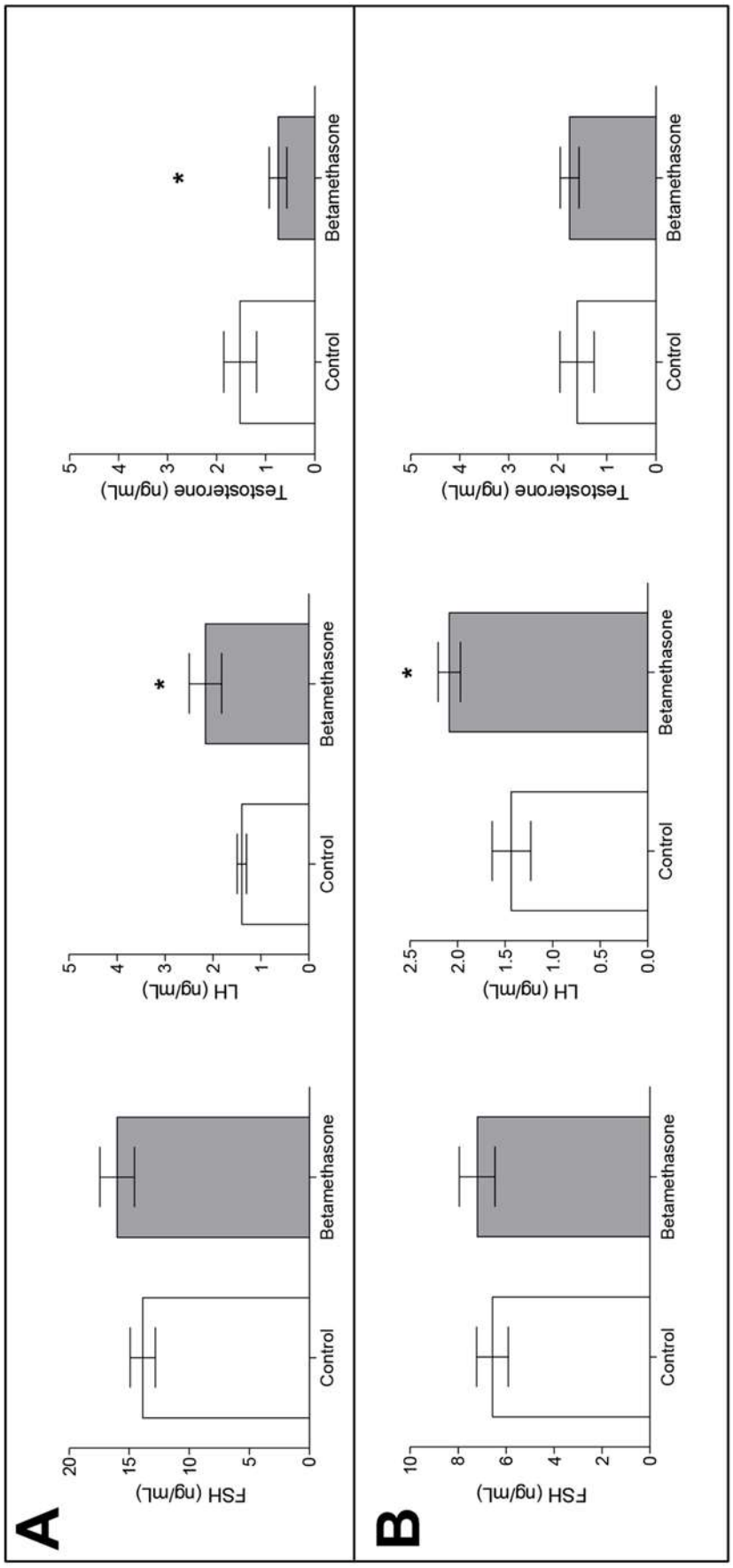

A
FSH (ng/mL)
20
15
10
5
0
Control
Betamethasone
LH (ng/mL)
5
4
3
2
1
0
*
Control
Betamethasone
Testosterone (ng/mL)
5
4
3
2
1
0
*
Control
Betamethasone
B
FSH (ng/mL)
10
8
6
4
2
0
Control
Betamethasone
LH (ng/mL)
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
Betamethasone
Testosterone (ng/mL)
5
4
3
2
1
0
Control
Betamethasone

Figure 2

Figure 3

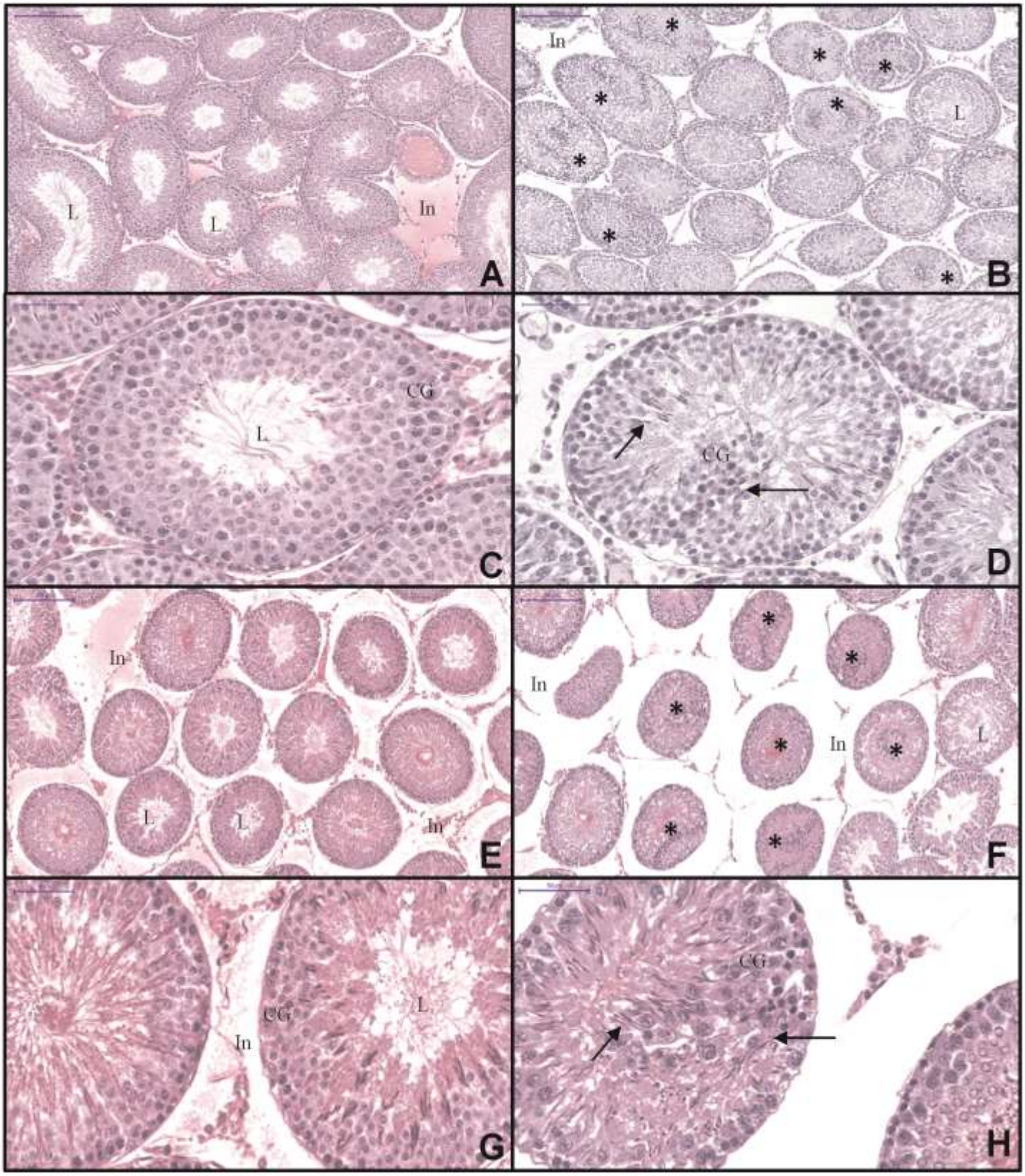


Figure 4

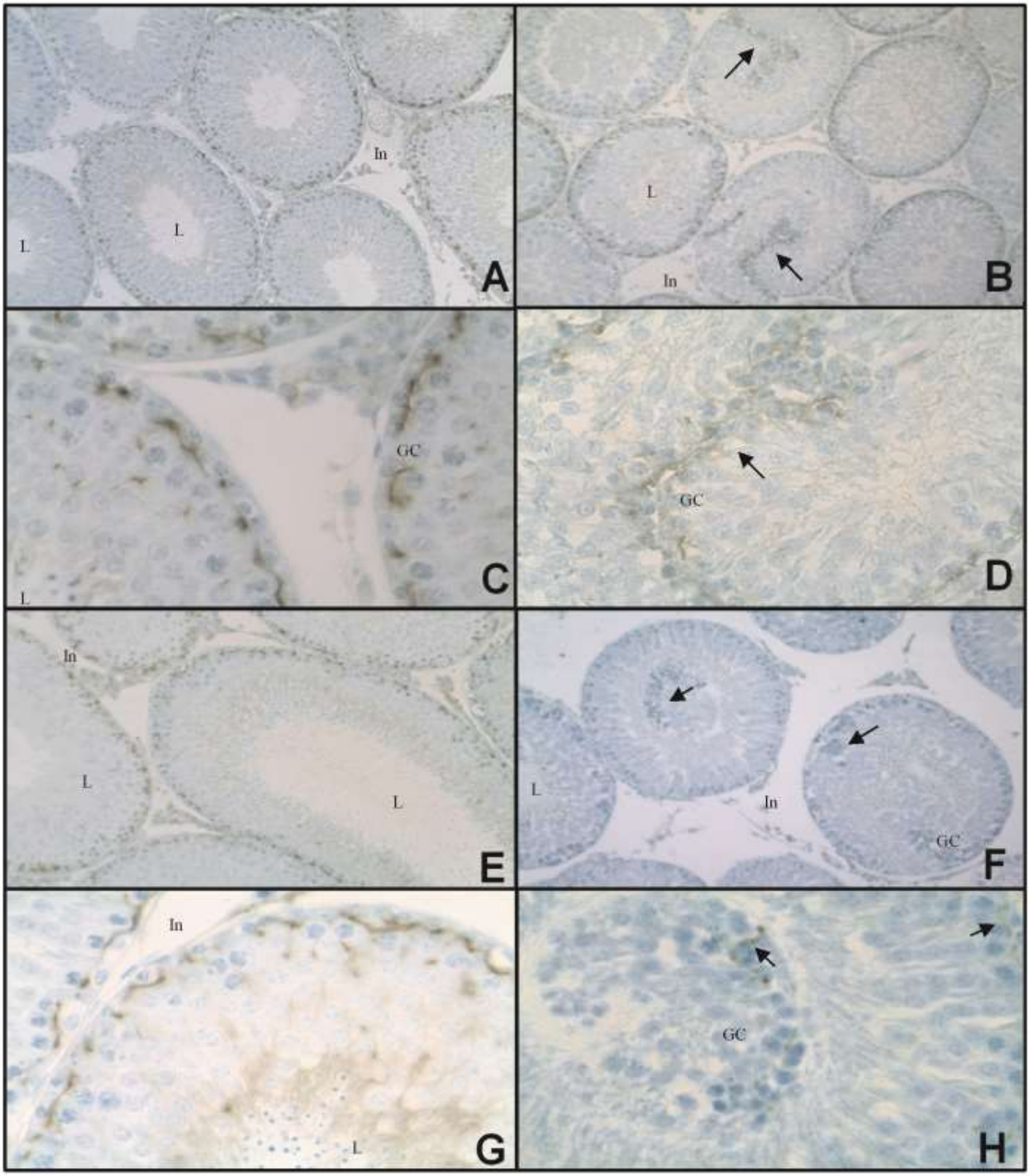
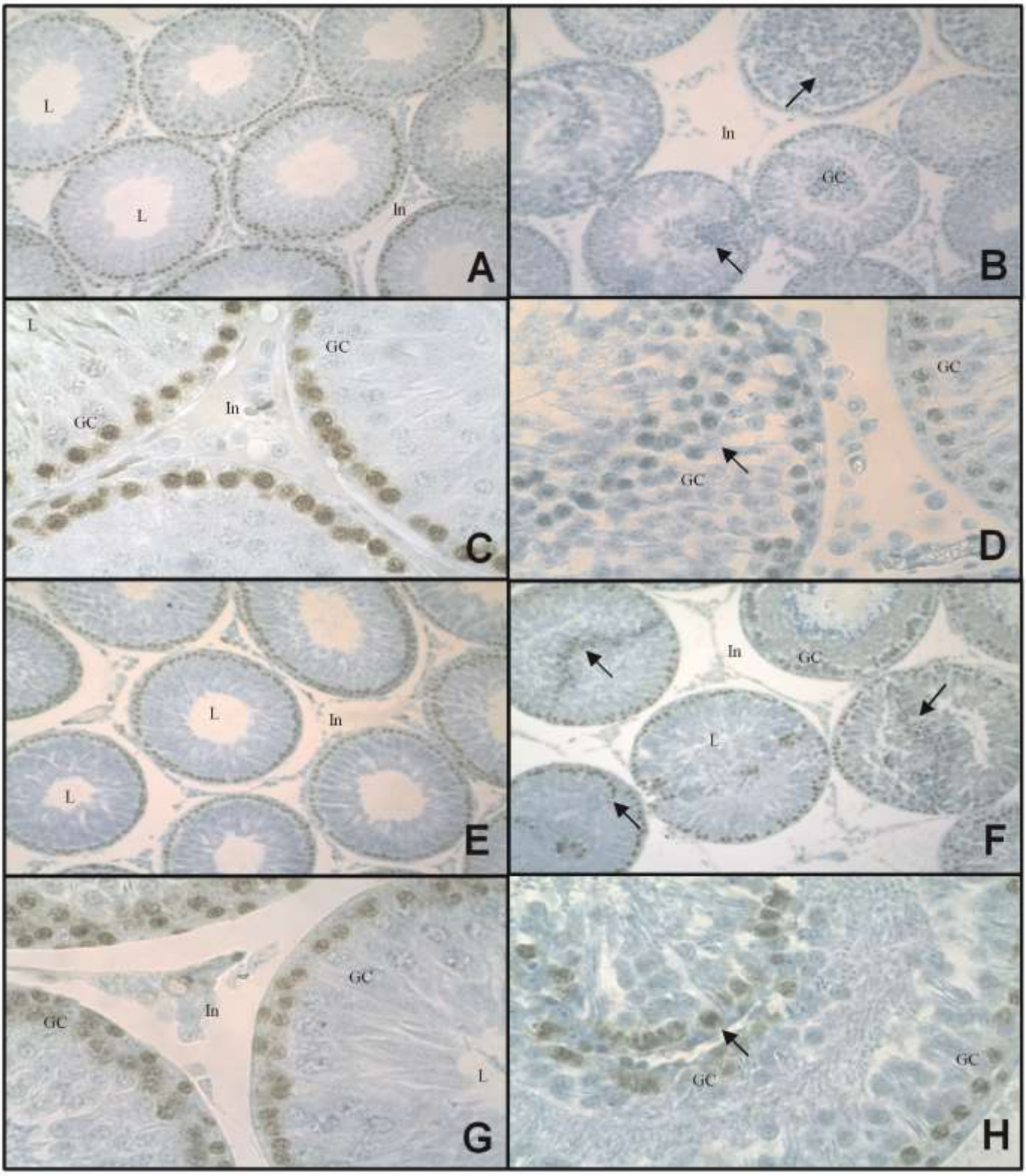


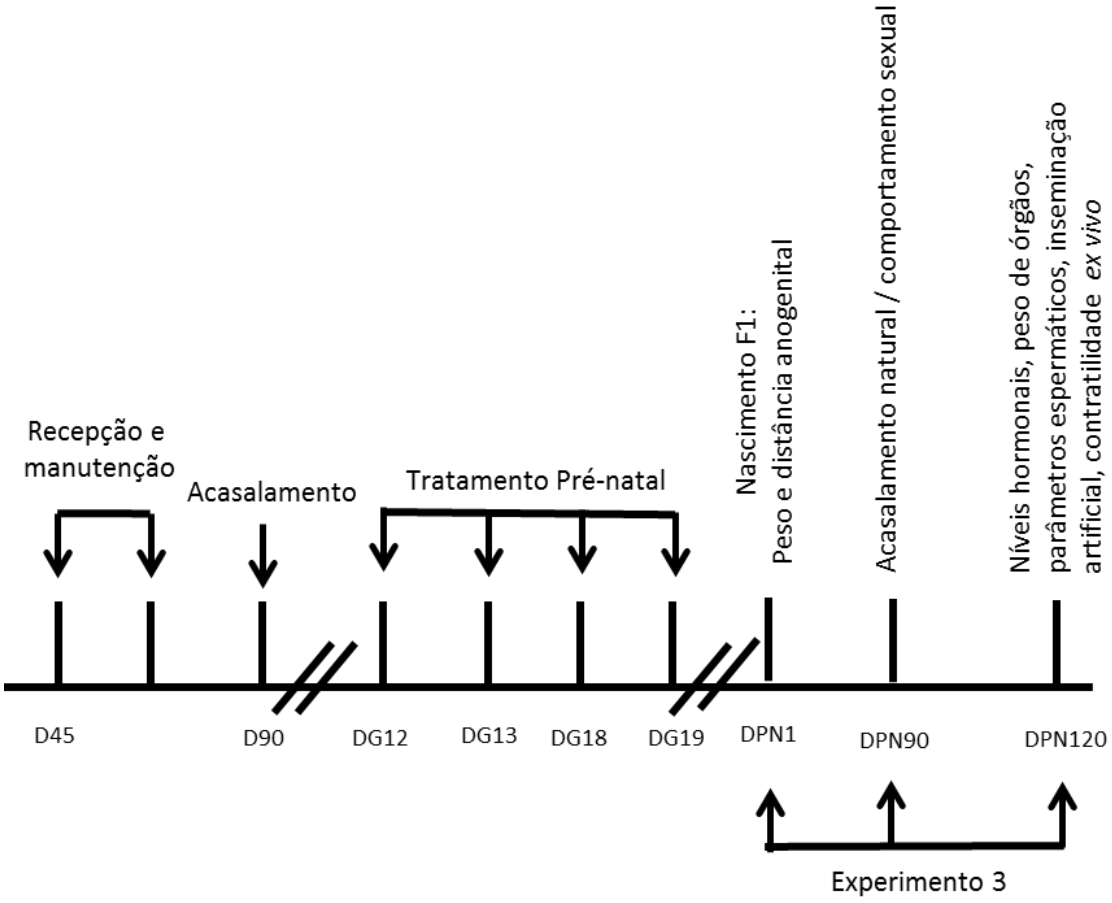
Figure 5



Capítulo 3

Este trabalho deu origem ao artigo “LONG-TERM ADVERSE EFFECTS ON REPRODUCTIVE FUNCTION IN MALE RATS EXPOSED PRENATALLY TO THE GLUCOCORTICOID BETAMETHASONE” que foi publicado no periódico “Toxicology” (fator de impacto: 3,817), http://dx.doi.org/10.1016/j.tox.2016.04.005.

Neste artigo foram avaliados os parâmetros espermáticos, o comportamento sexual e a fertilidade por meio da inseminação artificial *in utero* de parte da ninhada masculina (F1) exposta durante o período prenatal à betametasona como exemplificado no desenho experimental abaixo:



Nossos resultados demonstraram que a betametasona promoveu uma significante redução do peso ao nascimento da prole masculina, diminuição das concentrações de FSH e aumento das concentrações de LH. Além disso, houve um aumento no peso dos testículos e da próstata ventral, diminuição do peso da vesícula seminal seguido de um aumento de sua atividade contrátil. Os parâmetros espermáticos (motilidade, morfologia e contagem) foram alterados culminando em um baixo potencial fértil dos animais do grupo exposto a betametasona.

LONG-TERM ADVERSE EFFECTS ON REPRODUCTIVE FUNCTION IN MALE RATS EXPOSED PRENATALLY TO THE GLUCOCORTICOID BETAMETHASONE

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Abstract.

Betamethasone is the drug of choice for antenatal treatment, promoting fetal lung maturation, decreasing the incidence of respiratory distress syndrome and neonatal mortality. Previous studies reported that prenatal treatment with this drug reduced testosterone levels, sperm quality and fertility in adult rats. We aimed to further evaluate the reproductive consequences of prenatal betamethasone exposure in male rats. Pregnant Wistar rats (n = 13/group) were separated into two groups: control (vehicle) and betamethasone- treated (0.1 mg/kg IM) and rats were injected on gestational days 12, 13, 18 and 19. Body weight, sexual behavior, reproductive organ weights, serum hormone levels, accessory glands contractility, sperm parameters, and fertility after *in utero* artificial insemination were evaluated. Our results showed that prenatal betamethasone exposure provoked a significant reduction in body weight at PND 01 and, at adulthood, decrease in FSH levels, sperm motility and production. Furthermore, seminal vesicle weight was decreased while testicular and ventral prostate weights were increased. Serum LH levels and the percentage of abnormal sperm were significantly increased. Although sexual behavior was not altered, a significant reduction in fertility in the adult rats exposed prenatally to betamethasone was noted. We concluded that prenatal betamethasone exposure leads to long-term reproductive impairment in male rats. These results may have important implications for humans, considering the use of this glucocorticoid in pregnant women.

Keywords: betamethasone, fetal programming, sperm quality, fertility, sexual glands contractility.

1 - Introduction

Fetal lung maturation is important before birth to avoid the respiratory distress syndrome, a serious complication of preterm birth and the primary cause of early neonatal death (Jobe and Soll, 2004 and Roberts and Dalziel, 2007). The treatment used for women at risk of preterm birth to promote accelerating fetal lung maturation is denominated antenatal corticosteroid therapy, and the drug of choice is the glucocorticoid betamethasone (Drake et al., 2011).

Glucocorticoids play a key role in intrauterine programming, inducing permanent changes in physiological systems (Fowden and Forhead, 2004 and Moisiadis & Matthews, 2014). (Fowden and Forhead, 2004 and Moisiadis & Matthews, 2014). An important target of fetal programing is the reproductive system, once effects throughout the sexual development to adulthood can be observed.

The exposure to betamethasone *in utero* can promote changes in brain development (Yawno et al., 2014), renal function (Bi et al., 2014) and neuroendocrine alterations in different species (Matthews, 2000) as well as suppression of maternal and fetal adrenal (de Souza et al., 2001). Furthermore, the betamethasone was responsible for several alterations on male reproductive system, such as testis development in sheep (Pedrana et al., 2008 and Pedrana et al., 2013) and sperm quality, fertility and testosterone levels when rats were exposed *in utero* at gestational days 12,13, 18 and 19 (Piffer et al., 2009a and Piffer et al., 2009b).

It is known that glucocorticoids, at high concentrations, can reduce fetal testosterone, which is fundamental for sexual differentiation (Corbier et al., 1992, Hardy et al., 2005, Page et al., 2001 and Ward and Weisz, 1984). Thus, the present study investigated the long-term reproductive effects on male rats whose mothers were exposed to betamethasone during critical days of sexual development, with emphasis on sperm quality and fertility.

2. Materials and methods

2.1. Animals

Male (90 days old/300–350 g) and female (90 days old/225–230 g) Wistar rats were obtained from the Multidisciplinary Center for Biological Investigation, State University of Campinas and maintained under controlled conditions (25 °C, 30% air humidity, 12/12-h light/dark cycle) with food and water available ad libitum.

The experimental procedures used in this study were approved by the local Ethics Committee for the Use of Experimental Animals of the University of São Paulo State (protocol number 451-CEEA) in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health). All surgical procedures were performed under ketamine-xylazine anesthesia and euthanasia performed by decapitation following CO2 asphyxiation.

2.2. Experimental design

Two nulliparous female rats were mated with one male, during the dark cycle of the photoperiod. The detection of sperm in the vaginal smear of rats in estrus was considered as gestational day 1 (GD1). Pregnant and lactating rats were maintained in individual cages.

Pregnant female rats were randomly allocated into two experimental groups: control (treated with saline – vehicle, n = 11) and treated (0.1 mg/kg; Betamethasone 21-phosphate disodium – Sigma-Aldrich, St Louis, MO, diluted in vehicle, n = 13). This dose was selected based on the dose used in clinic for maternal treatment and modified to rodents (Piffer et al., 2009a and Piffer et al., 2009b). Rats received an intramuscular injection of vehicle or betamethasone on days 12, 13, 18 and 19 of pregnancy. The injection protocol was based on the maternal corticosteroid therapy adapted by de Souza et al. (2001) which takes into account differences in the sexual differentiation in male fetuses (Pereira and Piffer, 2005, Pereira et al., 2003, Piffer et al., 2009a and Piffer et al., 2009b).

After birth, on post-natal day (PND) 1, the litters were cutoff for 8 animals per dam, 4 males and 4 females at random. Then, the male offspring was weighted. At PND 21, rats were housed in separate cages (n = 4 males per cage). For the experiments herein presented two male rats from each litter were used, divided into two studies.

2.3. Study 1: sexual behavior, organ weights, hormones, sperm quality and fertility

2.3.1. Evaluation of male sexual behavior

At PND 90, one male per litter was held placed in a cage alone for 5 min prior to the introduction of a sexually receptive female (70 days old). Paired animals were observed during the dark cycle of the photoperiod. Males that did not mount the female in the first 10

min were considered sexually inactive. Male sexual behavior was evaluated for 40 min and included the following parameters: latency to the first mount; latency to the first intromission; latency to the first ejaculation; number of intromissions until the first ejaculation; latency of the first post-ejaculatory intromission; number of post-ejaculatory intromissions; and total number of ejaculations.

2.3.2. Organ weights

Thirty days later, these same animals tested for sexual behavior were weighed and euthanized by CO_2 asfixiation followed by decapitation. Then, blood was collected (9:00 and 11:30 AM) from the ruptured vessels and the right testis (intact and without the albuginea and fluid), epididymis, seminal vesicle (full and empty, without the coagulating gland), and ventral prostate were dissected out and weighed. The sexual glands were discarded. The parenchyma of right testis was obtained by cutting out the albuginea and removing the testicular fluid by centrifugation (3000 rpm) for 30 min at 4 °C and frozen at −20 °C for posterior sperm counts. The right epididymal cauda was used for sperm collection for intrauterine artificial insemination and the remainder tissue was frozen for sperm counts, as well as the epididymal caput/corpus. The left testis and epididymis were kept frozen for future studies.

2.3.3. Fertility assessment

Eight animals per group were used for fertility assessment using *in utero* artificial insemination (Kempinas et al., 1998a and Klinefelter et al., 1994). In brief, females in LHRH-induced proestrus were paired with sexually experienced vasectomized males for 1 h. Receptive females were selected for the insemination procedure. Sperm were released from the right proximal epididymal cauda, first site where fertile sperm is encountered in the rat, by nicking the duct and collecting the sperm in 1–2 ml of modified human tubular fluid (HTF) medium (Irvine Scientific). After a 10-fold dilution, sperm were counted and each uterine horn was injected with a volume containing 5 × 106 sperm. One female was inseminated per male and when insemination was completed, the abdominal musculature was sutured. All surgery was performed under ketamine-xylazine anesthesia, and all efforts were made to minimize suffering.

Twenty days later, the females were euthanized by decapitation to enable fertility evaluation. After collection of the uterus and ovaries the numbers of corpora lutea, implants and reabsorptions were recorded and the following endpoints determined: fertility potential (efficiency of implantation): implantation sites/corpora lutea × 100; rate of pre-implantation loss: (number of corpora lutea − number of implantations/number of corpora lutea) × 100; rate

4of post-implantation loss: (number of implantations − number of live fetuses)/number of implantations × 100.

2.3.4. Sperm motility

Sperm motility was evaluated in the same sperm sample used for artificial insemination. For this, an aliquot of 10 µl of sperm suspensions was immediately transferred to a Makler chamber maintained at 34 °C. Using a phase-contrast microscope (400× magnification), 100 sperm were counted and classified as Type A (mobile with progressive movement) Type B (mobile without progressive movement) and Type C (immobile).

2.3.5. Sperm morphology

An aliquot of 100 µl of the sperm suspension was added to 900 µl of formol saline. To analyze sperm morphologically, smears were prepared on histological slides that were left to dry for 90 min and 200 spermatozoa per animal were analyzed in a phase-contrast microscope (400× magnification). Morphological abnormalities were classified into two general categories: head morphology (without curvature, without characteristic curvature, pin head or isolated form, i.e., no tail attached) and tail morphology (broken or rolled into a spiral) (Filler, 1993). Sperm were also classified as to the presence or absence of the cytoplasmic droplet.

2.3.6. Serum hormonal levels

Serum was obtained by centrifugation (2400 rpm) for 20 min at 4 °C and stored at −20 °C. FSH and LH were assayed by double-antibody method with specific kits provided by the National Hormone and Peptide Program (Harbor-University of California at Los Angeles). The primary antibody for FSH was anti-rat FSH-S11, and the reference was FSH-RP2. The antiserum for LH was anti-rat LH-S10 and the reference was RP3. The lower limit of detection for FSH and LH were 0.2 and 0.04 ng/mL and the intra-assay coefficients of variation were 3% and 3.4%, respectively. Serum concentrations of testosterone were determined using specific kits provided by MP Biomedicals (Orangeburg, NY, USA). The lower limits of detection was 5 pg/mL and the intra-assay coefficient of variation was 4%.

2.3.7. Sperm counts, daily sperm production, and sperm transit time through the epididymis

Homogenization-resistant testicular spermatids (stage 19 of spermiogenesis) were counted as described previously (Fernandez et al., 2008 and Robb et al., 1978), with following adaptations: briefly, the testis, decapsulated and weighed soon after collection, was homogenized in 5 ml of NaCl 0.9% containing Triton X 100 0.5%, followed by sonication for 30 s. After a 10-fold dilution, one sample was transferred to Neubauer chambers (4 fields per

animal), and mature spermatids were counted. To calculate the daily sperm production (DSP), the number of spermatids at stage 19 was divided by 6.1, which is the number of days of the seminiferous cycle during which these spermatids are present in the seminiferous epithelium. The caput/corpus and cauda epididymidis that were frozen as mentioned previously were cut into small fragments with scissors and homogenized, and sperm counted as described for the testis. The sperm reservoir in the cauda region was calculated by combining the number of cells in the sperm suspension and the sperm count in the homogenized tissue. The sperm transit time through the epididymis was determined by dividing the number of sperm in each portion by DSP.

2.4. Study 2 – Pharmacological reactivity of isolated sexual glands

Five rats per experimental group (one per litter) were weighed and euthanized at PND 90 by decapitation. A section of basal ventral prostate and a lobule of seminal vesicle were prepared for the in vitro tension recording as described below (Nojimoto et al., 2009). Tissues were mounted in 10 ml organ baths in a modified Tyrode's solution at 30 °C under 9.8mN resting tension and allowed a 30 min equilibration period. After the resting period, the tissues were repeatedly challenged with 80 mM KCl every 30 min until two reproducible contractions were obtained. After that, a cumulative concentration–response curve to norepinephrine (NE, 10^{-8}M − 10^{-4}M) and carbachol (CCh, 10^{-8}M–3.10^{-4}M) were obtained at 30 min interval. The maximal tension developed (Emax, in milliNewtons, mN) and the potency of NE and CCh in developing tension (pEC50, the −log of agonist concentration inducing 50% of Emax) were evaluated.

2.5. Statistical analysis

Data are presented as mean ± standard error of mean (SEM) or median and interquartile range. Student́s t-test was used for comparison of parametric variables. Nonparametric data were compared using Mann-Whitney test. Differences were considered significant when $p \leq 0.05$. Statistical analyses were performed using the GraphPad InStat software (version 5).

3 - Results

Prenatal betamethasone exposure provoked a significant reduction of male offspring weight at PND 1 (Fig. 1A) that did not persist at PND 120 (Fig. 1B).

Sexual behavior was not significantly altered by betamethasone treatment (Table 1). On the other hand, there was a significant reduction in the fertility potential as well as an increase in pre-implantation loss after intrauterine insemination (Table 2).

The hormonal levels showed a significant increase in LH and a decrease in FSH levels in the betamethasone group (Fig. 2). Testicular and ventral prostate weights were significantly increased in the betamethasone group, while seminal vesicle weight was significantly decreased (Table 3). There was an increasing tendency, in the exposed group, in the difference between total testicular weight and testicular weight without the albuginea and fluid (control group: 0.47 ± 0.04; betamethasone group: 0.58 ± 0.03; $p = 0.07$).

In the bethametasone group there was a significant decrease in the percentage of progressive mobile sperm (Fig. 3A) and, consequently, an increase in percentage of mobile sperm without progressive trajectory in the betamethasone group (Fig. 3C). Furthermore, sperm with progressive motility and fertility potential were positively correlated (Fig. 3D). The percentages of morphologically abnormal sperm and sperm with cytoplasmic droplet increased (Table 4). The sperm transit time also increased in the betamethasone group (Table 5). On the other hand, daily sperm production and the number of sperm in the epididymal cauda were significantly decreased (Table 5).

Figure 4A shows that the potency of NE (adrenergic agonist) in inducing contractions in the isolated seminal vesicle was comparable between groups (control group: pEC50 = 5.431 ± 0.1618; betamethasone group: pEC50 = 5.478 ± 0.1235). However, the maximal tension developed (Emax) for NE in the betamethasone group was increased compared to the control ($p < 0.05$, Fig. 4B). The same pattern was registered for CCh (Fig. 4C and D) a cholinergic agonist (control group: pEC50 = 4.828 ± 0.1456; betamethasone group: pEC50 = 4.790 ± 0.1168).

For ventral prostate (Fig. 5) the potency of NE (control group: pEC50 = 6.287 ± 0.3704; betamethasone group: pEC50 = 6.199 ± 0.2846; $p > 0.05$) and CCh (control group: 4.618 ± 0.1251; betamethasone group: 4.842 ± 0.2281; $p > 0.05$) were similar. In the same way, the Emax for NE and CCh were comparable between groups.

4 - Discussion

In the past few years much has been discussed about the long-term effects caused by the intrauterine programming in physical and behavioral health of fetuses exposed to glucocorticoids, particularly betamethasone (Bi et al., 2014, Drake et al., 2011, Iqbal et al., 2012, Manojlović-Stojanoski et al., 2012, Pedrana et al., 2008, Pedrana et al., 2013, Piffer et al., 2009a, Piffer et al., 2009b and Yawno et al., 2014). In a previous work (Piffer et al., 2009a) we observed decrease in fertility after natural mating, lower sperm production, rate of normal morphology and motility, in adult rats whose mothers were exposed to betamethasone in critical periods of prenatal sexual differentiation. Considering the use of betamethasone as the drug of choice for antenatal treatment for fetal lung maturation, in the present work we further investigated long-term effects of intrauterine betamethasone exposure on reproductive parameters of adult male rats.

Rodents produce an excess of qualitatively normal sperm (Amann, 1982). Intrauterine artificial insemination is a technique that increases the sensitivity of fertility tests and sperm quality evaluation (Amann, 1986 and Kempinas and Klinefelter, 2010), once this technique excludes the interference of factors such as altered sexual behavior pattern and reduction in ejaculated sperm number (Fernandez et al., 2008). In the present work the reduction in fertility potential and an increase of pre-implantation loss were observed in the group exposed to betamethasone. These results were probably due to the decrease in sperm motility and normal morphology, as shown by the positive correlation between these parameters, corroborating previously data on the betamethasone negative impact on sperm quality and fertility (Piffer et al., 2009a).

Betamethasone acts, directly, on genes and, indirectly, through changes in the bioavailability of several types of hormones. Since hormones regulate fetal growth and the development of individual fetal tissues, glucocorticoids have a central role in intrauterine programming (Moisiadis & Matthews, 2014), promoting changes that can be transient or persist into postnatal life (Manojlović-Stojanoski et al., 2012 and Matthews, 2000).

Glucocorticoids, when applied during gestation, affect gonadal function at multiple levels in hypothalamo-pituitary-gonadal axis. In the hypothalamus, decreasing the synthesis and release of GnRH. Inhibiting the synthesis and release of LH and FSH in the pituitary gland, and directly in the testis, modulating steroidogenesis and gametogenesis (Whirledge and Cidlowski, 2010). These same authors (Whirledge and Cidlowski, 2013) showed that glucocorticoids can inhibit directly testicular LH receptor numbers.

In the present work FSH was decreased and LH increased after prenatal betamethasone exposure, although testosterone, measured at adulthood, did not change, as well as the sexual behavior. Thus, it is possible that, throughout development, by a mechanism of adaptation, the pattern of synthesis of LH hormone changed, in order to guarantee normal testosterone levels in adulthood. On the other hand, the reduction in FSH levels can be correlated with the decrease in sperm production in the betamethasone group. It is known that this hormone is important for spermatogenesis and acts optimizing germ cell production (O'Shaughnessy, 2014).

The first change promoted by intrauterine betamethasone exposure is lowering birth weight (Braun et al., 2015 and Fowden and Forhead, 2004). In the present work the decrease in body on PND 01 did not persist into adulthood, confirming data from the literature, in which fetuses with intrauterine growth restriction catchup development and accelerate weight gain during their postnatal development (Barker and Thornburg, 2013 and Moisiadis & Matthews, 2014).

Alteration of the reproductive organ weights, an indicator of a toxic effect on reproduction (Clegg et al., 2001), can influence directly male sperm quality and fertility.

Sperm motility and fertility capacity are acquired during epididymal transit, under the control of androgens and sympathetic innervations (Kempinas et al., 1998a, Kempinas et al., 1998b and Robaire et al., 2006). It is known that acceleration of epididymal sperm transit time can lead to lower sperm quality (Fernandez et al., 2008 and Klinefelter and Suarez, 1997). Thus, the reduction of sperm quality observed in the intrauterine betamethasone exposed group might be due to an alteration on luminal epididymal microenvironment (for review, see De Grava Kempinas and Klinefelter, 2014).

In this study, prenatal betamethasone treatment was associated with reduced seminal vesicle fluid with no alteration in the seminal vesicle parenchyma. During the emission phase of ejaculation, the seminal fluids produced by the seminal vesicles and prostate are deposited into the urethra by rhythmic contractions of smooth muscle induced by sympathetic and parasympathetic nervous system (Giuliano and Clement, 2005). These contractions are induced by activation of α1-adrenoceptors and muscarinic acetylcholine receptors by released norepinephrine and acetylcholine, respectively (Fedan et al., 1977 and Pennefather et al., 2000). Thus, the seminal vesicles from betamethasone-treated rats presented increased contraction to α1-adrenoceptor and muscarinic acetylcholine receptor activation suggesting

the decreased fluid amount remaining in the seminal vesicles could be a result, at least in part, of increased output during the ejaculation.

Interestingly, these effects of prenatal betamethasone on genital smooth muscle contractility seems to be organ-specific, since the prostate contraction to α1-adrenoceptor and muscarinic acetylcholine receptor activation were not changed. The increase of ventral prostate weight observed in this current work could be correlated to the higher LH levels, once this hormone acts on glandular epithelium increasing fluid production and/or promotes prostate hypertrophy (Ponglowhapan et al., 2012 and Reiter et al., 1999).

Glucocorticoids have been shown to program testicular function permanently (Pedrana et al., 2008 and Pedrana et al., 2013). In the current work, testicular weight was significantly increased in the betamethasone group. Taken into consideration that in these same animals there was a decrease in sperm production, it is possible this effect results from fluid accumulation, once we noted a difference between the total testicular weight and testicular parenchyma, but this hypothesis needs to be further investigated.

In conclusion, prenatal betamethasone exposure leads to long-term reproductive impairment in male rats. These results may be probably due to intrauterine programming and have important implications for humans, considering the use glucocorticoids in pregnant women.

5 - Acknowledgments

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7 – Figure legend

Figure 1 – Body weight of (A) the betamethasone-treated group (n = 13 litters) and control group (n = 11 litters) on PND 01 and (B) the betamethasone-treated group (n = 10) and control group (n = 10) on the PND 120. Values are expressed as mean ± SEM. ** $p \leq 0.01$ (Student´s t-test).

Figure 2 – Hormonal levels in the male offspring of treated and control groups on PND120. Values are expressed as mean ± SEM. * $p \leq 0.05$ (Student's t-test).

Figure 3 – Sperm Motility in male offspring of treated and control groups. (A) Type A: mobile with progressive trajectory, (B) Type B: mobile without progressive trajectory; (C) Type C: immobile. (D) Scatter plot depicting the correlation between fertility potential (%) and progressive sperm motility (%); n = 12 (6 animals of the control group and 6 animals of the betamethasone-treated group). Values are expressed as median and interquartile intervals (first to third). *$p \leq 0.05$, **$p \leq 0.01$ (Mann-Whitney test).

Figure 4. Seminal vesicle: effects of *in vitro* norepinephrine and carbachol. (A) Concentration-response curves to NE and (B) The maximal tension (Emax) developed in the presence of NE in the control (n=4) and betamethasone group (n=4). (C) Concentration-response curves to carbachol and (D) the maximal tension (Emax) developed in the presence of carbachol in the control (n=4) and betamethasone group (n=4). Values are expressed as mean ± SEM. * $p \leq 0.05$ (Student's t-test). Values of pEC_{50} were not different between NE curves (ANOVA, followed by Dunnett).

Figure 5. Ventral prostate: effects of *in vitro* norepinephrine and carbachol. (A) Concentration-response curves to NE and (B) The maximal tension (Emax) developed in the presence of NE in the control (n=4) and betamethasone group (n=4). (C) Concentration-response curves to carbachol in the control (n=4) and betamethasone groups (n=4). Values are expressed as mean ± SEM. * $p \leq 0.05$ (Student's t-test). Values of pEC_{50} were not different between NE curves (ANOVA, followed by Dunnett).

Table 1 - Male sexual behavior.

Parameters	Control (n=11)	Betamethasone (n=13)
Latency to the first mount (s)	79.00 (48.00 - 91.00) (n=11)	69.00 (46.00 - 105.00) (n=13)
Number of mounts until the first ejaculation	12.00 (6.00 - 19.00) (n=11)	13.00 (9.50 - 18.00) (n=13)
Latency to the first intromission (s)	156.00 (71.75 - 328.80) (n=10)	152.50 (81.00 - 244.80) (n=10)
Number of intromissions until the first ejaculation	46.00 (27,25 - 66.75) (n=10)	38.00 (28.75 - 47.00) (n=10)
Latency to the first ejaculation (s)	1581.00 (1173.00 - 1794.00) (n=9)	1124.00 (761.00 - 1467.00) (n=9)
Latency of the first post-ejaculatory mount (s)	240.50 (94.25 - 283.30) (n=8)	252.50 (198.00 - 290.30) (n=8)
Number of post-ejaculatory mounts	3.00 (1.00 - 4.00) (n=8)	3.00 (1.25 - 9.00) (n=8)
Latency of the first post-ejaculatory intromission (s)	243.50 (94.75 - 283.30) (n=8)	252.50 (201.00 - 290.80) (n=8)
Number of post-ejaculatory intromissions	9.00 (4.25 - 19.25) (n=8)	11.50 (8.50 - 13.75) (n=8)
Total number of ejaculations	1.00 (1.00 - 2.50) (n=9)	2.00 (1.00 - 3.00) (n=9)
Ejaculatory plugs (mg)	63.70 (56.05 - 86.50) (n=10)	68.30 (39.70 - 93.20) (n=11)

Values expressed as median and interquartile intervals (first to third). Mann-Whitney test.

Table 2 - Fertility after *artificial insemination*.

Parameters	Control (n=07)	Betamethasone (n=08)
[1] Pregnancy rate (%)	85.72	87.50
[1] Fertility potential (%)	70.59	52.13*
[1] Pre- implantation loss (%)	29.41	47.87*
[1] Post- implantation loss (%)	29.69	16.67
[2] Body weight of dams (g)	304.30±3.09	291.50±7.58
[2] Uterus weight with fetuses (g)	8.25±0.79	8.36±1.79
[2] Corpora lutea number	11.33±0.84	13.36±0.72
[2] Implantation number	7.92±1.04	6.93±1.44
[2] Reabsorption number	2.42±0.95	1.14±0.40
[2] Fetus weight (mg)	315.70±7.43	293.80±7.86[a]
[2] Placental weight (mg)	258.20±7.71	277.80±12.27

[1] Values expressed as percentage. *p< 0.05 (Chi-Square test). [2] Values expressed as mean ± SEM. (Student's t- test). [a] p= 0.0712

Table 3 – Body and organ weights.

Parameters	Control (n=11)	Betamethasone (n=13)
Final body weight (g)	488.30 ± 7.16	467.10 ± 10.60
Total testis (mg)	1793.00 ± 23.89	1858.00 ± 19.90*
Parenchyma of testis (mg)	1300.00 ± 50.00	1260.00 ± 41.00
Testis (%)	0.37 ± 0.01	0.41 ± 0.01**
Epididymis (mg)	556.20 ± 12.52	556.90 ± 11.12
Epididymis (%)	0.11 ± 0.004	0.12 ± 0.003
Ventral prostate (mg)	386.00 ± 15.34	457.40 ± 21.67*
Ventral prostate (%)	0.08 ± 0.003	0.10 ± 0.004**
Seminal vesicle (mg)	1266.00 ± 48.69	1163.00 ± 49.43*
Seminal vesicle (%)	0.26 ± 0.011	0.25 ± 0.007
Seminal vesicle fluid (mg)	923.10 ± 44.86	782.40 ± 40.35*

Values expressed as mean ± SEM. Student´s t-test. *$p \leq 0.05$; **$p \leq 0.01$

Table 4 – Sperm morphology.

Parameters	Control (n=10)	Betamethasone (n=10)
Normal sperm (%)	96.00 (95.00 – 98.00)	89.00 (86.00 – 92.50)*
Anormal sperm (%)		
Isolated head	1.00 (0.50 – 2.00)	4.00 (2.00 – 5.50)*
Head without curvature	2.00 (0.00 – 3.50)	5.00 (1.00 – 8.00)
Broken tail	0.50 (0.00 – 1.00)	1.50 (0.00 – 3.25)
Cytoplasmic droplet (%)	25.00 (17.50 – 26.00)	31.00 (28.50 – 36.50)*

Values expressed as median and interquartile intervals (first to third). Mann-Whitney test.

Table 5 - Sperm counts.

Parameters	Control (n=09)	Betamethasone (n=10)
Sperm count in the testis		
Mature spermatids number ($x10^6$/testis)	264.80 ± 8.35	197.30 ± 23.11*
Relative spermatids number ($x10^6$/g/testis)	203.70 ± 5.36	157.00 ± 19.13*
Daily sperm production ($x10^6$/testis/day)	43.35 ± 1.35	32.34 ± 3.78*
Relative daily sperm production ($x10^6$/testis/g/day)	33.39 ± 0.87	25.73 ± 3.14*
Sperm count in the epididymis		
Sperm number in caput/corpus ($x10^6$/organ)	95.09 ± 8.14	91.03 ± 7.42
Sperm number in cauda ($x10^6$/organ)	234.60 ± 11.28	182.80 ± 10.01*
Sperm transit time in caput/corpus (days)	2.24 ± 0.19	3.57 ± 0.77
Sperm transit time in cauda (days)	5.55 ± 0.33	7.71 ± 1.30
Total sperm transit time (days)	7.78 ± 0.46	12.08 ± 1.94 *

Values are expressed as mean ± SEM. * $p < 0.05$ (Student's t-test).

Figure 1

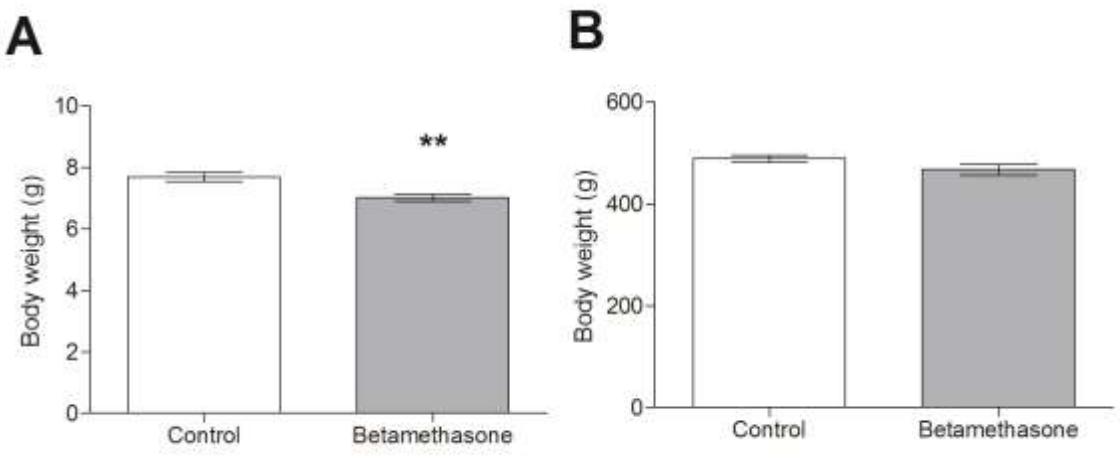

A
Body weight (g)
10
8
6
4
2
0
**
Control
Betamethasone
B
Body weight (g)
600
400
200
0
Control
Betamethasone

Figure 2

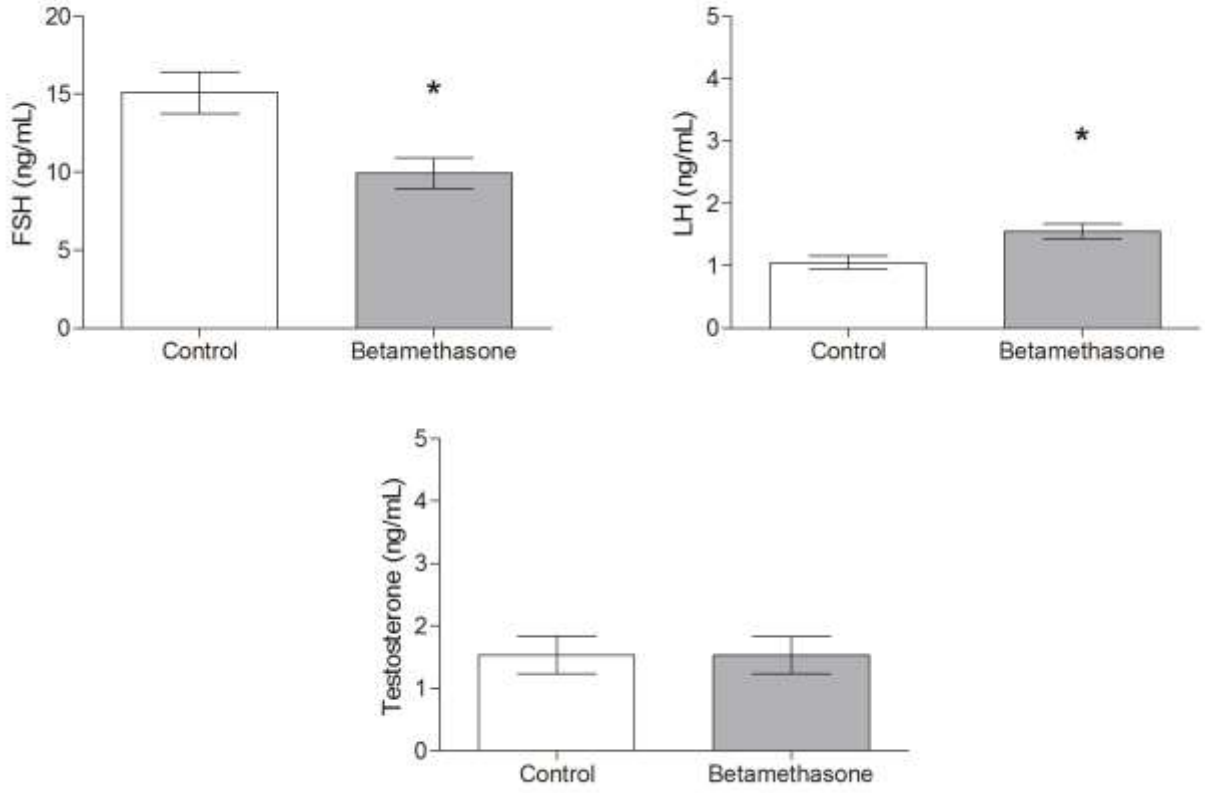

FSH (ng/mL)
20
15
10
5
0
*
Control
Betamethasone
LH (ng/mL)
5
4
3
2
1
0
*
Control
Betamethasone
Testosterone (ng/mL)
5
4
3
2
1
0
Control
Betamethasone

Figure 3

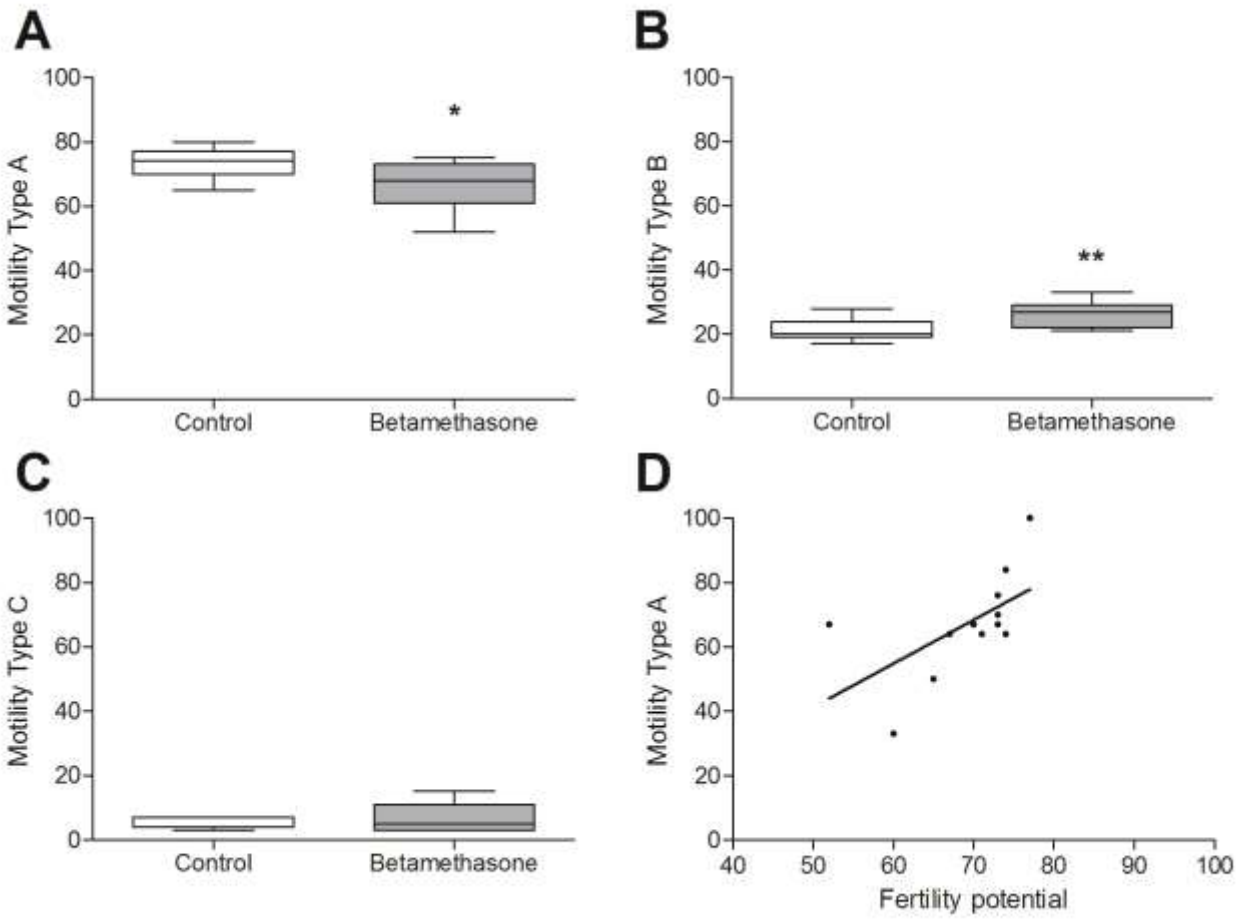

A
Motility Type A
100
80
60
40
20
0
*
Control
Betamethasone
B
Motility Type B
100
80
60
40
20
0
**
Control
Betamethasone
C
Motility Type C
100
80
60
40
20
0
Control
Betamethasone
D
Motility Type A
100
80
60
40
20
0
40
50
60
70
80
90
100
Fertility potential

Figure 4

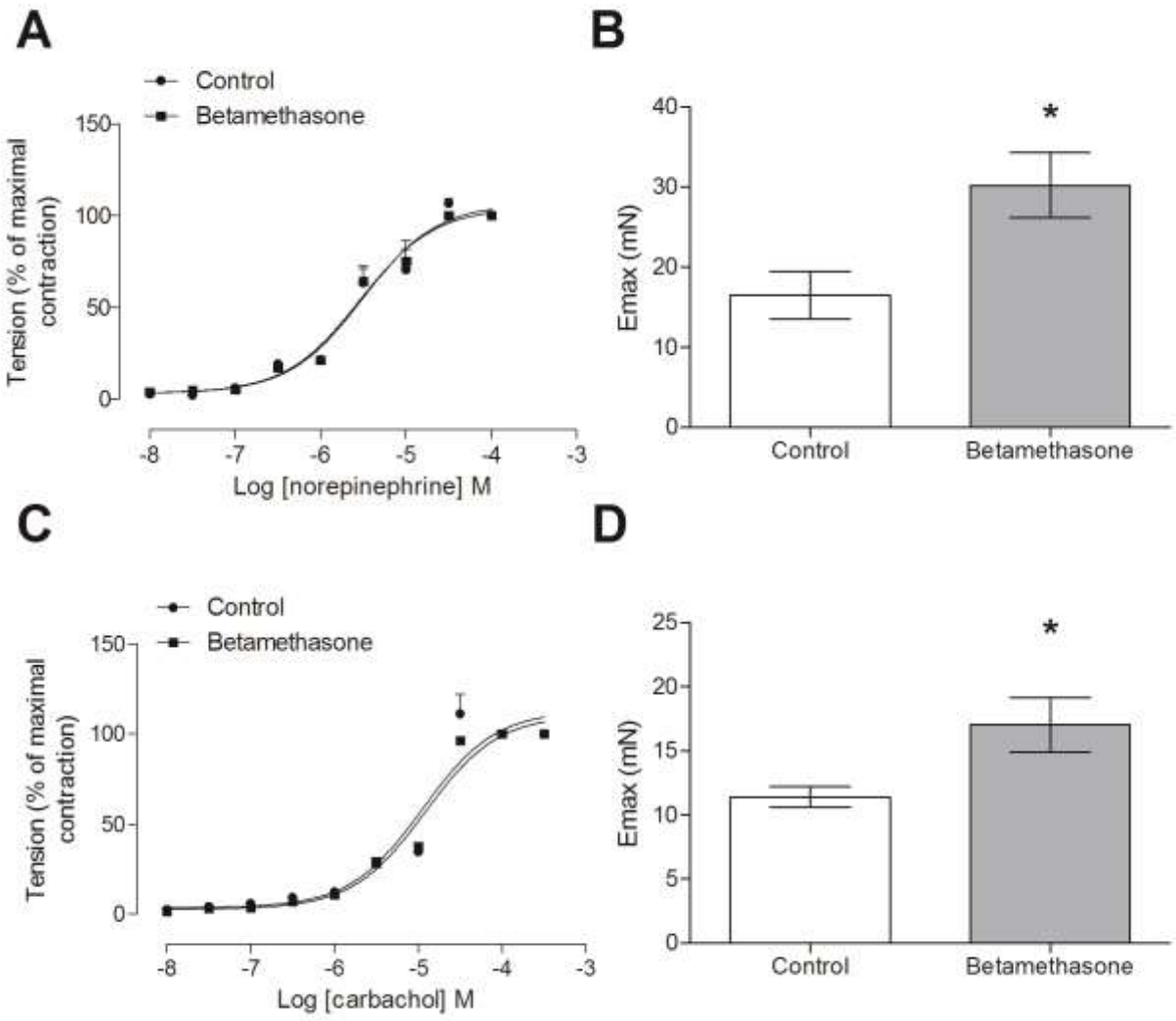
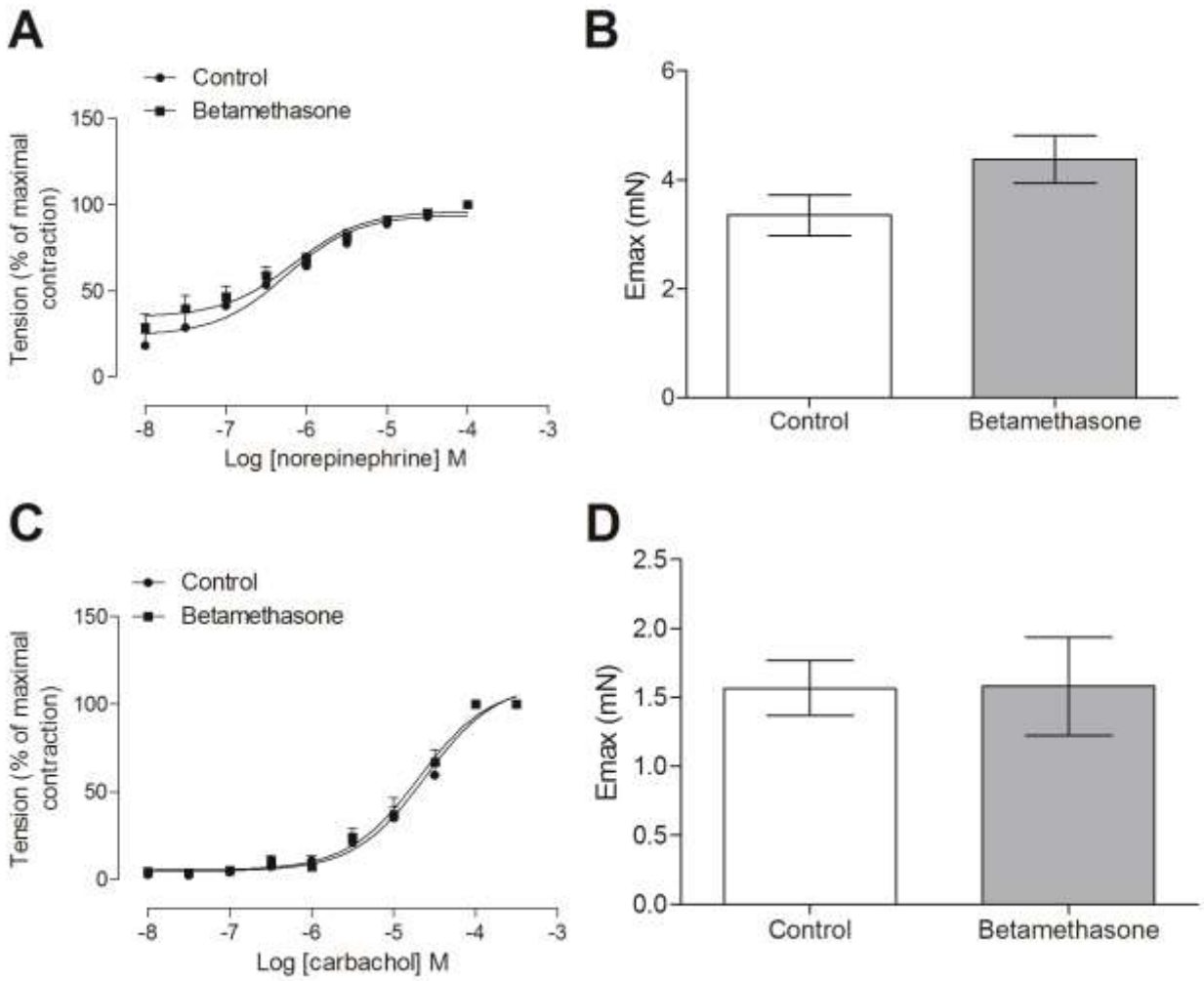

A
Control
Betamethasone
Tension (% of maximal contraction)
150
100
50
0
-8
-7
-6
-5
-4
-3
Log [norepinephrine] M
B
Emax (mN)
40
30
20
10
0
*
Control
Betamethasone
C
Control
Betamethasone
Tension (% of maximal contraction)
150
100
50
0
-8
-7
-6
-5
-4
-3
Log [carbachol] M
D
Emax (mN)
25
20
15
10
5
0
*
Control
Betamethasone

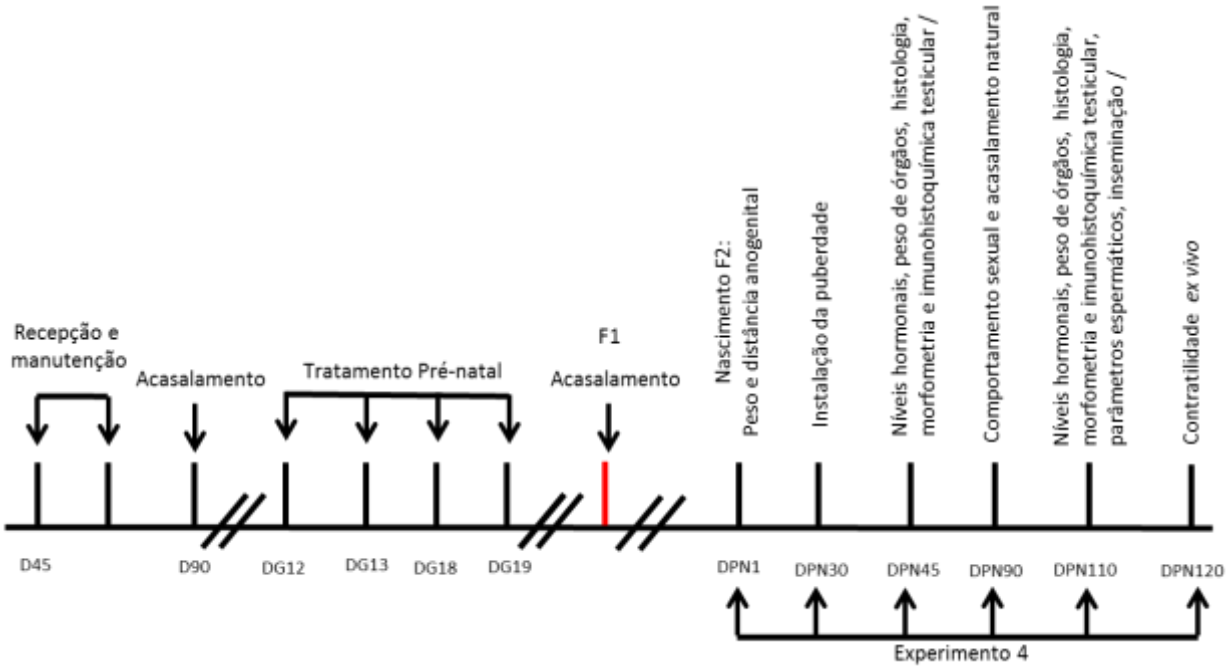
Figure 5


A
Control
Betamethasone
Tension (% of maximal contraction)
150
100
50
0
-8
-7
-6
-5
-4
-3
Log [norepinephrine] M
B
Emax (mN)
6
4
2
0
Control
Betamethasone
C
Control
Betamethasone
Tension (% of maximal contraction)
150
100
50
0
-8
-7
-6
-5
-4
-3
Log [carbachol] M
D
Emax (mN)
2.5
2.0
1.5
1.0
0.5
0.0
Control
Betamethasone

Capítulo 4

Este trabalho deu origem ao manuscrito “BETAMETHASONE CAUSES INTERGENERATIONAL REPRODUCTIVE IMPAIRMENT IN MALE RATS” que esta sob revisão no periódico “Reproductive Toxicology” (fator de impacto: 2,850).

Foram avaliados neste manuscrito todos os parâmetros de desenvolvimento sexual inicial, espermáticos (contagem, morfologia, motilidade e cometa), contratilidade das glândulas sexuais acessórias e fertilidade de ratos machos (F2) cujos pais foram expostos *in utero* à betametasona, segundo desenho experimental abaixo:



Nossos resultados demonstraram que a betametasona promoveu redução do peso da prole masculina ao nascimento, assim como atraso na instalação da puberdade. Foi observado alteração do peso do epidídimo e da vesícula seminal, esta ainda apresentou redução na sua atividade contrátil. As dosagens hormonais demonstraram redução nas concentrações de FSH e aumento nas concentrações de LH. Houve alteração dos parâmetros espermáticos, comportamento sexual e redução na fertilidade dos animais do grupo betametasona.

BETAMETHASONE CAUSES INTERGENERATIONAL REPRODUCTIVE IMPAIRMENT IN MALE RATS

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ABSTRACT

Prenatal betamethasone (BM) exposure in rats negatively impacts sperm quality and male fertility. Studies have shown that BM can cause multi-generational effects on the pituitary-adrenal-axis of rats. The objective of this study was to assess the reproductive development and fertility of male rats (F2) whose fathers (F1) were exposed to BM (0.1 mg/kg) on gestational days 12, 13, 18 and 19. In F2 rats, there was a significant reduction in body weights of the BM-treated group at PND 1 as well as delayed onset of puberty, and decreases in FSH levels, Leydig cell volume, sperm number and motility, seminal vesicle contractility and ejaculated volume. Furthermore, increased serum LH levels, sperm DNA damage and abnormal morphology were observed, resulting in reduced fertility. In conclusion, prenatal BM-treatment leads to intergenerational long-term reproductive impairment in male rats, raising concern regarding the widespread use of BM in preterm births.

Keywords: betamethasone, intergenerational effects, puberty onset, sperm quality, fertility, seminal vesicle contractility.

1. INTRODUCTION

Increased levels of glucocorticoids are necessary in the later stages of pregnancy to promote the maturation of several organs, including the lungs [1]. For pregnant women at risk of preterm birth, prenatal synthetic glucocorticoids, especially betamethasone (BM), is administered to decrease the incidence of neonatal mortality due to respiratory distress syndrome [2, 3].

Thus, glucocorticoids play an important role in promoting fetal lung maturation and reducing neonatal death, and because of this, have a fundamental role in determining fetal programming [4, 5]. Animal studies have demonstrated that fetal exposure to elevated doses of synthetic glucocorticoids at the end of the gestational period results in behavioral, endocrine, and metabolic abnormalities [6].

Our laboratory has previously demonstrated that, *in utero*, BM-treatment during two critical periods of development (days 12 and 13, corresponding to the period of germ cell migration and proliferation, and days 18 and 19, when testosterone levels increase) resulted in several important alterations to male sexual development, sperm quality and fertility. Furthermore, the treatment altered the normal pattern of Sertoli and germ cell organization in the testis of the rat, suggesting that reproductive programming may be dramatically altered by BM-treatment [7-10].

In the past several years, studies have attempted to demonstrate the influence of intra-uterine treatment of glucocorticoids using a multi-generational approach [5, 6]. Drake et al. [11, 12] observed changes in body weights, as well as in the expression of growth factors and hepatic proteins of rat fetuses exposed to dexamethasone during intrauterine development in both the first and second generation. Furthermore, prenatal exposure to BM resulted in trans-generational changes to the hypothalamic-pituitary-adrenal axis, resulting in altered ACTH (adrenocorticotropic hormone) secretion and changes in both glucocorticoid and mineralocorticoid receptors in the adrenal gland [13]. Thus, knowing the effects on the male reproductive tract in first generation [7, 8], the present study investigated the possible inter-generational reproductive impact of BM on sperm quality and fertility of second generation males whose fathers were exposed at critical stages of *in utero* sexual differentiation.

2. MATERIALS AND METHODS

2.1 ANIMALS

Male (90 days old/300–350 g) and female (90 days old/225–230 g) *Wistar* rats were obtained from the Multidisciplinary Center for Biological Investigation, State University of Campinas, and maintained under controlled conditions (25°C, 30% air humidity, 12/12-h light/dark cycle) with food and water available *ad libitum*.

The experimental procedures used in this study were approved by the local Ethics Committee for the Use of Experimental Animals under protocol number 451-CEEA in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health).

2.2 EXPERIMENTAL DESIGN

One nulliparous female rat was mated with a male during the dark cycle of the photoperiod (Figure 1). The detection of sperm in the vaginal smear of the female rat in estrus was considered as gestational day (GD) 1. Pregnant and lactating rats were maintained in individual cages.

Pregnant female rats were randomly allocated into two experimental groups: control (saline vehicle, n=11) and BM-treated (0.1 mg/kg; Betamethasone 21-phosphate disodium; Sigma-Aldrich, St Louis, MO, diluted in vehicle, n=13) using the same protocol previously described by Borges et al. [7, 8]. Rats received an intramuscular injection of vehicle (0.1 ml/kg) or BM on days 12, 13, 18 and 19 of pregnancy. Days 12 and 13 correspond to the period of germ cell migration and proliferation while days 18 and 19 are when gestational testosterone levels increase. After birth, one male per litter was kept until post-natal day (PND) 90 (n=11/group), and was mated with a non-treated female. The detection of sperm in the vaginal smear was considered as GD 1.

After birth, on PND 1, the number of pups per litter (F2) was randomized and redistributed in order to have 8 pups (4 males and 4 females) per lactating female. Rats were weaned at PND 21 and housed in separate cages (n=4 males per cage). For each study, one male rat from each litter was sampled in order to prevent potential variation due to intra-litter effects.

2.3 STUDY 1: INITIAL DEVELOPMENT AND SPERM QUALITY

2.3.1 Anogenital Distance and External Examination at Puberty

Rat pups (F2) were weighed and the anogenital distance was measured at PND 1 in four male rats per litter (n = 11 per group). The onset of puberty was determined by manual retraction of the prepuce [14].

2.3.2 Organ Weights

On PND 45 and PND 110 (n=10/ group), F2 rats were weighed and euthanized. Blood was then collected (9:00 and 11:30 AM) and the right testis, epididymis, seminal vesicle (full and empty, without the coagulating gland), and ventral prostate were dissected and weighed. The left testes and epididymides were fixed for histology and immunohistochemistry. For animals at PND 110, the parenchyma of right testis was obtained by cutting out the albuginea and removing the testicular fluid by centrifugation (3000 rpm) for 30 min at 4°C and frozen at -20°C for determining sperm counts. The right cauda epididymidis was used for sperm collection for intrauterine artificial insemination and sperm quality parameters. The remaining tissue was frozen and used for measuring sperm counts, as was the caput/corpus epididymidis.

2.3.3 Serum Hormonal Levels

Blood samples were allowed to coagulate and the serum obtained by centrifugation (2400 rpm) for 20 min at 4°C. Serum samples were subsequently stored at -20°C. Serum testosterone, FSH and LH levels were determined by radioimmunoassay. Testosterone levels (serum and intratesticular) were measured using the Coat-A-Count® assay (Diagnostics Products Corporation, Los Angeles, USA), while LH and FSH levels were measured using specific kits supplied by the National Institute of Arthritis, Diabetes and Kidney Diseases (NIADDK). Intra-assay variabilities were 3.4% for LH, 2.8% for FSH, and 4% for testosterone. Using the hormone values, Leydig cell function was determined by measuring intratesticular testosterone levels (ITT)/ LH levels (IT ratio) as described previously [15].

2.3.4 Fertility Assessment by *In Utero* Artificial Insemination

Rats (F2) euthanized on PND 110 were used for fertility assessment using *in utero* artificial insemination as described by Borges et al. [8]. Briefly, females in proestrus were paired with sexually experienced vasectomized males for 1 hour. Receptive females were selected for the insemination procedure. Sperm were released from the right proximal epididymal cauda and a volume containing 5×10^6 sperm was injected into each uterine horn. One female was inseminated per male.

Twenty days later, the females were euthanized to evaluate fertility. After collection of the uterus and ovaries, the number of corpora lutea, implanted fetuses, and reabsorption sites were recorded.

2.3.5 Sperm Motility

Sperm motility was evaluated in the same sperm sample used for artificial insemination [7]. An aliquot of 10 µL of sperm suspension was immediately transferred to a Makler chamber and maintained at 34^{o} C. Using a phase-contrast microscope (400 x magnification), 100 sperm were counted and classified as Type A (mobile with progressive movement), Type B (mobile without progressive movement), and Type C (immobile).

2.3.6 Sperm Morphology

An aliquot of 100 µl of the sperm suspension was added to 900 µl of formol saline (810 µl saline + 90 µl formol). To analyze sperm morphology, smears were prepared on histological slides that were left to dry for 90 min and 200 spermatozoa per animal were analyzed under a phase-contrast microscope (400X magnification). Morphological abnormalities were classified into two general categories: head morphology (without characteristic curvature, pin or isolated head) and tail morphology (broken or rolled into a spiral) [16]. Sperm were also classified as to the presence or absence of the cytoplasmic droplet.

2.3.7 Sperm Counts and Sperm Transit Time

Homogenization-resistant testicular spermatids (stage 19 of spermiogenesis) were counted as described previously [8]. Briefly, testes were decapsulated, weighed, and homogenized in 5 ml of NaCl 0.9% containing Triton X 100 0.5%, followed by sonication for 1 min. After a 10-fold dilution, one sample was transferred to Neubauer chambers (4 fields per animal), and mature spermatids were counted. To calculate the daily sperm production (DSP), the number of spermatids at stage 19 was divided by 6.1, which is the number of days of the seminiferous cycle during which these spermatids are present in the seminiferous epithelium [17]. The caput/corpus and cauda epididymidis were cut into small fragments with scissors and homogenized. Sperm were counted as described for the testis. The sperm reservoir in the cauda region of the epididymis was calculated by combining the number of cells in the sperm suspension and the sperm count in the homogenized tissue [7]. The sperm

transit time through the epididymis was determined by dividing the number of sperm in each region of the epididymis by the DSP [17].

2.3.8 Sperm DNA Fragmentation (Comet Assay)

Five F2 rats per experimental group (one per litter) were euthanized at PND 120. Sperm DNA fragmentation was evaluated by the comet assay as previously described [18] with the following modifications: frozen sperm samples from right cauda epididymides were thawed and diluted in low melting point agarose (0.5% at 37°C; LGC Laboratories, Sao Paulo, Brazil), at a concentration of 10^6 cells/mL. An aliquot (75µL) of the solution was placed on pre-coated slides with 1% normal melting point agarose (LGC Laboratories, Middlesex, UK). The slide was first immersed in lysis buffer (100mM Na_2-EDTA, 10mM TrisHCl, 2,5M NaCl, pH 11.0) containing 40mM DTT and 2% Triton X-100 for 1h at 4°C and subsequently in lysis buffer containing proteinase K (0.1 mg/ml), for 2.5hrs at 37°C. The DNA was fractionated by alkaline electrophoresis (300mM NaOH, 1mM Na2 EDTA, pH 13.0) at 3 V/cm and 270 mAmps for 45 mins. Finally, the slides were washing with water, fixed in absolute ethanol, air dried and stored in the dark until analysis. For microscopy, the slides were stained with SYBR® Gold (1:10,000; Invitrogen, Waltham, MA) and analyzed using the Comet Assay IV software (Comet Assay IV, Perceptive Instruments, Wiltshire, UK). DNA damage was measured by analyzing the tail intensity (% of migrated DNA). A total of 50 cells were analyzed per slide (2 slides/animal).

2.3.9 Histological Analyses

Left testes were fixed by immersion in Bouin's fixative, dehydrated, and embedded in Paraplast Plus® (P3683, Sigma-Aldrich). Tissues (n=5/ group) were sectioned (5µm), mounted on glass slides and stained with hematoxylin and eosin (HE) to evaluate testicular morphology. Sections destined for immunohistochemistry were mounted on silanized glass slides and stored at room temperature. Histopathology was analyzed in a blind assay using a Zeiss microscope (A1-Axio model) mounted with a digital camera and analysis software (ZeissAxio Vision, version 4.7.2). The histological analyses were performed as described previously [7]. Briefly, three non-serial testicular sections were taken for each animal in order to evaluate 100 seminiferous tubules. The interstitium, peritubular myoid cells and Leydig cells were also examined [19].

The diameter of the seminiferous tubules (stage IX of spermatogenesis) and thickness of germinal epithelium were evaluated in 10 sections of seminiferous tubules per animal. In

order to evaluate the degree of maturation of the seminiferous epithelium, 100 cross-sections of seminiferous tubules per animal were randomly evaluated by assigning values according to the abundance of mature germ cells in the epithelium [20]. The following scale was used: category 1 (I): young spermatids with rounded nucleus (stages 1–8 of the cycle of the seminiferous epithelium); category 2 (II): spermatids in maturation phase, with ovoid or elongated nucleus (stages 9–14); category 3 (III): spermatids in maturation phase, with elongated nucleus (stages 15–18); category 4 (IV): mature spermatids (stage 19) in small numbers; category 5 (V): mature spermatids (stage 19) in average numbers; category 6 (VI): mature spermatids (stage 19) in high abundance. Each symbol represents the number of tubules in the particular category. The "average category" was determined using the following mathematical formula: Average category = [(I x 1) + (II x 2) + (III x 3) + (IV x 4) + (V x 5) + (VI x 6)]/100.

In order to evaluate the dynamics of spermatogenesis, the relative frequency of stages was estimated: I-VI (two generations of spermatids), VII-VIII (mature spermatids), IX-XIII (only one generation of spermatids), XIV (secondary spermatocyte) in 100 cross-sections of seminiferous tubules per animal. The relative frequency of stages estimates the rate or duration of spermatogenic process [21].

Furthermore, the number of Sertoli cell nuclei and the volume of Leydig cells were also determined. Sertoli cell nuclei were determined in histological sections from 20 seminiferous tubules per rat testis at stage VII of spermatogenesis. The nuclear volume of Leydig cells was measured by randomly selecting 50 circular or elliptical cells, their diameters (D) were measured, and the volume obtained using the formula: V = [D x π}/6 [22].

2.3.10 Immunohistochemistry for Cx43 and PCNA in the Testis

Histological sections were deparaffinized and hydrated in graded ethanol and washed in TBST (Tris-Buffered Saline, 0.1% Tween-20). Sections were then subjected to antigen retrieval in citrate buffer (0.01 M, pH 6.0) and heated in a microwave at 70% power for 10 min. Slides were washed in ddH_2O, followed by hydrogen peroxide (3%) for 15 min, tap water (5 min), and then incubated in blocking solution (TBST, 5% BSA) for 1 hr at 37^{o}C in a humidified chamber. Sections were then incubated overnight with primary antisera as follows: anti-Cx43 (0.625 µg/ml, C-6219; Sigma-Aldrich); or anti-PCNA (4 µg/ml, SC-56/ PC-10; Santa Cruz Biotechnology). Sections were then washed 3 times in TBST and incubated with the appropriate secondary antibody for 1hr at 37°C (for Cx43: 1 µg/ml goat anti-rabbit HRP , Abcam; for PCNA: 1.6 µg/ml goat anti-mouse HRP ; Santa Cruz Biotechnology). Sections

were subsequently washed with TBST and incubated with diaminobenzidine (DAB) (0.5 mg/ml, Sigma-Aldrich) for 15 min, rinsed in tap water and counterstained with methylene blue for 5 min. Negative control sections did not receive primary antibody.

2.4 STUDY 2: SEXUAL BEHAVIOR AND NATURAL MATING

2.4.1 Evaluation of Male Sexual Behavior

At PND 90, one F2 male per litter (n=11/ group) was placed in a cage alone for 5 min prior to the introduction of a sexually receptive female (in estrous phase determined by previous vaginal smears). Paired animals were observed manually during the dark cycle of the photoperiod. Males that did not mount the female within the first 10 min were considered sexually inactive. Male sexual behavior was evaluated for 40 min and included the following parameters: latency to the first mount; latency to the first intromission; latency to the first ejaculation; number of intromissions until the first ejaculation; latency of the first post-ejaculatory intromission; number of post-ejaculatory intromissions; and total number of ejaculations. The female rats used for sexual behavior were kept with a male for 3 hrs after the end of sexual behavior. After that, the male and female were removed and the ejaculatory plugs were collected and weighted.

2.4.2 Fertility Evaluation after Natural Mating

The female rats used for sexual behavior were allocated in individual cages and vaginal smears were collected to confirm whether or not insemination occurred. Females naturally inseminated by rats from the control and treated groups were euthanized 20 days after mating to assess male fertility. A median laparotomy was done and the uterus and ovaries were collected. The parameters observed were the same as those described in section 2.3.4 [8].

2.5 - STUDY 3 – CONTRACTILITY OF ISOLATED SEMINAL VESICLE

Five F2 rats per experimental group (one per litter) were weighed and euthanized at PND 120. A lobule of seminal vesicle was prepared for the *in vitr*o tension recording as described below [23]. Tissues were mounted in 10ml organ baths in a modified Tyrode's solution at 30ºC under 9.8 mN (milliNewtons) resting tension and allowed to equilibrate for 30 min. After the resting period, tissues were repeatedly challenged with 80 mM KCl every

30 mins until two reproducible contractions were obtained. Cumulative concentration–response curves to norepinephrine (NE, 10^{-8} M - 10^{-4} M) and carbachol (CCh, 10^{-8} M – 3.10^{-4} M) were obtained at 30 min intervals. The maximal tension developed (Emax,) and the potency to NE and CCh in developing tension (pEC50, the –log of agonist concentration inducing 50% of Emax) were evaluated.

2.6 - STATISTICAL ANALYSIS

Data are presented as mean ± standard error of mean (SEM) or median and interquartile range. A Student´s t-test was used for comparison of parametric variables. Non-parametric data were compared using a Mann-Whitney test. Differences were considered significant when $p \leq 0.05$. Statistical analyses were performed using the GraphPad InStat software (version 5; La Jolla, CA).

3. RESULTS

3.1 Study 1: Initial development and sperm quality

At PND 1 a significant reduction of F2 male offspring weight was observed in the BM-group (Figure 2A) without any changes in relative anogenital distance (Figure 2B). At PND 21, the difference in body weight between the experimental groups were maintained (Figure 2C).

In the BM-group, there was a significant delay in the onset of puberty as determined by preputial separation (Figure 2D). Circulating levels of FSH were significantly lower while LH levels were significantly higher at both ages (PND 45 and PND 110). Serum testosterone levels were unaltered (Figure 3). Interestingly, ITT ratios were lower, although these were not statistically significant (Figure 3).

Testicular and epididymal weights were significantly higher in the BM-group while seminal vesicle weights were significantly lower at PND 45; however at PND 110 both organ weights were similar between groups (Table 1).

There were no alterations in histopathologic and morphometric analysis of the testis between the control and BM-treated group (Table 2); however, Leydig cell volume and function, according to the ITT ratio, were significantly decreased in BM-treated group at PND 110 (Figure 4). Immunostaining for Cx43 and PCNA in the testis was also similar between groups (data not shown).

In the BM-treated group, there was a significant decrease in daily sperm production and the number of sperm in the caput/corpus and cauda epididymidis (Table 3). The percentage of both immobile sperm (Figure 5) and morphologically abnormal sperm were significantly increased. Most of the abnormalities in the sperm were due to alterations in sperm heads (Table 4).The sperm samples from the BM-treated group showed an increase percentage of DNA damage (tail intensity), when compared with the control group (Figure 6).

Fertility potential, after *in utero* artificial insemination, was significantly reduced in the BM-group and, consequently, pre-implantation loss was significantly increased (Table 5).

3.2 Study 2: Sexual behavior and Natural Mating

Sexual behavior was altered in the F2 BM-treated group, which displayed a significant delay prior to the first mount (Table 6). Few of these animals ejaculated in the females (approximately 9%) during sexual intercourse. In contrast, the control animals showed an ejaculation frequency of almost 55% (Table 6). Furthermore, the weight of the ejaculatory

plugs obtained after the end of the mating period was reduced by approximately 31% in the BM-treated group as compared to controls.

After natural mating, the fertility potential was reduced and pre-implantation loss significantly increased in the BM-treated group (Table 7). These results are similar to those observed after *in utero* artificial insemination (Table 5).

3.3 Study 3: Contractility of Isolated Seminal Vesicles

As shown in Figure 7A, the potency of NE (adrenergic agonist) in inducing contractions in the isolated seminal vesicle was comparable between groups (control group: pEC50 = 5.75 $\pm$ 0.07; BM group: pEC50 =5.68 $\pm$ 0.19). However, the maximal tension developed (Emax) for NE in the BM-treated group was significantly decreased as compared to controls (Figure 7B). The same pattern was observed for CCh (Figures 6C and 6D), a cholinergic agonist (control group: pEC50 = 4.79 $\pm$ 0.09; BM group: pEC50 = 4.80 $\pm$ 0.16).

4. DISCUSSION

Betamethasone is one of the most prescribed compounds for promoting fetal development at critical periods of intrauterine development, and is known to cause permanent physiological alterations [24, 25]. Several studies have demonstrated the impact of intrauterine BM-treatment during long-term development, with emphasis on neuronal, endocrine, renal and reproductive problems [3, 7-10, 26, 27].

Indeed, in recent years, several studies have reported on the impact of prenatal glucocorticoid treatment not only on the exposed offspring, but also on subsequent generations, resulting in altered protein expression, growth factors, and hypothalamic-pituitary-adrenal axis [6, 11-13]. The present study indicates that prenatal BM exposure during critical periods of male fetal sexual development can induce alterations in the reproductive system of the second generation males, and that this impact can be observed from birth to adulthood.

Birth weight reduction is an important target that may be correlated with fetal programming and can have lasting impacts over the life of the individual [28]. In the present study, we observed a reduction in the birth weight of F2 BM-treated group, which is similar to previous findings [11, 13]. This reinforces the possible inter-generational impact of BM treatment. We previously observed a similar effect, in which male fetuses exposed to BM during intrauterine development displayed reduced weight [8]. Moreover, these fetuses displayed other alterations such as poor weight gain during early stages of postnatal development and delayed puberty onset [9, 29-31].

Our data indicate that the weight of the epididymis was significantly increased while seminal vesicle weights were decreased only at PND 45. The reasons for these changes are difficult to explain, since both organs are androgen-dependent, and in this case the differences are contrary to expected changes. It is possible that changes in weights of these organs reflect a specific effect of BM. Further studies are needed to fully understand this observation.

Circulating levels of FSH were decreased in F2 rats at both ages, while LH levels were increased without alterations to serum testosterone levels. We previously observed a similar pattern of circulating hormone levels in rats treated *in utero* to BM [7, 8]. Interestingly, this effect is maintained in the second generation. Previous studies by Iqbal et al. [13] reported that BM altered the hypophyseal-pituitary-adrenal axis over two generations. The present observations suggest that a similar effect may be occurring in the hypophyseal-pituitary-testicular axis. This may, in part, explain the decreased levels of FSH. Lower levels of FSH

can, in turn, be correlated with the decreased sperm production via its actions on spermatogenesis [8, 32].

While testosterone levels were unaltered by BM, serum LH levels were increased and the ITT ratio of Leydig cells was decreased. Since the Leydig cell volume was decreased, and this is generally correlated with lower testosterone production [33], the higher LH levels may reflect the need for a greater stimulation of the Leydig cells in order to maintain circulating levels of testosterone [15].

Changes in sexual behavior were observed in rats from the BM-treatment group. These rats displayed a significant decrease in sexual interest with respect to the time of first mount and decreased number of ejaculations, in which only 9% of the animals were able to ejaculate. Disruption of male sexual behavior has been described after prenatal exposure to stress or to several compounds, such as glucocorticoids, that alter fetal programming, [9, 34, 35]. Male sexual behavior in adult mammals is modulated by testosterone; however, this hormone is not the only factor that regulates sexual performance, which also requires normal function of the hypothalamic–pituitary–testicular axis and neuronal control [35]. Pereira et al. [34] demonstrated that after testosterone replacement, male rats continue to exhibit sexual behavior dysfunction, suggesting that other factors contribute to the impairment of sexual interest. These may include hormonal imbalance, as suggested by the high levels of LH, or changes in neurotransmitter responses in the sexual reproductive organs. Interestingly, in our previous study [8], the BM-treated group did not show alterations in sexual behavior. Hence, this effect may only manifest itself as an inter-generational effect.

During the emission phase of ejaculation, 70-90% of the ejaculate consists of seminal fluid from seminal vesicle, which is dependent on testosterone levels [36] and rhythmic contractions of smooth muscle induced by the sympathetic and parasympathetic nervous system [37]. Pereira and Arena [34] reported that intrauterine glucocorticoids reduce the contraction of seminal vesicles in adults. This may explain the reduced ejaculated plug weights observed in BM-treated F2 rats.

Fertility tests performed on the same animals as those used for sexual behavior showed a significant reduction in fertility potential [38]. However, the decreased ability to ejaculate and the reduced ejaculated plug weights observed in the F2 BM-treated animals could have interfered with the interpretation of fertility results, and have directly resulted in reduction of fertility potential. To circumvent this, *in utero* artificial insemination was performed [38-41]. The results indicated that artificial insemination still resulted in decreased

fertility potential in the second generation BM-treated rats, thereby suggesting that the actual quality of the semen, or gametes, was greatly reduced in these animals.

To further examine the quality of semen samples in BM-treated F2 animals, sperm morphology, motility and sperm DNA damage were analyzed [38, 42-44]. Considering that both motility and morphology are important for assessing sperm quality and can be directly correlated to sperm fertile capacity [45], our data indicate that the reduction on sperm fertility in the second generation of BM-treated rats is due to, in part, to a reduction in motility as well as an increase in sperm morphological defects.

Comet assays indicated an increase in the percentage of DNA damage in the BM-treated group. Since sperm DNA damage may be related to an increase in premature embryo losses as reflected by pre-implantation and/or initial post-implantation loss [46], these results might explain the increased pre-implantation loss observed in the BM-treated group. These data, along with the fertility assays, support the notion that semen quality was seriously compromised and maintained over two generations in BM-treated rats.

In conclusion, prenatal betamethasone exposure leads to inter-generational long-term reproductive impairment in male rats and these findings raise concern for humans, considering the use of BM in pregnant women.

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7. CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

8. FIGURE LEGENDS

Figure 1 – Experimental design of animals whose fathers were exposed to vehicle (controls) or BM during the intrauterine development.

Figure 2 –Body weights at PND 1 (A) and relative anogenital distance (B) of control (n = 11 litters) and BM-treated rats (n = 11 litters). Body weights on the day of weaning (C). Age of complete preputial separation in control and BM-treated litters (D). Data are expressed as the mean ± SEM. * indicates $p \leq 0.05$ (Student T-test).

Figure 3 – Hormone levels in male offspring of BM-treated and control rats at PND 45 and PND 110. Data are expressed as the mean ± SEM. * indicates $p \leq 0.05$ (Student T-test).

Figure 4 – (A) Leydig cell volume and (B) ITT ratio, expressed as the ITT (pg/mL)/ LH levels (pg/mL), in male offspring of control and BM-treated rats. Data are expressed as the mean $\pm$ SEM. * indicates $p \leq 0.05$. (Student T-test).

Figure 5 – Sperm Motility in male offspring of control and BM-treated groups (A) Type A: mobile with progressive trajectory, (B) Type B: mobile without progressive trajectory and (C) Type C: immobile. Data are expressed as median and interquartile intervals. * indicates $p \leq 0.05$. (Mann-Whitney test).

Figure 6 – Sperm DNA fragmentation in male offspring of control and BM groups observed by percentage of tail intensity *** indicates $p \leq 0.001$. (Mann-Whitney test).

Figure 7 - Seminal vesicle contraction stimulated *in vitro* with either norepinephrine (NE) or carbachol. (A) Concentration-response curves to NE and (B) maximal tension (Emax) developed in the presence of NE in the control (n=5) and BM-treated groups (n=5). (C) Concentration-response curves to carbachol and (D) the maximal tension (Emax) developed in the presence of carbachol in the control (n=5) and BM-treated groups (n=5). Data are expressed as the mean ± SEM. * indicates $p \leq 0.05$(Student's t-test).

Table 1 - Reproductive organ weights

Parameters	PND 45		PND 110	
	Control (n=10)	Betamethasone (n=10)	Control (n=10)	Betamethasone (n=10)
Final body weight (g)	216.20 ± 3.27	214.10 ± 4.20	385.20 ± 7.20	364.50 ± 12.06
Organ weights				
Testis (g)	1.13 ± 0.02	1.17 ± 0.02	1.78 ± 0.03	1.71 ± 0.03
Epididymis (mg)	125.20 ± 4.39	143.30 ± 6.79*	455.90 ± 0.01	437.80 ± 0.01
Ventral prostate (mg)	99.44 ± 4.42	96.07 ± 5.40	281.50 ± 13.63	253.30 ± 16.52
Seminal vesicle (mg)	132.70 ± 9.39	108.80 ± 5.85*	710.40 ± 26.61	688.60 ± 34.46
Relative organ weights				
Testis (mg/100g)	521.10 ± 8.59	546.60 ± 8.27*	0.46 ± 0.01	0.47 ± 0.01
Epididymis (mg/100g)	57.97 ± 2.02	67.88 ± 3.90*	118.70 ± 2.86	120.80 ± 4.28
Ventral prostate (mg/100g)	45.94 ± 1.74	44.98 ± 2.61	72.92 ± 2.75	69.24 ± 3.82
Seminal vesicle (mg/100g)	62.22 ± 3.69	51.57 ± 3.28*	184.60 ± 6.46	189.10 ± 8.17

Data expressed as the mean ± SEM *$p \leq 0.05$. (Student T-test).

Table 2 – Histological and morphometric assays (n=5/group).

Parameter	Control	Betamethasone
[1]Normal tubules at PND45 (%)	89.00 (84.00 – 94.50)	87.0 (80.00 – 89.50)
[1]Normal tubules at PND110 (%)	88.00 (87.00 – 92.50)	84.0 (77.00 – 88.50)
[2] Category of cell maturation (1-5)	3.30 (3.16 – 3.39)	3.14 (2.83 – 3.29)
[2] Spermatogenesis dynamics		
I – VI (%)	28.00 (28.00 – 98.50)	33.00 (27.50 – 35.00)
VII – VIII (%)	32.00 (27.00 – 35.50)	30.00 (24.50 – 35.50)
IX – XIII (%)	29.00 (28.50 – 30.50)	30.00 (27.50 – 31.50)
XIV (%)	8.00 (5.50 – 8.00)	8.00 (7.00 – 11.00)
[2]Tubule diameter PND45 (µm)	257.80 $\pm$ 2.78	257.10 $\pm$ 4.37
[2]Epithelial cell height at PND45 (µm)	76.98 $\pm$ 1.89	79.57 $\pm$ 0.83
[2]Tubule diameter at PND110 (µm)	258.30 $\pm$ 3.38	250.00 $\pm$ 5.19
[2]Epithelial cell height at PND110 (µm)	86.96 $\pm$ 1.70	82.98 $\pm$ 1.77
[1]Sertoli cell number at PND110	18.45 $\pm$ 0.82	18.36 $\pm$ 0.50

[1]Data are expressed as the median and interquartile intervals (Mann-Whitney test). [2] Data are expressed as the mean $\pm$ SEM. *$p \leq 0.05$. (Student T- test)

Table 3 - Sperm counts and sperm epididymal transit time at PND110.

Parameter	Control (n=10)	Betamethasone (n=10)
Sperm count in the testis		
Spermatid Number ($x10^6$/testis)	239.00 ± 6.72	210.90 ± 11.65*
Relative Spermatid Number ($x10^6$/g/testis)	164.20 ± 4.84	152.20 ± 6.58
Daily Sperm Production ($x10^6$/testis/day)	39.18 ± 1.10	34.58 ± 1.91*
Relative Daily Sperm Production ($x10^6$/testis/g/day)	26.76 ± 0.82	24.95 ± 1.08
Epididymal Sperm Count		
Sperm number in caput/corpus ($x10^6$/organ)	158.10 ± 4.49	130.50 ± 8.29*
Sperm number in cauda ($x10^6$/organ)	231.40 ± 5.72	204.30 ± 12.32*
Sperm transit time in caput/corpus (days)	4.02 ± 0.21	3.83 ± 0.26
Sperm transit time in cauda (days)	5.95 ± 0.21	5.96 ± 0.31

Data are expressed as the mean ± SEM. *$p \leq 0.05$ (Student T-test).

Table 4 – Analysis of sperm morphology at PND110.

Parameters	Control (n=10)	Betamethasone (n=10)
Normal sperm (%)	97.50 (96.25 – 99.00)	94.00 (90.00 – 96.25)*
Abnormal sperm (%)		
Isolated head	2.00 (0.62 – 2.50)	3.50 (2.00 – 6.25)*
Broken tail	0.50 (0.00 – 1.00)	1.00 (0.50 – 1.50)
Cytoplasmic droplet (%)	25.00 (20.75 – 26.25)	26.50 (20.50 – 30.25)

Data are expressed as the median and interquartile intervals. *$p \leq 0.05$ (Mann-Whitney test).

Table 5 - Fertility assays following *in utero* artificial insemination at PND110.

Parameter	Control (n=10)	Betamethasone (n=10)
[1] Pregnancy rate (%)	50.00	90.00
[1] Fertility potential (%)	75.00	53.33*
[1] Pre- implantation loss (%)	25.00	46.67*
[1] Post- implantation loss (%)	40.00	25.00
[2] Body weight of the dams (g)	298.80±10.82	308.50±6.56
[2] Uterus weight with fetuses (g)	8.55±1.29	8.42±1.39
[2] Corpora lutea number	11.80±1.11	13.89±0.54
[2] Implantation number	9.60±1.89	7.22±1.05
[2] Reabsorption number	2.60±0.68	1.78±0.49
[2] Fetus weight (mg)	310.00±16.43	333.80±23.45
[2] Placental weight (mg)	270.00±8.37	277.50±31.15

[1] Data are expressed as percentage (Chi-Square test). [2] Data are expressed as the mean ± SEM. *$p \leq 0.05$ (Student T-test).

Table 6 - Male sexual behavior at PND90.

Parameters	Control (n=11)	Betamethasone (n=11)
[1]Latency to the first mount (s)	92.00 (56.00 - 104.00) (n=11)	116.00 (87.00 - 262.00) (n=11)*
[1]Number of mounts until the first ejaculation	14.00 (9.00 - 20.00) (n=11)	10.00 (6.00 - 14.00) (n=11)
[1]Latency to the first intromission (s)	172.00 (108.30 – 215.50) (n=10)	173.50 (127.00 – 383.30) (n=8)
[1]Number of intromissions until the first ejaculation	33.50 (17.50 – 45.00) (n=10)	27.50 (12.00 - 50.00) (n=8)
[2]Frequency of ejaculations (%)	54.54	9.09*
[1]Ejaculatory plugs (mg)	248.50 (190.00 – 281.00) (n=9)	171.90 (79.45 – 211.10) (n=6)*

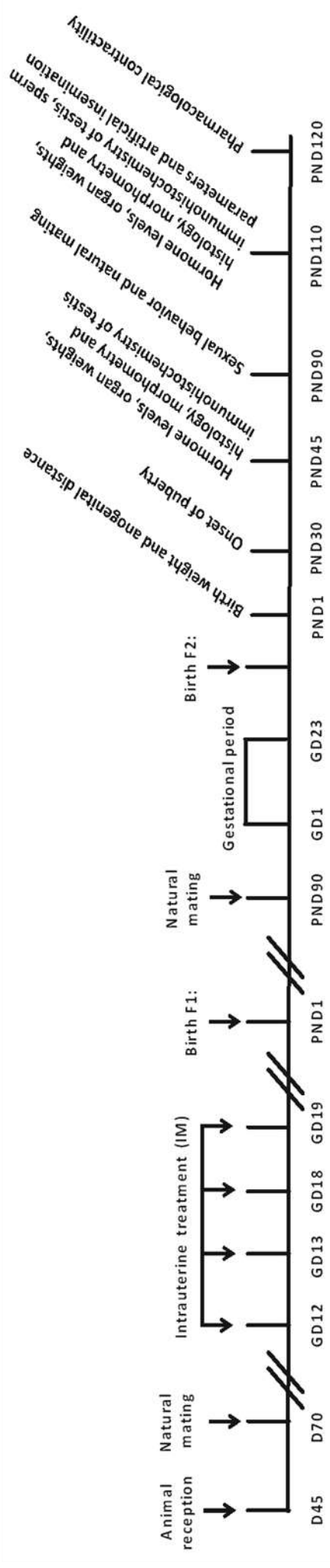
[1]Values expressed as median and interquartile intervals (Mann-Whitney test). [2] Values expressed as percentage (Chi-Square test).
*$p \leq 0.05$.

Table 7 - Fertility assay after natural mating in animals at PND90.

Parameters	Control (n=9)	Betamethasone (n=9)
Pregnancy rate (%)	81.81	81.81
[1] Fertility potential (%)	100.00 (91.77 – 100.00)	87.50 (59.60 – 100.00)*
[1] Pre- implantation loss (%)	0.00 (0.00 – 8.33)	12.50 (0.00 – 40.00)*
[1] Post- implantation loss (%)	0.00 (0.00 – 8.10)	0.00 (0.00 – 13.95)
[2] Body weight of dams (g)	355.30±8.10	344.50±10.99
[2] Uterus weight with fetuses (g)	56.47±1.65	53.03±6.22
[2] Corpora lutea number	12.56±0.24	13.78±0.78
[2] Implantation number	12.11±0.35	10.78±1.46
[2] Reabsorption number	0.44±0.24	0.67±0.29
[2] Fetus weight (g)	2.87±0.06	2.83±0.06
[2] Placental weight (g)	0.49±0.01	0.55±0.06

[1]Values expressed as median and interquartile intervals (Mann-Whitney test). [2] Values expressed as mean ± SEM (T-Student test). *$p \leq 0.05$.

Figure 1:


Animal reception
Natural mating
Intrauterine treatment (IM)
Birth F1:
Natural mating
Gestational period
Birth F2:
D45
D70
GD12
GD13
GD18
GD19
PND1
PND90
GD1
GD23
PND1
PND30
PND45
PND90
PND110
PND120
Birth weight and anogenital distance
Onset of puberty
Hormone levels, organ weights, histology, morphometry and immunohistochemistry of testis
Sexual behavior and natural mating
Hormone levels, organ weights, histology, morphometry and immunohistochemistry of testis, sperm parameters and artificial insemination
Pharmacological contractility

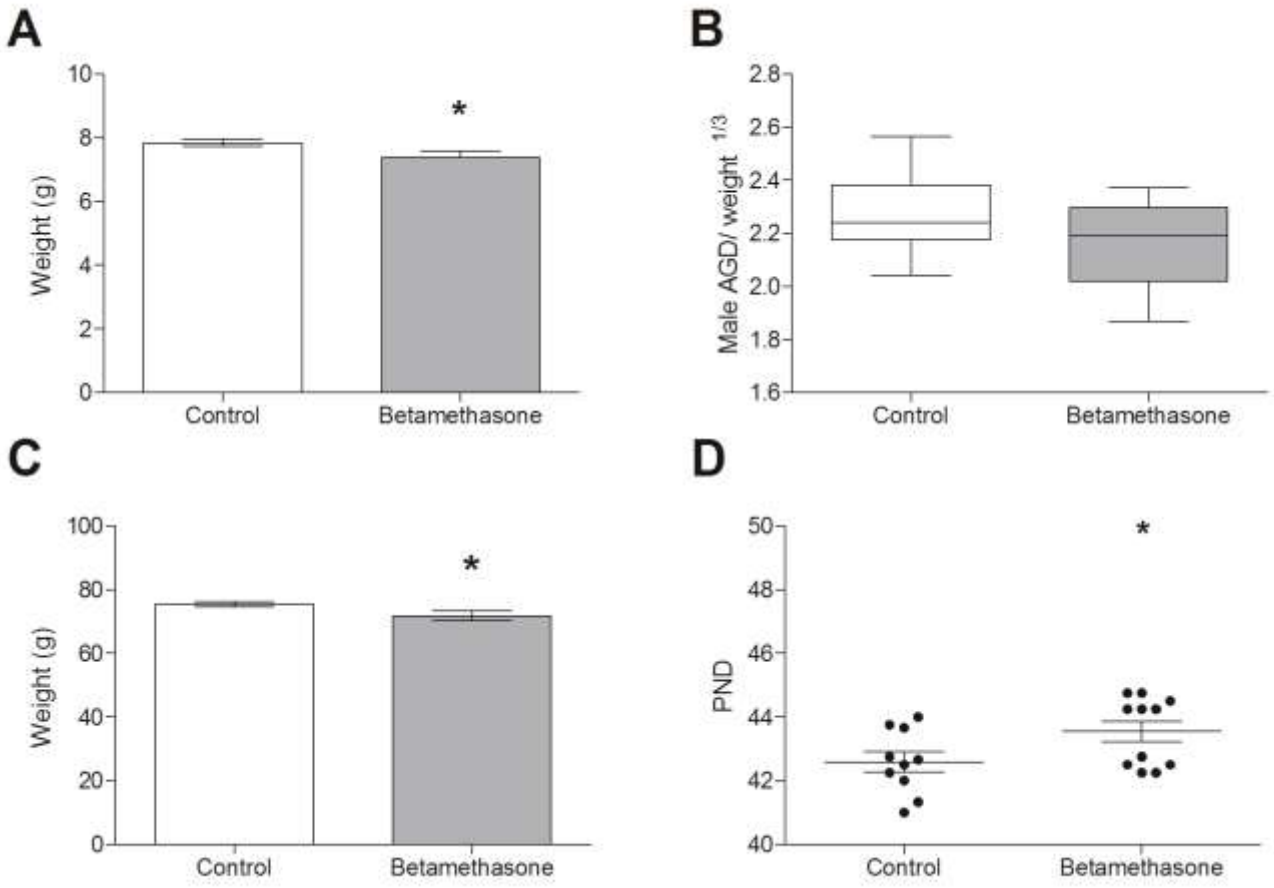

A
Weight (g)
10
8
6
4
2
0
*
Control
Betamethasone
B
Male AGD/ weight 1/3
2.8
2.6
2.4
2.2
2.0
1.8
1.6
Control
Betamethasone
C
Weight (g)
100
80
60
40
20
0
*
Control
Betamethasone
D
PND
50
48
46
44
42
40
*
Control
Betamethasone

Figure 2:

Figure 3:

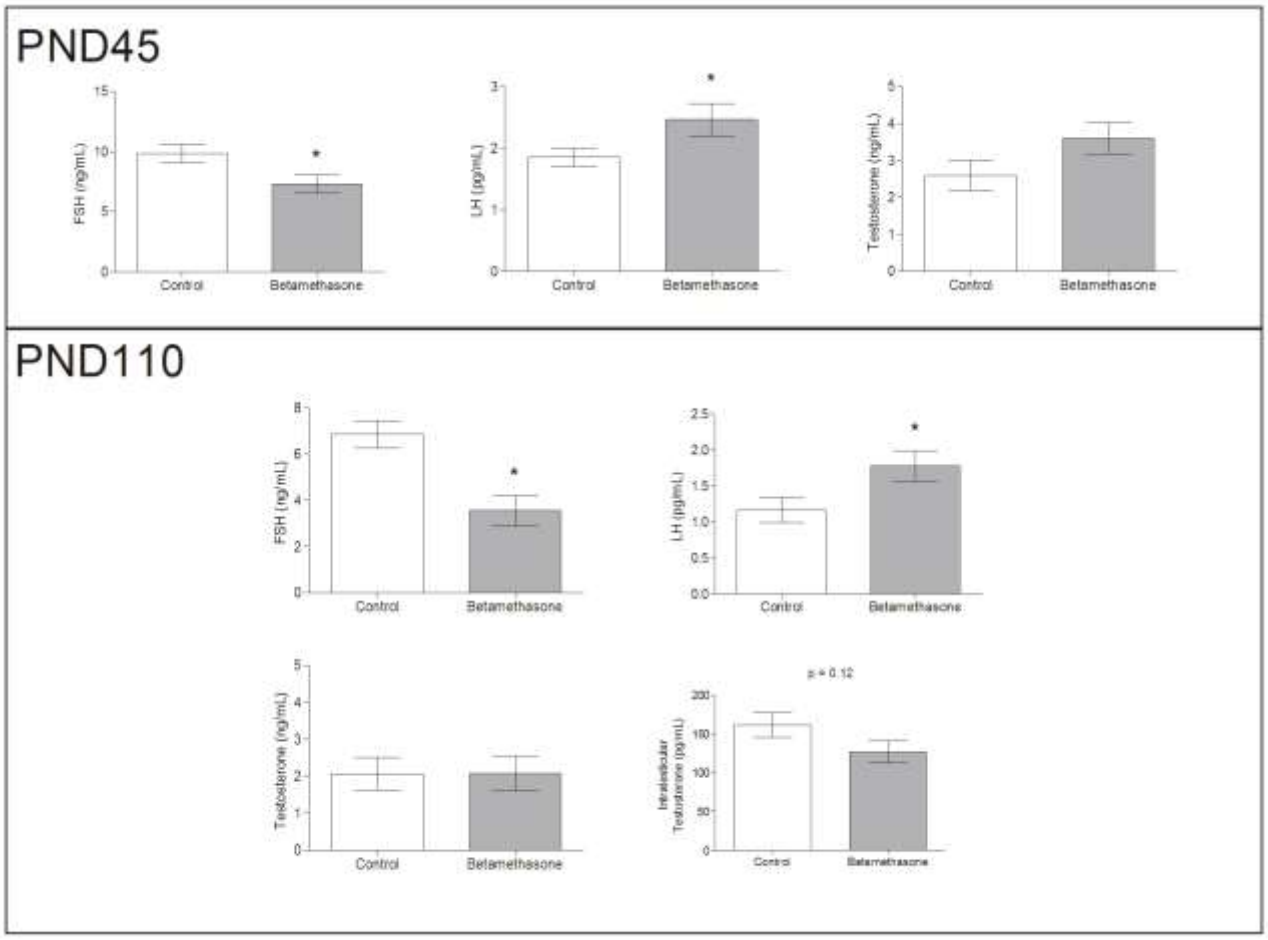

PND45
FSH (ng/mL)
Control
Betamethasone
LH (pg/mL)
Control
Betamethasone
Testosterone (ng/mL)
Control
Betamethasone
PND110
FSH (ng/mL)
Control
Betamethasone
LH (pg/mL)
Control
Betamethasone
Testosterone (ng/mL)
Control
Betamethasone
p = 0.12
Intratesticular Testosterone (pg/mL)
Control
Betamethasone

Figure 4:

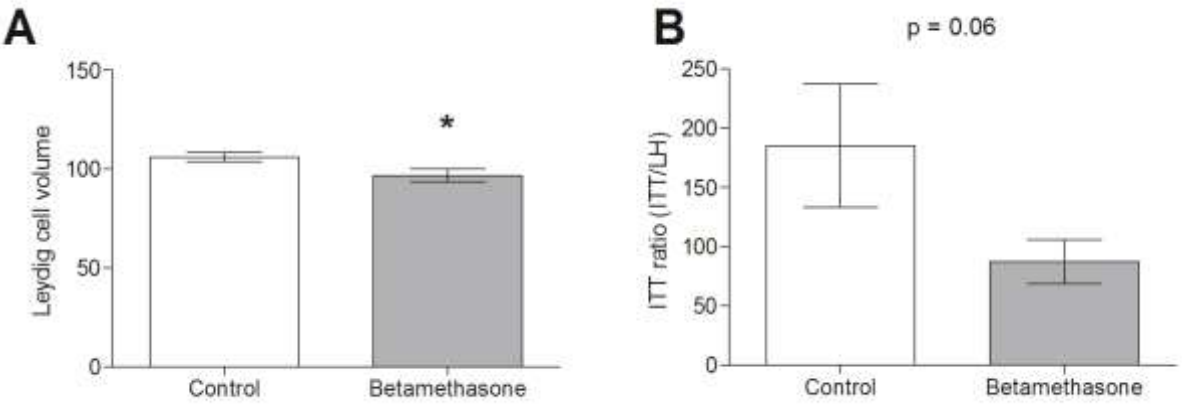

A
Leydig cell volume
150
100
50
0
*
Control
Betamethasone
B
p = 0.06
ITT ratio (ITT/LH)
250
200
150
100
50
0
Control
Betamethasone

Figure 5:

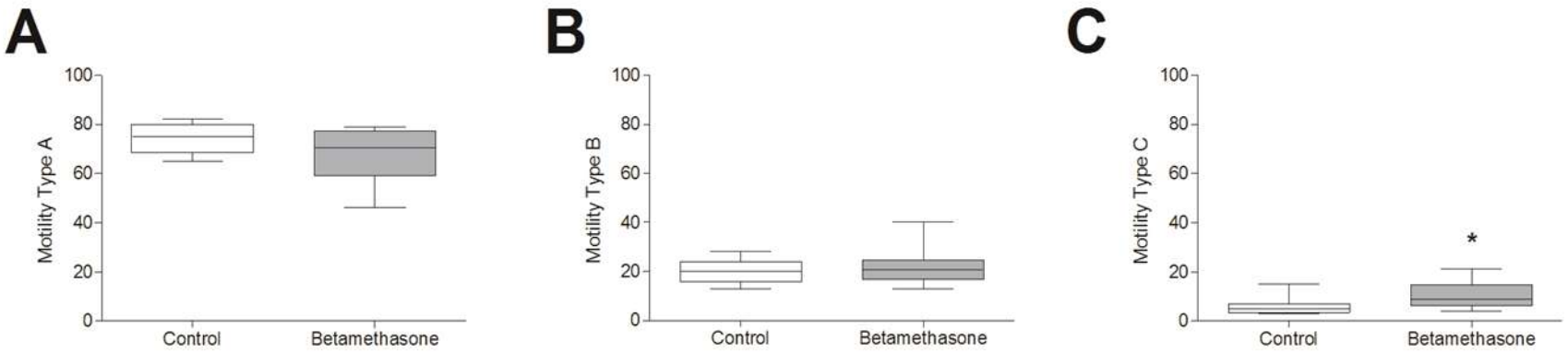

A
Motility Type A
100
80
60
40
20
0
Control
Betamethasone
B
Motility Type B
100
80
60
40
20
0
Control
Betamethasone
C
Motility Type C
100
80
60
40
20
0
*
Control
Betamethasone

Figure 6:

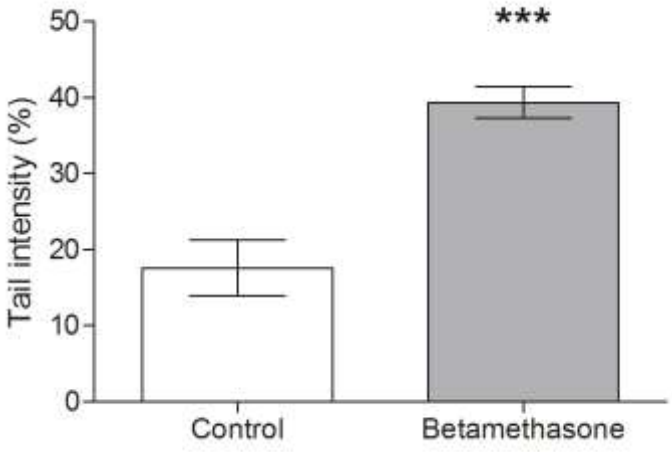
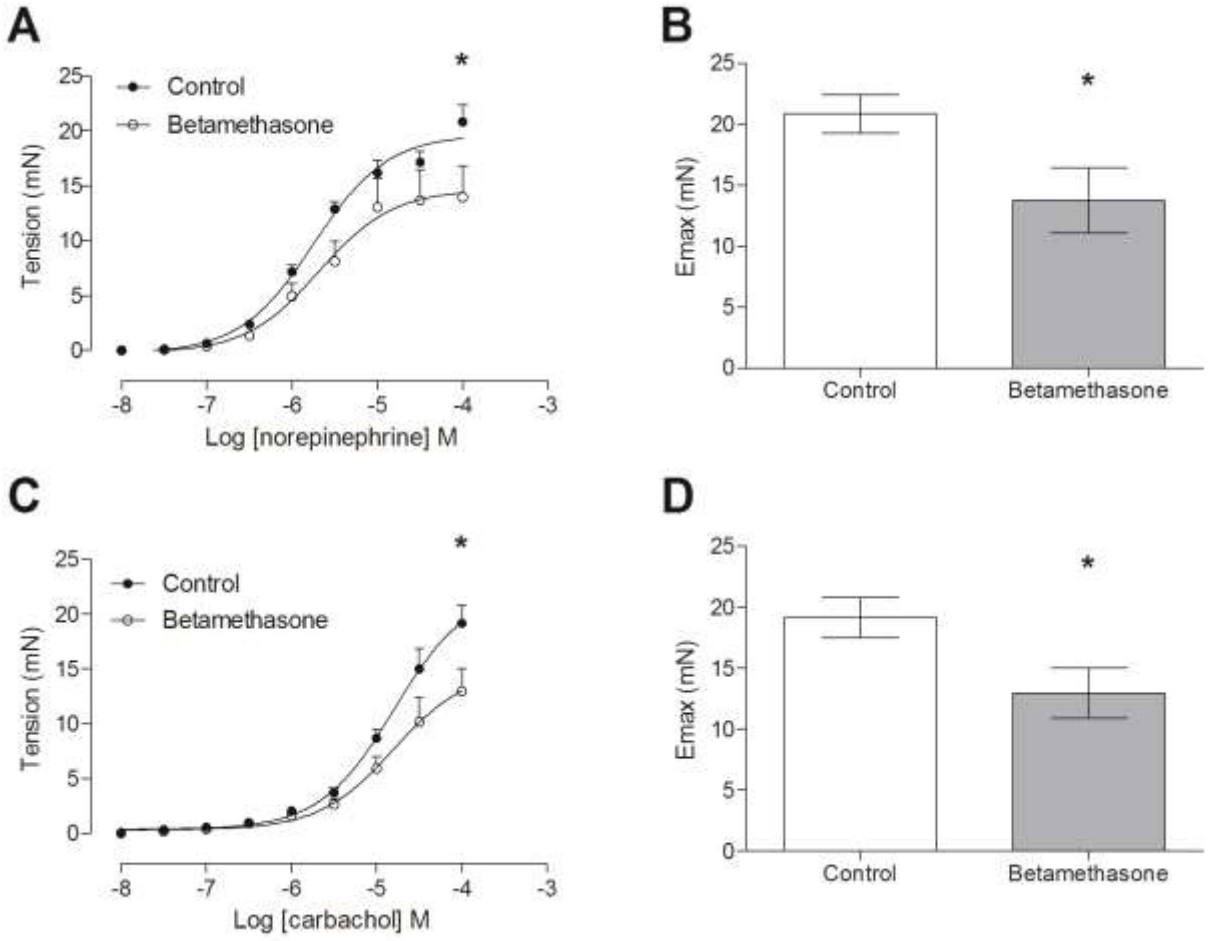


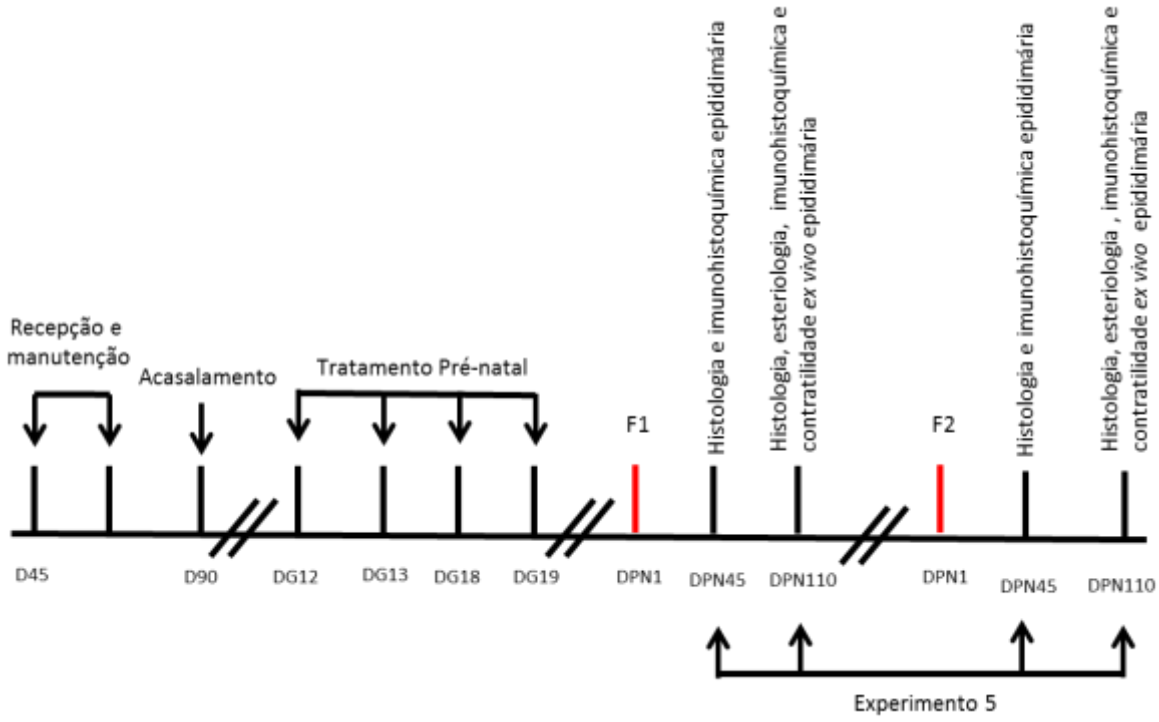
Figure 7:


A
Control
Betamethasone
Tension (mN)
Log [norepinephrine] M
B
Emax (mN)
Control
Betamethasone
C
Control
Betamethasone
Tension (mN)
Log [carbachol] M
D
Emax (mN)
Control
Betamethasone

Capítulo 5

Este trabalho deu origem ao manuscrito “PRENATAL EXPOSURE TO BETAMETHASONE HAS MARKED AND MULTIGENERATIONAL EFFECTS ON THE DEVELOPMENT AND STRUCTURE OF THE RAT EPIDIDYMIS” que será submetido ao periódico “Journal of Toxicology and Environmental Health, Part A” (fator de impacto: 2,243).

Foram avaliados neste manuscrito a histologia, esteriologia, imunomacação para as proteínas Cx43 e p63 nos cortes histológicos do epidídimo de ambas as idades, púberes e adultos, assim como a contratilidade do ducto epididimário tanto da F1 quanto da F2 (observe desenho experimental abaixo).



Nossos resultados demonstraram que a betametasona promoveu na F1 um atraso na diferenciação das células epiteliais do ducto epididimário. Foi observado alteração na imunomarcação da proteína Cx43 (fracamente marcada) e p63 (fortemente marcada) e redução do volume epitelial do epidídimo dos animais adultos. Na F2, houve o mesmo padrão de atraso do epitélio epididimário assim como na imunomarcação da proteína Cx43.

PRENATAL EXPOSURE TO BETAMETHASONE HAS MARKED AND MULTIGENERATIONAL EFFECTS ON THE DEVELOPMENT AND STRUCTURE OF THE RAT EPIDIDYMIS

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Running Title: Epididymis after prenatal betamethasone exposure

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ABSTRACT

There is a body of literature showing that high corticosteroid levels during gestation leads to fetal androgen depletion. In human clinic antenatal betamethasone therapy is used for accelerating fetal lung maturation in women at risk of preterm birth. We previously reported testicular morphological dysfunction and altered sperm quality and fertility in adult rats following intrauterine exposure to betamethasone. Sperm parameters and fertility impairment were also observed in the next generation. Given the essential role of the epididymis for sperm maturation, the present study was carried out to investigate epididymal morphology and contractility in the F1 and F2 male offspring of female rats treated with betamethasone during gestation. The results depicted herein show, for F1 and F2, that prenatal betamethasone exposure promoted a delay in the differentiation of the cauda epididymal epithelium and weaker or absent Cx43 immunostaining. On the other hand, p63 appeared to be more intense in basal cells of F1. At postnatal day 110 the epithelial compartment was reduced in the betamethasone exposed rats. The contractile activity of the isolated epididymal duct was comparable between groups. We concluded that prenatal betamethasone exposure leads to multigenerational impairment in the development and structure of the rat epididymis. Our study potentially shed light on the possible long-term impact of betamethasone antenatal therapy on the human epididymal biology and, consequently, sperm maturation process.

INTRODUCTION

There is a body of literature showing that high corticosteroid levels during gestation leads to fetal androgen depletion (Corbier et al., 1992, Hardy et al., 2005). In male rats, testosterone surges markedly on gestational days (GD) 18–19 (Weisz and Ward 1980; Ward and Weisz 1984; Lalau et al., 1990; Hardy et al., 2005) when brain sexual differentiation starts taking place.

The epididymis is composed by a single highly convoluted duct that connects the efferent ducts to the vas deferens (Rodriguez, 2002; Hermo and Robaire, 2002; De Grava Kempinas and Klinefelter, 2014) and plays an important role on transport, protection, storage, concentration and sperm maturation (Cuasnicú et al., 2002; Robaire et al., 2006; Fernandes et al., 2008). The sperm maturation process occurs along the sperm passage by the epididymal duct with particular luminal environment necessary for the proper functioning of the epididymis (Rodriguez, 2002; Hermo and Robaire, 2002; França et al., 2005; Sullivan et al., 2005; De Grava Kempinas and Klinefelter, 2014). Factors that accelerate transit time compromise both sperm quality and quantity (Klinefelter and Suares, 1997; Fernandez et al., 2008; De Grava Kempinas and Klinefelter, 2015). Indeed, increased contractility of the epididymal duct is related with lower sperm quality and fertility (Borges et al., 2013).

On GD 15 the rat epididymis starts to differentiate from the mesonephric duct under the influence of testosterone (You and Sar, 1998; Robaire et al., 2006). Postnatal development events in the epididymis have been well characterized in undifferentiated period, period of differentiation and period of expansion (Sun and Flickinger, 1979). In rats, cellular differentiation occurs between postnatal day (PND) 16 to PND 44 (Hermo et al., 1992; Marty et al., 2003). Thus, the epididymal duct with only one kind of cells (columnar cells) acquires all types of functional cells for sperm maturation (principal, basal, apical, halo, narrow and clear cells) and also the proteins necessary for the formation of the epididymal-blood barrier (Cyr, 2011). On PND 45 the cell types of the epididymal epithelium are expected to be fully differentiated (Hermo et al., 1992; Marty et al., 2003; Robaire et al., 2006).

Among several important proteins that are expressed during epididymal development connexin 43 (Cx43), a gap junction protein, plays an important role in the communication between adjacent cells (Cyr, 2011). The transcription factor tumor protein 63 (p63) is a proliferative protein expressed only in basal cells which are considered adult stem cells in the epididymis (Mandon et al., 2015). Changes in hormonal control during critical periods of epididymal development may compromise the organ differentiation, with possible long-term effects on its structure and function.

Betamethasone (BM) is a glucocorticoid used for antenatal treatment in human clinic, promoting fetal lung maturation (Jobe and Soll, 2004; Drake et al., 2011). Previous studies showed that *in utero* BM exposure causes fetal programming not only in the first generation, but also in the subsequent offspring (Fowden and Forhead, 2004; Drake et al., 2011; Crudo et al., 2012; Moisiadis and Matthews, 2014). We showed in recent studies that *in utero* BM exposure promoted reproductive programming of rat male offspring, altering reproductive development and impairing sperm quality and fertility of these animals (Piffer et al., 2009a/b; Borges et al., 2016; Borges et al., 2017).

Thus, knowing that BM exposure during intrauterine life may reduce fetal testosterone and directly impact reproductive organs development (Borges et al., 2016) the present study investigated long-term epididymal structure and ductal contractility in the F1 and F2 male offspring of female rats treated with BM during gestation.

MATERIAL AND METHODS

Ethics Statement

The experimental procedures were approved by the local Ethics Committee for the Use of Experimental Animals of the University of São Paulo State (protocol number 451-CEEA) and are in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health). The euthanasia was performed by decapitation and all efforts were made to minimize suffering.

Drugs and Solutions

Betamethasone 21-phosphate disodium (BM) was obtained from Sigma (St. Louis, MO, USA). The drug was diluted in vehicle (saline) in concentration of 0.2mg/ml and administered once a day in 0.5ml/kg.

Animals

Male (90 days old/300–350 g) and female (90 days old/225–230 g) Wistar rats were obtained from the Multidisciplinary Center for Biological Investigation, State University of Campinas and maintained under controlled conditions (25°C, 30% air humidity, 12/12-h light/dark cycle) with food and water available ad libitum.

Two nulliparous female rats were mated with one male, during the dark cycle of the photoperiod. The detection of sperm in the vaginal smear of rats in estrus was considered as GD 1. Pregnant and lactating rats were maintained in individual cages.

Treatment

Pregnant female rats were randomly allocated into two experimental groups (5 per group): control (treated with vehicle) and BM (treated with 0.1 mg/kg BM). Rats received an intramuscular injection of vehicle or BM on days 12, 13, 18 and 19 of pregnancy. This dosing paradigm was previously adopted (Souza et al., 2001; Piffer et al., 2009a/b; Borges et al., 2016; Borges et al., 2017), and takes into account the beginning of the critical window of male rat reproductive tract development (McIntyre et al., 2002; Wilson et al., 2004) and brain sexual differentiation in male fetuses (Pereira et al., 2003; Pereira et al., 2005; Piffer et al., 2009a/b).

Experimental design

First generation (F1): on PND 1 the number of pups per litter was randomized and redistributed in order to have 8 pups (4 males and 4 females) per lactating female. Rats were weaned at PND 21 and housed in separate cages according to sex. A subset of two males from each litter (n=5) were used for the present experiments, one euthanized on PND 45 (pubertal animals). The other rat was kept until PND 90 when was mated with a non-treated female to obtain the next generation. Twenty days later these animals were sacrificed (adult animals). The remainder males and all the females were utilized in other studies (Borges et al., 2016; Borges et al., 2017; Borges et al., submitted manuscript).

Second generation (F2): the same protocol described for the F1 was applied for F2, except for the mating protocol on PND 90.

Histological Analyses

On PND 45 and 110 one of the epididymides from each rat was removed and fixed by immersion in Bouin's fixative, dehydrated, and embedded in Paraplast Plus® (P3683, Sigma-Aldrich). Tissues were sectioned (5µm), mounted on a glass slide and stained with hematoxylin and eosin (HE) to evaluate epididymal morphology. Sections destined for immunohistochemistry were mounted on silanized glass slides and stored at room temperature. Histopathology was analyzed in a blind assay using a Zeiss microscope (model Scope A1-Axio) assembled with a digital camera and analysis software (ZeissAxio Vision, version 4.7.2).

Morphometric-stereological analysis

Morphometric-stereological analysis was performed on adult animals in 30 random histological cross-sections of the epididymal cauda 6A region (Reid and Cleland, 1957; Garcia et al., 2012). The epithelial, luminal, and interstitial epididymal duct volumes were calculated using a x10 objective lens connected to a computer image analysis system (Image Pro Plus 4.5, Media Cybernetics, Inc.).

Immunohistochemistry for Cx43 and p63 in the epididymis

Histological sections (n=5 animals per group) were deparaffinized and hydrated in graded ethanol and washed in TBST (Tris-Buffered Saline, 0.1% Tween-20). Sections were then subjected to antigen retrieval in citrate buffer (0.01 M, pH 6.0) and heated in a microwave. Slides were washed in ddH_2O, followed by hydrogen peroxide (3%) for 15 min,

tap water (5 min), and then incubated in blocking solution (TBST, 5% BSA) for 1 hr at 37^{o}C in a humidified chamber. Sections were then incubated with primary antisera. Sections were incubated overnight with anti-Cx43 (0.625 µg/ml, C-6219; Sigma-Aldrich); or with anti-p63 (8 µg/ml, SC-25268, Santa Cruz Biotechnology). Slides were then washed 3 times in TBST and incubated with the appropriate secondary antibody for 1 hr at 37°C (for Cx43: goat anti-rabbit HRP (1 µg/ml, C# 6721;Abcam); for p63: goat anti-mouse HRP (1.6 µg/ml SC- 2005; Santa Cruz Biotechnology). Sections were subsequently washed with TBST and incubated with diaminobenzidine (0.5 mg/ml, Sigma-Aldrich) for 15 min, rinsed in tap water and counterstained with methylene blue for 5 min. Negative control sections did not receive primary antibody.

Contractile activity of isolated epididymal duct

In the adult animals the left epididymides were carefully excised and duct segments (1.5 cm) were dissected from the distal cauda to record *in vitro* isometric tension as previously described (Bellentani et al., 2011). After a 30 minutes stabilization period, the tissues were repeatedly challenged with 80 mM KCl to evaluate tissue viability and maximal response stabilization. After the stabilization period and challenge with 80 mM KCl a cumulative concentration–response curve to NE (10^{-9}M - 10^{-3}M) was obtained and the maximal tension (Emax) and the potency of NE in developing tension of epididymal duct (pEC_{50}) were evaluated.

Statistical Analysis

Data are presented as mean ± standard error of mean (SEM) or median and interquartile range. Student t-test was used for comparison of parametric variables. Non-parametric data were compared by Mann-Whitney test. Differences were considered significant when $p \leq 0.05$. Statistical analyses were performed using the GraphPad InStat software (version 5).

RESULTS

BM prenatal exposure provoked significant delay in the differentiation of the caudal epididymal epithelium, characterized by the increase in cribriform changes on PND 45 in both F1 and F2 (Figures 1 and 2, respectively). In the adult animals the structure of the epididymis was comparable between groups.

Cx43 was localized in the basal region of the epithelium and appeared to be present at the margins between basal and principal cells. In the BM group the Cx43 immunostaining was absent or weaker compared to control in the F1 and F2 at both ages (Figures 3 and 4). Immunostaining for p63, however, appeared to be more intense in basal cells of BM F1 compared to controls (Figure 5).

The volumetric density of epididymal cauda epithelial cells was significantly lower in BM F1 compared to the control group at PND 110 (Figure 6A).

There were no intergroup differences in the potency of NE in the tension of the epididymal duct for F1 (control group: pEC50 = 5.442 $\pm$ 0.095; BM: pEC50 = 5.470 $\pm$ 0.096) and F2 (control group: pEC50 = 5.973 $\pm$ 0.114; BM: pEC50 = 6.060 $\pm$ 0.248).

DISCUSSION

This work is part of a comprehensive study we are performing concerning the effects of prenatal BM exposure on the female and male offspring of rats, and other reproductive outcomes, published elsewere, were reported in the rats of the present study. For instance, in the BM F1 (Borges et al., 2016) and F2 (submitted manuscript) there was a significative delay in puberty onset and an increase in LH levels in pubertal and adult rats. At puberty a significative decrease in testosterone levels and epithelial testicular injury were observed in the BM F1. This adverse effect on the testis persisted until adulthood (Borges et al., 2016). In other rats from the same BM litters sperm number and quality were lower compared to the control group in both F1 and F2 (Borges et al., 2017; Borges et al., submitted manuscript). Likewise, BM prenatal exposure provoked multigenerational changes (Crudo et al., 2012; Iqbal et al., 2012; Moisiadis et al., 2014) to the hypothalamic-pituitary-adrenal axis, resulting in altered ACTH (adrenocorticotropic hormone) secretion and changes in both glucocorticoid and mineralocorticoid receptors in the adrenal gland.

Findings from the present work document multigenerational adverse effects of prenatal betamethasone exposure on the development and structure of rat epididymis.

BM is a potent glucocorticoid used in the third trimester of gestation for antenatal therapy (Fowden and Forhead, 2004), and it is known that it promotes reduction in fetal testosterone levels, important to masculinization and defeminization processes (Ward and Weisz, 1984; Corbier et al., 1992; Page et al., 2001; Hardy et al., 2005).

The adult epididymis is a highly convoluted duct important for sperm protection, maturation, concentration and storage (for references, see Robaire & Hinton, 2002). The development of this organ is essential to male fertility and occurs under the influence of androgens and testicular growth factors, during intrauterine life until puberty (Robaire et al., 2006; Murashima et al., 2015).

On PND 45 the cell types of the epididymal epithelium are expected to be fully differentiated (Hermo et al., 1992; Marty et al., 2003; Robaire et al., 2006). In the present work there was a delay in the epididymal epithelial differentiation in the BM F1 and F2, indicated by the increase in cribriform aspect, that is a folding of the epithelium on itself and apparent formation of pseudoglandular structures (Creasy et al., 2012). Cribriform aspect has been associated with delayed epididymal epithelial development (Leite et al., 2014), testicular toxicity (De Grava Kempinas and Klinefelter, 2015), androgen depletion (Butterworth and Bisset, 1992) and epithelial growth factors (Calder, 1993).

In order to further investigate the delay in the epididymal epithelial development after BM prenatal exposure, immunolocalization of the Cx43 was performed. Connexins are gap junction androgen-dependent proteins and their expression is dependent on epididymal cellular complete differentiation (Cyr et al., 1996). Cx43 has been shown to be expressed between principal and basal cells, clear and basal cells and also between myoid cells, particularly in the distal regions of epididymal duct (Cyr, 2011). In the present study, the Cx43 immunostaining appeared to be absent or less intense in BM group, at both ages and in both generations and, therefore, this weak immunostaining reinforces the adverse effect of BM prenatal exposure on the epithelial cell differentiation in the epididymis. It is important to highlight that the first BM injection occurred on GD 12, when the primordia of the hemato-epididymal barrier starts to be formed in the mesonephric duct cells, as the first occlusive junctions appear at this gestational age (Suzuki & Nagano, 1978).

Knowing that the differentiation of basal cells occurs around PND 28 (Robaire et al., 2006), and that the transcription factor tumor protein 63 (tp63) is required for their differentiation (Murashima et al., 2012), we performed the immunolocalization for p63, a proliferative protein expressed in basal cells (Mandon et al., 2015). Differently from the observed for Cx43, the immunostaining for p63 was stronger in the BM F1 on PND 45. It is known that the basal cells could be stem cells in the epididymis (Mandon et al., 2015) and also can regulate intercellular communication (Cyr, 2011). This more marked expression of p63 might indicate also a delay in the differentiation of basal cells, that were normal at adulthood. These results, at a molecular level, are in agreement with the increase in cribriform aspect, and reinforce the impact of BM on the cellular differentiation of the epididymal epithelium.

Although the general histological aspect of the epididymis of adult rats were similar in the control and BM-exposed rats, the epididymal epithelium volume density was significantly decreased in BM F1. It is known that changes in the proportions of the epididymal compartments volumes can impact the normal function of the organ (Saade et al., 2008).

Taking into account the importance of the sperm transit time in the epididymis for sperm maturation (for references see De Grava Kempinas and Klinefelter 2014), the contractile activity of the isolated epididymal duct was investigated. No differences were found when both groups were compared, so we can infer that prenatal BM exposure did not interfere with the smooth muscle activity of the epididymal duct.

Thus, prenatal BM exposure lead to delayed differentiation of the epididymal epithelium and compromised the expression of key proteins for cell proliferation and

communication. At least part of these changes were persistent until adulthood and passed to the next generation, with possible impact on organ functions, but not on the ductal contractility.

We recognize strengths and weaknesses in our work. First, it reports new data on the effects of gestational exposure to BM on critical windows of epididymal development. Also, the dosing paradigm of two separated courses of BM intramuscular injections was enough in order to elicit permanent epididymal developmental abnormalities. Carruthers & Foster (2005) reported similar effect in the rat reproductive tract after prenatal exposure to phthalate. On the other hand, and considering the use of BM in antenatal therapy for pregnant women at preterm birth, it does not parallel, necessarily, with gestational and developmental events in humans.

In summary, data reported herein show that prenatal BM exposure leads to multigenerational impairment of the development and cellular structure and density of the rat epididymis. Our study potentially shed light on the possible long-term impact of BM antenatal therapy on the human epididymal biology and, consequently, sperm maturation process.

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. Representative histological aspect of the epididymis of F1 on PDN 45 and PND 110. Spz: spermatozoa; Ep: epithelium; In: interstitial tissue. Note, in the BM group, the delay in the differentiation of the cauda, characterized by the infoldings (arrow) of the epithelium (cribriform aspect) on PND 45. Final magnification: 400x.

Figure 2. Representative histological aspect of the epididymis of F2 on PDN 45 and PND 110. Spz: spermatozoa; Ep: epithelium; In: interstitial tissue. Note, in the BM group, the delay in the differentiation of the cauda, characterized by the infoldings (arrow) of the epithelium on PND 45. Final magnification: 400x.

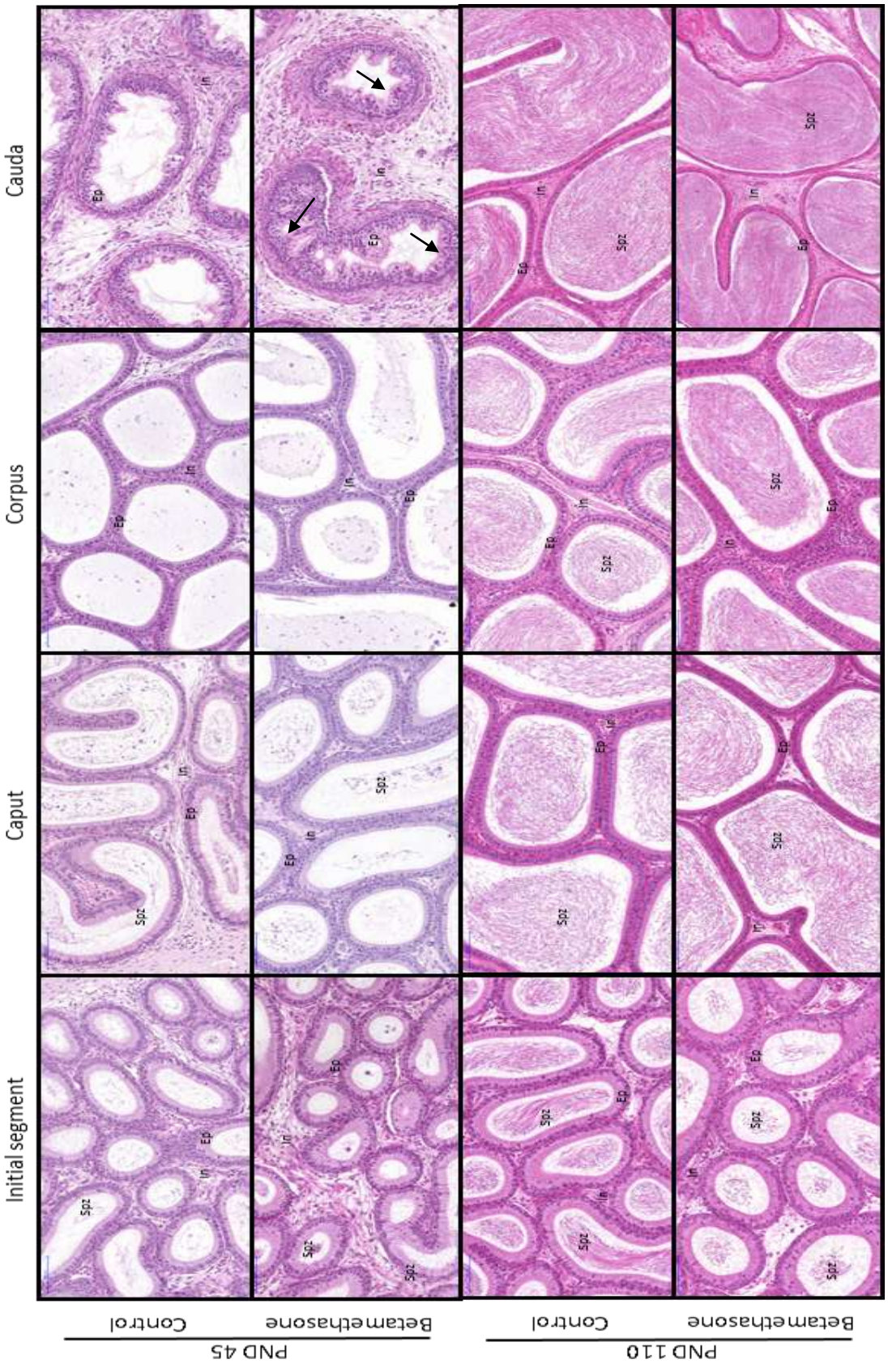
Figure 3. Representative immunostaining for Cx43 in epididymis of F1 on PND 45 and PND110. Observe typical immunostaining in basal cells (detailed in the inserts). Note that in the BM group, at both ages, the immunostaining was absent or weaker than in the control group. Final magnification: 640x.

Figure 4. Representative immunostaining for Cx43 in epididymis of F2 on PND 45 and PND110. Observe typical immunostaining in basal cells (detailed in the inserts). Note that in the BM group, at both ages, the immunostaining was absent or weaker than in the control group. Final magnification: 640x.

Figure 5. Representative immunostaining for p63 in epididymis from animals of F1 on PND 45 and PND 110. Observe typical immunostaining in basal cells (detailed in the inserts). Note stronger reaction in the BM group at both ages. Final magnification: 640x.

Figure 6. Epididymal stereology from animals of **(A)** F1 and **(B)** F2 on PND 110 (n=5/group). Ep: epithelium; In: interstitial tissue and L: lumen. Data are expressed as median and interquartile intervals. * $p \leq 0.05$ (Mann-Whitney test).

Figure 1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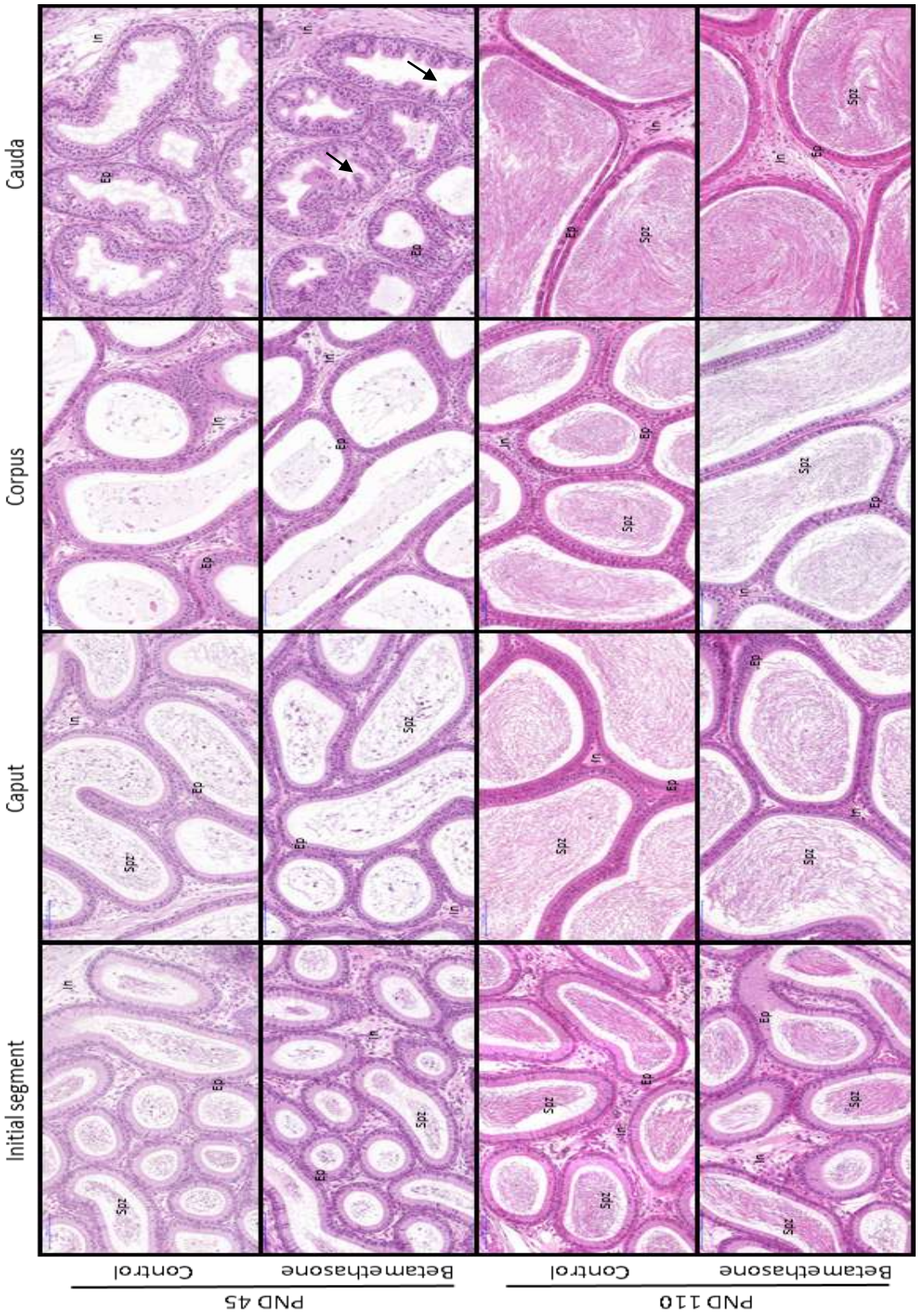
Corpus
Caput
Initial segment
Control
Betamethasone
PND 45
Control
Betamethasone
PND 110
Ep
In
Spz

Figure 2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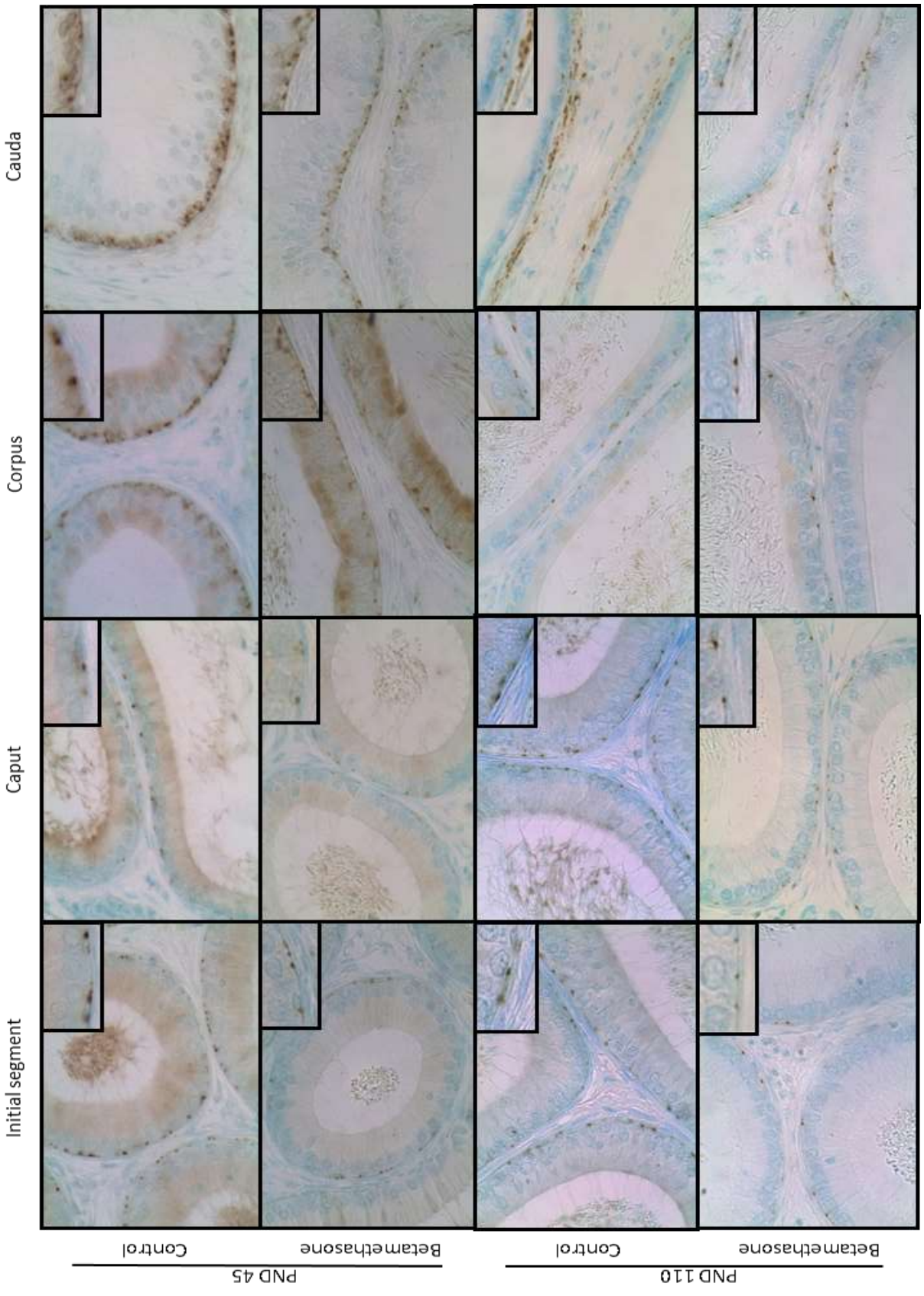
Initial segment
Control
Betamethasone
PND 45
Control
Betamethasone
PND 110
Ep
Spz
In

Figure 3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Corpus
Caput
Initial segment
Control
Betamethasone
PND 45
Control
Betamethasone
PND 110

Figure 4

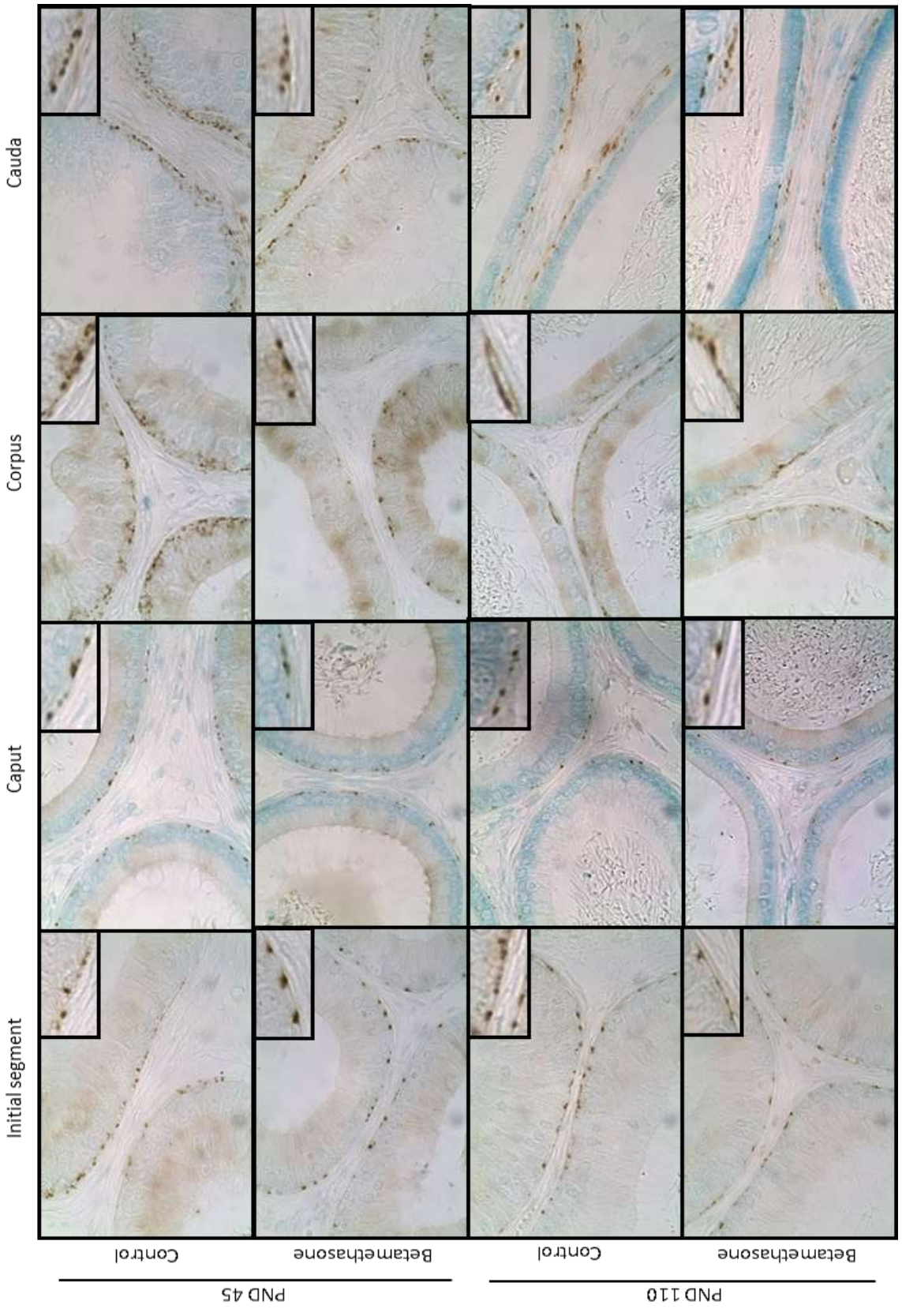
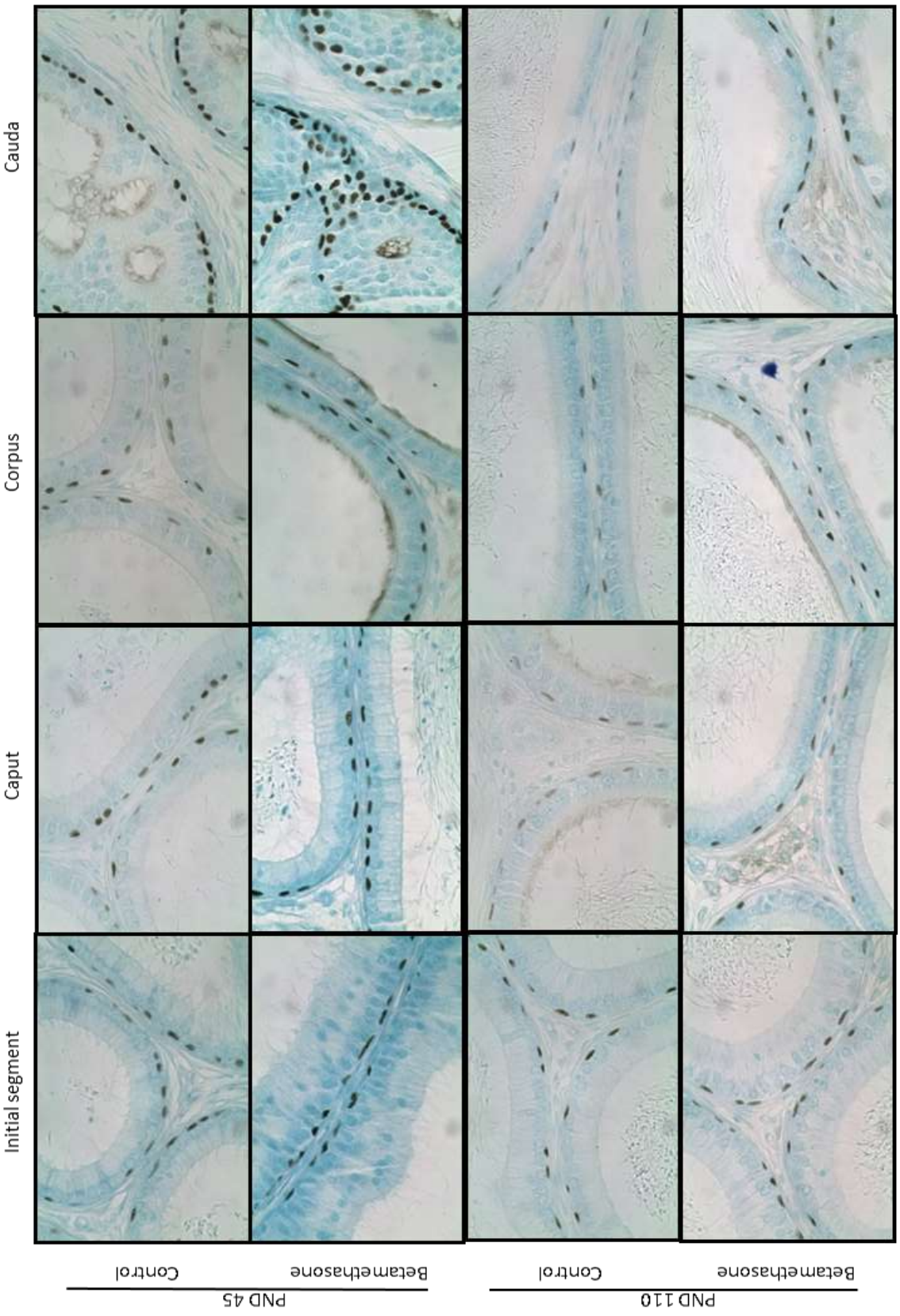

Cauda
Corpus
Caput
Initial segment
Control
Betamethasone
Control
Betamethasone
PND 45
PND 110

Figure 5


Cauda
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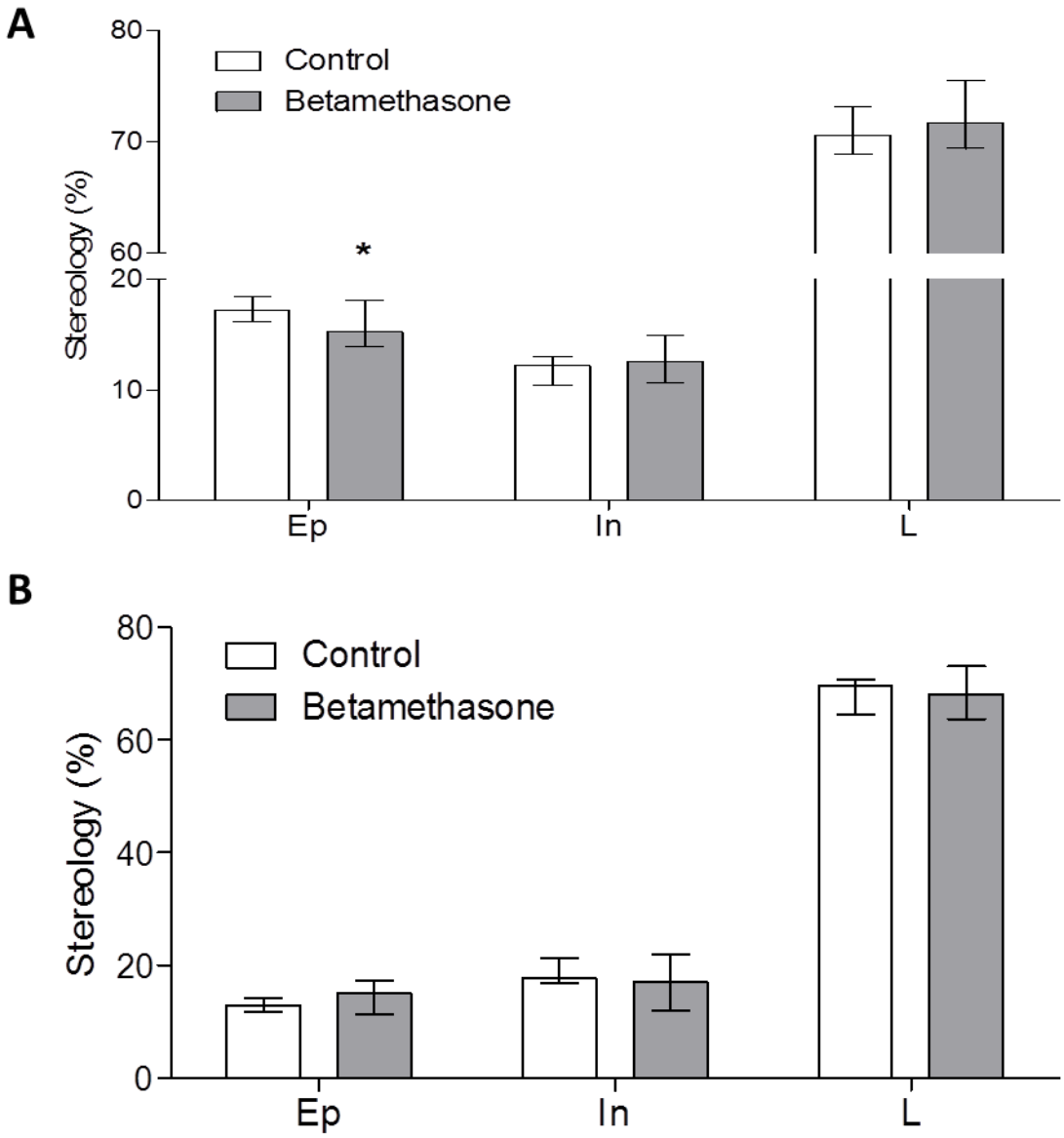
Control
Betamethasone
PND 45
Control
Betamethasone
PND 110

Figure 6


A
80
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Stereology (%)
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Betamethasone
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80
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Stereology (%)
Control
Betamethasone
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Sumário dos Resultados

A administração de betametasona em ratas prenhes nos dias gestacionais 12, 13, 18 e 19 promoveu as seguintes alterações:

Nas ratas tratadas:

- Redução do consumo de ração durante o tratamento;
- Redução do ganho de peso durante o tratamento.

Na prole feminina (F1):

- Redução de peso ao nascimento;
- Atraso na instalação da puberdade;
- Aumento dos concentrações de LH;
- Aumento da duração do ciclo estral e diminuição do número de estros;
- Redução do quociente de lordose;
- Aumento da área do miométrio e diminuição da área endometrial;
- Aumento do número de reabsorções.

Na prole masculina (F1) sexualmente imatura (do nascimento até DPN 45):

- Redução do peso ao nascimento;
- Atraso na instalação da puberdade;
- Atraso na maturação das células germinativas;
- Atraso na diferenciação do epitélio epididimário;
- Redução dos concentrações de testosterona durante a puberdade;
- Alteração na expressão das proteínas Cx43 e p63.

Na prole masculina (F1) sexualmente madura (DPN 90 até DPN 120)

- Aumento das concentrações de LH e redução das concentrações de FSH;
- Alteração da dinâmica espermatogênica;
- Alteração na expressão das proteínas Cx43 (testículo e epidídimo) e p63 (epidídimo);
- Alteração do padrão de distribuição das células germinativas e de Sertoli;
- Redução do diâmetro dos túbulos seminíferos;
- Redução do volume epitelial no epidídimo;
- Diminuição da produção diária espermática;
- Aumento da porcentagem de espermatozoides malformados;

- Aumento da porcentagem de espermatozoides imóveis;
- Redução do potencial fértil e
- Aumento da atividade contrátil da vesícula seminal.

Na prole masculina (F2) sexualmente imatura (do nascimento até DPN 45):

- Redução do peso ao nascimento;
- Atraso na instalação da puberdade;
- Aumento das concentrações de LH e redução das concentrações de FSH;
- Atraso na diferenciação do epitélio epididimário;
- Alteração na expressão da proteína Cx43.

Na prole masculina (F2) sexualmente madura (DPN 90 até DPN 120)

- Aumento das concentrações de LH e redução das concentrações de FSH;
- Comportamento sexual prejudicado;
- Redução do peso dos plugs ejaculatórios;
- Diminuição da atividade contrátil da vesícula seminal;
- Alteração na expressão das proteínas Cx43 (epidídimo);
- Redução do volume das células de Leydig;
- Diminuição da produção diária espermática;
- Aumento da porcentagem de espermatozoides malformados;
- Aumento da porcentagem de espermatozoides imóveis;
- Aumento na porcentagem de danos no DNA espermático e
- Redução do potencial fértil.

Conclusões

Nossos resultados demonstram, neste modelo experimental, que a exposição pré-natal ao glicocorticoide betametasona promoveu efeitos adversos sobre o desenvolvimento sexual inicial, parâmetros reprodutivos e a fertilidade da prole tanto feminina quanto masculina, assim como da geração subsequente. Do ponto de vista translacional os resultados obtidos indicam que se a betametasona, embora eficaz na clínica humana como terapia antenatal, agir no organismo humano como no de roedores, pode promover reprogramação fetal, alterando padrões normais do desenvolvimento sexual, do trato genital e fertilidade dos indivíduos expostos no ambiente intrauterino.

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Apêndices

Comissão de Ética

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Certificado

Certificamos que o Protocolo nº **451-CEUA**, sobre "Efeitos da exposição *in utero* à betametasona sobre aspectos reprodutivos de ratos machos, com ênfase na qualidade espermática e fertilidade: uma abordagem multigeracional", sob a responsabilidade de **Wilma De Grava Kempinas**, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado "*Ad referendum*" da ***COMISSÃO DE ÉTICA NO USO DE ANIMAIS*** (CEUA), nesta data.

Botucatu, 20 de fevereiro de 2013.

Prof. Dr. Wellerson Rodrigo Scarano
Presidente da CEUA

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